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# Measures of Denitrification in Selected South Dakota Semi-permanent Prairie Pothole Wetlands

Beverly S. Klein
*South Dakota State University*

# MEASURES OF DENITRIFICATION IN SELECTED SOUTH DAKOTA SEMI-PERMANENT PRAIRIE POTHOLE WETLANDS

BY

Beverly S. Klein

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Bruce H. Bleakley, Ph.D.
Thesis Advisor

Date

Dale J. Gallenberg
Head, Plant Science Department

Date

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# ABSTRACT

MEASURES OF DENITRIFICATION IN SELECTED SOUTH DAKOTA SEMI-PERMANENT PRAIRIE POTHOLE WETLANDS

Beverly S. Klein

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Wetlands are an integral part of agricultural systems in the prairie pothole regions of the North Central United States and Canadian Provinces. Little research has been done on denitrification in prairie potholes, and a better understanding of their denitrifying capability could aide in optimizing management practices near pothole areas. Most probable number (MPN) and denitrifying enzyme activity (DEA or Phase I) assays were conducted. Most probable number (MPN) measures were used to give an estimate of dissimilatory nitrate reduction to ammonia (DNRA) and denitrifying populations present in the wetlands. This study involved 3 selected semi-permanent prairie pothole wetlands near Madison, South Dakota on farms with different farming practices which were conventional (CON), transitional no till (TNT) and organic (ORG). Phase I denitrifying enzyme activity assays were used to give an estimate of the soil's existing denitrifying enzymes, representing the denitrifying activity history of the soil. In addition to Phase I assays, an amendment-modified Phase I assay was used to test for whether carbon or nitrate was limiting to denitrification. Phase I assays by depth were done using soil from semi-permanent wetland 8 located on a farm using organic farm-management practices. In addition to the depth study, a natural denitrification rate potential study was done to

examine natural denitrification rates without exogenous substrate addition. The effect of varying chloramphenicol concentrations on DEA assays was also examined. The original chloramphenicol concentration used in the study was 1.0 g/L, as per recommendations in the literature. This concentration was later found to be inhibitory to existing denitrifying enzyme activity. Chloramphenicol concentrations for DEA assays in this study were then adjusted to a lower concentration of 0.25 g/L.

The MPN study showed DNRA bacteria to be more numerous in the wetlands than denitrifiers. DNRA populations dominated both the upland and lowland sites, and there were temporal variances for both DNRA bacteria and denitrifiers. Phase I rates for denitrifying bacteria were higher in the lowland and exhibited temporal variances. The Phase I rates-by-depth study showed much of the denitrification activity to be in the top 0-5 cm depth segment, and the 5-10 cm segment had higher rates than the 10-15 cm depth segment. Soil tests were completed for pH, nitrate, soluble salts, organic matter, potassium and phosphorus. Correlations between soil test and Phase I rates by depth were done and a model estimating 35% of the variance in $N_2O$ rates was proposed.

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# GENERAL INTRODUCTION

Prairie potholes are wetlands located in the Midwestern United States and Canada and are unique because of their dense pattern in the landscape and transitory nature (Leitch, 1989). Prairie potholes were formed over 12,000 years ago. Glaciers that covered North America consisted of three lobes and several glacial stages (Flint, 1971). The ice-sheet lobes that covered the Dakotas were called the Pre-Late Wisconsin and Late Wisconsin glaciers (Figure 0.1).

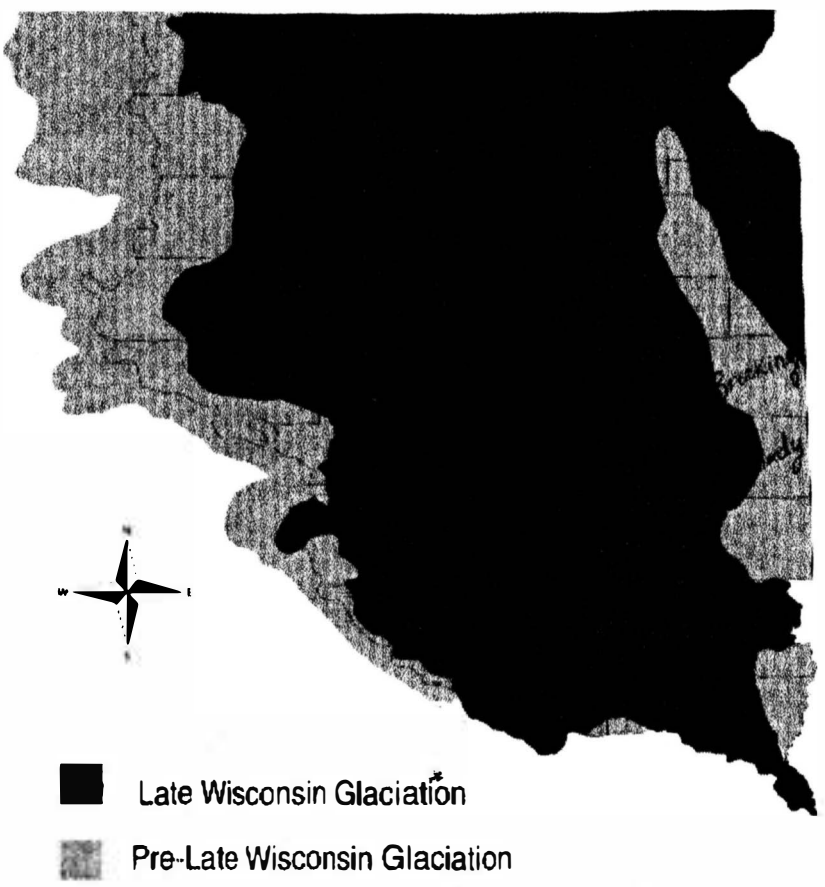



Figure 0.1 Position of Late Wisconsin Glaciation
(taken from Johnson and Higgins, 1997).

The pre-Wisconsin ice sheets stretched south picking up (superglacial) and pushing (englacial) debris; then, upon retreat and stagnation they dropped sediment, sand and gravel (moraine) to form much of what is called the Prairie Coteau (Johnson and Higgins, 1997). Under the Prairie Coteau lies a hard bedrock core that (with the deposits of the pre-Wisconsin glacier moraine) caused the late Wisconsin glacier to fracture and split into two lobes called the James River Lowland (which flows to the James River) and the Des Moines Lobe, which traveled into southern Minnesota and Iowa (Johnson and Higgins, 1997 )(Figure 0.2).

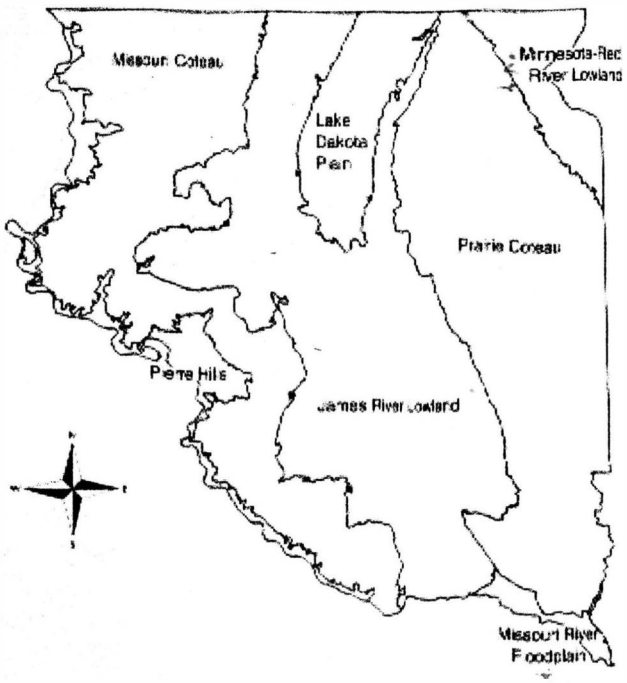



Figure 0.2. Prairie Coteau
(Taken from Johnson and Higgins, 1997)

When the ice sheets swelled or shrunk fractures in the ice sheet occurred that caused pieces of ice to break away from the sheet and become stagnant. The amount of sand, gravel and sediment left behind depended on the thickness of the ice sheet, speed of

glacial movement and amounts of superglacial and englacial debris (Johnson and Higgins, 1997). Larger chunks of ice that were left exposed usually produced a pothole/kettle. Smaller chunks of ice were covered with till and basins were created as the ice melted (Flint, 1971).

The Prairie Pothole Region (PPR) covers areas in five states and three provinces (Figure 0.3). Stewart and Kantrud (1971) divide the wetlands in the PPR into 7 classes, using vegetation to assign zones. A wetland can have two or more zones depending on its depth. The vegetation of the deepest, center, part of the basin is an indicator of the permanency of water in that zone. Five zones are recognized: which are low-prairie, wet meadow, shallow marsh, deep marsh, and permanent open water. The five corresponding classes of freshwater wetlands assigned by vegetation zones are Class I ephemeral ponds (low-prairie central zone); Class II, temporary ponds (wet-meadow central zone and a low-prairie zone); Class III, seasonal ponds and lakes (shallow marsh central zone, wet-meadow zone and a low-prairie zone); Class IV, semipermanent ponds and lakes (deep-marsh central zone, shallow marsh zone, wet-meadow zone and a low-prairie zone); and Class V, permanent ponds and lakes (permanent open-water central zone, deep-marsh zone, shallow marsh zone, wet-meadow zone and a low-prairie zone ). Two saline wetlands included in the classes are class VI, alkali pond or lake (intermittent alkali center zone, shallow marsh zone with two fen zones, wet-meadow zone and a low-prairie zone) and Class VII, fen pond (fen central zone, deep-marsh zone, wet-meadow zone and a low-prairie zone) (Stewart and Kantrud, 1971).

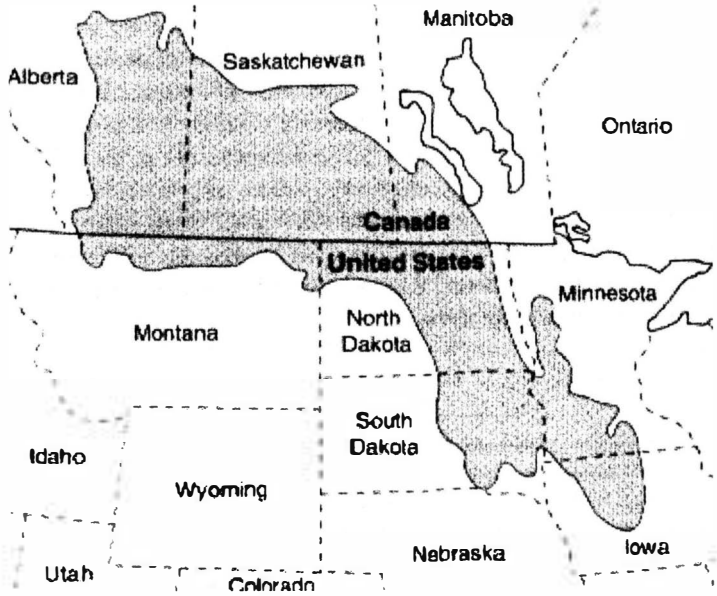



Figure 0.3. Prairie Pothole Region
(taken from the National Research Council, 1995).

Potholes can also be divided into the categories of ground water recharge, flow-through, and discharge (Winter and Rosenberry, 1995). Groundwater recharge potholes lie above the water table, so groundwater flows out of the wetland. Most of the surface water accumulated in this type of wetland is by snowmelt. Recharge wetlands recharge groundwater and often are oligosaline (800-8,000 μS; given as electrical conductivity units of siemens per meter, where 1 S $m^{-1}$ equals 10 mmho $cm^{-1}$). Groundwater flow-through wetlands receive seepage from groundwater and lose water back to the groundwater pool on the low end of groundwater gradients (National Research Council, 1995). Groundwater flow- through wetlands can be considered mixosaline or fresh (< 800 μS). Groundwater discharge wetlands receive upwelling groundwater and lose water by evapotranspiration. Groundwater discharge potholes become highly saline where

recharge and discharge are meso- or hypersaline (8,000 to >60,000 μS)(National Research Council, 1995).

Semi-permanent prairie pothole wetlands are Class IV wetlands by Stewart and Kantrud's definition; semipermanent ponds with a deep-marsh central zone, shallow marsh zone, wet-meadow zone and a low-prairie zone. Semi-permanent prairie pothole wetlands usually contain water. They can, however, become dry during droughts, and the amount of water present will vary from year to year or by seasons. Water input for the wetland comes from precipitation, snowmelt, run off and groundwater. In South Dakota precipitation inputs come mainly in spring and summer, with June usually being the wettest month (National Research Council, 1995). Semi-permanent wetlands can receive substantial water from snowmelt in years with large snow accumulation and from run off. Most of these wetlands are at least moderately saline (National Research Council, 1995).

Protecting wetlands preserves wetland function and values (National Research Council, 1995). Wetlands function to provide biodiversity, recharge ground water, regulate flood control, catch sediments, retain nutrients, remove toxins, and provide wildlife habitats (Miller and Gardiner, 1998). Wetlands are an integral part of agricultural systems in the Prairie Pothole Region of the North Central United States and Canadian Provinces. Governing of wetlands dates back to the mid 1800's. The federal government, at this time, encouraged through the Swamp Lands Acts (1849, 1859, 1860) the conversion of inland wetlands to agricultural land (Salverson, 1990). An estimated 50% of U. S. wetlands were destroyed. In 1899 the River and Harbor Act gave the U. S. Army Corp of Engineers control and responsibility of keeping U. S. waterways open and navigable. No dredging or discharging in the waterways was allowed without a permit.

In the early 1900's the main concern for the U.S. Army Corp of Engineers was dam building, canal digging, and shipping canal and harbor maintenance. In the 1960's environmental awareness was on the rise and the U.S. government passed a number of acts to protect the environment, including wetlands. Regulation of wetlands currently can be administered at both federal and state levels (National Research Council, 1995).

The National Flood Insurance Act (1968) provided financial incentives for communities to accept federal flood plain management programs. This indirectly affected wetlands because many wetlands occur in flood plains (National Research Council, 1995).

In 1969 the National Environmental Policy Act was enforced to resolve conflicts between community growth and environmental protection issues. This act required federal agencies to think about environmental impacts in a proposed project before its initiation. The consideration had to be submitted in the form of an environmental impact statement or an environmental assessment for all federally permitted projects (National Research Council, 1995).

In 1973 the Endangered Species Act went into effect. This act prohibited any action that proposed harm to an endangered species. This indirectly protected wetlands because wetlands provided habitat that supported many such species (National Research Council, 1995).

The Clean Water Act of 1977 was a major act affecting wetlands. Section 404 of the act disallowed the filling of waters of the U. S. without a permit from the Corp. Section 404 is subdivided into five sections that define its role in wetland protection.

Section 404:

(a) Allows the Corp to issue permits for filling waters, including wetlands.

(b) States the Corp are required to follow the U.S. Environmental Protection Agency's (EPA) guidelines when issuing a permit.

(c) Allows the EPA to veto permits issued by the Corp to fill wetlands.

(f) Exempts normal farming, silviculture, minor drainage, or upland soils and water conservation practices if they were preexisting activities.

(g) Allows states to issue permits if the EPA approved their program.

Sub-section (f) of Section 404 of the Clean Water Act and "Swamp buster" were the Acts that most affected the agricultural community (National Research Council, 1995).

The conservation compliance (Swamp buster) and wetland conservation provisions (Wetland Reserve Program) of the Farm Bills of 1985 and 1990 required agricultural producers to protect wetlands or lose certain USDA farm program benefits. Farmland converted by drainage, leveling, or other means prior to 1985 was not regulated by the Section 404 Act or Swamp buster (National Research Council, 1995).

The development of wetland policies has necessitated definition and delineation of wetlands. Wetlands by definition are " transitional between terrestrial and aquatic systems where the water table is usually at or near the surface or the land is covered by shallow water. For purposes of this classification wetlands must have one or more of the following three attributes: (1) at least periodically, the land supports predominantly hydrophytes, (2) the substrate is predominantly undrained hydric soil, and (3) the substrate is nonsoil and is saturated with water or covered by shallow water at some time during the growing season of each year" (Johnson and Higgins, 1997). The USDA

under conditions of saturation, flooding, or ponding long enough during the growing season to develop anaerobic conditions in the upper parts." Hydric soils are usually poorly drained soils, many having a histic epipedon, sulfidic material, gleyed horizon, with chroma less than 2, an aquic moisture regime, and low redox potential (Miller and Gardiner, 1998). In this type of environment, at times, the microbial oxygen demand leads to anaerobic conditions, allowing facultative and obligate anaerobic bacteria to reduce and oxidize forms of nitrogen, iron, manganese and other compounds. The reduction of iron and manganese oxides produces a color change in the soil (National Research Council, 1995). These mottles are an indication of hydric soils. Salts or carbonate (lime) deposits existing in the soil are white in color. Rust mottles indicate a soil that has had inadequate aeration while bluish, grayish, and greenish sub soils (gleying) with or without mottles indicate longer periods each year of waterlogged conditions and inadequate aeration (Miller and Donahue, 1995). Dark soils often have high levels of soil organic matter, but when interpreted with consideration of where the soil profile is on the landscape, dark color can be a general indicator of reduced forms of iron and manganese oxides that producing colors characteristic of hydric soils (National Research Council, 1995). Soil color determination is standardized and determined by comparing the color to a Munsell color chart. The Munsell color chart divides color notation into three parts: hue, value, and chroma. Hue refers to the dominant spectral or "rainbow" color (red, yellow, blue, and green). Value refers to the amount of light reflected and is noted as the amount of blackness or whiteness in the soil. The value range is 0 (black and no reflection of light) to 10 (white and the most light reflected). Chroma refers to the purity of the "color". Chroma is noted in values of 0 (neutral) to 10

Chroma refers to the purity of the "color". Chroma is noted in values of 0 (neutral) to 10 (a select portion of wavelengths, called brilliance). A chroma value of less than 2 is indicative of wetland/hydric soil (Miller and Donahue, 1995).

Water bodies such as lakes and streams can be polluted by non-point pollution sources like highway or commercial development and agricultural practices (Linder and Hubbard, 1982). Nitrates can enter wetlands through surface runoff and ground water discharge (Downing and Peterka, 1978). When nitrate is in aqueous form, it can move from wetland to groundwater or from soil water to the wetland. Wetlands slow the water flow, permitting the settling of suspended materials and filtering the water as it passes through the wetland's littoral layer (Linder and Hubbard, 1982). The levels of C, N, P, S and other nutrients in Prairie Pothole Region wetlands and the water that passes through them will be affected by microorganisms, particularly anaerobes, and by the presence of wetland plants and animals (Bleakley et al., 1995; Dumas, 1990).

Many biogeochemical processes take place in wetlands. Oxygen diffuses approximately 10,000 times more slowly in water than air (Paul and Clark, 1989). Biological activity in wetland sediments must tolerate low oxygen concentrations or anaerobic conditions (Dunn et al., 1979). Among the anaerobic microbial processes that often occur in wetland sediments are denitrification, sulfate reduction, and methanogenesis (Conrad, 1996).

Denitrification is a microbial process occurring in soil or sediment during periods of anoxia and reduces nitrate to nitrogenous oxides and/or dinitrogen gas via the denitrification pathway (Figures 1.1; 3.4). Organisms that use this pathway are facultatively anaerobic bacteria and therefore prefer to use $O_2$ as their terminal electron

acceptor. However, when $O_2$ is not available denitrifiers can use nitrogen oxides as respiratory electron acceptors.

Denitrification in wetlands within an agricultural setting has just begun to be understood. Extreme fluctuations in denitrifying activity can have negative effects. When too high it can limit the amount of nitrate available for plant growth (Rolston et al., 1978), and when it is too low excess amounts of nitrate can move through the soil profile and contaminate groundwater, rendering it unsafe for drinking at levels of 4-5-mg of nitrate per liter (Mellor et al., 1992).

Numerous factors that affect wetland functions will need to be correlated and applied to understand denitrification in prairie pothole wetlands. Other anaerobic processes that may occur in these wetlands are dissimilatory reduction of nitrate to ammonia and sulfate reduction. In 1996 another member of our laboratory, Tom Botel, assayed oxidation and reduction (redox) potential values and compared the anaerobic measures taken between three farming management systems to see if significant differences existed

Semi-permanent prairie wetlands used in this study were ground water discharge sites (Bleakley et al., 1995). The landscape surrounding wetland 5 was organically farmed (Table 0.1). Wetland number 8 was located on a farm using a transitional-no-till (TNT) management style (Table 0.1), and wetland number 13 was located on a farm using conventional (CON) management style (Table 0.1). These farms near Madison, South Dakota were part of a study whose findings have been documented elsewhere (Bleakley et al., 1995).

Table 0.1 Summary of Management Style Differences (based on Bleakley et al., 1995).

| Farm management Style | Fertilizer | Primary weed control | Pesticide | Crop rotation (year) | Management style's highest priority |
|---|---|---|---|---|---|
| Conventional | Synthetic | Tillage but chemical is secondary | Chemical | (1) corn (2) soybean (3) small grain with alfalfa (4-6) alfalfa | ---------- |
| Transitional – no-till | Synthetic | Chemical, tillage only if chemical fails | Chemical | (1) corn (2) soybean | Crop residue management |
| Organic | Manure | Tillage, crop rotation and hand weeding are secondary | ---------- | Strict adherence to: (1) small grain with alfalfa (2&3) alfalfa (4) soybeans (5) corn (6) soybeans | Weed control management |

Botel found redox potentials of lowland sites in three semi-permanent wetlands to be significantly different, with the organic wetland consistently at a lower redox potential compared to the conventional or transitional no-till semi-permanent wetlands on the dates studied.

This study uses information gained from the Botel study (1996) and networked information from the North Central Region ACE program Annual reports dating from 1993 to 1995, whose information paralleled the data collection seasons of this study.

The main purpose of the projects included in this thesis was to (1) assay for possible differences in dissimilatory nitrate reduction to ammonia (DNRA) and denitrifying bacteria population sizes as well as location differences (upland vs. lowland); (2) screen values for population size according to farm management styles; (3) determine denitrifying enzyme activity (DEA) values of lowland verses upland soil; (4) examine if any correlation between MPN values and DEA values exist; (5) screen for differences in DEA values according to farm management styles; and (6) assay DEA by depth.

It is important to remember the variability that exists in soil, both temporally and spatially. Also, the number of semi-permanent wetlands studied with management style differences was very small. Results and their interpretation should be considered preliminary and aiding the formation of future hypotheses and more elaborate studies of prairie-pothole wetlands and the effects that different farm management styles may have on them.

# CHAPTER 1

# ESTIMATION OF POPULATIONS OF DENITRIFIERS AND DISSIMILATORY NITRATE REDUCERS TO AMMONIA ASSOCIATED WITH PRAIRIE-POTHOLE SOILS IN DIFFERENT AGRICULTURAL MANAGEMENT SYSTEMS

## INTRODUCTION

The most probable number (MPN) method for estimating microbial populations, discussed by Halvorson and Ziegler (1933) and Cochran (1950), gives an estimate of microbial populations without actually counting single cells or colonies. Most-probable-number analysis does not indicate the number of metabolically active cells at the time of sampling but estimates the number of viable cells present at that time. The values obtained from MPN analysis may not be indicative of denitrification or dissimilatory nitrate reduction of ammonia (DNRA) processes at wetland sample sites, but indicate the number of viable cells of these organisms present in the sample. The MPN method is based on a determination of the presence or absence of microorganisms in several consecutive dilutions of soil or other material. It can be used as an index of "activity or catalytic potential" of a soil sample (Davidson et al. 1985). In this chapter, MPN estimates were made of denitrifiers and DNRA bacteria in selected prairie-pothole soils.

Microorganisms can channel the fate of nitrate into three different pathways (Figure 1.1). The pathways are assimilatory and dissimilatory reduction of nitrate to ammonia, and denitrification (Tiedje, 1980).

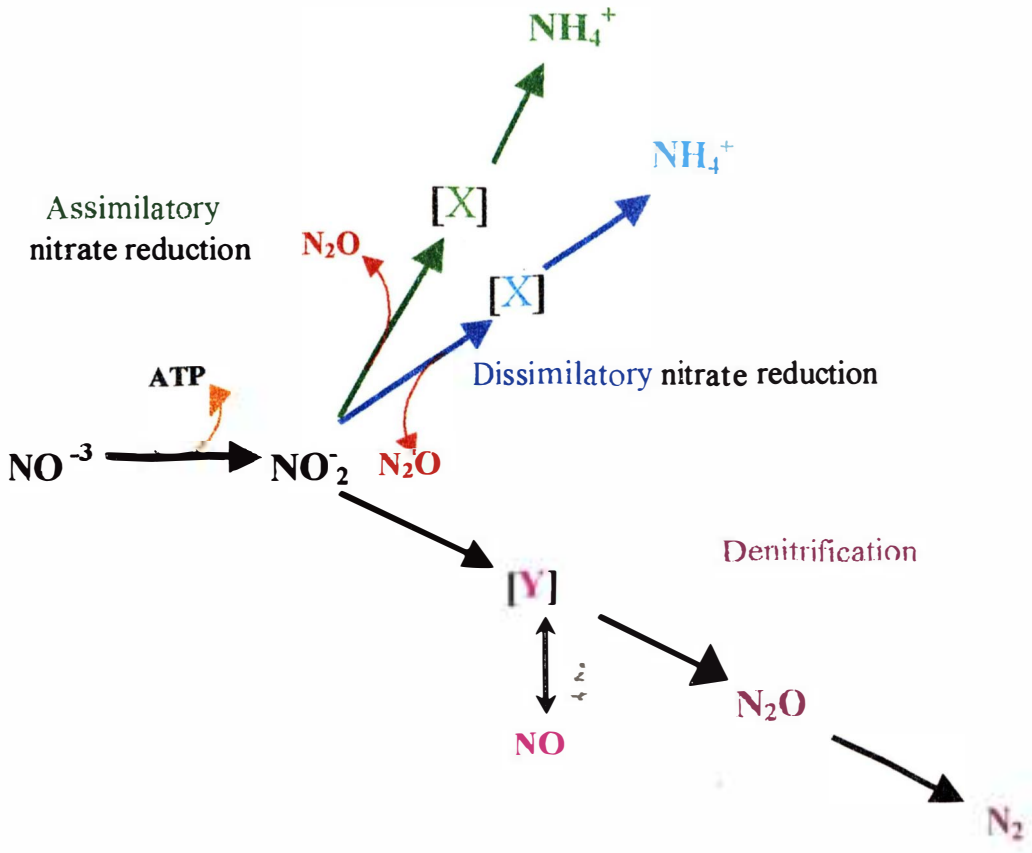



Figure 1.1.
Three pathways of nitrate reduction and free intermediates.
[X] is a membrane-bound enzyme for dissimilatory and assimilatory nitrite reduction.
[Y] is a membrane-bound enzyme for denitrification.
(Adapted from Tiedje, 1988)

Nitrate assimilatory pathways are regulated by the amount of ammonia present, and are insensitive to atmospheric oxygen concentrations. Assimilatory reduction of nitrate to ammonia is balanced with growth of the microbe and involves soluble enzyme systems including a soluble nitrate reductase. Because the cell uses nitrate for biosynthesis it only reduces as much nitrate as is needed for growth (Tiedje, 1982). Denitrification is a type of anaerobic respiration, where nitrate is reduced to gaseous nitrogen species, especially dinitrogen and nitrous oxide. It is the major type of dissimilatory nitrate reduction in soil. This nitrate reduction is coupled to ATP production via oxidative (electron transport) phosphorylation (Myrold, 1998).

DNRA involves the total reduction of nitrate to ammonium. Several bacterial genera can do this under anaerobic conditions. Some DNRA bacteria that have been isolated from soil are *Bacillus* strains (most prevalent), with *Enterobacter* and *Citrobacter* strains being less prevalent (Tiedje, 1988). The first step is the reduction of nitrate to nitrite, which is coupled to ATP production via oxidative phosphorylation. Most DNRA bacteria do not obtain any additional ATP from the ensuing reduction of nitrite to ammonium. Because of little or no energy gain from this step, it may allow detoxification of nitrite and/or regeneration of reducing equivalents via reoxidation of NADH. Ecologically, DNRA bacteria are more numerous and probably more active than denitrifiers in organic carbon-rich environments, including sediments and sewage sludge, while denitrifiers are more dominant in soil and other typically carbon-poor environments (Myrold, 1998).

The lack of energy conservation in the nitrite to ammonium step probably explains why the organisms accumulate nitrite under carbon-limited conditions. ATP is produced during the nitrate-to-nitrite step but not from nitrite reduction, so it would be more beneficial for the organism to direct its electron flow to the energy-producing step. But if nitrate is the limiting factor then the need for a high-capacity electron sink is more important, so the reduction to ammonium pathway would be more beneficial to the DNRA organism (Table 1.1)(Tiedje, 1988).

In dissimilatory pathways nitrogenous compounds are not taken up for biosynthesis but used as electron acceptors that allow the organism to conserve energy, grow more efficiently and possibly control toxic levels of nitrite in the cell's environment (Thauer et al., 1977). The dissimilatory pathway functions in the absence of oxygen, the preferred electron acceptor for facultative anaerobes. Because of this, $O_2$ is the main regulator of DNRA activity and enzyme synthesis. The organism itself is basically unaffected by the amounts of $NH_4^+$ present. DNRA will convert some nitrite to $NH_4^+$ but because this pathway is electron/energy expensive the preferred pathway would be the ATP- yielding, nitrate-to-nitrite, energy-producing step and nitrite would accumulate as long as the organism was carbon-limited. If conditions are nitrate-limited, the DNRA bacteria will need a high-capacity electron sink, and then reduction of nitrate to ammonium is a more probable outcome. The accumulation of nitrite under carbon-limiting conditions is the critical step often used in DNRA bacterial identification (Tiedje, 1980). As previously mentioned the reduction of nitrate-to-ammonium is linked to energy conservation in DRNA, indicating that at least some of the enzymes are

imbedded in the cell membrane. When this is the case energy conservation happens by electron transport phosphorylation (ETP) involving obligate membrane-bound redox reactions (Gottschalk, 1986). Other than for energy, a cell can benefit by using the nitrite-to-ammonium conversion as an electron sink that allows the reoxidation of NADH. In this case the nitrogenous oxides serve as acceptors in NADH oxidation, which allows substrate-level phosphorylation to continue in glycolysis (Tiedje, 1988).

| Reaction | $\Delta G^{\circ\prime}$ (kcal/mole) $H_2$ | $NO^-_3$ | Electrons accommodated per $NO^-_3$ |
|---|---|---|---|
| Dissimilatory $NO^-_3 \rightarrow NH^+_4$ | | | |
| $NO^-_3 + 4H_2 + 2H \rightarrow NH^+_4 + 3\ H_2O$ | -35.8 | -143.3 | 8 |
| Denitrification | | | |
| $2NO^-_3 + 5\ H_2 + 2H^+ \rightarrow N_2 + 6H_2O$ | -53.6 | -133.9 | 5 |

Table 1.1. Theoretical energy yield and electron accepting capacity of dissimilatory nitrate reduction to ammonium and denitrification. (Adapted from Tiedje, 1980).

This electron sink for NADH oxidation would be able to accept eight electrons per nitrate reduction, making it the most favorable to facultative anaerobes faced with a shortage of electron acceptors even though the free energy change is approximately the same (Tiedje, 1988).

Not all DNRA organisms will use the same energy-conservation mechanisms. Some strains of *Clostridium* have no ETP coupled with nitrate reduction to ammonium,

but they use the dissimilatory pathway and experience increased growth because of substrate-level phosphorylation. Three genera of microorganisms that conserve energy with the reduction of nitrate to ammonium by membrane-bound nitrite reductases are *Campylobacter sputorum*, *Desulfovibrio gigas*, and *Wolinella succinogenes* (Tiedje, 1988). If the nitrite reductases are not membrane-bound they are soluble and therefore not expected to conserve energy. Because of this nitrite-to-ammonium step most DNRA organisms tend to accumulate nitrite under carbon-limited conditions (whereby 1 glucose + 12 $NO_3^- \rightarrow$ 6 $CO_2$ + 6 $H_2O$ + **12$NO_2^-$** with $\Delta G'^\circ$ of $^-$422 kcal, (Gottschalk, 1986). ATP is produced from the nitrate-to-nitrite step but not from nitrite reduction, so the organism would prefer to divert its limited electron flow to the energy-producing step. But if in a nitrate-limited environment the organism's need for a high-capacity electron sink would be more important, and reduction to ammonium would occur (whereby 1 glucose + 3 $NO_3^-$ + $3H^+ \rightarrow$ 6 $CO_2$ + **3$NH_3$** + 3$H_2O$ with $\Delta G'^\circ$ $^-$429 kcal, (Gottschalk, 1986).

Using carbon-versus nitrate-limiting conditions, Dunn et al. (1979) showed that two strains of *Klebsiella* shift the nitrate reduction products from ammonium to nitrite as carbon becomes limiting. Then in 1981 Smith and Zimmerman showed the same type of shift with seventy soil isolates when each was grown in carbon-rich versus a carbon-poor medium. It is the non-energy-linked nitrate-to-ammonia producers that are thought to be the more dominant DNRA type found in nature.

Depending on the denitrifying microorganism, denitrification produces some or all of the gases NO, $N_2O$ and $N_2$. Nitrous oxide ($N_2O$) is a freely diffusible, obligate intermediate of the denitrification pathway. Nitric oxide (NO) is thought to be

exchanged out of cells in proportion to the NO that is added and found to be in steady state concentrations in all denitrifier pure cultures examined as long as nitrate and an enzyme-bound intermediate [Y] are present (Figure 1.1) (Tiedje, 1980; Zumft, 1997).

Denitrification is regulated by the presence of oxygen gas. When oxygen ($O_2$) is depleted and a state of anoxia or near anoxia (2% oxygen levels) is obtained, denitrifiers will use certain nitrogenous compounds as their terminal electron acceptor. Denitrifiers are widely distributed throughout nature. Some major genera of denitrifiers found in soil and aquatic environments are *Pseudomonas* and *Alcaligenes* (Tiedje, 1988). Sugahara et al. (1986) found 98.1% of a total of 264 denitrifying organisms isolated were *Pseudomonas* species, with *Alcaligenes* isolates being second in dominance.

A major controlling factor of denitrifier populations is not their denitrifying capabilities, but their ability to compete for natural carbon substrates. Myrold and Tiedje (1985) showed that when alfalfa was added to soil, denitrifier biomass increased. This study was done in aerobic soil, so denitrification was probably not a controlling factor. The survival and growth of the denitrifying population were probably due to their ability to compete for carbon sources.

Since many pathways can produce $N_2O$, including that which produces ammonium from nitrate, incorrect interpretations about denitrification could be made if $N_2O$ production is the sole measure for judging whether denitrification is occurring. The competition for nitrate between the two dissimilatory pathways occurs under the same oxygen-limited conditions. The denitrification pathway will convert nitrogen to gaseous forms unavailable to most biological species, and the DNRA pathway will produce

ammonium that many living things can assimilate. Because of this competition it is important to estimate populations of denitrifiers and DNRA bacteria in soil environments, and see if the populations correlate with measures of denitrification in soil, such as the denitrification enzyme assay (DEA) analysis. The hypothesis to be tested in this thesis was that DNRA bacteria populations would be present in greater numbers than denitrifiers in sediment soil samples because they are carbon-rich environments often in a state of anoxia, and to screen for differences in population values by farm management style. In carbon-rich environments where nitrate is not limited, DNRA bacteria could out-compete denitrifiers for carbon substrate based on the amount of energy gained per nitrate ion available in the soil (whereby 1 glucose + **12 $NO_3$-** $\rightarrow$ $6CO_2 + 6H_2O$ + 12 $NO_2$- with $\Delta G^{\circ'}$ of -429 kcal for dissimilatory nitrate reduction, vs. 1 glucose + **4.8 $NO_3$-** + $4.8H^+ \rightarrow 6CO_2$ + 2.4 $N_2$ + $8.4H_2O$ with $\Delta G^{\circ'}$ of -638 kcal for denitrification) (Gottschalk, 1986). In the upland soils, which are more often carbon-limited, the situation would be reversed compared to the lowland soil. The competition between denitrifiers and DNRA bacteria could be based in part on the organisms' differing enzyme $K_m$ thresholds when nitrate concentration falls below the $K_m$ threshold (Tiedje, 1988; Zumft, 1997). Some examples of $K_m$ thresholds for denitrifiers and DNRA bacteria are $NO_3$-/ $NO_2^-$ = 0.5-7nM, $NO_2$-/ NO = 7-31nM, NO/ $N_2O$ = ~400nM, and $NO_3^-$/ $NH_4^+$ = 0.4mM. Another way to look at the differences between denitrifiers and DNRA bacteria is by their redox potentials. Some redox potentials for enzyme activity are $NO_3$-/ $N_2$ = + 0.74 v, $NO_2$-/ $NH_2OH$ = +0.066 v, $NO_3^-$/ $NO_2$ = + 0.43 v, $NH_2OH$/ $NH_3$ = +0.08 v, $NO_2^-$/ NO = +0.37 v, NO/ $N_2O$ =

+1.18 v, and $N_2O/ N_2$ =+0.35 v (where positive potentials are the strongest oxidants having greatest tendency to accept electrons)(Gottschalk, 1986; Zumft, 1997). Upland agricultural soils would be expected to have higher concentrations of nitrates due to fertilization, and periodically when fields reached water saturation and became anoxic denitrifier numbers could exceed those of DNRA. When substrates are not limiting DNRA bacteria should predominate based on reaction rates. If carbon supplies are limiting denitrifiers should predominate, all else being equal.

## MATERIALS AND METHODS

### Soil Sampling

Samples were taken from three semi-permanent wetlands. Each wetland represented a different farm management practice. The farm management styles used were; wetland 5, organically farmed (Table 0.1); wetland number eight, a transitional-no-till (TNT) management style (Table 0.1); and wetland number thirteen, conventional (CON) management style (Table 0.1)). Farmland soils were similar, Egan / Ethan, classes e1, e2 or e3. Egan soils are silty clay loams having medium to high fertility while Ethan soils are a silty clay loam that has medium to low fertility. The "1-3" defines land capacity classes and "e" refers to a subclass that means erosion potential or slope, where generally classes e1-e4 are considered suitable for cropland. Wetland soils were classified as marsh soil. Marsh soils are hydric soils and include Worthing (Typic Haplustolls), Tetonka (Typic Argiaquolls), and Baltic and Lamo (Cumulic

Haplaquolls)(Kringen, 1998). These farms near Madison, South Dakota were part of a study whose findings have been documented elsewhere (Bleakley et al., 1995).

At each of the three wetlands, soil cores, 2.0 cm in diameter and 31.0 cm in depth, were collected using a model DB soil-sampling auger (Oakfield Apparatus, Inc. Oakfield, WI). On each wetland in 1993, upland, lowland and check samples were taken. The check sites were located on the north-east side of wetland 13 approximately thirty paces from the 1994 upland sample site 1; 30 paces north of 1994 site 2 upland for wetland 8; and 30 paces north of the 1994 site 2 upland sample for wetland 5 (Figure 1.2 and 1.3). The check samples were used to add a control for the upland sample sites. Data for check samples were not included in statistical analysis of MPN numbers, but instead used to compare MPN counts between upland and check-sample areas. At each upland, lowland and check site three sub-sample points were taken, two to three feet apart in a triangular pattern, and placed in a sterile Whirl-pak bag (Figure 1.2). The bags were marked as lowland site sample 1;2,3; upland site sample 1,2,3 or as a check site sample 1,2,3 and then placed on ice until returning to the laboratory within a few hours after sampling.

Second year (1994) soil cores, 2.0 cm in diameter and 31.0 cm in depth, were collected at each of three sampling points, with three sub-sample points taken two to three feet apart in a triangular pattern. These were selected along the lowland perimeter of the wetland for a total of 9 individual lowland sites. The upland core sites, three sample points with three sub-samples per point taken two to three feet apart in a

triangular pattern, for a total of nine individual upland sites, were located 50 paces away from each lowland site (Figure 1.3).

Each of the 18 locations was numbered and sampled repeatedly throughout the study. For example, a sample labeled "wetland 5, site 1a (sub-sample), lowland", refers to one of six cores taken at any given sampling date. The auger was washed with water (deionized $H_2O$ brought to the wetland from the laboratory) between sampling sites, and a test-tube brush was used to remove all visible soil adhering to the inside and outside of the sampling tube.

Once in the laboratory the soil sub-samples for each site (upland and lowland, sites 1,2 and 3) were pooled. Approximately 10 g (wet weight) of each sample site were placed into separate 6 oz. sterile, polypropylene Whirl-pak bags (Nasco, Fort Atkinson, WI). Bags were stored at 4 degrees Celsius until time of analysis.

Collection of soil samples was usually every two weeks (bi-weekly) from August of 1993 through November 6, 1993. Bi-weekly soil core collection resumed in April of 1994 and continued until September 17, 1994. On each sampling date nine sites with three sub-samples each were involved, with soil being collected from each of the three semi-permanent wetlands. In addition to sites mentioned above check sites were sampled. Check sites were located on the north-east side of wetland 13 approximately thirty paces from the upland sample point site 1; 30 paces north of site 2 for wetland 8; and 30 paces north of the site 2 upland sample point for wetland 5 (Figure 1.2 and 1.3).

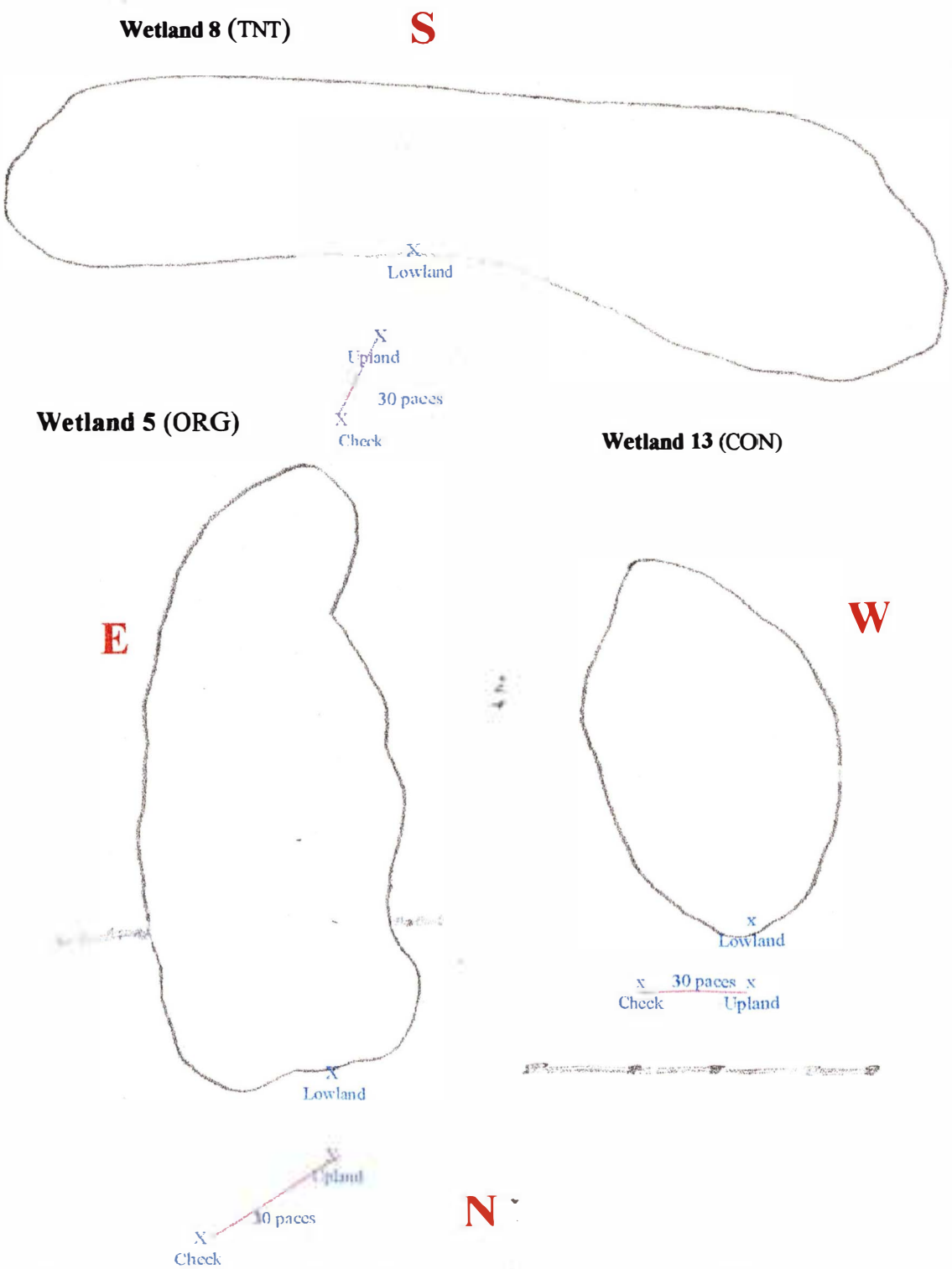



Figure 1.2: Aerial view of the wetland soil sampling schemes for 1993. Transitional no-till (TNT), organic (ORG), and conventional (CON) management systems were involved.

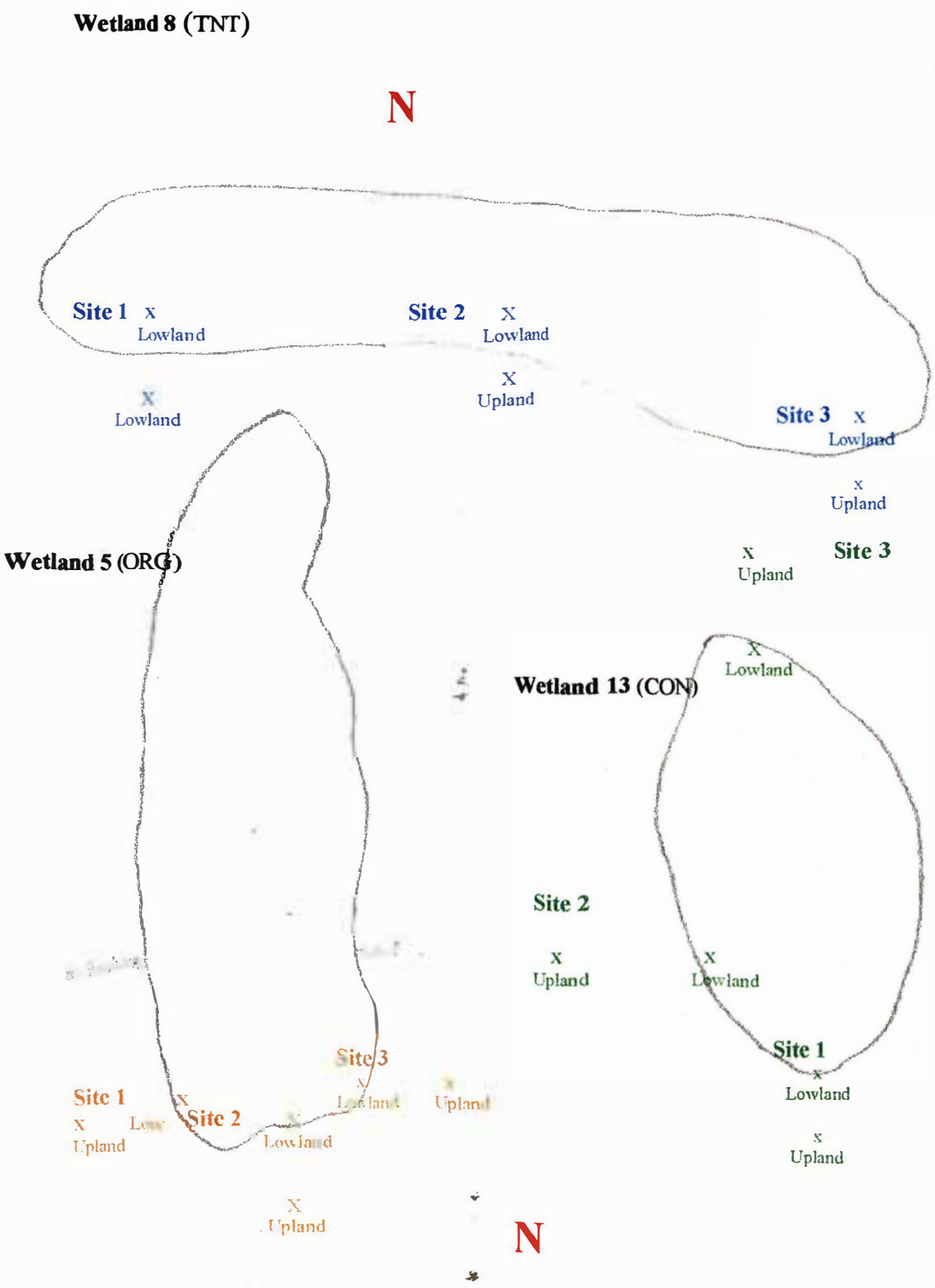



Figure 1.3. Aerial view of the wetland soil sampling schemes for 1994. Transitional no-till (TNT), organic (ORG), and conventional (CON) management systems were involved.

The check sites added a control for the upland sample sites. The MPN data from these sites were not included in statistical analysis of MPN numbers, but instead used to compare MPN counts between upland and check-sample areas. Each of the 18 locations was numbered and sampled repeatedly throughout the study. For example, a sample labeled "wetland 5, site 1a (sub-sample), lowland," refers to one of six cores taken at any given sampling date.

MPN Media Preparation

The media used in estimating DNRA bacteria and denitrifying populations were nutrient broth and tryptic soy broth. Of the two media nutrient broth (NB) is thought to be the best for enumeration of denitrifiers (Tiedje, 1982). Tryptic soy broth (TSB), a richer medium, favors the growth of dissimilatory $NO^-_3$ reducers over denitrifiers (Tiedje, 1982; , Højberg 1994).

Nutrient broth was prepared according to Tiedje and Dazzo (1982) and formulated as 0.8% (wt/vol) nutrient broth (Difco) amended with 5 mM $KNO_3$. Tryptic soy broth was prepared as 1.5% (wt/vol) tryptic soy broth (Difco) with 0.25% (wt/vol) glucose (Sigma) amended with 5 mM $KNO_3$. Each medium was dissolved in deionized water ($dH_2O$), and then dispensed in 10.0 ml amounts into screw-cap test tubes (16 mm X 125 mm) fitted with black screw-caps. Screw-caps were loosely fitted on the tubes placed in test-tube racks, and then sterilized by autoclaving at $121^0$C for 22 minutes at 15 p.s.i. (standard temperature and pressure for autoclaving or STP). Screw-caps were tightened after autoclaving and all other solutions or materials in this thesis that were

sterilized by autoclaving were sterilized at the STP conditions described above unless otherwise noted.

Diluent to suspend soil in was prepared according to Tiedje and Dazzo (1982), consisting of 0.85% (wt/vol) NaCl dissolved in $dH_2O$. To prepare a dilution bottle, 93.0 ml of this saline solution was added per 150 ml milk-dilution bottle fitted with a screw-cap. After adding saline to each bottle, one controlled drop of Tween 80 (Difco Laboratories, Detroit MI.) was added per bottle via disposable Pasteur pipette, then screw-caps were loosely fitted on the bottles and the meniscus of each bottle was marked with a Sanford Sharpie permanent marker. To prepare smaller tubes of diluent 4.5 ml of saline was added per standard size test tube (16 mm X 125 mm). Test-tubes were capped with standard plastic sleeve-type stoppers and the meniscus of each tube was marked. Bottles or test-tubes of diluent were autoclaved, and screw-caps on bottles were tightened after autoclaving. The final volume of diluent per bottle after autoclaving was 90.0 ml.

To inoculate a MPN assay, 10 g (wet-weight) of soil was added to an autoclaved, sterile glass blender cup, along with 90.0 ml of sterile saline diluent from a dilution bottle. Soil was dispersed in diluent by blending with a blender with pulsed bursts for two minutes. Dispersed soil was aseptically poured back into the dilution bottle. Ten-fold serial dilutions were prepared by adding 0.5 ml of suspension to the first tube of a dilution series via a sterilized 1.0 ml glass pipette, thus creating a $10^{-2}$ dilution, and the test tube was then mixed by vortexing. Then 0 .5 ml of this dilution was added to the next tube, creating a $10^{-3}$ dilution that was then mixed by vortexing and so forth. Dilutions were continued in this manner until a $10^{-7}$ dilution was obtained (Figure 1.4).

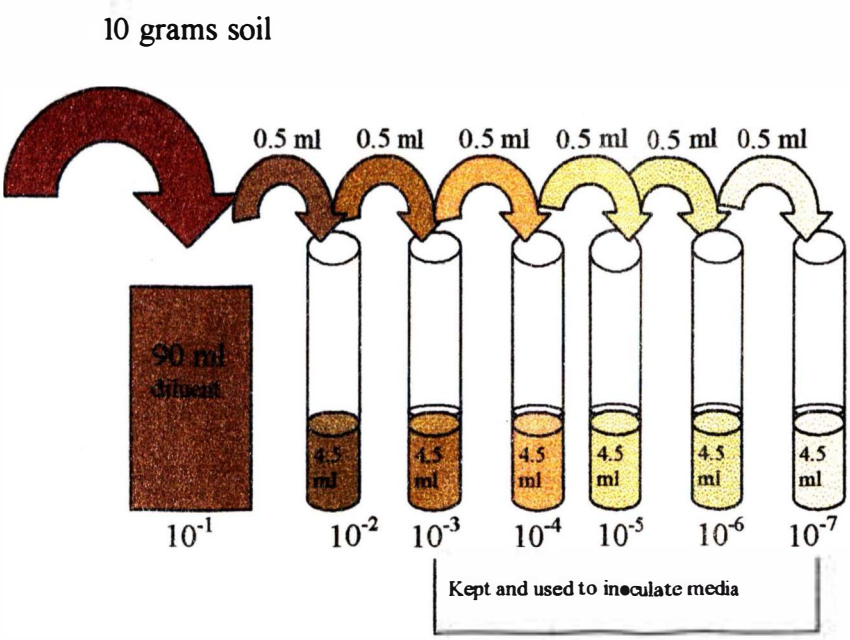



Figure 1.4. Serial dilution series of soil used for inoculating media in most-probable-number assays.

Dilution series tubes ranging from $10^{-3}$ to $10^{-7}$ dilutions were used to inoculate NB or TSB tubes. To inoculate, 0.1 ml of diluted soil suspension was added, via sterile 1.0 ml pipette, to each of five tubes of broth medium per dilution. Inoculated screw-cap tubes were mixed by vigorously shaking by hand then placed in racks and incubated at 25°C for 14 days (Figure 1.5). One row of five uninoculated tubes was present in each test-tube rack as a control.

Oven-dry-weights of soils were determined by weighing out five grams (wet weight) of soil for each soil sample that was then placed in an aluminum weigh boat and dried in an oven at 75° C for three days, then placed in a desiccator to cool before weighing. Weight loss of water was used to calculate oven-dry weights of soil. The MPN results were all expressed on an oven-dry weight basis.

At the end of the incubation period tubes were mixed well and contents poured out. To the few drops remaining in the bottom of the tube a diphenylamine reagent was added drop-wise and visually observed for color changes. If a blue color was observed it was recorded as a (-) meaning there was $NO_3^-$ or $NO_2^-$ still present. If remaining contents of the tube were colorless after diphenylamine reagent addition it was recorded as a (+) meaning no $NO_3^-$ or $NO_2^-$ remained in the solution, so the tube was positive for denitrification or DNRA depending on which medium was used.

The diphenylamine reagent used for $NO^-_3$ detection was prepared according to Tiedje and Dazzo (1982). To a milk dilution bottle, equipped with a magnetic stir bar, 100.0 ml of concentrated $H_2SO_4$ (sulfuric acid) was added. As the concentrated $H_2SO_4$ stirred, 0.2 grams of diphenylamine (Sigma Chemical, St. Louis, MO) was added. This solution was allowed to mix until completely dissolved, and the bottle was covered with aluminum foil and refrigerated in darkness when not in use.

Tables and graphs were set up on Microsoft Works spreadsheets (Microsoft Works Suite 2000, Microsoft Corporation, 1999). Data were fitted to an analysis of variance (ANOVA) model (SAS Institute Inc., 1997) to investigate significant differences ($P<0.05$) in denitrifiers and DNRA bacteria by date, position and site.

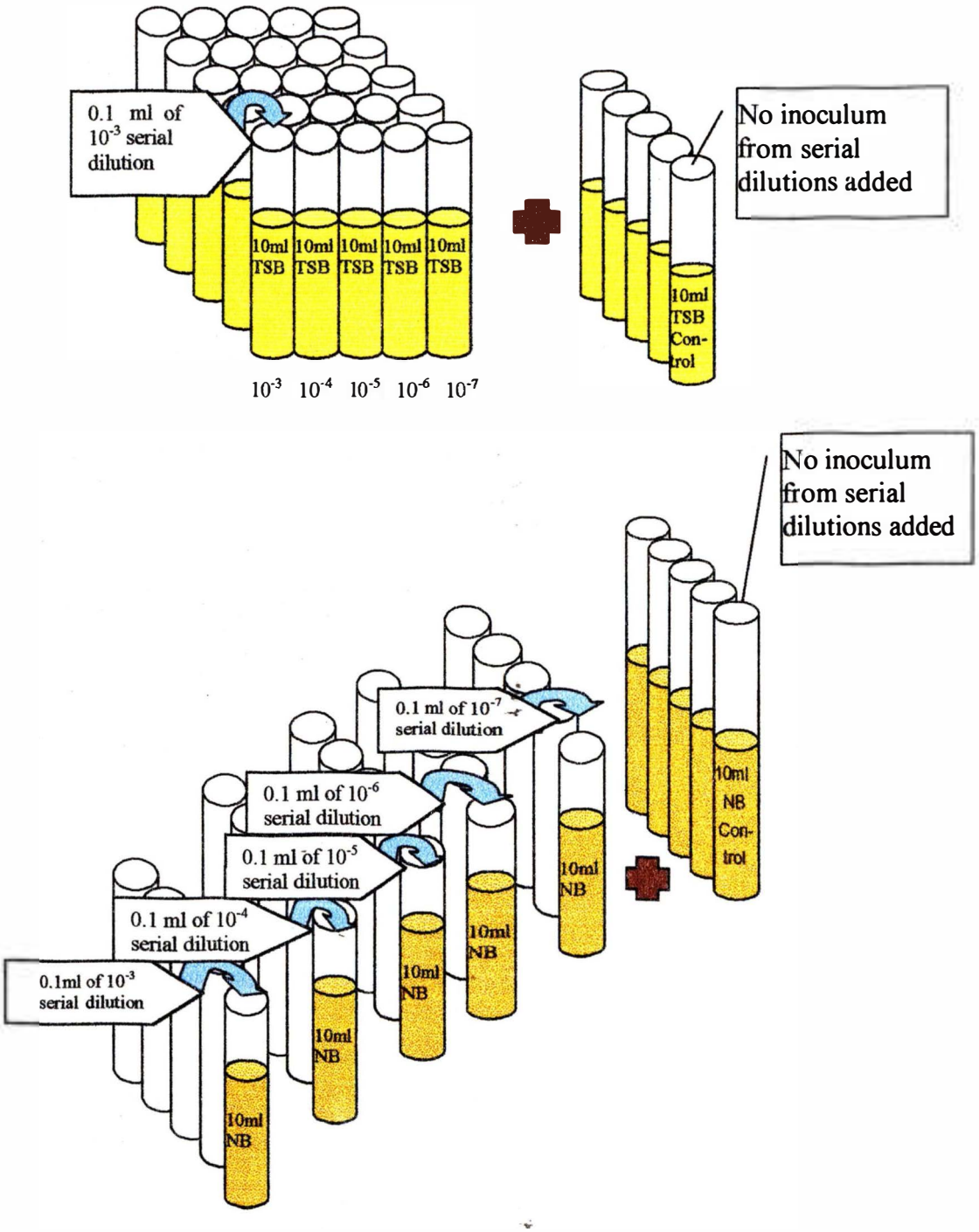



Figure 1.5. Inoculation of tryptic soy broth (TSB) and nutrient broth (NB) media in most-probable-number assays.

## RESULTS AND DISCUSSION

The type of media to use in the MPN analysis was determined by the nutritional needs of the organisms studied. DNRA and denitrification are both processes that reduce nitrate. While DNRA will reduce nitrate to ammonia and denitrifiers will reduce nitrate to nitrous oxide and dinitrogen gas, analyzing tubes for nitrate disappearance alone may not give a clear interpretation for the fate of nitrate in the MPN analysis. Nitrate and nitrite losses do not always produce dinitrogen or nitrous oxide, so measurements will not necessarily accurately enumerate bacteria either. However, the NB and TSB were found in previous work to select for denitrifiers and DNRA bacteria respectively (Højberg, 1994).

As an example, using actual data from wetland 5 upland, in the $10^{-5}$ dilution there were 5 NB tubes that scored positive for denitrification, and 5 TSB tubes that were positive for DNRA. At the $10^{-6}$ dilution there were 4 NB tubes that scored positive for denitrification and 4 TSB tubes that were positive for DNRA; and at the $10^{-7}$ dilution 1 NB tube scored positive for denitrification and 4 TSB tubes were positive for DNRA. When looking at Table 1.2 under $\mathbf{p}_1$ only the section labeled 5 would be considered for both denitrifying and DNRA bacteria, under $\mathbf{p}_2$ only the section labeled 4 would be considered for both denitrifying and DNRA bacteria, and under $\mathbf{p}_3$ the section labeled 1 would be considered for denitrifying bacteria and section 4 would be considered for the DNRA bacteria. Following the table for the denitrifiers a value of 1.7 as the most

Table 1.2. Table of most probable numbers for use with 10-fold dilutions and 5 tubes per dilution (Cochran, 1950; Alexander, 1982 ).

| | | Most probable number for indicated values of $P_3$ | | | | | |
|---|---|---|---|---|---|---|---|
| $P_1$ | $P_2$ | 0 | 1 | 2 | 3 | 4 | 5 |
| 0 | 0 | ---- | 0.018 | 0.036 | 0.054 | 0.072 | 0.090 |
| 0 | 1 | 0.018 | 0.036 | 0.055 | 0.073 | 0.091 | 0.11 |
| 0 | 2 | 0.037 | 0.055 | 0.074 | 0.092 | 0.11 | 0.13 |
| 0 | 3 | 0.056 | 0.074 | 0.093 | 0.11 | 0.13 | 0.15 |
| 0 | 4 | 0.075 | 0.094 | 0.11 | 0.13 | 0.15 | 0.17 |
| 0 | 5 | 0.094 | 0.11 | 0.13 | 0.15 | 0.17 | 0.19 |
| 1 | 0 | 0.020 | 0.040 | 0.060 | 0.080 | 0.10 | 0.12 |
| 1 | 1 | 0.040 | 0.061 | 0.081 | 0.10 | 0.12 | 0.14 |
| 1 | 2 | 0.061 | 0.082 | 0.10 | 0.12 | 0.15 | 0.17 |
| 1 | 3 | 0.083 | 0.10 | 0.13 | 0.15 | 0.17 | 0.19 |
| 1 | 4 | 0.11 | 0.13 | 0.15 | 0.17 | 0.19 | 0.22 |
| 1 | 5 | 0.13 | 0.15 | 0.17 | 0.19 | 0.22 | 0.24 |
| 2 | 0 | 0.045 | 0.068 | 0.091 | 0.12 | 0.14 | 0.16 |
| 2 | 1 | 0.068 | 0.092 | 0.12 | 0.14 | 0.17 | 0.19 |
| 2 | 2 | 0.093 | 0.12 | 0.14 | 0.17 | 0.19 | 0.22 |
| 2 | 3 | 0.12 | 0.14 | 0.17 | 0.20 | 0.22 | 0.25 |
| 2 | 4 | 0.15 | 0.17 | 0.20 | 0.23 | 0.25 | 0.28 |
| 2 | 5 | 0.17 | 0.20 | 0.23 | 0.26 | 0.29 | 0.32 |
| 3 | 0 | 0.078 | 0.11 | 0.13 | 0.16 | 0.20 | 0.23 |
| 3 | 1 | 0.11 | 0.14 | 0.17 | 0.20 | 0.23 | 0.27 |
| 3 | 2 | 0.14 | 0.17 | 0.20 | 0.24 | 0.27 | 0.31 |
| 3 | 3 | 0.17 | 0.21 | 0.24 | 0.28 | 0.31 | 0.35 |
| 3 | 4 | 0.21 | 0.24 | 0.28 | 0.32 | 0.36 | 0.40 |
| 3 | 5 | 0.25 | 0.29 | 0.32 | 0.37 | 0.41 | 0.45 |
| 4 | 0 | 0.13 | 0.17 | 0.21 | 0.25 | 0.30 | 0.36 |
| 4 | 1 | 0.17 | 0.21 | 0.26 | 0.31 | 0.36 | 0.42 |
| 4 | 2 | 0.22 | 0.26 | 0.32 | 0.38 | 0.44 | 0.50 |
| 4 | 3 | 0.27 | 0.33 | 0.39 | 0.45 | 0.52 | 0.59 |
| 4 | 4 | 0.34 | 0.40 | 0.47 | 0.54 | 0.62 | 0.69 |
| 4 | 5 | 0.41 | 0.48 | 0.56 | 0.64 | 0.72 | 0.81 |
| 5 | 0 | 0.23 | 0.31 | 0.43 | 0.58 | 0.76 | 0.95 |
| 5 | 1 | 0.33 | 0.46 | 0.64 | 0.84 | 1.1 | 1.3 |
| 5 | 2 | 0.49 | 0.70 | 0.95 | 1.2 | 1.5 | 1.8 |
| 5 | 3 | 0.79 | 1.1 | 1.4 | 1.8 | 2.1 | 2.5 |
| 5 | 4 | 1.3 | 1.7 | 2.2 | 2.8 | 3.5 | 4.3 |
| 5 | 5 | 2.4 | 3.5 | 5.4 | 9.2 | 16 | -- |

| Number of tubes per dilution (n) | Factor for 95% confidence limits with indicated dilution ratios | | | |
|---|---|---|---|---|
| | 2 | 4 | 5 | 10 |
| 1 | 4.00 | 7.14 | 8.32 | 14.45 |
| 2 | 2.67 | 4.00 | 4.47 | 6.61 |
| 3 | 2.23 | 3.10 | 3.39 | 4.68 |
| 4 | 2.00 | 2.68 | 2.88 | 3.80 |
| 5 | 1.86 | 2.41 | 2.58 | 3.30 |
| 6 | 1.76 | 2.23 | 2.38 | 2.98 |
| 7 | 1.69 | 2.10 | 2.23 | 2.74 |
| 8 | 1.64 | 2.00 | 2.12 | 2.57 |
| 9 | 1.58 | 1.92 | 2.02 | 2.43 |
| 10 | 1.55 | 1.86 | 1.95 | 2.32 |

Table 1.3. Prepared confidence limits tables for most probable numbers (Cochran, 1950; Alexander, 1982).

Dividing this value by the soil dry weight value gives a microorganism per gram oven-dry soil count as the MPN value for the original sample. The 95% confidence limits for MPN values can be obtained from existing tables (Table 1.3). To use the table multiply by the value listed on the table. To find the lower limit, divide the MPN value by the factor. Using the examples given previously in the paragraph, 1.7 would be multiplied by 3.30 to yield a value of 5.61 for the upper confidence limit. Using the same examples the lower limits would be found by dividing 1.7 by 3.30 to yield a value of 0.52. This 95% confidence level means that the MPN value for these microorganisms is between 561,000 and 52,000. It is obvious that MPNs alone have a low order of precision. If more precise measurements are desired large numbers of tubes must be inoculated at each dilution. When doing so the confidence intervals will be narrowed, but decreasing the dilution

ratio can also narrow confidence levels, such as by using two-fold dilutions rather than ten-fold dilutions (Alexander, 1982).

The results of the MPN analysis for DNRA bacteria and denitrifying bacteria in soils collected from the three semi-permanent wetlands in 1993 are shown in Table 1.4 (Appendices A) and Figures 1.11-1.12 (Appendices A). The MPN analysis results from 1994 are shown in Table 1.6a and b (Appendices A) and Figures 1.14-1.19 (Appendices A). The temporal variability of DNRA bacteria and denitrifiers in soil was obvious in both years' results proving significant in an analysis of variance model ($Pr > F = 0.0001$; Table 1.8 and 1.9), which is discussed below.

Differences between DNRA bacteria and denitrifier population numbers was examined and proven non-significant (Figure 1.6). However when least mean square (LMS) values were graphed, by date, DNRA bacterial data points indicated DNRA bacteria were present in slightly greater numbers overall. Only one data point showed otherwise (for 6-19-94) (Figure 1.6).

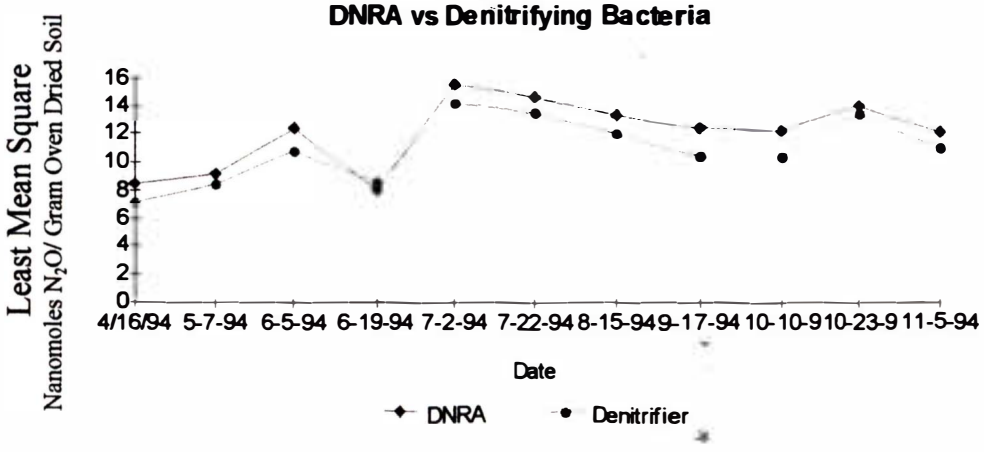



Figure 1.6. Mean comparisons of DNRA bacteria and denitrifier populations.

This graph's data presented a familiar "M" pattern similar to many of the individual data graphs for DNRA bacteria and denitrifiers (Appendices A Figures 1.11,1.12 and 1.14-1.19) and basically correlates with precipitation and water table levels. Precipitation and water table level graphs were published in the North Central Region ACE progress Report, 1994 (Bleakley et al., 1995)(Figure 1.7). These suggest that population size could be sensitive to precipitation and water table level fluctuations.

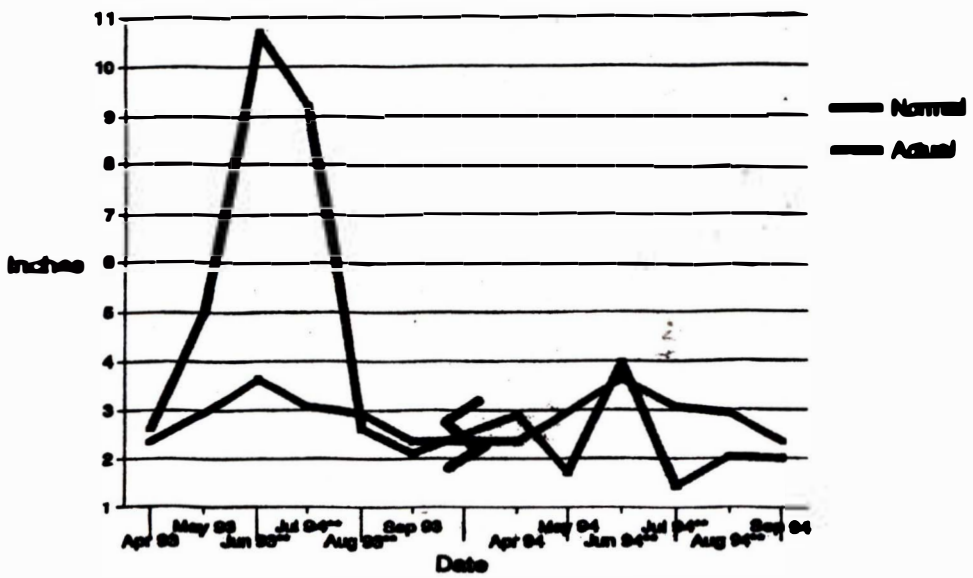



Figure 1.7. Monthly precipitation (April-September, 1993-94) Madison, SD weather reporting station and on-site locations
(Taken from Bleakley et. al., 1994).

Literature suggests that in nitrate limiting environments the nitrate $K_m$ of the DNRA organisms is high enough to enable DNRA bacteria to be present in as much as 10 fold greater population numbers than are denitrifiers (Tiedje, 1988). However, it is possible that the increased numbers of DNRA bacteria present in the soil are because some DNRA bacteria can be spore-formers. This could skew MPN readings by giving

higher than actual numbers of DNRA organisms than were active at the time of sampling.

Differences in sampling position were slight and not significant (Pr >F = 0.9549, denitrifiers; Pr >F = 0.6346, DNRA)(Tables 1.8 and 1.9). The differences by farm management systems, in this study, for sampling position were suggestive for denitrifiers (Pr > F = 0.1044)(Table 1.8).

Many parameters need to be considered when comparing farming systems and it is possible that because of the interaction of such parameters, differences by farming systems were diminished. Such parameters include biomass, soil water, water table levels and water quality (i.e. nitrate, orthophosphate and pesticide presence). Biomass for this study, as shown in Figure 29 of the North Central Region ACE progress report (Bleakley et al., 1994)(Figure 1.8), included crop, weed, and wetland plant species.

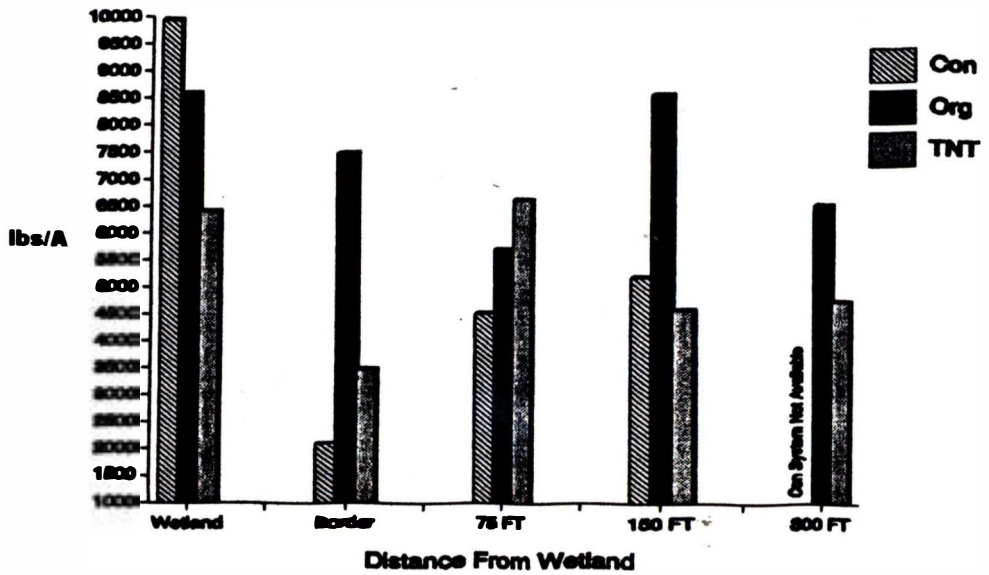



Figure 1.8. Above-ground biomass production, 1993-94 average, for farming systems as influenced by landscape position. (Figure 29 taken from Bleakley et al., 1994)

Wetland biomass included emergent wetland vegetations, while border biomass included crop areas bordering wetland vegetation. The distances 75 ft, 150 ft, and 300 ft refer to the piezometer locations at each individual wetland (Bleakley et al., 1994). Above-ground biomass is important not only because it is an indicator of fertility but also for its contribution to soil organic matter content which is important for replenishing of soil carbon supplies.

Soil water values in 1993 and 1994 at the 0-12" were not influenced by farm systems, 12"-24" soil moisture ranked TNT>CON>ORG. No-till management ranked first because it reduces soil moisture losses due to evaporation and improves water infiltration, which can result in additional soil water storage. The organic farm system was ranked last because a large part of the crop is alfalfa. Alfalfa has a high water requirement that can reduce soil moisture content (Machacek, 1995; Kringen, 1998).

Water table levels as shown in Figure 42 of the North Central Region ACE progress report (Bleakley et al., 1994)(Figure 1.9) gradually declined over winter months and rose as ground water thawed in the spring.

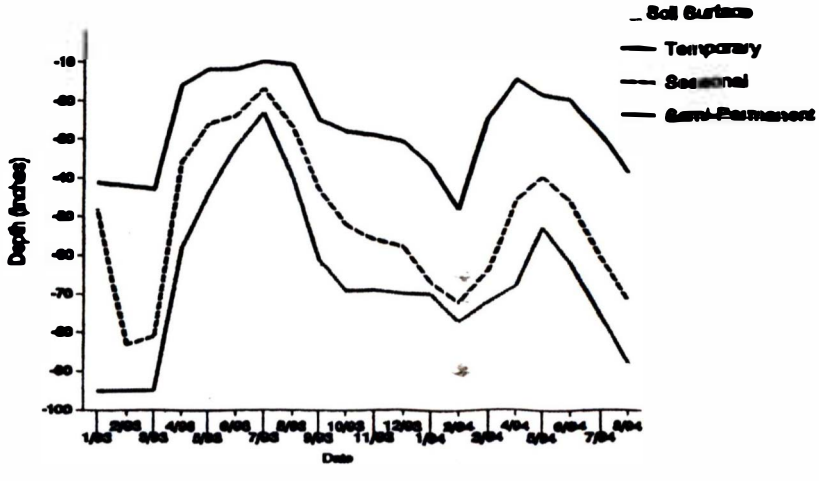



Figure 1.9. Upland water table level, wetland classification comparison. (Figure 42 taken from Bleakley et al., 1994)

The 1994 precipitation levels were more normal than for 1993 (which was an extremely wet year). Surface water run-off was tested for nitrate and orthophosphate and pesticides. The North Central Region ACE progress Report (Bleakley et al., 1994), documents monitoring and testing of the run-off in several agricultural fields that included temporary, seasonal, and semi-permanent wetlands. Of those three the semi-permanent wetlands contained higher nitrate concentrations. This could be in part because denitrification favors wet/dry cycles (typical of seasonal wetlands) and is effective in reducing $NO_3^-$ concentrations of wetland surface water; i.e., concentrations remained low in wetland surface water while 1994 upland water $NO_3^-$ exceeded drinking water standards (at 17 ppm in semi-permanent wetlands)(Kringen, 1998).

Orthophosphate concentrations were higher in seasonal wetlands. A possible explanation for this could be that they do not have significant vegetative border as a buffer, whereas semi-permanent wetlands have wider vegetative borders. These buffer strips trap sediments before reaching the wetland and are sites for potential denitrification (Kringen, 1998).

Water quality differences are shown in the North Central Region ACE project Figure 43 (Bleakley et al., 1994)(Figure 1.10). Farming systems showed highest nitrate concentrations in upland samples of the organic wetland. This could be because of alfalfa crops and manure applications with insufficient crop rotations with legumes (which require higher nitrate levels)(Kringen, 1998; Bleakley et al. 1995).

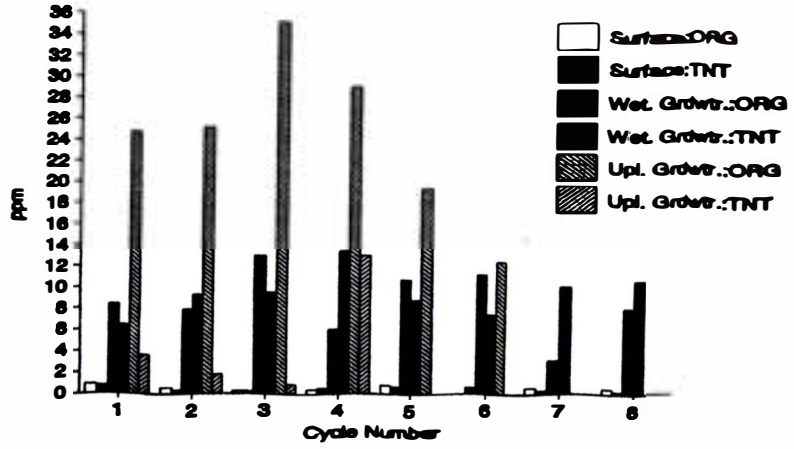



Figure 1.10. Nitrate concentrations (ppm) in semi-permanent wetlands in transitional no-till (TNT) and organic (ORG) farming systems. (Figure 43 taken from Bleakley et al., 1994)

Pesticides were found in water during early spring, presumably from snow thaw, and increased at the June sample date (June 27). Organic and no-till management systems had detectable pesticides, indicating an aerial drift or transport via ground water. This suggested a need for watershed and/or aquifer based research and management (Bleakley et al., 1994).

For this thesis wetland 5 (ORG) LMS values were more uniform with less extreme variations than those of either wetland 8 or wetland 13, possibly due to manure application and alfalfa related nitrogen available throughout the growing season. Wetland 8 and 13 may fluctuate more in nitrogen and moisture due to fertilizer application differences, crop residue management and water table level variances.

In this study there were no differences in numbers of DNRA bacteria by farm management systems (Pr >F = 0.5048)(Table 1.9), where denitrifier numbers suggested a difference (Pr >F = 0.1044)(Table 1.8), but neither was significant by the 95% rule.

| *Source* | *NDF** | *DDF*** | *Type III**** | *Pr > F***** |
|---|---|---|---|---|
| **Site** | 2 | 6 | 3.37 | 0.1044 |
| **Position** | 1 | 6 | 0 | 0.9549 |
| **Site * Position** | 2 | 6 | 1.76 | 0.2503 |
| **Date** | 10 | 48 | 20.42 | 0.0001 |
| **Site * Date** | 20 | 48 | 1.75 | 0.0576 |
| **Position * Date** | 10 | 48 | 1.21 | 0.3102 |
| **Site * Position* Date** | 20 | 48 | 1.06 | 0.4193 |

**Table 1.8** Analysis of Variance (ANOVA) for Denitrifying Bacteria Populations

**Table 1.9** Analysis of Variance (ANOVA) for DNRA Bacteria Populations

| *Source* | *NDF** | *DDF*** | *Type III**** | *Pr > F***** |
|---|---|---|---|---|
| **Site** | 2 | 6 | 0.77 | 0.5048 |
| **Position** | 1 | 6 | 0.25 | 0.6346 |
| **Site * Position** | 2 | 6 | 1.03 | 0.4115 |
| **Date** | 10 | 48 | 35.82 | 0.0001 |
| **Site * Date** | 20 | 48 | 1.87 | 0.0388 |
| **Position * Date** | 10 | 48 | 1.91 | 0.0666 |
| **Site * Position* Date** | 20 | 48 | 1.61 | 0.0887 |

*NDF is the numerator of degrees of freedom.

**DDF is the denominator degrees of freedom.

***Type III equals a type III sum of squares. In this type data have been adjusted for all other-effects in the model and is used to analyze data sets with missing data.

****Pr > F is the probability that the calculated F value is going to be greater than the F table value.

# CHAPTER 2

# DENITRIFICATION ENZYME ACTIVITY ASSOCIATED WITH PRAIRIE-POTHOLE SOILS IN DIFFERENT AGRICULTURAL-MANAGEMENT SYSTEMS

## INTRODUCTION

There are many important biological, physical, and chemical processes in soil. The nitrogen cycle is one of these, playing an important part in soil fertility by affecting cation exchange capacities, soil pH, and redox potentials (Evangelou, 1998)(Figure 2.1). Interfering with or altering one or more steps of the nitrogen cycle could cause an excess of one or more products. Excess of nitrates in the soil could be undesirable, as when nitrates build up to levels where the soil could become unsuitable for agricultural plants (Evangelou, 1998), if increased trace gas ($N_2O$, NO) emission from the soil to the atmosphere occurs (Conrad, 1996), or if nitrogen compounds cause pollution of the groundwater and surface-water (Zumft, 1997).

Soil organic matter consists largely of plant material, which many soil bacteria can use as an electron donor source, to drive respiration, which reduces oxygen concentration (Ambus and Christensen, 1993). Microbial decomposition of organic matter supplies the majority of the soluble carbohydrates and proteins necessary for microbial growth (Fazzorlari et al., 1990; Paul and Clark, 1989; Tiedje, 1988). Many organisms have the capacity to reduce nitrate. Denitrifiers are a small percentage of this total and therefore must compete for available carbon with other microbial groups (Tiedje, 1980).

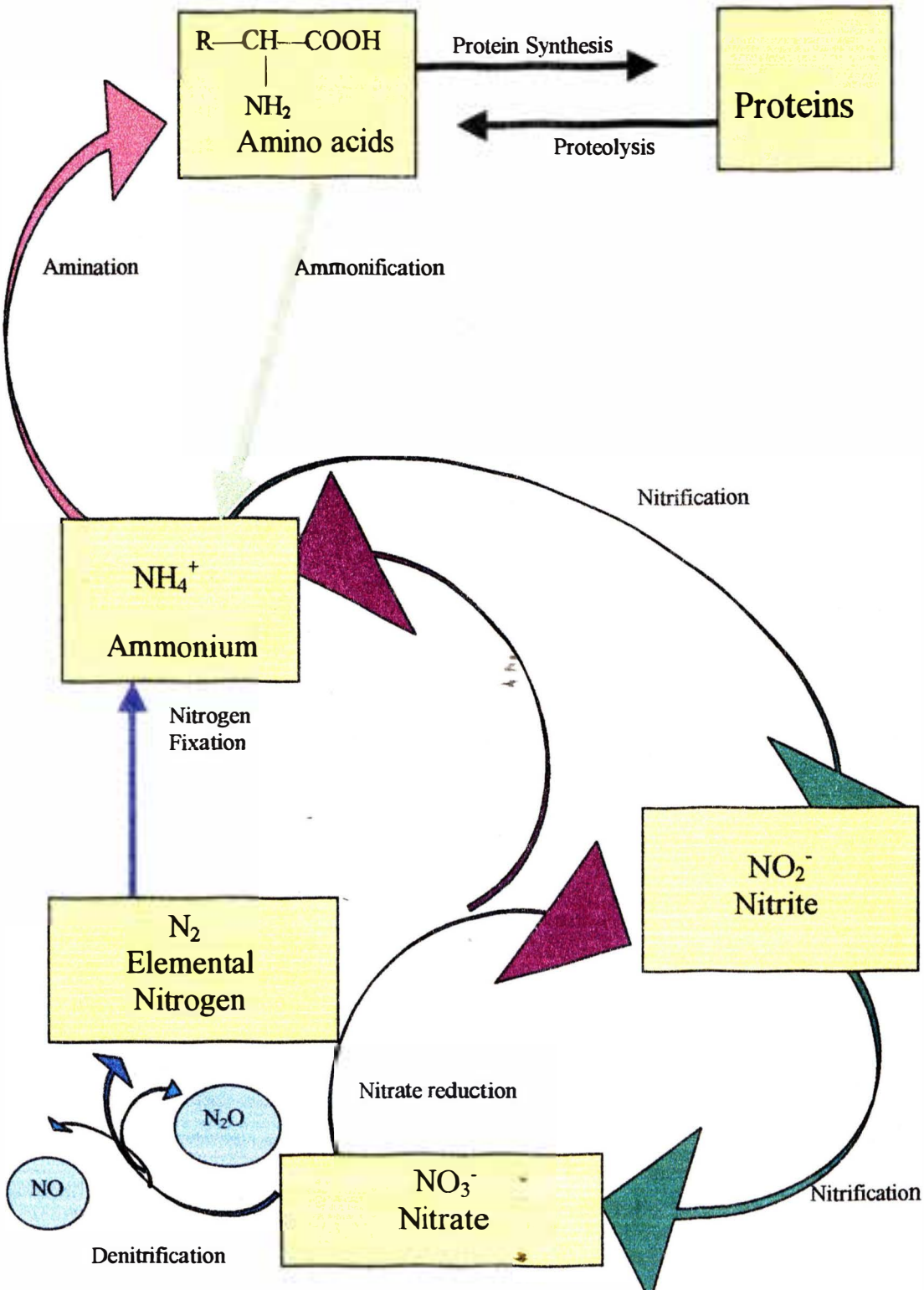



Figure 2.1. Nitrogen cycle (Adapted from Evangelou, 1998)

Under anoxic conditions, where obligate aerobes can no longer compete, more soil organic carbon is available to the denitrifiers giving them a competitive edge.

Fates of soil nitrate can include an aerobic process (assimilatory reduction) and an anaerobic process (dissimilatory reduction) (see Chapter 1). Research has investigated carbon effects on denitrification in various environments (Slater and Capone, 1987; Højberg et al., 1994; Gordon et al., 1986; Tiedje, 1988; Myrold and Tiedje, 1985) finding that carbon availability and $NO_3^-$ have major effects on denitrifying organisms. Other important determinants of denitrifying rates in soil are soil moisture, soil aeration, soil pH and temperature.

Soil water can slow aerobic processes in soil by greatly restricting $O_2$ diffusion (by approximately 10,000 times) through soil pores (Paul and Clark, 1989). This change in oxygen diffusion creates a favorable environment for denitrification. The rhizosphere of some aquatic plants can allow aerobic bacteria to thrive by creating an oxic zone within the anoxic zone (Figure 2:2)(Conrad, 1996). Soil texture (affecting soil water holding capacities), rainfall and plants (affecting evapotranspiration) influence the soil water content (Tiedje, 1988).

In general soil pH that best supports microbial growth is around pH 6-8. Biological denitrification is possible at a pH of 5 but becomes negligible or nonexistent below a pH of 4. Soil pH can influence the cation exchange in soil, which in turn influences the abilities of soils to store products of mineralization and maintain favorable fertility (Evangelou, 1998).

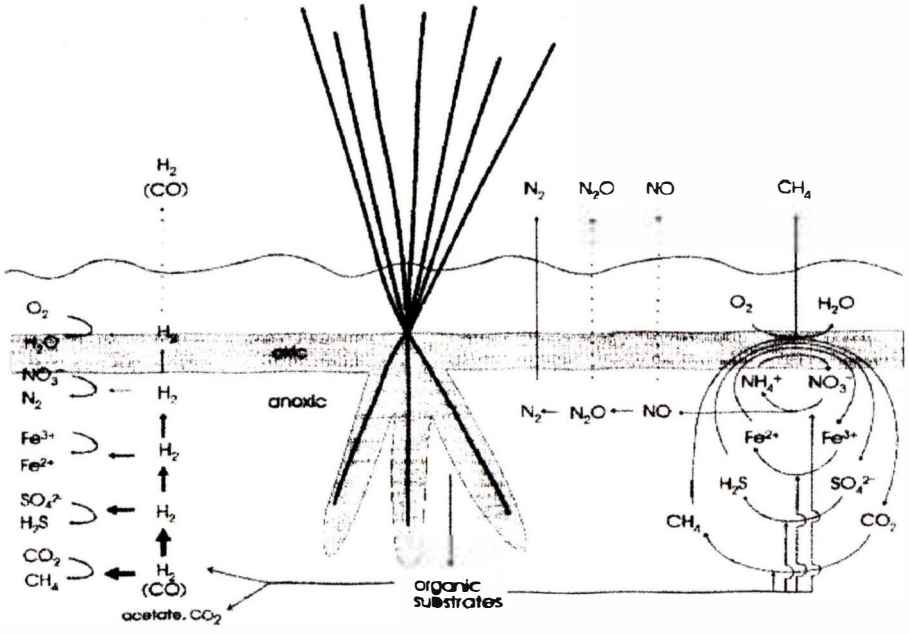



Figure 2.2. Redox reactions that influence denitrification and oxic zones within the rhizosphere of aquatic plants. (Conrad, 1996)

A microbe will thrive at its optimal temperature provided adequate nutrients are available. As temperature increases an exponential increase in microbial activity can occur (Paul and Clark, 1989). Because temperature affects oxygen solubility and diffusion in water it indirectly affects denitrification. The minimum temperature for biological denitrification is about 5°C; and the maximum temperature is about 75°C, due to thermophilic denitrifying bacteria. Above 50°C nitrite and most enzyme reactions become unstable and enzymes denature (Paul and Clark, 1989). As temperature increases proteins unfold, enzymes are deactivated, and weak hydrogen bonding is broken. In addition, cytoplasmic membranes can collapse causing cell lysis and death (Brock et al., 1994).

The soils that were sampled in this study were agricultural cropland soils (Egan/Ethan types) and wetland hydric soils. Most cropland soils are generally not thought of as being anaerobic. They can however have some anaerobic sites that form as soil particles clump around organic matter and form organic matter-mineral complexes (Paul and Clark, 1989)(Figure 2.3). The pore neck size of the aggregate will determine accessibility by organisms according to their size. Smaller aggregates (50-250 μm) that are filled with water support soil microorganisms(Paul and Clark, 1989). The bacteria in the pore space use the organic matter as their carbon source and can, through respiration, create an anaerobic environment.

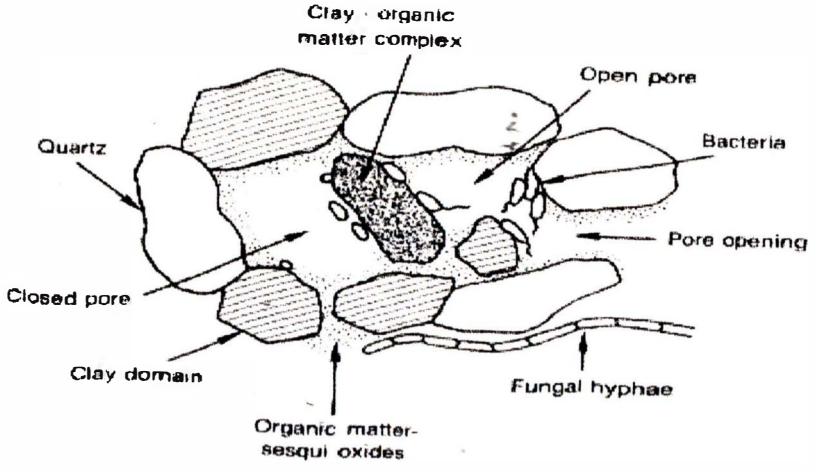



Figure 2.3. Soil aggregate with soil organic matter and bacteria within the pore space (Paul and Clark, 1989).

Lowland soils of wetlands are often water-saturated. This means the pore spaces are filled with water and the diffusion rate for oxygen is approximately 10,000 times slower than it is for air, so the environment becomes anaerobic. In wetlands, some aquatic plants have rhizospheres that supply oxygen to an otherwise anaerobic soil zone (Conrad, 1996)(Figure 2.4, Appendices B). Also in wetlands, the disruption of surface

litter by wetland microfauna that mix the litter layer or burrow tunnels in the soil can cause aeration of what would otherwise be anaerobic soil (Tiedje and Dazzo, 1982).

The rate of $N_2$ production from denitrification in the soil is difficult to measure, considering the atmosphere contains as much as 80% $N_2$. In many situations a more indirect method of measuring $N_2$ evolution is necessary. The acetylene-inhibition method is based on the principle that HC≡CH (acetylene) is similar in structure to N=N=O (nitrous oxide) and will block the reduction of nitrous oxide, allowing it to accumulate (Tiedje, 1988; Tiedje et al., 1989). Acetylene also blocks nitrification by irreversibly reacting with the active site of ammonium monooxygenase, the enzyme that oxidizes ammonia for the nitrifiers. When acetylene is removed from the system, new enzymes are synthesized (Tiedje, 1988). Acetylene can be biodegraded, but in natural soils few organisms are capable of this and it is not an issue in short-term incubations. Commercial grades of acetylene can contain acetone and CO. These contaminants can be removed by diverting the gas flow through a gas-scrubbing train (Walter et al., 1979).

The phase I assay, or denitrifying enzyme activity (DEA) assay, was first developed by Smith and Tiedje (1979) and is based on the acetylene-block method to allow for the accumulation of $N_2O$ in the soil sample. Excess carbon substrate in the form of glucose, and excess electron acceptor in the form of $KNO_3$ are added to an anaerobic soil slurry containing chloramphenicol. Chloramphenicol is an antibiotic used to prevent the synthesis of protein and thus blocks the synthesis of new denitrifying enzymes. The DEA assay is designed to measure levels of functional denitrifying enzymes already present in the soil at the time of sampling. By using chloramphenicol

the denitrifiers' biomass is kept from increasing during the assay. Phase I refers to the linear part of a growth curve that constitutes the first hour or two of the phase I incubation. The amount of $N_2O$ produced by soil during this time is indicative of the concentration of denitrifying enzymes present, which in turn is representative of the environmental history of the soil sample (Tiedje et al., 1989).

The substrate additions and sample environment of the DEA assay are an effort to optimize activity of denitrifying enzymes in the soil sample, to obtain an estimate of the potential a soil microbial community has to denitrify. Natural rates of denitrification are not measured by this assay, but the assay instead estimates how much denitrification could happen at this particular site if conditions for denitrification were optimal. Optimal requirements would include availability of nitrate, a carbon source, anaerobic conditions and the necessary denitrifying enzymes (Tiedje et al., 1989).

In this investigation the DEA assay was used to estimate the denitrification potential of wetland-associated soils of the farms representing three farming practices described in Chapter 1. Phase I activities over time by particular wetland and site were examined. The average DEA values of upland and lowland areas associated with each wetland were assessed and expressed as nanomoles $N_2O$ per gram oven dried soil. The hypothesis that lowlands would have greater DEA values when compared to upland soil sample sites was investigated. Second, the hypothesis that organic farming practices have increased soil DEA values when compared to transitional no-till and conventional farming practices was also tested.

# MATERIALS AND METHODS

## Soil Sampling

Soil samples were taken from three semi-permanent wetlands. Each wetland represented a different farm management practice. Wetland number 5 was located on a farm using organic (ORG) management, wetland number 8 was located on a farm using a transitional-no-till (TNT) management style, and wetland number 13 was located on a farm using a conventional (CON) management style (Table 0.1).

At each of the three wetlands, soil cores, 2.0 cm in diameter and 31.0 cm in depth, were collected using a model DB soil-sampling auger (Oakfield Apparatus, Inc. Oakfield, WI). Soil cores were collected at each of three sampling points, with three sub-sample points taken two to three feet apart in a triangular pattern. These were selected along the lowland perimeter of the wetland for a total of 9 individual lowland sites. The upland core sites, three sample points with three sub-samples per point taken two to three feet apart in a triangular pattern, for a total of nine individual upland sites, were located 50 paces away from each lowland site (Figure 1 of Chapter 1).

Each of the 18 locations was numbered and sampled repeatedly throughout the study. For example, a sample labeled "wetland 5, site 1a (sub-sample), lowland", refers to one of six cores taken at any given sampling date. The auger was washed with water (deionized $H_2O$ brought to the wetland from the laboratory) between sampling sites, and a test-tube brush was used to remove all visible soil adhering to the inside and outside of the sampling tube.

Cores were placed on ice and transported to the laboratory. Once in the laboratory the soil sub-samples for each site (upland and lowland, sites 1,2 and 3) were pooled. Approximately 10 g (wet weight) of each semi-permanent lowland sample site or upland sample site were placed into separate 6 oz. sterile, polypropylene Whirl-pak bags (Nasco, Fort Atkinson, WI). Bags were stored at 4 degrees Celsius until time of analysis. Collection of soil samples was conducted from June 5 of 1994 through November 5, 1994 in an approximate bi-weekly manner. Soil core collection resumed in 1995 with a sample taken June 15, and on September 24.

On each sampling date nine sites with three sub-samples each were involved, with soil being collected from each of the three semi-permanent wetlands. This study attempted to monitor denitrification activity throughout two years to monitor the rate of change.

Soil Storage

Sample storage times before Phase I analysis were as follows: for the June 5, 1994 date, 2 years 2 months; for the June 19, 1994 date 2 years 2 months; for September 17, 1994, 1 year 11 months; for October 10, 1994, 1 year 10 months; for October 23, 1994, 1 year 11 months; for November 5, 1994, 2 years; for June 15, 1995, 10 months; and for September 24, 1995 it was 11 months.

Plant Community

Plant community studies for species identification and seed bank analysis were previously compiled for the North Central Region ACE program. The study utilized a

standard canopy coverage technique and compiled a species list by percent frequency. For wetland 13 the perimeter dominant plant species were (from most common to least); star duck seed (*Lemna tuvionifera*), river bulrush (*Scirpus fluviatilis*), reed canary grass (*Phalaris arundinacae*), giant bureed (*Sparganium eurycarpium*), white top (*Scolochloa fescutacea*), bladderwart (*Utriculari vulgaris*), aster (*Aster hesperius*), and slough sedge (*Carex atheroides*). Wetland 5 perimeter dominant plant species included; bladderwart (*Utriculari vulgaris*), star duck seed (*Lemna tuvionifera*), cattail (*Typha spp.*), slough sedge (*Carex atheroides*), thistle (*Cirsium arvense*), reed canary grass (*Phalaris arundinacae*), river bulrush (*Scirpus fluviatilis*), aster (*Aster hesperius*), and smooth brome (*Bromus inermis*). Wetland 8 perimeter dominant plant species included; cattail (*Typha spp.*), quack grass (*Agropyron repens*), aster (*Aster hesperius*), slough sedge (*Carex atheroides*), bladderwart (*Utriculari vulgaris*), river bulrush (*Scirpus fluviatilis*), and reed canary grass (*Phalaris arundinacae*)(Hubbard, personal communication, 2000).

Media and Flask Preparation

The Phase I protocol used to measure DEA was described by Smith and Tiedje (1979), and was later modified by Tiedje et al. (1989). To conduct the Phase I assay, 25 grams (fresh weight) of soil from each of the eighteen sites were weighed out in triplicate, and each 25 gram aliquot was placed in each of three 125 ml-Erlenmeyer flasks containing 25 ml of assay liquid medium. The liquid medium in the final soil slurry contained 1 mM D-glucose, 1 mM $KNO_3$, and 0.025 g chloramphenicol, all dissolved in sterile $dH_2O$.

The 125 ml-Erlenmeyer flasks were modified by a glass blower (Allen Scientific Glass, Boulder CO) to have a side arm consisting of a Hungate tube (Bellco Glass, Vineland, NJ) cut at its midpoint using the top portion (having screw threads and an opening for a rubber Hungate septum and plastic screw cap) and attached to the Erlenmeyer flask. The final modified 125-Erlenmeyer flask had two openings, one standard mouth opening (for No. 5 stoppers, but No. 6 sized stoppers were used instead) used to add soil and substrate, and the other situated at a 40-degree angle from the flask for drawing gas samples and to pressurize and evacuate the flasks via needles.

The flasks were made anaerobic by evacuating and flushing with argon gas four times, using a gassing manifold which could alternately pressurize and evacuate or obtain a vacuum on the flasks. Twenty one-gauge, 1-inch disposable needles (Becton-Dickinson, Franklin Lakes, NJ) were used on the gas manifold needle housing apparatus and placed through the Hungate septum on the flask. The flasks were then evacuated to a constant pressure of approximately –20 psi, followed by flushing to a constant pressure of approximately +12 psi. Following the first evacuation and flushing the flask was left attached to the gassing station with an over pressure of +12 psi and tested for leaks. If the pressure decreased, the septum and /or stopper were tightened or replaced until a steady state was obtained. Once a steady state had been obtained the flasks were flushed and evacuated two additional times, while being shaken by hand to aid in removing oxygen gas and having a final pressure of +12 psi.

Once flasks were anaerobic a 25-gauge, 1-inch long disposable needle (Becton-Dickinson, Franklin Lakes, NJ) and 25 ml plastic syringe were used to remove 11 ml of

argon gas from the flask's headspace. This was then replaced with 11 ml of purified acetylene gas. Commercial acetylene gas can be contaminated with unknown amounts of acetone and CO, which will interfere with denitrification. To correct this the procedure of Walter et al. (1979) was used. First the acetone was removed by passing it through a solution containing 15 g $CuCl_2 \bullet 2H_2O$ in 150 ml concentrated (36.5-38.0%) HCl. The next phase involved passing the gas through an equal amount of $dH_2O$ to remove CO. Finally, the gas was passed through a ¼ inch, 120 cc Gas Purifier cartridge (Alltech, Deerfield, IL, Cat. No. 8126) to remove moisture, oil and other foreign materials before being collected in a 1.3 liter gas sampling bag (Alltech, Deerfield, IL, Cat. NO. 42202). Acetylene was used to inhibit $N_2O$ reductases and allow $N_2O$ to accumulate in the flask.

After acetylene addition, the flasks were put on a rotary shaker set at 90 rpm in a 27°C incubator. Each flask was removed from the incubator for no more than 90 seconds while 0.4 ml of headspace gas was being withdrawn at 30, 60, 90, 105, and 120 minutes. Gas samples were taken using a 25-gauge, 1-inch long deflected point, stainless steel needle (Popper and Sons, New Hyde Park, NJ) and disposable Glaspak ½ ml glass syringes (Becton-Dickinson, Rutherford, NJ). Teflon tape was used to secure the seal between each syringe and needle. In an effort to keep the flasks anaerobic, the syringes were flushed three times with argon contained in a gas-sampling bag prior to putting the syringe needle through the septum of the flask's sidearm. Each syringe was left with approximately 0.5 ml of argon to be expelled just prior to inserting the syringe needle into the flask's sidearm septum to withdraw the gas sample. Once inserted through the sidearm septum, the sampling syringe plunger was plunged back and forth three times

before extracting a gas volume of 0.4 ml. After withdrawal from the flask the needlepoint of each syringe containing gas samples was inserted into No. 10 rubber stoppers to contain the gas samples until they could be analyzed by gas chromatography. About 0.2 ml of each gas sample was expelled from the gas-sampling syringe just prior to injecting the remaining volume of gas, 0.2 ml, into the gas chromatograph.

Modified Phase I assays were conducted (Smith and Tiedje, 1979; Dendooven and Anderson, 1995; Fazzolari, et al., 1990) to see what factors might be limiting to denitrification. Soil cores were collected and prepared, except for substrate amendments, in the same manner as described in Chapter 1. The soils used in the analysis were from wetland 13 site 2 lowland, wetland 8 site 2 lowland, and wetland 5 site 2 lowland. Amendments were prepared for Phase I analysis as follows: Eight flasks per wetland were prepared for Phase I analysis. With the exception of "chloramphenicol" flasks each amendment was prepared in replicates of two. Amendments consisted of; Treatment 5: "chloramphenicol" flasks (meaning 25 grams of soil, 0.025 g chloramphenicol and 25 ml of sterile water were added to the flask and made anaerobic, then normal phase I protocol was followed); Treatment 4: "chloramphenicol and glucose" flasks (contained 1 mM glucose, 0.025 g chloramphenicol and 25 ml sterile water); Treatment 3: "$KNO_3$, glucose and chloramphenicol" flasks (contained 1mM nitrate, 1mM glucose and 0.025 g chloramphenicol); Treatment 2: " $KNO_3$ and chloramphenicol" flasks (contained 1 mM $KNO_3$ and 0.025 g chloramphenicol); and Treatment 1: "$KNO_3$ and glucose " (contained 1mM $KNO_3$ and 1mM glucose). Following the substrate amendment additions, and

chromatographic analysis the soil slurries were poured into sterile 50 ml polypropylene, wide-mouth centrifuge tubes (Nalgene, style 3140), labeled and weighed.

Samples of "like" weights were placed across from each other in a Beckman J2 series centrifuge, then spun for 30 minutes at 12,000 x g. The filtrate was poured into sterile disposable, graduated, Nalgene centrifuge tubes.

Tubes were labeled according to amendment manipulation and frozen until glucose, ammonia, and nitrate analysis by Oscar E. Olson Biochemistry Laboratories (Analytical Services Laboratory, SDSU).

Varian Gas Chromatograph

A Varian 3700 Gas Chromatograph equipped with a $^{63}Ni$ electron-capture detector (ECD)(Charles 1967) and a Spectra-Physics 4270 integrator (Thermo Separation Products, San Jose, CA) were used to measure the amount of nitrous oxide in flasks. The Deactiglas glass column used in the chromatograph was six feet in length, and 4 mm in diameter (Alltech, Cat. No. 6192) and packed with Porapak Q (Thusse, 1978). The column oven temperature was maintained at 30°C with an electron-capture carrier gas mix of 95% argon and 5% methane delivered at a flow rate of 60 ml/min. This was slightly faster than the suggested elution time for $N_2O$, but it allowed a change in integrator response and elution of $N_2O$ moved from 8 minutes to approximately 4 minutes (Burford and Bremner, 1972; Umbreit, 1979). The detector temperature was set to 300°C (Thusse, 1978), and the attenuation of both the gas chromatograph and integrator were set at 4. In addition, the peak onset (PO), peak threshold (PT), and peak width (PW) of the integrator were set to 4, 100, and 6, respectively.

Standard nitrous-oxide samples were run on the same day and prior to the soil-slurry headspace gases. The nitrous oxide standards (Scotty II type cylinders) were obtained from Scott Specialty Gases (Troy, MI) in the following concentrations: 0.502 ppm (with a percent accuracy of +/- 2%); 0.966 ppm (+/- 5%); 10.4 ppm (+/- 5%); 104 ppm (+/- 2%); and 1020 ppm (+/- 2%). Three injections of each standard concentration were made, and the results were averaged and reported as one value on the standard curve.

Following the 120-minute sampling the stoppers, septum and screw caps were removed. The flasks were then placed in a drying oven set at 75°C for three days. It was determined previously that, for each flask, after three days of oven drying the soil weights remained the same. The flasks with soil were placed in desiccators and allowed to cool to room temperature, then weighed. The soil was then removed from the flask, all soil residue was washed from the flask and the flask was placed on a drying rack. Once dried, the flasks were reweighed and the dry weights of the soil were calculated for each individual flask.

**Calculations**

The total $N_2O$ present in the flasks was determined by using Henry's Gas Law ($C_g = kP_g$), where k is a constant at a certain temperature for a certain gas, $P_g$ is the pressure of the gas in atmospheres, and $C_g$ is the concentration of gas dissolved in water. Based on this, the following equation was used to determine the total amount of $N_2O$ present in each flask:

$M = C_g (V_g + (V_l)(\alpha))$

Where M is the total amount of $N_2O$ in the aqueous plus gas phases, $C_g$ is the concentration of $N_2O$ in the gas phase, $V_g$ is the volume of the gas phase, $V_l$ is the volume of liquid phase, $\alpha$ is the Bunsen adsorption coefficient.

The Bunsen absorption coefficient ($\alpha$) (which is ml of gas at 0°C and 760 mm of Hg (STP) that is absorbed by 1 ml of water)(Tiedje and Dazzo, 1982) was used to correct for dissolved $N_2O$ in the slurry. The $\alpha$ value is calculated from a third order polynomial $[(-8.2256 \times 10^{-6}) T^3] + [(9.6068 \times 10^{-4}) T^2] + [(4.824 \times 10^{-2}) T] + 1.2777$. At 25°C the $\alpha$ value is, $[(-8.2256 \times 10^{-6}) 15{,}625] + [(9.6068 \times 10^{-4}) 625] + [(4.824 \times 10^{-2}) 25] + 1.2777 =$ 0.544 (personal communication, Parkin 1994). $C_g$ was the concentration in nm of $N_2O$ in the 0.2 ml injected into the gas chromatograph. The value ($C_g$) is the x in the equation of the line ($y = mx + b$), where y is measured in integrator units and the slope (m) and y-intercept (b) values are obtained from the known concentration standards by linear regression. Linear regressions and the calculation described above were done using Corel Quattro Pro 7.0.

The first step in obtaining the y-intercept and slope values, was to convert ppm of the standard to nanomoles (i.e. 0.513 ppm $N_2O$ standard= (0.513/1,000,000) x 100 = $0.0000513\% = 5.13 \times 10^{-7}$ ml pure $N_2O$ /100 ml total volume). If 0.2 ml of 0.513 ppm is injected for analysis, using

$V = 0.0002 \times 10^{-3} L \times 5.13 \times 10^{-7} = 1.026 \times 10^{-10} L\ N_2O.$

Then using, $PV = nRT$ where $T = 298°K$

Then $(1 \text{ atm})(1.026 \times 10^{-10} L\ N_2O) = n\ (0.08205 \text{ L atm/g mol°K})(298\ °K)$

So, $\frac{1.026 \times 10^{-10} \text{ L } N_2O}{24.4509} = n$

$4.1962 \times 10^{-12} = n$

$0.0041962 \times 10^{-9} =$

n = 0.0042 nm of pure $N_2O$ is in 0.2 ml injection for 0.513 ppm $N_2O$ standard.

Calculations for standards of 0.5, 1.0, 10, 100, and 1000 ppm (+/- 4) were integrated into a table (via cell formulas) labeled "standard curve information."

Then 0.2 ml of each standard was injected into the Varian gas chromatograph with three injections for each standard. The three-peak base areas were averaged and entered in the appropriate column. Liner regression tables via Quattro Pro software were calculated. The slope and y-intercept were used from the Quattro-Pro standard regression table, to convert integrator units to nanomoles $N_2O$ per 0.2 ml of headspace gas in the unknown sample.

Table 2.1. Standard curve information

| ppm | % $N_2O$ (ml/ml) | $N_2O$/L | moles/0.2 ml | | | nm $N_2O$/0.2ml (taken as the x value) | Total amount of $N_2O$ (taken as y value) |
|---|---|---|---|---|---|---|---|
| 0.503 | 5.03E-05 | 1.006E-10 | 4.114367978 27483E-12 | | | 0.0041 | 2451.33 |
| 1 | 0.0001 | 2E-10 | 8.179658008 49867E-12 | | | 0.0082 | 7056.66 |
| 10 | 0.001 | 2E-09 | 8.179658008 49867E-11 | | | 0.0818 | 55077.33 |
| 100 | 0.01 | 2E-08 | 8.179658008 49867E-10 | | | 0.8180 | 610216 |
| 1000 | 0.1 | 2E-07 | 8.179658008 49867E-09 | | | 8.1797 | 4629924 |

Table 2.2. Linear regression and coefficient table

| | | | |
|---|---|---|---|
| Constant | | 37663.3417322844 | (Y-intercept)(b) |
| Std Err of Y Est. | | 73445.8289163674 | |
| R Squared | | 0.99900046814624 | |
| No. Of Observations | | 5 | |
| Degrees of Freedom | | 3 | |
| X Coefficient(s) | 562755.095195328 | (Slope)(m) | |
| Std Err of Coefficients | 10279.3555553921 | | |

Using the above standard curve information (Table 2.1) and linear regression and coefficient table (Table 2.2) the total $N_2O$ concentration "M" (in the liquid and headspace phases) can be calculated for flask 1 at zero minutes using integrator units (41395)(Table 2.3), with the formula $[N_2O] = M = C_g (V_g + (V_l)(\alpha))$ (Table 2.4)

Table 2.3. Phase I integrator units.

| | 0 min | 30 min | 60 min | 90 min | 105 min | 120 min |
|---|---|---|---|---|---|---|
| flask 1 | 41395 | 234481 | 386810 | 419449 | 598421 | 537763 |
| flask 2 | 64647 | 274899 | 253313 | 514614 | 551073 | 732988 |
| flask 3 | 61027 | 259293 | 509792 | 522484 | 693877 | 986012 |
| flask 4 | 70813 | 236941 | 497220 | 674954 | 853702 | 1108961 |
| flask 5 | 71703 | 195320 | 359959 | 464504 | 729314 | 905548 |
| flask 6 | 66825 | 128236 | 236924 | 423368 | 185937 | 450808 |
| flask 7 | 54430 | 117155 | 91390 | 255665 | 415102 | 471179 |
| flask 8 | 69215 | 118553 | 96523 | 393657 | 601804 | 672567 |
| flask 9 | 58882 | 101822 | 244458 | 362952 | 498206 | 539334 |

Table 2.4. $[N_2O] = M = C_g (V_g + (V_l)(\alpha))$ calculations.

| | Time | nm $N_2O$/0.2ml | $C_g$ (nm $N_2O$/ml) | $V_g$ (ml) | $V_l$ (ml) | $\alpha$ (25°C) | M Total $[N_2O]$ (nm) | g.o.d. soil per flask | nm $N_2O$ per g. o.d. soil |
|---|---|---|---|---|---|---|---|---|---|
| Flask 1 | 0 min | 0.006631051 | 0.033155259 | 110.1 | 42 | 0.544 | 4.407925468 | 11.83 | 0.372605703206209 |

Flask one at zero time had 41395 (Table 2.3) measured integrator units for $N_2O$. The y-intercept (Table 2.2) , via cell formulas, would be subtracted from the integrator units of an unknown sample (41395 – 37663) and divided by the slope (562755) (Table 2.2) to equal 0.006631 nm $N_2O$ per 0.2 ml injection (Table 2.4). $C_g$ is a per milliliter concentration so 0.006631 is multiplied by 5 to convert the concentration to the proper per milliliter form. $V_g$ is the volume of the gas phase of the 125 ml -Erlenmeyer flask. This was calculated by weighing the flasks with stoppers, screw-caps and septum in place, then filling the flask with $dH_2O$, removing any air bubbles and reweighing the flask. Subtracting empty from full flask mass gives a gram value for volume content (since 1.0 g of $H_2O$ is one milliliter), minus the soil slurry volume ($V_l$) leaves a 110.1 value for $V_g$. M is equal to 4.408 and when divided by the oven dried soil weight of 11.83 will give 0.3726 nm $N_2O$ per gram oven dried soil (Table 2.4). When the table was filled in for all the flasks and time intervals the nm $N_2O$ per gram oven dried soil values were put through linear regressions and from the slope denitrification rates were calculated.

The tables and graphs were created on Quattro Pro for DOS ver. 7.0 (Corel Corporation Limited, Dublin, Ireland) and Microsoft Works spreadsheets (Microsoft Works Suite 2000, Microsoft Corporation, 1999).

Data were analyzed by an ANOVA procedure for split-plot arrangement. Wetland sites (upland or lowland) were used as whole plots with site 1, 2, and 3 as repetitions and the sampling dates as a subplot (SAS Procedures Guide, 1990)(SAS/STAT ® Software, 1997).

## RESULTS AND DISCUSSION

Research conducted by other workers has found denitrification activity to be most prevalent during the spring and fall with little activity during the summer (Tiedje et al., 1989). Another study in our laboratory (Boetel, 1996) looked at differences in denitrification rates between semi-permanent and seasonal wetlands during the spring, summer and fall and found these temporal variables to be significant. In my study the temporal variables were again significant ($Pr > F$ 0.0656)(Table 2.9). Most of the graphs showed a temporal familiar "M" shaped pattern (Chapter 1). Temporally the high peaks of the "M" were consistent with increased soil water content from the spring thaw and with fall frost (where plants would die off and denitrifiers would not have to compete with them for nitrate). The low peak of the "M" could be due to plant competition for water and/or nitrate or be due to plant root's contribution of oxygen to root zones in the soil (Conrad, 1996).

Substrate Amendments

The analysis of substrate additions was to determine whether one of the amendments in the assay was limiting to denitrification. To see if sufficient carbon (or nitrate) substrate was present in soil, the substrate remaining in the filtrate following a phase I assay was determined. Also, the effect of chloramphenicol on the phase I assay results was examined and compared. The results for the substrate experiment are reported in Table 2.5 and Figure 2.7 (Appendices B). Each substrate manipulation was assigned a treatment number to aid in graphing. Each data entry was an averaged mean of the phase I assay replicates of each amendment.

In treatment 1 (Table 2.5, Appendices B) chloramphenicol was omitted and the increased rates were probably due to "new" enzyme synthesis if glucose and $KNO_3$ were not a limiting factor. According to the Olson Biochemistry Laboratory report glucose and $KNO_3$ amendments remaining in the filtrate were 118.00 to 130.00 parts per million and 5.26 to 8.7 parts per million respectively. Concentrations in the "blank" control filtrate (sterile and no soil additions) were 211.00 and 23.00 parts per million. In soil incubations, half to near half of the glucose amendments were metabolized where approximately two thirds of the nitrate substrate were metabolized.

In treatment 2 and treatment 3 the amount of nitrate consumed was approximately the same. The amount of ammonia present was highest in treatment 2 and second highest in treatment 3. Carbon-limited environments may favor denitrification and nitrate limited environments may favor dissimilatory nitrate reduction to ammonium,

all other factors being equal (Smith and Tiedje, 1979; Tiedje, 1988). Comparison of these two treatments does not agree with this. This may be because the addition of chloramphenicol actually favors dissimilatory reduction of nitrate to ammonium (DNRA), or chloramphenicol concentrations may have been too high and existing denitrifying enzymes may have been repressed or inhibited. DNRA bacteria populations may have been more prevalent in the soil's history and as stated in Chapter 1 they might out-compete denitrifiers by differences in $V_{max}$ and $K_m$ values. In treatment 2 DNRA bacteria may have been most active but this is not certain. If stressed long enough cells could die and lyse, releasing amino acids and proteins into the soil environment. When observing the graph data (Table 2.5, Appendices B) the later probability seems likely. The $N_2O$ production rates of treatment 2 and 3 were similar.

Treatments 4 and 5 had the lowest denitrification rates of all treatments examined. Treatment 4 was meant to mimic a nitrate-limited environment, which might favor the DNRA pathway. In this treatment exogenous glucose levels were the least metabolized of all glucose treatments, the presence of ammonia was low (when omitting wetland 8, 2$^{nd}$ replication), and nitrate levels second lowest (with only existing soil nitrate present). In treatment 5 both amendments of nitrate and carbon were omitted. The modified phase I assays suggested that, for the site 2 lowland soils examined, wetland 8 was nitrate limited and glucose limited. The wetland 5 and 13 sites were not apparently nitrate or glucose limited.

Percent Water Content

One of the variables that most influenced denitrification was soil water content. Because it is an important regulator of oxygen transport in non-saturated soils it causes much of the temporal and spatial variability of soil by creating oxygen gradients (Tiedje, 1988; Conrad, 1996). The percent water content of soil is reported in Table 2.7 and Figures 2.8-2.10 (Appendices B). With the exception of an occasional outlier point, the lowland water content was higher than that of the upland. Lowland percent water levels were as high as 44.28 % on 4-16-94 and the upland percent water as high as 28.8% on 7-22-94, 6-15-95, 6-5-94, and 9-24-95.

Phase I Assay

Denitrification rates for Phase I assays are reported in Table 2.8 (Appendices B) and graphed in Figures 2.11 thru 2.13 (Appendices B). Lowland samples (over all) showed consistently higher DEA rates than did the upland (Pr > F 0.0001; Table 2.9)(Figure 2.4; 2.5; 2.6).

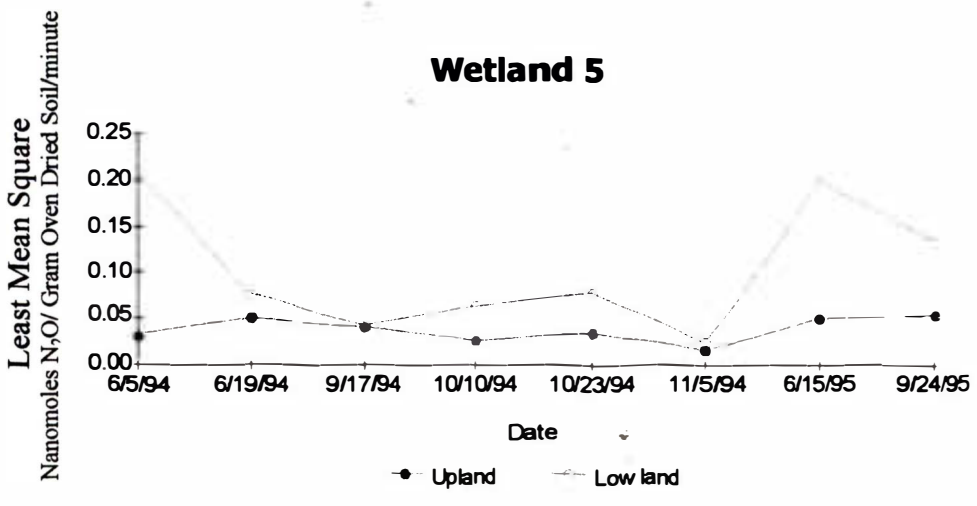



Figure 2.4. Least mean square for wetland 5 $N_2O$ rates.

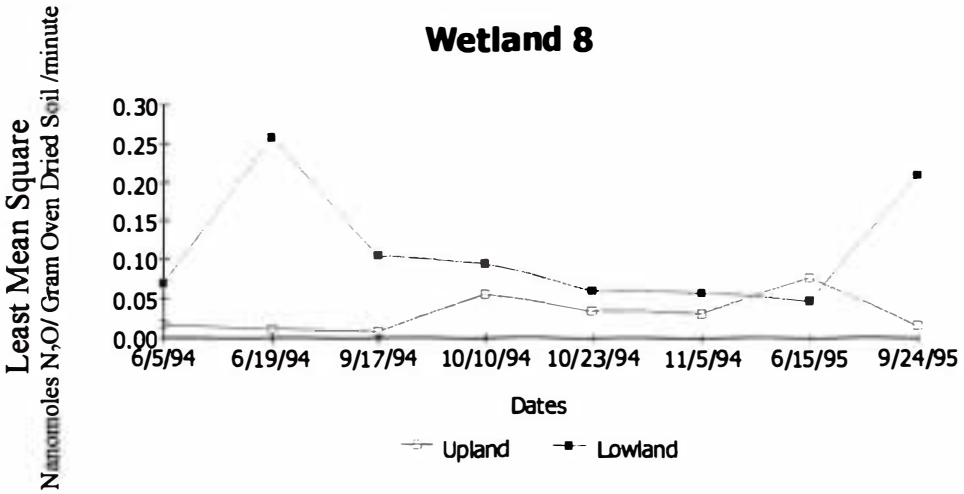



Figure 2.5. Least mean square for wetland 8 $N_2O$ rates.

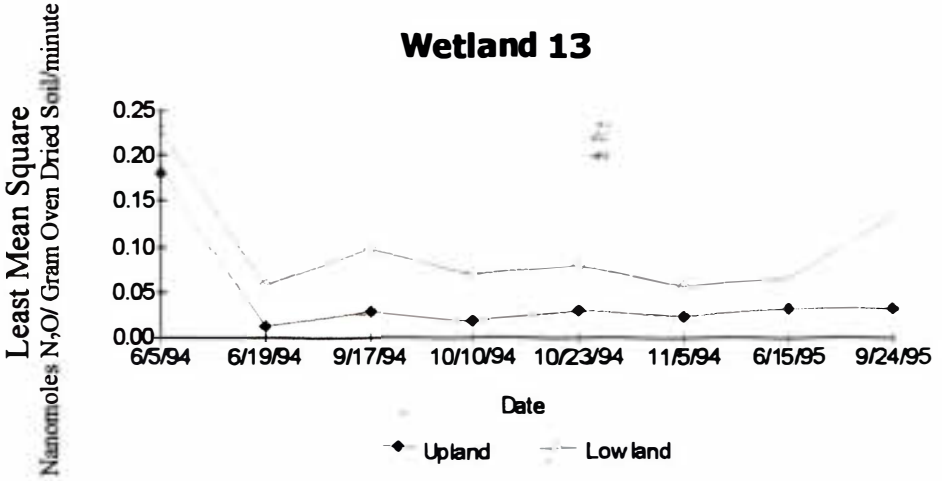



Figure 2.6. Least mean square for wetland 13 $N_2O$ rates.

Percent water-content tables and graphs showed the same correlation, with the lowland consistently showing higher percent water-content than that of upland soil samples (Figure 2.8-9-10; Appendices B). Lowland samples also showed increased DEA activity in the spring and fall. Upland samples showed similar increases but at lower magnitudes. Percent water-content graphs showed the temporal changes in percent

water content also. Correlation statistics were done between denitrification rates and percent water content. Statistically 3% of the variability of $N_2O$ was explained by the soil sample's percent water content at the time of analysis (Table 2.10). The rates for denitrification seemed lower in 1994 than in 1993 or 1995. Soil sample storage time may have affected the rates (i.e. changes in percent water content could be one of the variables altered)(Dendooven et al., 1996). However, because lowland and upland samples were stored in the same manner, data may still give comparable estimates of potential DEA in both positions. The data support the first hypothesis that DEA would be higher in the lowland samples (Pr > F 0.0001), but not the second hypothesis that the organic farming practice would promote higher overall DEA (Pr >F 0.9959)(Table 2.9).

Table 2.9. Analysis of Variance for 1994 Phase I Rates.

| | *NDF* | *DDF* | *Type III* | *Pr > F* |
|---|---|---|---|---|
| **Wetland** | 2 | 96 | 0.00 | 0.9959 |
| **Position** | 1 | 96 | 22.51 | 0.0001 |
| **Wetland * Position** | 2 | 96 | 0.35 | 0.7054 |
| **Date** | 7 | 96 | 1.98 | 0.0656 |
| **Wetland * Date** | 14 | 96 | 1.44 | 0.1504 |
| **Position * Date** | 7 | 96 | 0.82 | 0.5705 |
| **Wetland * Position* Date** | 14 | 96 | 1.01 | 0.4542 |

*NDF is the numerator of degrees of freedom.

**DDF is the denominator degrees of freedom.

***Type III equals a type III sum of squares. In this type data have been adjusted for all other-effects in the model and is used to analyze data sets with missing data.

****Pr > F is the probability that the calculated F value is going to be greater than the F table value.

Table 2.10. Correlation Analysis Between the Variables $N_2O$ production and Percent Water Content.

Correlation Analysis

2 'VAR' Variables: N2O PERWATER

Simple Statistics

| Variable | N | Mean | Std Dev | Sum | Minimum | Maximum |
|---|---|---|---|---|---|---|
| N2O | 3150 | 3.06499 | 9.61977 | 9655 | -6.71289 | 331.38664 |
| PERWATER | 3150 | 27.24441 | 8.31698 | 85820 | 7.60000 | 51.44000 |

Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N = 3150

| | N2O | PERWATER |
|---|---|---|
| N2O | 1.00000 | 0.17704 |
| | 0.0 | 0.0001 |
| PERWATER | 0.17704 | 1.00000 |
| | 0.0001 | 0.0 |

# CHAPTER 3

# DENITRIFYING ENZYME ACTIVITY VERSUS DEPTH IN A PRAIRIE POTHOLE SEMI-PERMANENT WETLAND

## INTRODUCTION

The availability of nitrate is a crucial factor affecting soil denitrification activity, since nitrate is the first N-oxide in the denitrification pathway. Carbon compounds provide the energy for denitrifying bacteria and are crucial in determining the denitrification rate of soil. Oxygen and nitrate affect short-term denitrification rates and carbon compounds are the primary regulators of soil denitrification over time scales of days to months (Tiedje, 1988). Complex plant material, when decomposed, releases simpler compounds that support microbial populations (Højberg et al., 1994; *DE*Catanzaro et al., 1987). This produces great spatial and temporal variability in occurrence of carbon compounds in soil, based on where and when plant material is decomposed. In areas where plant material is being decomposed, elevated levels of available carbon would likely be present and denitrifying enzyme activity values would be expected to be greater than in areas lacking such plant-based carbon. Seasonal patterns of denitrification have been shown to exist (Højberg et al., 1994; *DE*Catanzaro et al., 1987). The greatest denitrification rates have usually been measured in the spring and fall, when soil moisture is high and plant competition with denitrifying bacteria


1. Nitrate reductase
2. Nitrite reductase
3. Nitric oxide reductase
4. Nitrous oxide reductase

Figure 3.1. Denitrification pathway and enzymes involved.

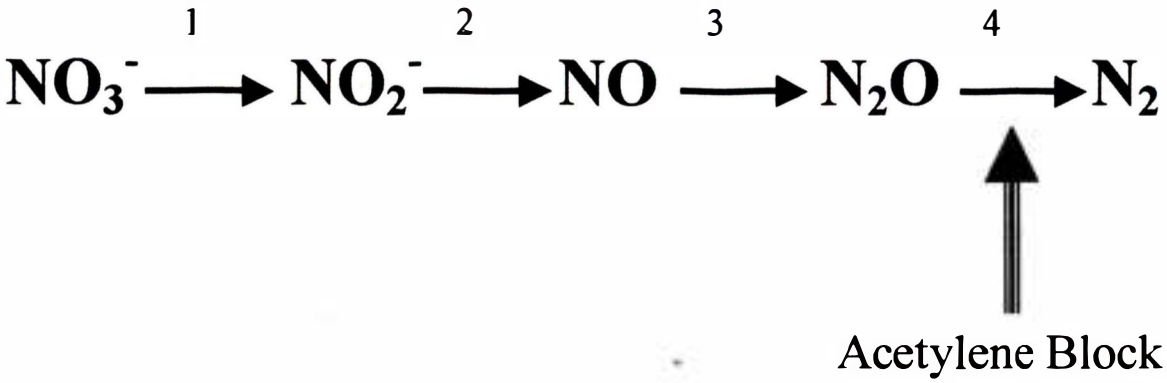



Figure 3.2 Acetylene block of denitrification pathway

for available moisture and nitrates is low (Tiedje et al., 1989). Carbon supplies are also high at these two times of the year. In the spring, freezing and thawing events can disrupt soil aggregates and lyse microbial cells, causing an increase of available carbon (Tiedje, 1988). In the fall, plant material that has fallen to the ground and started to decay can provide high levels of available carbon to soil microbes. By some estimates, 80% of N loss at a soil site over a year's time, as a result of denitrification, occurs during a 3-6 week period during the spring and fall. Some examples of bacteria capable of denitrification are *Paracoccus denitrificans*, *Thiobacillus denitrificans*, and several types of *Pseudomonas* (Tiedje, 1988). Evolution of gaseous nitrogenous oxides by these organisms can be measured by gas chromatography.

Acetylene block methods are used in gas chromatography because dinitrogen is hard to measure against the high background of atmospheric nitrogen (Figure 3.2). The acetylene will interfere with nitrous oxide reductase and allow $N_2O$ to accumulate. Acetylene has a triple bond and can bind to nitrous oxide reductase's active site to prevent further enzyme activity (Matthees, 1998; Tiedje et al., 1989; Miller et al., 1993; Sorensen, 1978; Conrad, 1996).

Previous work in our laboratory (Boetel, 1996) has shown that significant numbers of denitrifiers and sulfate reducing bacteria are present in the top layers of lowland sediments of semi-permanent prairie pothole wetlands. Other research has also shown this in other wetland types (Macfarlane and Herbert, 1984; Sorensen, 1978). Carbon and nitrate, both being major controlling factors of denitrification rates, should be more plentiful in the top layers of soil due to microbial metabolism of organic matter and

applied chemical fertilizers that have entered the wetland (Slater and Capone, 1987). The top layers of soil also can have alternating wet and dry cycles. These cycles may provide a more hospitable environment for denitrifying bacteria than a continuously wet or dry habitat (Tiedje, 1988).

Denitrifying enzyme activity may vary across a wetland due to site variation, such as at a wetland's perimeter versus deeper in the wetland's interior. Water levels at the shoreline of the semi-permanent wetland studied in this chapter experienced wetting and drying cycles (Figure 3.3). This allowed assay of denitrifying enzyme activity when oxygen diffusion to the soil pore water had less resistance than other sample dates, and also provided a chance to look at the activity deeper in the wetland waters where the sediment usually was not exposed directly to atmospheric oxygen.

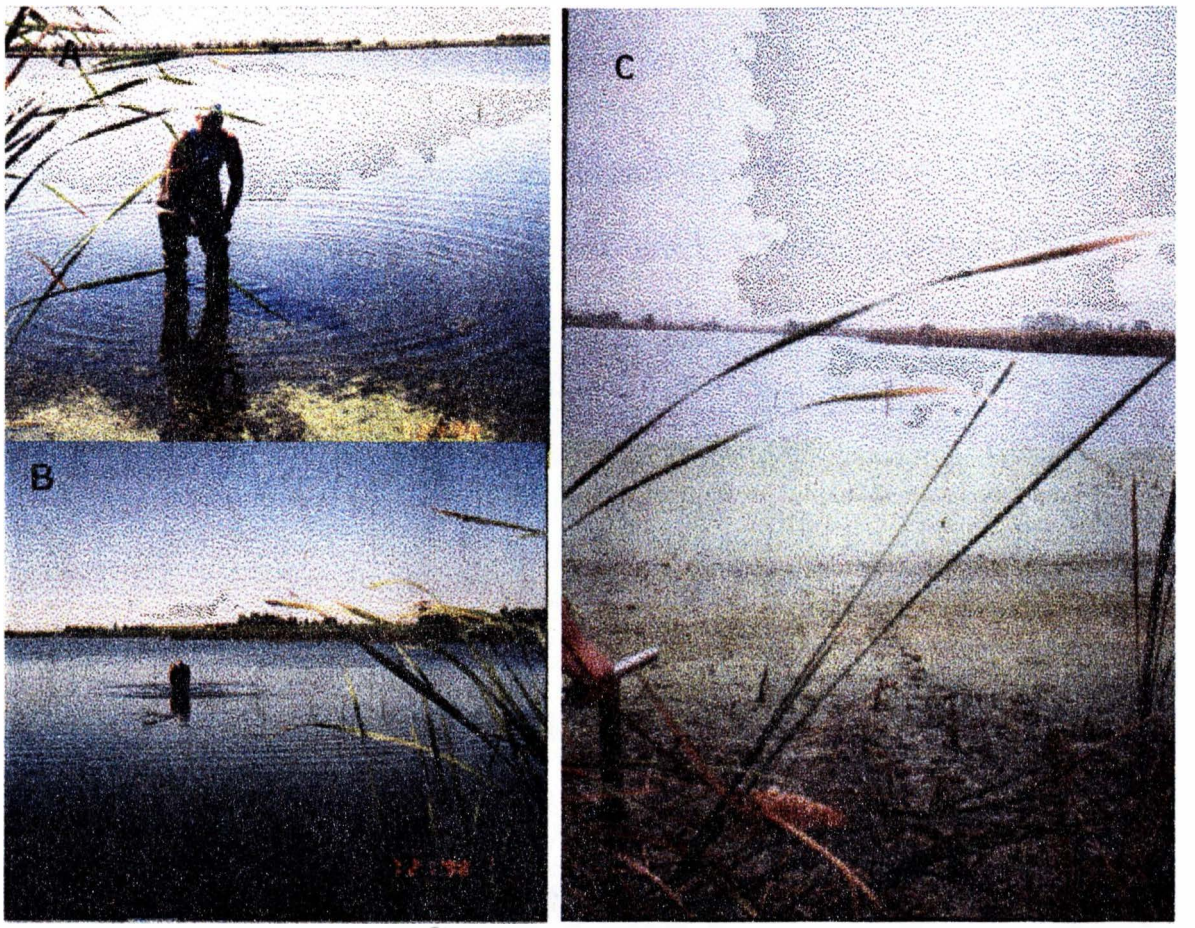
One method to attempt measurement of natural denitrification rates is with unamended phase I, acetylene block assays. Potential denitrification rates are assays conducted under optimum conditions. The capacity measurement method is a potential rate assay that varies oxygen, carbon and nitrate to determine their influence on denitrification (Tiedje et al., 1989). The denitrifying enzyme activity (DEA) assay is also a potential rate assay. DEA assays measure potential activity in soils at the time of collection. The result of this assay gives an estimate of the environmental history of soil and is relatively short in length (Tiedje et al., 1989).

This study hypothesized that substantially more DEA activity would be present in the top layers of the wetland sediment, and that denitrifiers would decrease by depth increments and be influenced by wet-dry cycles near the shoreline. DEA values in

Chapter 2 of this thesis did not take depth into consideration, but did look at two dates for DEA values of a pooled soil sample from the top1-2 inches of a 31.0 cm soil core. A study of this semi-permanent wetland by depth would give a better understanding of denitrifying enzyme activity at the 0-5 cm, 5-10 cm, and 10-15 cm depths.

Results would help evaluate a prairie pothole wetland's possible capabilities in handling nitrogen loads. Indirectly this study could give indication as to the placement of denitrifiers within the soil profile.

Besides assaying DEA by depth, differences in soil organic matter, pH, soluble salts, nitrate, phosphorus and potassium were examined.




**A.** Shoreline with approximately 12 inches of standing water.

**B.** Deeper in the wetland where surface is almost always covered with standing water.

**C.** Shoreline drying out and exposed to atmospheric oxygen.

**Figure 3.3. Wetland site variations for semi-permanent organic wetland number 5.** (Photographs were taken on July 21, 1998)

# MATERIALS AND METHODS

## Soil Core Collection

Semi-permanent wetland number 5 (described in chapters 1 and 2) located on a farm using an organic management system was selected (Figure 3.3 through 3.5).

Figure 3.4. Wetland 5 (photographed April 4, 1999).

Organic farm management systems use no synthetic fertilizers and generally no chemical pesticides. Within this system, there is a strict adherence to crop rotation among row crops, small grains and cover crops that are used to protect the soil (Bleakley et al, 1993).

Three cores, 5.0 cm in diameter and 30 cm in depth were usually taken bi-weekly (every two weeks) from May 1998 through October 1998 at six different positions at two different sites (Figure 3.4). Three replicates of site 1 ($A_1$, $B_2$, $C_3$) were located along the shoreline (where the water level at maximum was 18 inches and at minimum was 0.5 inch (Figure 3.3), and three ($A_4$, $B_5$, $C_6$) replicates for site 2 were located 60 feet deeper in the wetland interior (Figure 3.5). Water levels at site 2 never exceeded 4 ft in depth (Figure 3.3).

Three soil cores, 0.5 cm in diameter and 20 cm in depth, were collected at each sampling point and date. The cores were taken from the wetland using a JMC concentric sampling tube soil-sampling auger with polyethylene terephthalate, glycol modified (PETG) co-polyester plastic liners (Clements Associates Inc., Newton, IA). Sampling was done in a triangular manner where sides of the triangle were 2 to 4 feet between corners. Plastic liners were capped at both ends and labeled according to sample core position, site and depth increment (example: site 1 $A_1$, site 1 $A_2$ and site 1 $A_3$). After collection, cores were placed in an upright position, on ice, until returning to the laboratory. In the laboratory, cores were sectioned into 5-centimeter depth increments (within two hours of sampling).

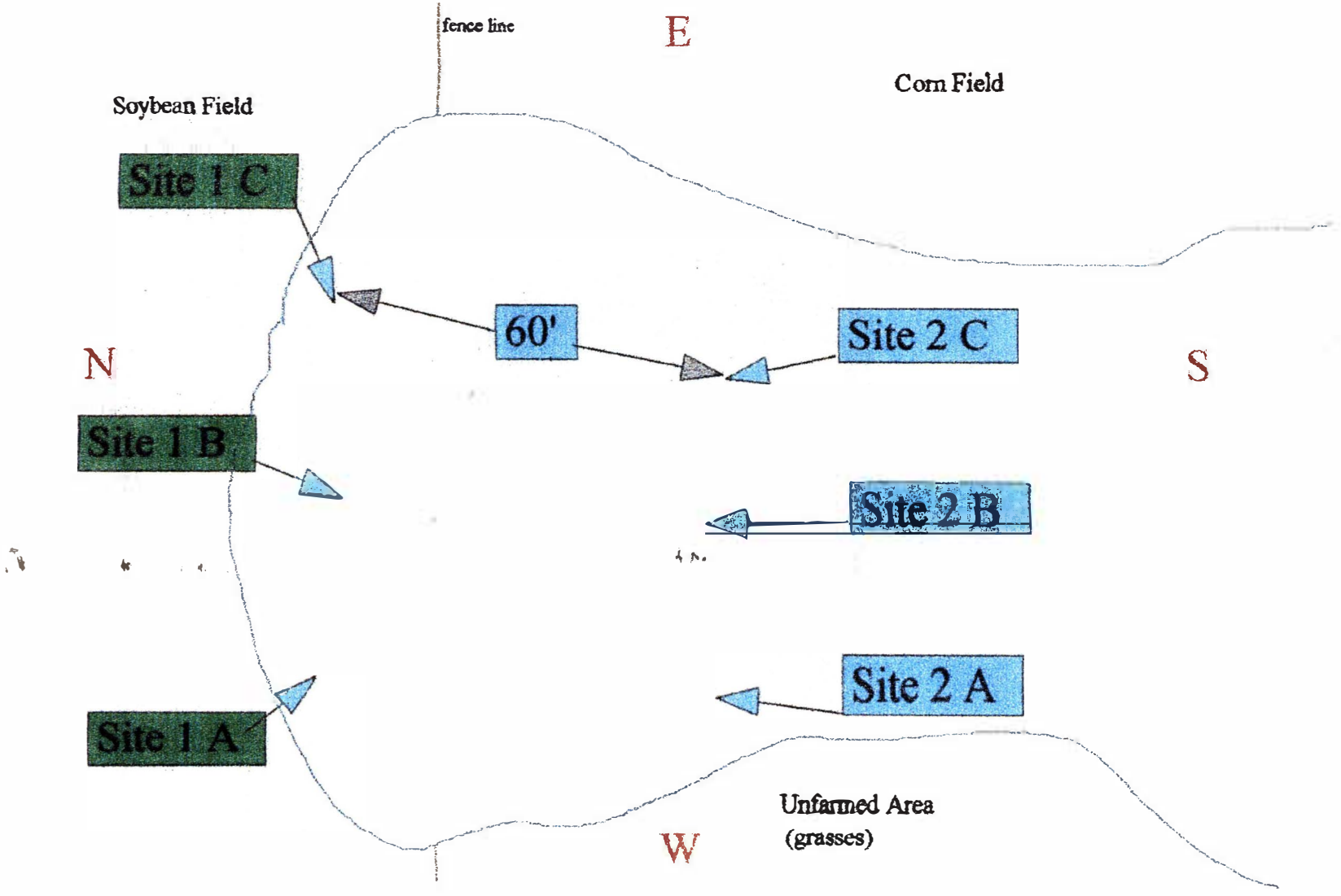



Figure 3.5 1998 aerial view of sampling scheme for wetland 5.

Core Preparation

Core sectioning began by placing a clean $5\frac{1}{2}$" x $5\frac{1}{2}$" square VWR brand weigh paper on an inverted plastic autoclave basin. One of the core sample liners without caps and an interior cap (red) placed just inside the bottom end of the liner were pressed against the surface of the soil core. Then depth measurements were determined (via ruler) and marked with a Sharpie marker at each of three 5 cm depth increments starting from the top of the inserted cap (Figure 3.6).

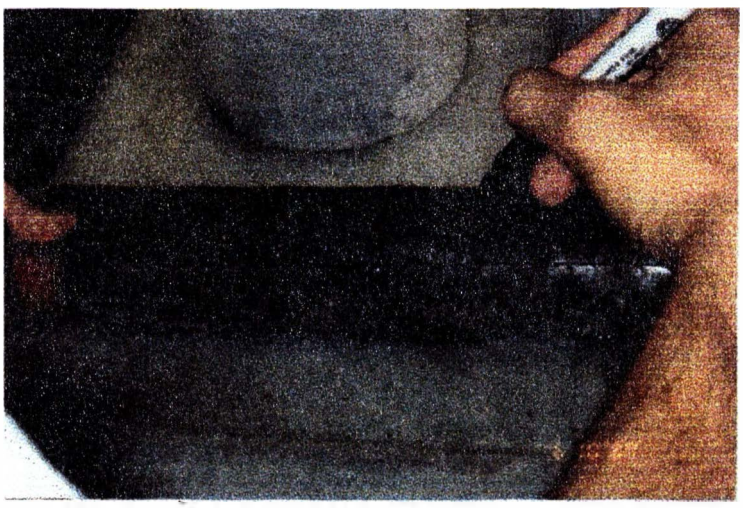


Figure 3.6. Marking for depth increments.

Once depth increments were marked, a large wooden dowel was inserted on the underside of the red cap and even pressure was applied to the soil core (via wooden dowel and cap) until the front cap edge lined up with the first 5 cm increment. At the other end of the liner, 5 cm of the top of the soil core was extended beyond the edge of the liner. This 5 cm of extruded soil was then cut off via a standard laboratory spatula (Figure 3.7.).

completed (such as Site 1 $A_1$ plus Site 1 $A_2$ and Site 1 $A_3$). Now each 600 ml Nalgene beaker contained three 5 cm sections per depth increment. These were thoroughly mixed manually using a large metal spoon (Figure 3.8)

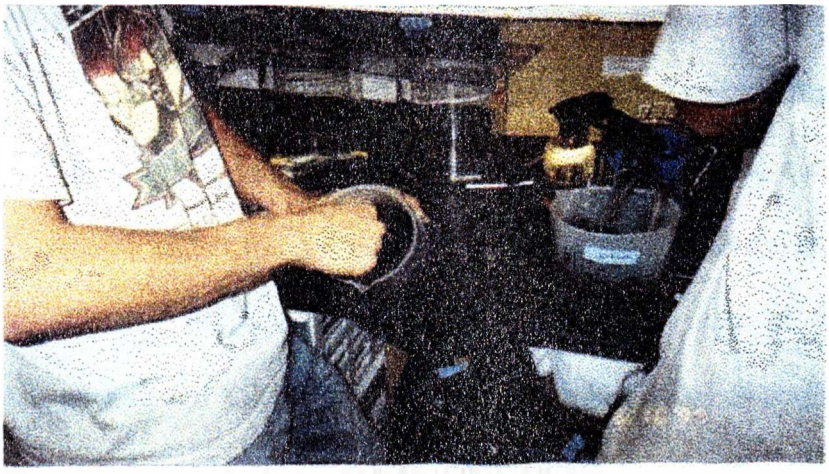


Figure 3.8. Mixing of depth segments.

Each depth mixture was placed in a sterile polyethylene Whirl-Pak bag (Nasco, Fort Atkinson, WI), and labeled according to the depth increment and date of the sample. The samples were then stored at 4°C until time of analysis. Phase I analysis for May 21 samples was done on May 28th, June 2nd and June 9th. Other sampling dates included June 24th, 1998 with phase I analyses done on June 29th, 30th, and July 1st, 1998; July 7th, 1998 with phase I analyses done on July 13th, 14th and 15th; October 2nd, 1998 with phase I analyses done on October 12th, 14th, and 19th; and October 15th, 1998 with phase I analyses done on October 22nd, 23rd and 24th.

Phase 1 Assay and Natural Denitrification Rate Potential Assays

25 grams of soil (fresh weight) were weighed out in triplicate and each 25-gram sample was placed in a clean $3\frac{1}{2}$" x $3\frac{1}{2}$" x 1" deep polystyrene weigh boat that was zeroed

prior to each soil addition and reweighed after placing the soil in a 125 ml Erlenmeyer flask that contained 25 ml of assay solution. The weight of the polystyrene dish, after soil was removed, was calculated and subtracted from the original 25 grams and then recorded by flask number and sample information. The assay solution in the final soil slurry contained 1mM D-glucose, 1 mM $KNO_3$, and 0.00625 grams chloramphenicol. The concentration of chloramphenicol in this study was decreased from the concentration used in Chapter 2. Several studies have reported undesired inhibitory action due to chloramphenicol (Pell et al., 1996; Wu and Knowles, 1995; Dendooven et al., 1994; Brooks et. al., 1992; Murray and Knowles, 1999). Chloramphenicol is an antibiotic, preventing the synthesis of proteins by inhibiting the 70S ribosome and thus production of additional denitrifying enzymes. The purpose of chloramphenicol in a Phase I assay is to keep bacterial biomass from increasing. This is important because concentration of enzyme is indicative of denitrifier biomass in the soil sample. However, it is undesirable to inhibit existing enzymes in a Phase I assay (Pell et al., 1996; Murray and Knowles, 1999). Concentrations greater than 0.1 g/L have been found to decrease DEA by as much as 61% and the only step in the denitrification pathway not affected by chloramphenicol concentration was the NO to $N_2O$ step (Figure 3. 9). Separate assays were conducted varying the chloramphenicol concentrations to determine a concentration suitable for the prairie pothole soils used in this study.

Natural denitrification-rate potential assays were carried out in the same manner as described for the Phase I assay, only without substrate additions or chloramphenicol.

The only exogenous substance included after making slurry flasks anaerobic was acetylene to allow for $N_2O$ accumulation.

The 125 ml sidearm Erlenmeyer flasks were as described in Chapter 2. The flasks were made anaerobic by evacuating and flushing with argon gas as described in Chapter 2. Purification of acetylene, its addition to flasks, and the steps for flask incubation and gas sampling of flask headspace were as described in Chapter 2.

Varian Gas Chromatograph

Gas chromatograph, detector, column and column packing material, oven temperature, carrier gas and its flow rate were as described in Chapter 2. The detector temperature was modified from settings described in Chapter 2 and set to 270°C. Dr. Duane Matthees (personal communication, 1998) suggested a lower temperature since nitrogenous gases are volatile at temperatures slightly above room temperature. The attenuation of both the gas chromatograph and integrator were set at 1. In addition, the peak onset (PO), peak threshold (PT), and peak width (PW) of the integrator were set as previously described.

Standard nitrous oxide samples were injected and run on the same day as Phase I assays and prior to the soil-slurry headspace gas injections. The nitrous oxide standards (Scotty II type cylinders) were obtained from Scott Specialty Gases (Troy, MI) in the following concentrations: 0.503 ppm (with a percent accuracy of +/- 2%), 1.0 ppm (+/- 5%), 10.0 ppm (+/- 5%), 100.0 ppm (+/- 2%) and 1000.0 ppm (+/- 2%). Three injections of each standard concentration were made, and the results were averaged and reported as one value on the standard curve.

Following the 120-minute sampling, flasks and their soil were processed as described in Chapter 2 to obtain soil oven-dry weights. After the Phase I assay for an entire sample date was completed, the remaining soil was dried at 75°C for three days and ground by mortar and pestle, bagged, labeled (according to depth, site, replication, and date) and sent to the Soil Testing Laboratory, Plant Science Department, SDSU for analysis. Soil samples were analyzed for percent organic matter, nitrates, pH and on one occasion for potassium, phosphates and salt content.

Calculations

Calculations for Phase I analyses and linear regression were as described in Chapter 2.

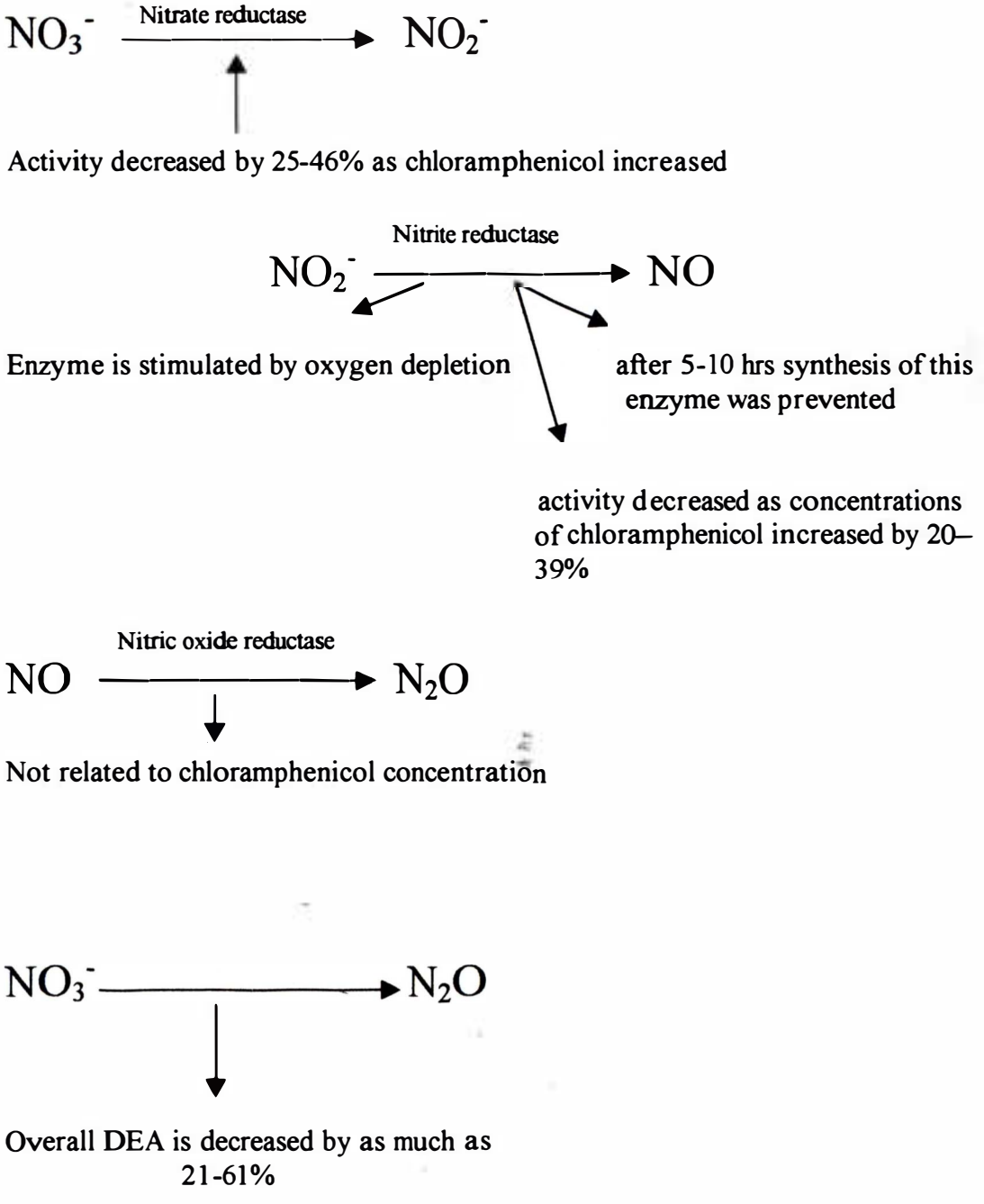



Figure 3.9. Overview of the effects of chloramphenicol concentration on the denitrification pathway.
(Based on Murray and Knowles, 1999)

## RESULTS

On May 21, 1998, 18 to 20 dead salamanders were found floating in the water and the shores were littered with a large number of dead snails (Figure 3.10). This was the only date where such large numbers of dead salamanders and snails were noted.

Figure 3.10. Dead snail littered shoreline (site 1).

Natural denitrification potential results for sets 1-6 (for soil sampled on July 7, 1998)(Table 3.1, Figure 3.23 and 3.24; Appendices C) showed the 10-15 cm depth increment values were higher than the 0-5 or 5-10 cm increments. This may indicate that denitrification successfully competes at this depth for existing nitrate and that periods of anoxia are longer, promoting higher denitrifying enzyme concentrations. The 0-5 cm

depth increment had the lowest values of the three depth increments, possibly indicating shorter periods of anoxia and more competition for nutrients and electron acceptors. A natural denitrification potential, with a 0-120 minute gas-sampling period (set 5), showed the 5-10 cm segments with values above that of the 0-5 or 10-15 cm depth segments. The 0-5 cm depth increment maintained the lowest values of the three depth segments over time. The analysis showed that in a shorter time interval the 5-10 cm segment had larger values than did the other two segments, but in longer time intervals the order was 10-15 cm > 5-10 cm > 0-5 cm. Another two sets of natural denitrification potentials (set 7 and 8)(Figure 3.24) also showed 10-15 cm > 5-10 cm > 0-5 cm, but this date showed the 5-10 cm depth increment values closer to those of the 10-15 cm depth values. When linear regressions were run on the values to determine natural rates of the potential results and graphed (Table 3.2 and Figure 3.25; Appendices C), the order was 10-15 cm > 5-10 cm > 0-5 cm. The 0-5 cm depth increment was variable in natural denitrification potential. At times, its rate reached that of the 10-15 cm rates and at other times it was similar to or less than that of the 5-10 cm rate, but overall was the lowest rate of the three depth increments. This analysis would need to be run over several seasons to see if this trend is consistent and to monitor how the profile reacts to drought or extremely wet years. The analysis described here did show differences by depth in the amount of denitrifying enzymes. Further analysis for organisms present by depth should be performed to ascertain what denitrifiers are present within each depth increment.

A study by Dendooven et al., (1994) suggested care in choosing chloramphenicol concentrations and for considering different soil types when selecting a chloramphenicol

concentration for individual studies. Several investigators have noticed the effects of chloramphenicol on existing enzyme activities (Murray and Knowles, 1999; Pell et al., 1996; Brooks et al., 1992; Wu and Knowles, 1995). Optimum concentrations vary according to author. In my study chloramphenicol concentration analyses for 1998 showed a steady decrease in activity as chloramphenicol concentration increased. Concentrations of 0.0 g/l, 0.05 g/l and 0.075 g/l had increased activity compared to concentrations of 0.1 g/l, 0.15 g/l and 0.25 g/l. The 0.5 g/l, 0.75 g/l and 1.0 g/l concentrations showed the least activity (Table 3.3 and Figure 3.27a, 3.27b and 3.27c; Appendices C). The 1998 activity for each individual chloramphenicol concentration analysis is graphed in Figure 3.27a,b, and c (Appendices C). The 1998 and 1999 rates for each chloramphenicol concentration analysis are graphed in Figure 3.28, 3.29 (Appendices C) and Table 3.4 (Appendices C). A steady decrease is shown with the greatest drop in rates shown from zero concentration to 1.5 g/l and a lesser decrease between 2.5 to 1.0 g/l. The effects of chloramphenicol can be seen when comparing 1994 Phase I rates with those of 1998. The 1998 10-15 cm depth increment rates were the lowest for the 1998 depth study and comparable to the whole-core Phase I rates obtained in 1994. However, both analyses showed changes in DEA rates by season. When observing 1994 and 1998 rates it was noted that both years had rate increases in June and October (see Chapter 2).

Regular Phase I rates showed increases in June and October of 1998. In June the percent water content of the soil was probably higher than in July or August due to spring thaw. As temperatures rise so does biological activity. Because the growing season had

just begun, plants would not be extensively utilizing soil nitrate, which could create a favorable environment for denitrification. In October the rise in activity could be attributed to post-frost die-off of plants. In this situation soil microbes would not be as restricted in nitrate availability and also would have ample organic matter as carbon source. October's rates were such that graphs were reported using different scales (Figure 3.29 and Table 3.5; Appendices C). For each site the 0-5 cm rates were the highest with 5-10 cm rates second and 10-15 cm rates being close to the 5-10 cm rates but still notably lower (Figure 3.30-32; Appendices C). Of the 0-5 cm rates, those of site 2 were higher than those of site 1 (Figure 3.30-32; Appendices C).

When comparing site one with site two, site two rates graphically appeared to be greater than those of site one in the 0-5 cm depth increment. This was not supported statistically with the site-by-site comparison where the Pr > F equaled 0.3525. As stated previously, site 2 did not experience wetland perimeter drawback. The soil at site 2 was continually covered with water and a thick mat of plant material. Soil microorganisms at the surface would have sufficient carbon supply (organic matter) and experience more frequent periods of anoxia during the growing season, which could help explain a slightly higher enzyme concentration in the fall sampling periods.

An ANOVA analysis showed significant differences among rates by depth (Pr > F = 0.0502; Table 3.13)(Figure 3.11), by date (Pr > F = 0.0119; Table 3.13)(Figure 3.12), depth by date (Pr > F = 0.0025; Table 3.13)(Figure 3.13), and site by depth by date (Pr > F = 0.0992; Table 3.13)(Figure 3.14).

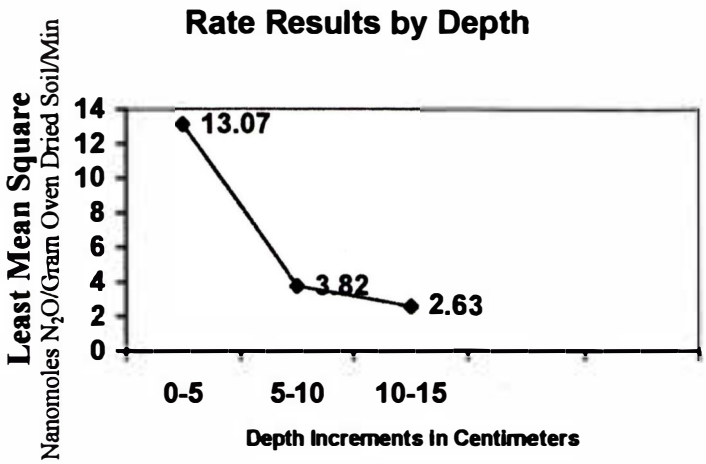



Figure 3.11. SAS mean comparison for $N_2O$ rates by depth.

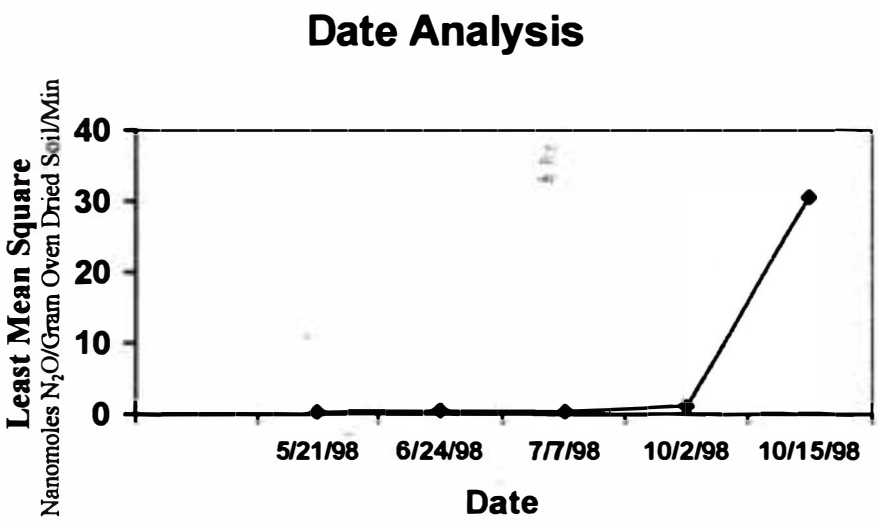



Figure 3.12. SAS mean comparison for $N_2O$ rates by date analysis.

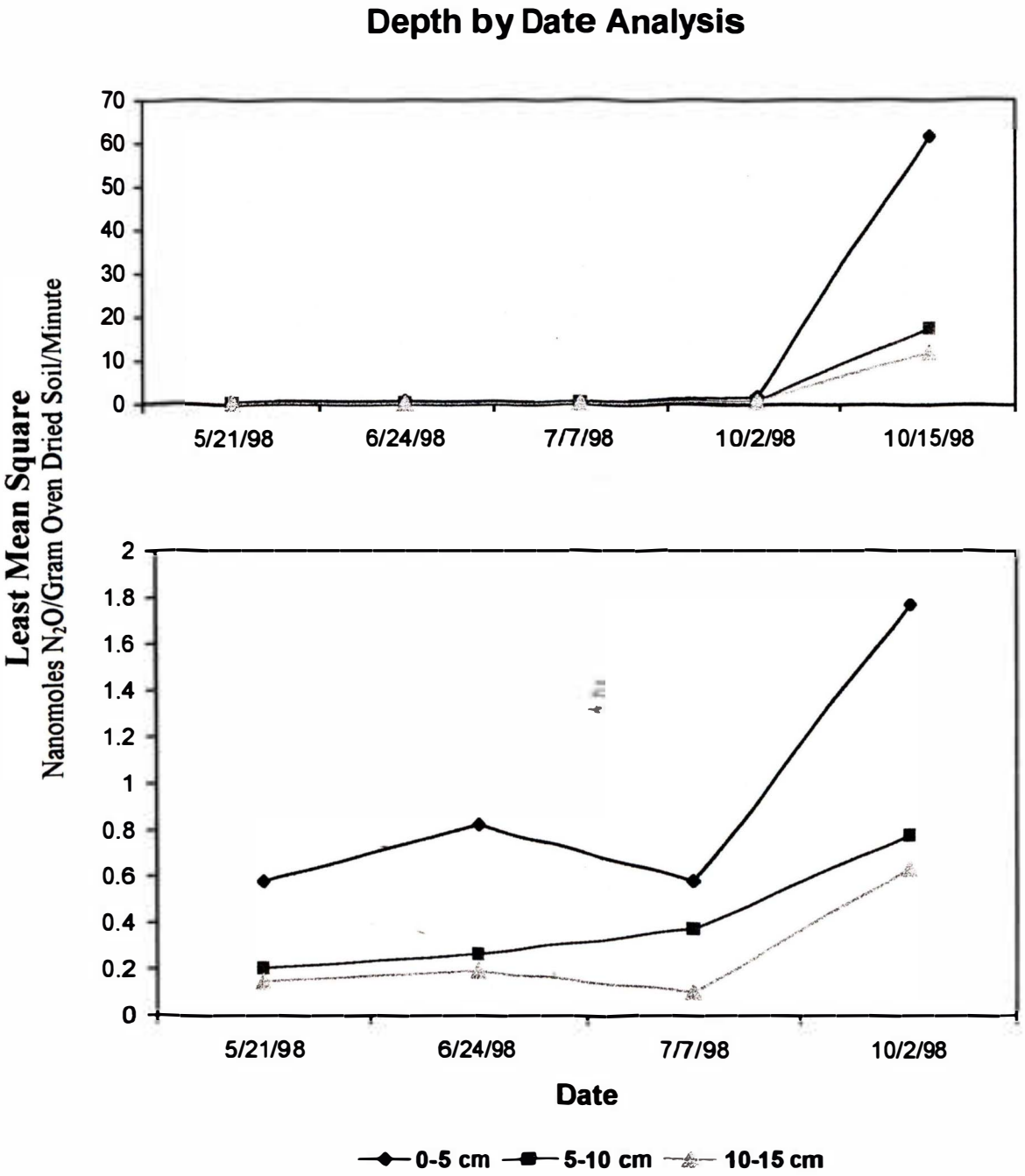
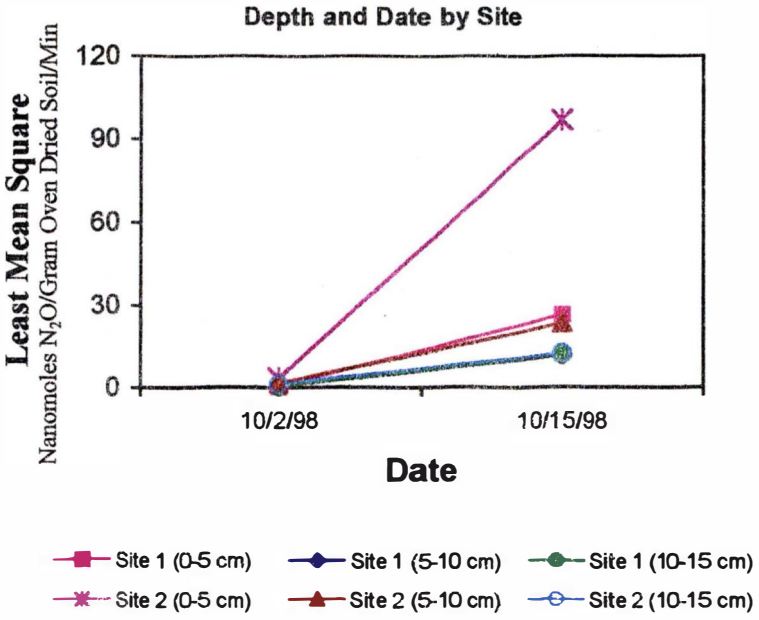



Figure 3.13. SAS mean comparison for depth by date. $N_2O$ rate analysis on two different rate scales for more detail on May through October 2nd 1998 results.



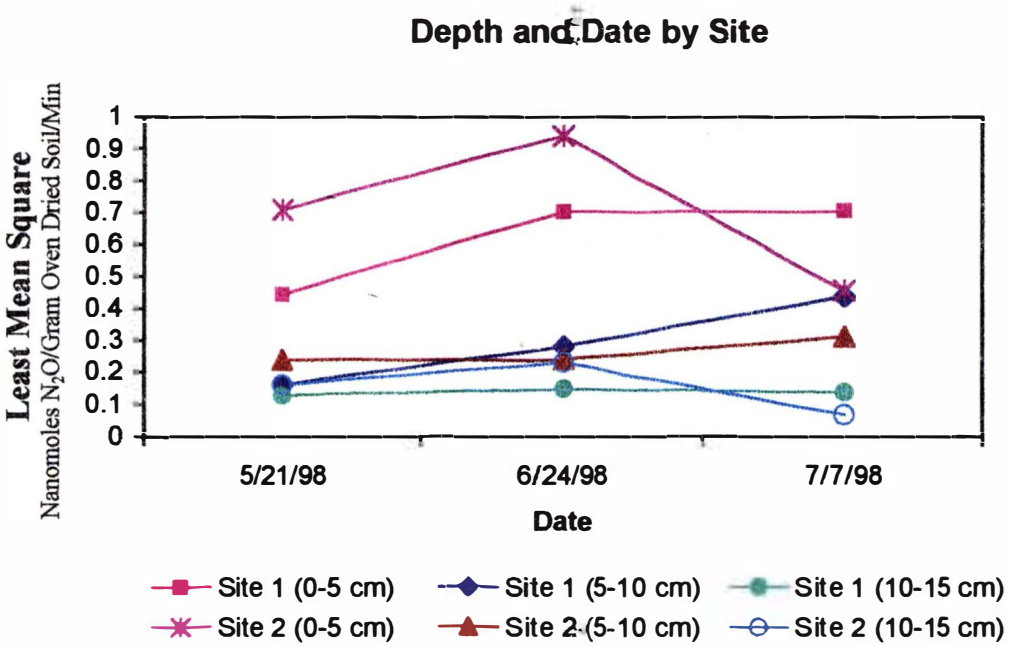




Figure 3.14. SAS mean comparison for depth and date by site results. $N_2O$ rates are on two different rate scales for more detail on May through July 7th, 1998 results.

Percent water content was analyzed for wetland 5 soil by depth (Table 3.6). The 0-5 cm increment always contained more water by weight than the other increments. The lowest percent water by weight value was 20.4 (on 10-2-98) and was found in the 10-15 cm depth increment. The highest water percent by weight was 77.1 (on 6-24-98) and was found in the 0-5 cm depth increment (Figure 3.33-35). Correlations between Phase I rates and percent water contents were analyzed and found to explain 3% of the variability in soil $N_2O$ production (Table 3.17).

Nitrate analyses showed nitrate presence and availability were highest in May and in August with the largest decreases in concentration in June and October (Figure 3.36 and Table 3.7; Appendices C). Other studies (Kringen, 1998) also reported nitrate increases in wetland surface and groundwater during the months of May and June 1994 and 1995, with nitrate concentration decreasing steadily throughout the growing season and into the fall. The increase in nitrate concentration could be due to fertilizer applications. This study looked at soil nitrate concentration by depth and noted a decrease in concentration by depth. Nitrate concentrations reached 26 ppm and had an average of 11 ppm in the 0-5 cm increment, and averaged 8 ppm in the 5-10 cm depth increment and 7 ppm in the 10-15 cm depth increment. All depths experienced a decrease in concentration during the months of June through October (Figure 3.36; Appendices C). The Kringen study (1998) noted the same trend in wetland surface and groundwater nitrate concentrations that could be due to biological activity. In the spring temperatures of soil and water would rise, water levels would increase due to spring thaw, and field application of nitrate or applications of manure combined with increased

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precipitation and runoff would replenish nitrate supplies for denitrifying organisms. SAS analysis confirmed the nitrate differences with depth (Pr > F = 0.0007; Table 3.16)(Figure 3.15), by date (Pr > F = 0.0001; Table 3.16)(Figure 3.16), and with depth by date (Pr > F = 0.0039; Table 3.16)(Figure 3.17).

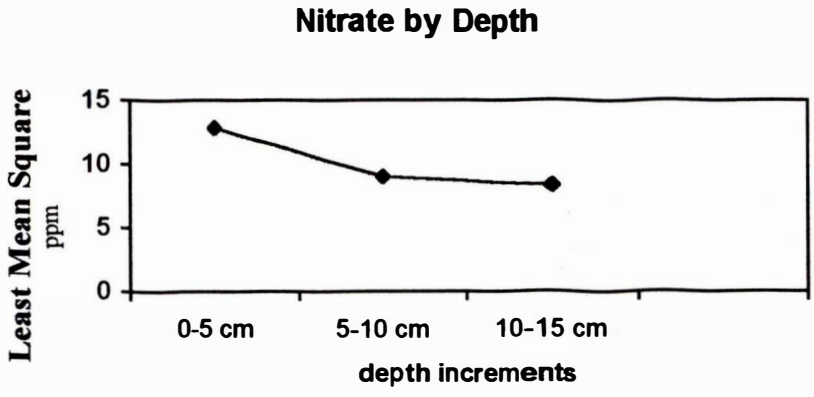


Figure 3.15. SAS mean comparison for nitrate by depth

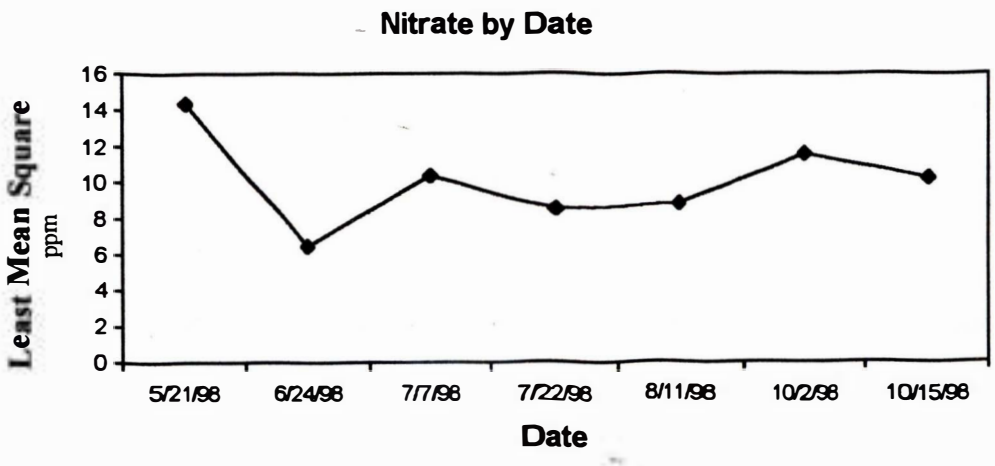


Figure 3.16. SAS mean comparison for nitrate by date.

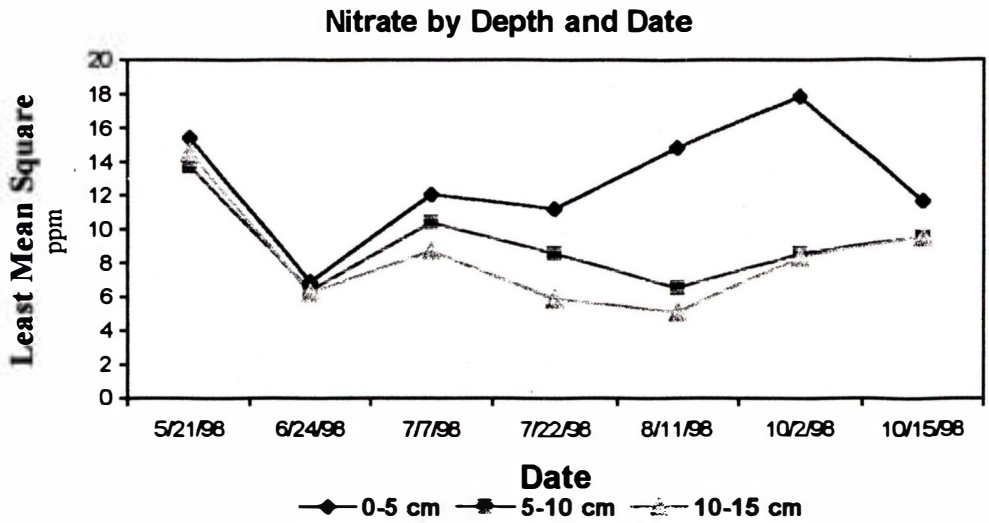


Figure 3.17. SAS mean comparison for nitrate by depth and date.

Soil organic matter showed decreases by depth on the dates it was analyzed and little difference over the sampling period (Figure 3.37 and Table 3.8; Appendices C). The decrease in organic matter by depth could be attributed in part to fewer roots by the 10-15 cm depth. Differences by site and date could be attributed in part to plant differences by site. Plants found at Site 1 were largely cattail and duckweed, and Site 2 was mostly a thick mat of pondweed. Cattail plant structure is tougher and possibly harder for microbes to degrade than the pondweed's structure, which is much softer. All factors combined could account for the site by depth by date differences. SAS analysis confirmed the depth differences with a Pr > F of 0.0001(Table 3.15)(Figure3.18), a site by date significance with a Pr > F of 0.0016 (Table 3.15)(Figure 3.19), and a site by depth by date significance with a Pr > F of 0.0232 (Table 3.15)(Figure 3.20).

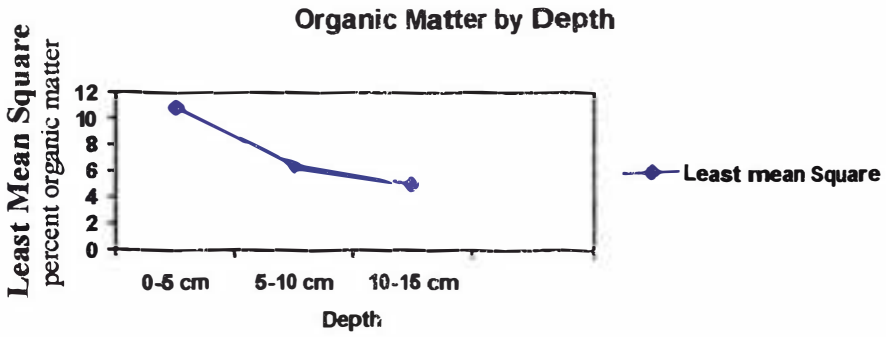


Figure 3.18. SAS mean comparison for organic matter by depth.

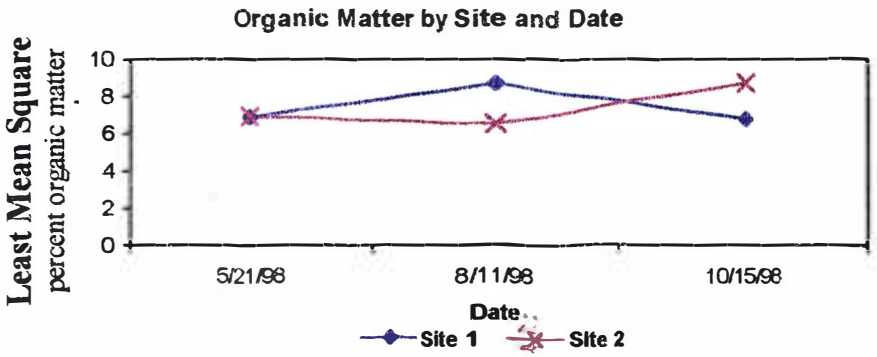


Figure 3.19. SAS mean comparison for percent organic matter by site and date.

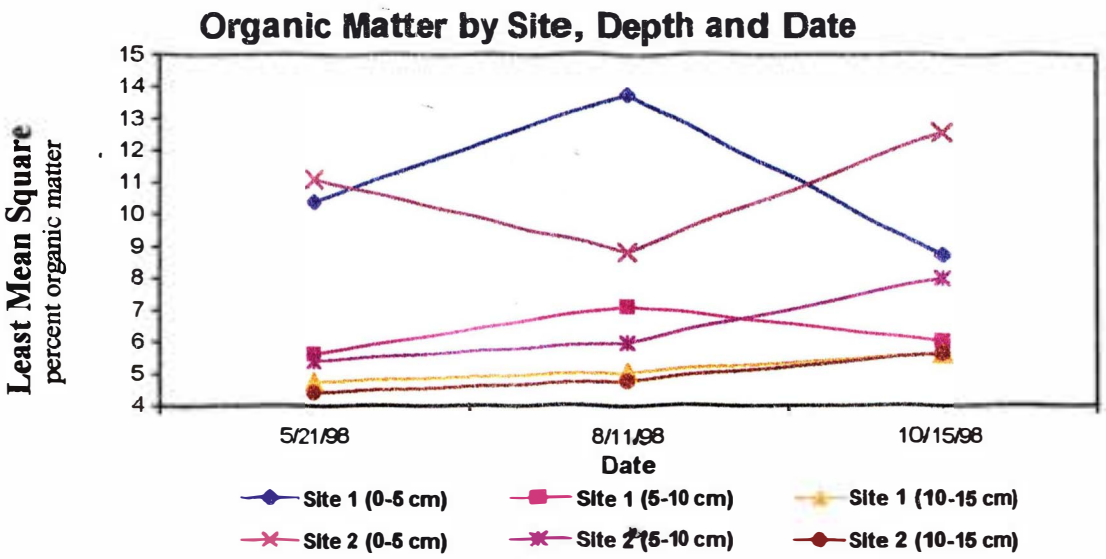


Figure 3.20. SAS mean comparison for percent organic matter by site, depth and date.

Soil pH was at or near pH 7 for the month of May with little or no difference by depth. For the August and the October 2nd sampling dates the pH had dropped by half a unit and showed a rise in pH by one half to three quarters of a unit on the October 15th sampling date (Figure 3.38 and Table 3.9; Appendices C). Differences in pH could be due to the amount and/or type of organic matter being degraded and water content of the soil. As organic matter is decomposed different organic compounds are formed which, depending on their identity, could either raise or lower pH (Paul and Clark, 1989). These differences were confirmed by SAS analysis where Pr > F = 0.0001(Table 3.14)(Figures 3.21 and 3.22).

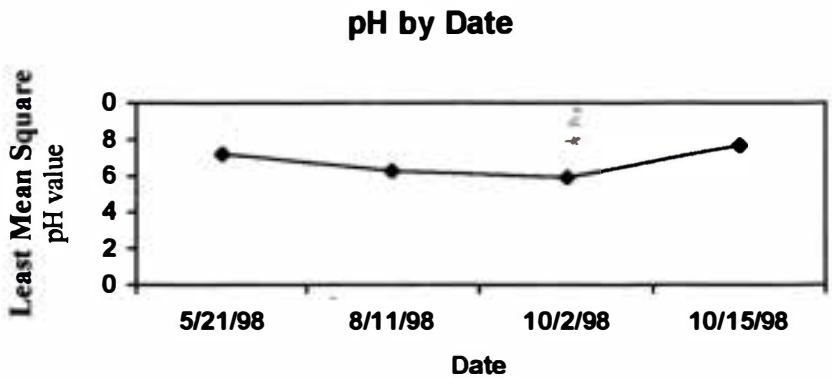


Figure 3.21. SAS mean comparison for pH by date.

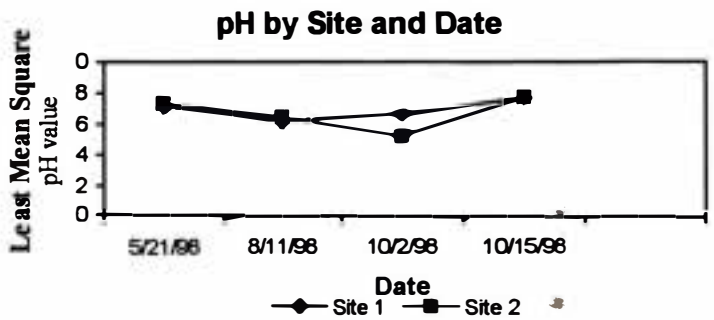


Figure 3.22. SAS mean comparison for pH by site and date.

Salts, potassium and phosphorus concentrations were analyzed for the May 21, 1998 sampling only. Salt concentrations generally showed an increase as depth increased (Table 3.10; Figures 3.39; Appendices C). This follows the trend for flow-through wetlands. Semi-permanent wetlands can exhibit both recharge and discharge characteristics. Water movement in a recharge wetland is both horizontal and vertical in soil on its borders. Water movement for discharge is an inward seepage from surrounding soil environments. As the water flows through calcium carbonate moves with the water and is deposited at waterfronts. In this case the calcium carbonate is moved down and out through the soil profile (Machaeck, 1995).

Potassium concentrations according to the chart below were very high. Phosphorus concentrations were within the medium to high range (Figure 3.40 and Table 3.12; Appendices C) and were supported by an earlier study (Kringen, 1998) that reported a phosphorus value of 29 to 43-mg/L for wetland 5 soil cores at the 0-15 cm depth. Approximate nutrient levels found in a variety of soils are listed below (Table 3.11).

Table 3.11. Soil Phosphorus and potassium test levels
(Adapted from Miller and Donahue, 1995)

| P lb/a | K lb/a | Soil Test Level |
|---|---|---|
| 0-10 | 0-80 | Very Low |
| 11-20 | 81-150 | Low |
| 21-30 | 151-210 | Medium |
| 31-70 | 211-300 | High |
| 71+ | 301+ | Very High |

High organic matter content of wetland sediments and application of manure or fertilizer may explain the relatively high levels of phosphorus and potassium.

Correlations between pH, organic matter, salt, percent water content, and nitrate were sought and presented in Table 3.17. All correlations were found to be significant with 2.09% of the variability found in the effect of pH concentrations, 0.6 % variation due to salt concentration, 24.5% of the variability due to organic matter variations, 0.004% of the variability due to the nitrate concentration, and 25.26 % of the variation due to percent water content. From this information, a five-variable model (Table 3.17) for predicting the variable's effects on $N_2O$ rates was obtained:

$\widehat{N_2O}$ = - 63449 + 8017 * pH –979* salt + 375 * organic matter + 266 * nitrate + 66* percent water

This model may explain 35 % of the variability found in the Phase I denitrification rates. Further experimentation to test the model to see if it has predictive power would be desirable.

The data support the hypotheses that DEA rates would differ by depth increments and that DEA rates would differ by season. The data did not indicate significant differences between site one and site two. Further work should be done to profile potential denitrifiers and denitrification in the soil by depth. This could give a better understanding of the fate of nitrates as they pass though the soil profile in such wetlands and sometimes enter groundwater.

Table 3.13. SAS results for regular phase I by depth results

# Phase 1 Rates

## Analysis of Variance
(ANOVA)

| Source | NDF[1] | DDF[2] | Type III F[3] | Pr > F[4] |
|---|---|---|---|---|
| Site | 1 | 4 | 1.11 | 0.3525 |
| Depth | 2 | 8 | 4.45 | 0.0502 (95%) |
| Site* Depth | 2 | 8 | 2.06 | 0.1903 |
| Date | 4 | 16 | 4.57 | 0.0119 (98.8%) |
| Site*Date | 4 | 16 | 0.95 | 0.4633 |
| Depth*Date | 8 | 32 | 3.92 | 0.0025 (99.75%) |
| Site*Depth*Date | 8 | 32 | 1.87 | 0.0992 (90.08%) |

[1]NDF is the number of degrees of freedom.

[2]DDF is the denominator degrees of freedom.

[3]Type III F equals a type III sum of squares. In this type data have been adjusted for all other-effects in the model and are used to analyze data sets with missing data and calculate a F value.

[4]Pr > F is the probability that the calculated F value is going to be greater than the F table value.

Table 3.14. SAS results for pH

**pH**

**Analysis of Variance**
(ANOVA)

| Source | NDF[1] | DDF[2] | Type III F[3] | Pr > F[4] |
|---|---|---|---|---|
| Site | 1 | 12 | 1.06 | 0.3243 |
| Depth | 2 | 12 | 1.13 | 0.3545 |
| Site* Depth | 2 | 12 | 0.65 | 0.5419 |
| Date | 3 | 36 | 10.03 | 0.0001 (99.99%) |
| Site*Date | 3 | 36 | 2.34 | 0.0901 (90.99%) |
| Depth*Date | 6 | 36 | 0.72 | 0.6398 |
| Site*Depth*Date | 8 | 32 | 1.87 | 0.8623 |

[1]NDF is the number of degrees of freedom.

[2]DDF is the denominator degrees of freedom.

[3]Type III F equals a type III sum of squares. In this type data have been adjusted for all other-effects in the model and are used to analyze data sets with missing data and calculate a F value.

[4]Pr > F is the probability that the calculated F value is going to be greater than the F table value.

Table 3.15. SAS results for percent organic matter

## Percent Organic Matter

### Analysis of Variance
(ANOVA)

| Source | NDF[1] | DDF[2] | Type III F[3] | Pr > F[4] |
|---|---|---|---|---|
| Site | 1 | 12 | 0.01 | 0.9207 |
| Depth | 2 | 12 | 77.95 | 0.0001 (99.99%) |
| Site* Depth | 2 | 12 | 0.09 | 0.9129 |
| Date | 2 | 24 | 1.54 | 0.2352 |
| Site*Date | 2 | 24 | 8.54 | 0.0016 (99.84%) |
| Depth*Date | 4 | 24 | 0.62 | 0.6538 |
| Site*Depth*Date | 4 | 24 | 3.45 | 0.0232 (97.68%) |

[1]NDF is the number of degrees of freedom.

[2]DDF is the denominator degrees of freedom.

[3]Type III F equals a type III sum of squares. In this type data have been adjusted for all other effects in the model and are used to analyze data sets with missing data and calculate a F value.

[4]Pr > F is the probability that the calculated F value is going to be greater than the F table value.

Table 3.16. SAS results for soil nitrate

**Soil Nitrate in ppm**
**Analysis of Variance**
(ANOVA)

| Source | NDF[1] | DDF[2] | Type III F[3] | Pr > F[4] |
|---|---|---|---|---|
| Site | 1 | 12 | 3.01 | 0.1081 |
| Depth | 2 | 12 | 14.36 | 0.0007 (99.92%) |
| Site* Depth | 2 | 12 | 0.70 | 0.5145 |
| Date | 6 | 72 | 10.94 | 0.0001 (99.99%) |
| Site*Date | 6 | 72 | 1.11 | 0.3656 |
| Depth*Date | 12 | 72 | 2.76 | 0.0039 (99.61%) |
| Site*Depth*Date | 12 | 72 | 0.69 | 0.7554 |

[1]NDF is the number of degrees of freedom.

[2]DDF is the denominator degrees of freedom.

[3]Type III F equals a type III sum of squares. In this type data have been adjusted for all other effects in the model and are used to analyze data sets with missing data and calculate a F value.

[4]Pr > F is the probability that the calculated F value is going to be greater than the F table value.

Table 3.17. Correlation analysis between the variables pH, organic matter, salt and nitrate (SAS Institute Inc. 1990).

## Correlation Analysis

5 'WITH' Variables: pH Salt Organic matter Nitrate Percent water
1 'VAR' Variables: N2O

Simple Statistics

| Variable | N | Mean | Std. Dev | Sum | Minimum | Maximum |
|---|---|---|---|---|---|---|
| PH | 342 | 7.13 | 0.29 | 2440.10 | 5.30 | 7.50 |
| Salt | 1008 | 3.36 | 0.65 | 3385.60 | 2.00 | 4.30 |
| Organic Matter | 342 | 6.87 | 3.24 | 2350.60 | 3.90 | 14.50 |
| Nitrate | 1008 | 10.28 | 4.14 | 10363 | 1.00 | 26.00 |
| Percent Water | 972 | 48.31 | 12.60 | 26958 | 31.00 | 79.12 |
| $N_2O$ | 972 | 24.83 | 37.05 | 24140 | -3.93 | 253.32 |

Pearson Correlation Coefficients/ Prob > /R/ under Ho: Rho=O / Number of Observations

| | $N_2O$ |
|---|---|
| pH | -0.14461<br>0.0091<br>324 |
| SALT | -0.07753<br>0.0156<br>972 |
| ORGANIC MATTER | 0.49456<br>0.0001<br>324 |
| NITRATE | 0.0697<br>0.8281<br>972 |
| PERCENT WATER | 0.50258<br>0.0001<br>972 |

## GENERAL DISCUSSION AND CONCLUSIONS

The major purpose of this study was to investigate denitrification processes in selected South Dakota prairie pothole wetlands. Initially MPNs were used to give an estimate of DNRA and denitrifying populations present in the three selected semi-permanent prairie pothole wetlands associated with different farming practices. It was hypothesized that DNRA bacteria would be present in higher numbers in the lowland sediment soil because of the increased carbon supplies available there compared to upland soils. The results from this study indicated that from 8-12-93 to 11-6-93 there was a steady decrease in DNRA bacterial population. DNRA bacteria numbers were higher in the upland and lowland soils compared to the denitrifying bacteria numbers. In 1994 MPN counts were higher overall (possibly due to 1993 being an extremely wet year) than the 1993 counts. Still the study showed that DNRA bacteria were present in higher numbers compared to the denitrifying bacteria and did not support the original hypothesis. Percent water contents were examined in an effort to explain temporal differences in wetland microbial populations. Correlations were found and percent water content explained 3 % of the variability. Possibly temporal differences were also due to different optimal temperatures for microorganism growth, differences in amounts of organic matter available, and reduced competition for nitrate between microbes and wetland plants at certain times of the year.

The next phase of this study investigated the denitrification enzyme activity of prairie pothole soils in the different agricultural systems. It was hypothesized that lowland soils would have greater DEA values compared to upland soil, and that organic

farming practices would support increased DEA activity compared to the TNT and CON farm management types.

Modified Phase I assays tested if carbon or nitrate were limiting to the DEA activity present in the wetland soils. Results suggested site 2 lowland soils of wetland 8 were nitrate limited and glucose limited. These limitations were not apparent for sites on wetland 5 or 13.

Regular Phase I assays were conducted according to established protocol (Tiedje, 1982). In these assays acetylene blocked the $N_2O$ reductase step, and chloramphenicol prevented new protein synthesis in the soil slurry. Phase I assays only measure the soil's existing denitrifying enzyme activity. During the sampling period the lowland soil samples consistently showed higher DEA values compared to upland soils for all three wetlands, and a temporal increase in rates was apparent for all wetlands and sites. The differences in lowland versus upland values may be due to the increased organic matter carbon and nitrate available to the bacteria and the longer periods of anoxia experienced in lowland sediment compared to upland soil. No significant differences were noted between farming practices.

The final phase of this study looked at denitrifying enzyme activity by depth in one prairie pothole semi-permanent wetland. The hypothesis tested was that DEA activity would be higher in the top layers, would decrease by depth and be influenced by wet-dry cycles near shore (site 1). Previous work in our laboratory (Boetel, 1996) and other investigators (Macfarlane and Herbert, 1984; Sorensen, 1978) have shown increased numbers of denitrifiers present in the top layers of lowland sediments. In

addition site variation was investigated, with site 1 near shore and site 2 in the lowland interior. Site 1 experienced wet-dry cycles that could provide a more hospitable environment for denitrifying bacteria than site 2, and possibly create a rate difference between shoreline and deeper sites.

Natural denitrification potentials were assayed at 0, 4.5, 19.5, 24, and 28 hour sampling periods with the exception of one assay set (set 5) that was carried out with time intervals similar to Phase I assay times. In this set the 5-10 cm segment had larger DEA values. In other sets with larger time intervals the rate order was 10-15 cm > 5-10 cm > 0-5 cm. Differences in organic matter composition with depth might help account for this. On the sediment surface soil fauna activity can increase the amount of oxygen supplied to the littoral layer, causing incomplete anoxia. Denitrifying bacteria could be present but not yet forced to use the denitrification pathway. Hemicelluloses from plant material are degraded faster than cellulose. Because of the heterogeneity of hemicelluloses not all may be consumed in the top layer. It is possible for hemicelluloses to move through the soil profile with the water to the 5-10 cm and 10-15 cm depths (Evangelou, 1998). In July 1998 as depth increased pH levels decreased (from pH 6.5 to 5.3) and soluble salts increased. Oxygen levels were probably reduced as depth increased, forcing denitrifiers to denitrify.

Chloramphenicol concentration used in the first Phase I analyses was 0.025 grams per liter. Literature published after my work suggested inhibitory effects to existing denitrifying enzymes at this chloramphenicol concentration (Murray and Knowles, 1999; Pell et al, 1996; Brooks et al., 1992; Dendooven et al., 1994; Wu and Knowles, 1995).

Chloramphenicol is supposed to stop new protein synthesis, and it also may inhibit existing enzymes at concentrations greater than 0.1 g/L. Phase I assays with varying chloramphenicol concentrations were set up for wetland 5 soil. The concentrations used were 0.0, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, and 1.0 g/L. Results showed rates to vary at different chloramphenicol concentrations, with similar DEA rates for (0.0, 0.05,0.075 g/L), similar rates for (0.1, 0.15, 0.25 g/L), and similar rates for (0.5, 0.75, 1.0 g/L). Rates were graphed and two best-fit lines were drawn. The concentration that was the cross point for both lines was the 0.25 g/L concentration and so chosen for this study.

Regular phase I assay results were graphed using two different y-axis rate scales. October samples were at least one order of magnitude higher than other sample dates at all depth increments. The 0-5 cm depth for both sites had higher rates (nearly one order of magnitude higher than other depth increments). These rates were comparable to the 6-5-96 rates and also were in agreement with Boetel's research. The 5-10 cm depth rates were higher than the 10-15 cm rates but not by an order of magnitude. Percent water content decreased by depth. Correlations analysis found percent water content to account for 3% of the $N_2O$ rate variability. Denitrification overall was higher in May and June and decreased through summer, then rates increased in the fall. This correlates with nitrate levels in the wetland. In May nitrate levels were higher and denitrification rates were also higher. In June nitrate concentrations decreased and denitrification values were high. Possibly plant species were not yet competing for soil nitrates. The decrease in denitrification during the summer may have been due in part to plant uptake of available nitrates. In August and October (10-2-98) nitrate concentrations increased. This could

be due to plant die-off and less plant competition for soil nitrates. The second October date (10-15-98) showed a small decrease in nitrates, possibly due to microbial activity increasing, as plant competition for nitrates was probably no longer a factor. SAS analysis of data confirmed the differences of nitrate concentrations by depth and date (Pr>F= 0.0007; 0.0001), denitrification by depth (Pr>F= 0.0502) and date (Pr>F= 0.0119) and organic matter by depth (Pr>F= 0.0001), and site by date (Pr>F= 0.0016).

In water saturated soil pH can become more neutral, while phosphorus, potassium, and salts can become more soluble, and $N_2$ – fixation can increase (Miller and Donahue, 1995). Salt and pH were not found to be rate limiting for denitrification.

Soil pH varied little by depth. Temporally pH decreased from May (pH 7) to August (pH 6.0 to 5.3), and increased in the fall (pH 7.5 - 7.8) in the 0-5 cm depth. In the 10-15 cm segment the pH changes had less distinct patterns, possibly suggesting a buffering zone in the soil by depth. The temporal differences for pH were found significant by SAS analysis (Pr>F= 0.0001).

Salt concentrations increased by depth as would be expected for flow-through sites. As water moves through the soil profile salts leach with it. Lowland potassium concentrations for wetland 5 were higher than upland values and similar to potassium values reported in other research for wetland 5 (Kringen, 1998). Phosphorus concentrations in wetland sediment were medium to high compared to upland soils. At pH 6 to 7 phosphorus availability for plant uptake is at its highest, and next to nitrogen phosphorus is most important for plant growth (Brady, 1974).

Correlations between pH, organic matter, salt, percent water content, and nitrate were sought and found to be significant. Correlation table information suggests a five variable model to explain 35% of the variability in $N_2O$ rates within wetland 5 (organic farm management practices). The data gathered in this study support the hypothesis that denitrification rates would decrease by depth and differ by season.

# FUTURE DIRECTIONS

Certain topics relating to denitrification in South Dakota prairie pothole wetlands should be studied in greater detail.

1. Further testing of the model, presented in this study, is necessary to see if it has predictive power.
2. Future work to investigate potential denitrification needs to be done for the prairie pothole wetlands. Natural denitrification potential assays were done mostly for July samples. Other measures in May, June, August and November would give a full sample season to compare rates.
3. Future investigation to profile potential denitrifiers in soil by depth would be beneficial in understanding the fate of nitrate as it passes through the soil profile and enters groundwater.

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# Appendices

# A

# Chapter 1 Research Results Data

Table 1.4. Most-probable-numbers data for 1993

| | NB* Media | | | | TSB* Media | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cells per gram oven dried soil | | | | Cells per gram oven dried soil | | |
| Date | Wetland | Upland | lowland | check | Wetland | Upland | lowland | check |
| 8-12-93 | 5 | 170000 | 180000 | 920000 | 5 | 350000 | 1800000 | 1800000 |
| | 8 | 2800 | 40000 | 1710 | 8 | 930000 | 49000 | 1600000 |
| | 13 | | | 550000 | 13 | | | 1800000 |
| 8-21-93 | 5 | 280000 | 24000 | 177000 | 5 | 1600000 | 3800 | 364000 |
| | 8 | 36000 | 17000 | 9420 | 8 | 290000 | 7760 | 287000 |
| | 13 | 920000 | 33000 | | 13 | 430000 | 24000 | |
| 9-4-93 | 5 | 9350 | 37200 | 569 | 5 | 3910 | 31000 | 3250 |
| | 8 | 33100 | 31900 | 227 | 8 | 414000 | 507000 | 20400 |
| | 13 | 1650 | 643 | 327 | 13 | 20000 | 11300 | 280000 |
| 9-18-93 | 5 | 3110 | 876 | 697 | 5 | 200000 | 4380 | 180000 |
| | 8 | 503 | 581 | 310 | 8 | 14400 | 18800 | 310000 |
| | 13 | 1590 | 831 | 442 | 13 | 79500 | 27700 | 57000 |
| 10-10-93 | 5 | 317 | 1470 | 91500 | 5 | 79400 | 29300 | 197000 |
| | 8 | 456 | 446 | 105 | 8 | 38600 | 323000 | 2490 |
| | 13 | 3250 | 116000 | 338 | 13 | 16200 | 283000 | 5920 |
| 10-23-93 | 5 | 32300 | 68200 | 3230 | 5 | 1850000 | 390000 | 2080 |
| | 8 | 514 | 1050 | 276 | 8 | 4310 | 50900 | 32200 |
| | 13 | 777 | 858 | 520 | 13 | 25500 | 33700 | 82700 |
| 11-6-93 | 5 | 3500 | 8990 | 126 | 5 | 15400 | 3500 | 19500 |
| | 8 | 241 | 2480 | 259 | 8 | 9530 | 24800 | 22200 |
| | 13 | 277 | 4490 | 501 | 13 | 12700 | 35300 | 39800 |

* NB is nutrient broth; TSB is Tryptic soy broth.

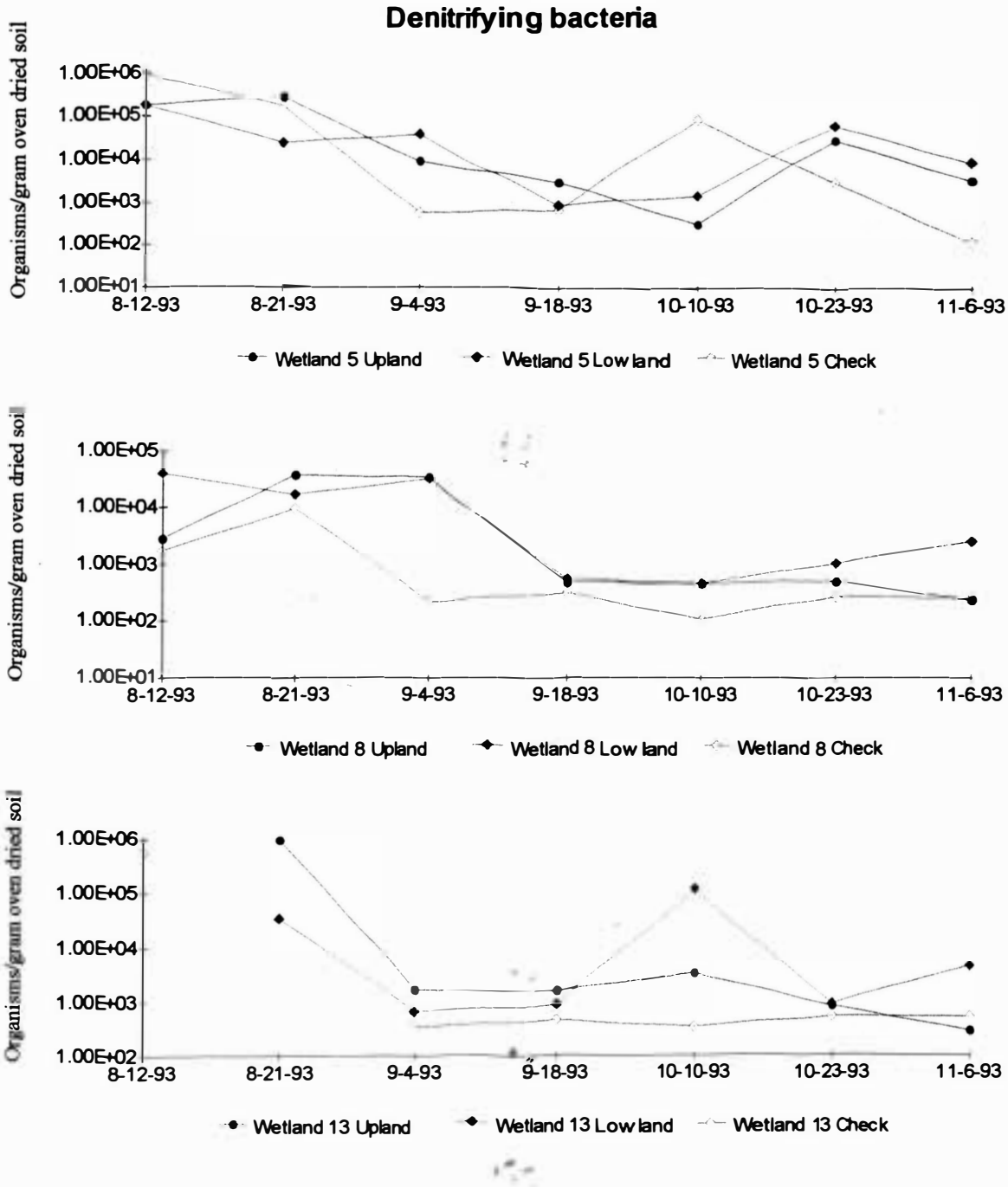



Figure 1.11. Most-probable-numbers for denitrifying bacteria in wetlands 5, 8, and 13 during the 1993 sampling season.

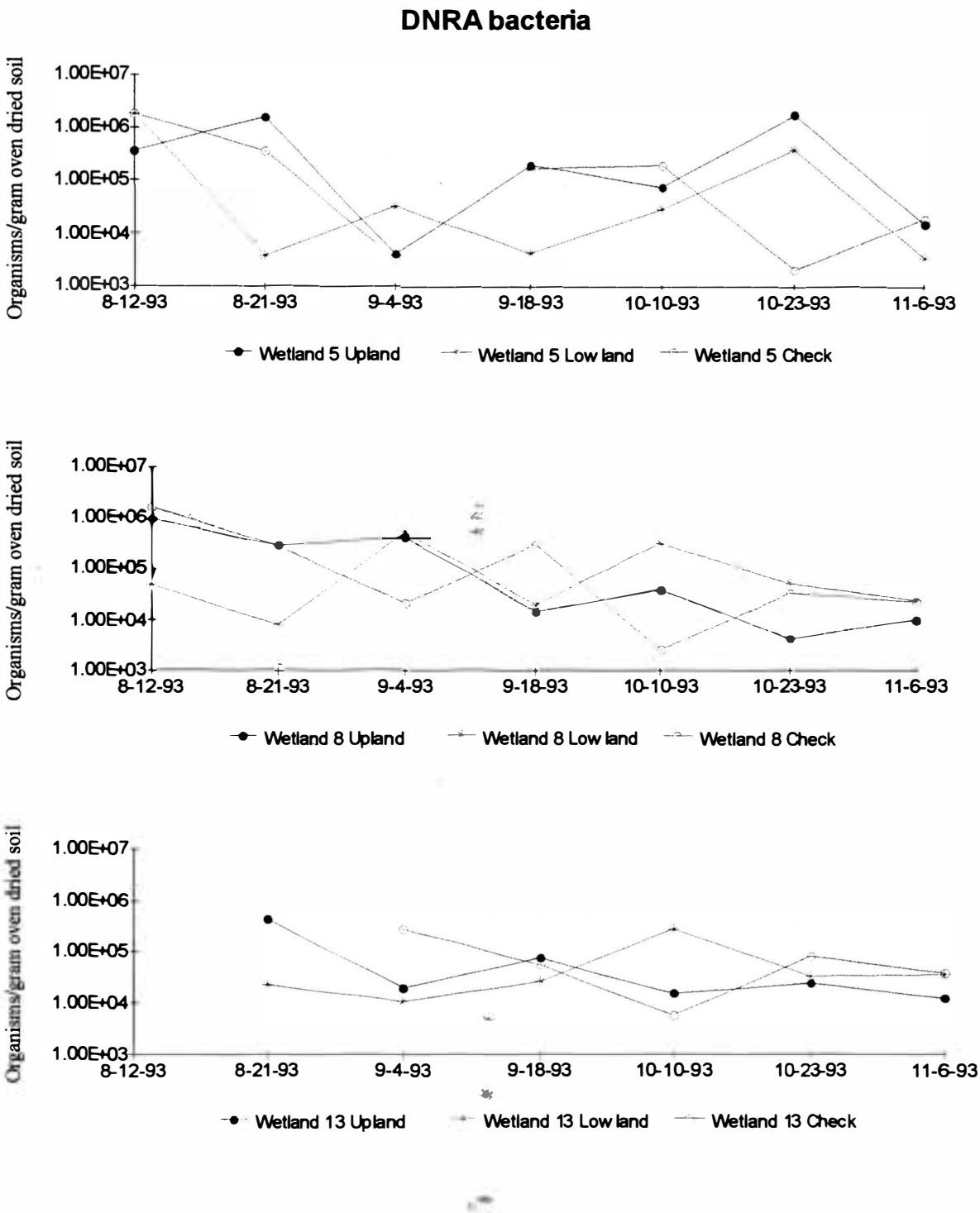



Figure 1.12. Most-probable-numbers for dissimilatory nitrate reduction to ammonia (DNRA) bacteria in wetlands 5, 8, and 13 during the 1993 sampling season.

Table 1.5. Percent water content at time of analysis for soil samples used in 1993 most-probable-number (MPN) analyses.

| | | 1st Year (1993) | | | |
|---|---|---|---|---|---|
| Date | Wetland | Upland | Lowland | Check | |
| 8-12-93 | 5 | 0.43 | 9.47 | 0.63 | |
| | 8 | 1.4 | 12.87 | 0.75 | |
| | 13 | | | 1.84 | |
| 8-21-93 | 5 | 1.3 | 7.73 | 3.92 | |
| | 8 | 1.85 | 17.56 | 2.33 | |
| | 13 | 2.32 | 24.41 | | |
| 9-4-93 | 5 | 15.5 | 24.8 | 13.9 | |
| | 8 | 14.6 | 31 | 11.7 | |
| | 13 | 15.2 | 37.8 | 14.4 | |
| 9-18-93 | 5 | 10 | 20.1 | 22.5 | |
| | 8 | 10.6 | 32 | 9.6 | |
| | 13 | 11.9 | 35 | 14 | |
| 10-10-93 | 5 | 11.8 | 25 | 13.7 | |
| | 8 | 14.2 | 35 | 11.6 | |
| | 13 | 13.8 | 39.9 | 17.2 | |
| 10-23-93 | 5 | 13.4 | 28.2 | 13.3 | |
| | 8 | 18.7 | 31.2 | 13 | |
| | 13 | 17.6 | 34.7 | 15.4 | |
| 11-6-93 | 5 | 20 | 28.8 | 12.7 | |
| | 8 | 17.1 | 31.5 | 19 | |
| | 13 | 13.4 | 37.7 | 12.1 | |

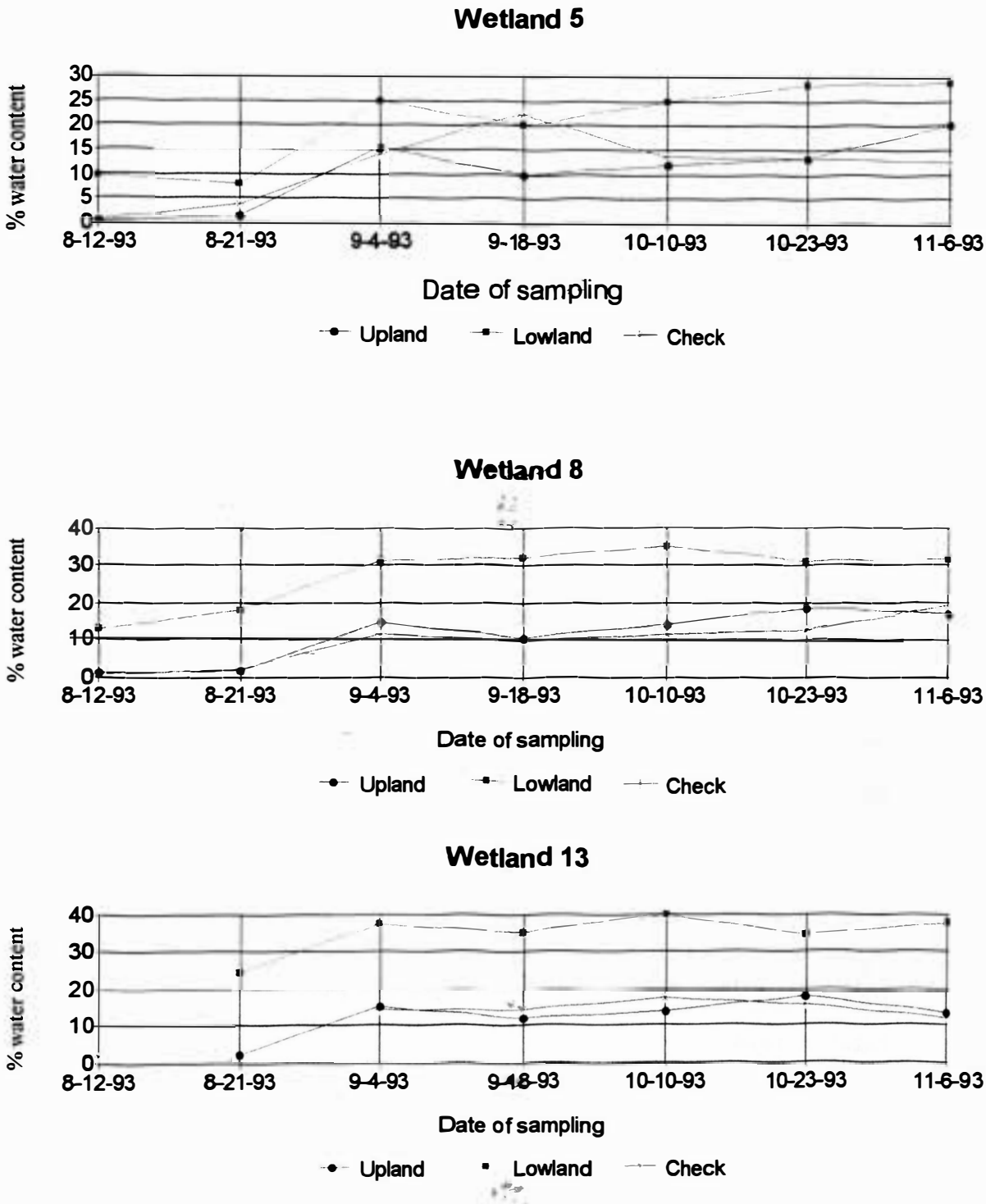



Figure 1.13. Percent water content for soils in wetlands 5, 8, and 13 during the 1993 sampling season.

Table 1.6a. Most-probable-numbers data for 1994 denitrifying bacteria

| NB* Media | | | | | | | |
|---|---|---|---|---|---|---|---|
| all units in per gram oven dried soil | | | | | | | |
| | | Upland | Lowland | Upland | Lowland | Upland | Lowland |
| Date | Wetland | Site 1 | | Site 2 | | Site 3 | |
| 4-16-94 | 5 | 5320 | 6090 | | 3550 | | |
| | 8 | 1320 | 625 | | 577 | | |
| | 13 | 215 | 2410 | | 653 | | |
| 5-7-94 | 5 | 5370 | 2500 | | 2020 | | |
| | 8 | 4190 | 281 | | 2650 | | |
| | 13 | 14000 | 3900 | | 143000 | | |
| 6-5-94 | 5 | 46100 | 1580000 | 24600 | 43000 | 150000 | 16200 |
| | 8 | 145000 | 22500 | 96000 | 22700 | 16800 | 21600 |
| | 13 | 10700 | 70300 | 41400 | 66000 | 32800 | 19600 |
| 6-19-94 | 5 | 46600 | 265 | 560 | 38500 | 354000 | 178000 |
| | 8 | 2980 | 678 | 2680 | 901 | 2280 | 859 |
| | 13 | 371 | 44117.6 | 381.5 | 18927.44 | 962.24 | 22606.38 |
| 7-2-94 | 5 | 2010000 | 691000 | 15000000 | 1090000 | 8620000 | 674000 |
| | 8 | 44600 | 2320000 | 26500 | 309000 | 31500 | 1840000 |
| | 13 | 7560000 | 292000 | 38600 | 21200 | 807000 | 46000 |
| 7-22-94 | 5 | 1320000 | 1320000 | 1970000 | 9960000 | 22600 | 77300 |
| | 8 | 193000 | 184000 | 804000 | 1000000 | 3720000 | 4480000 |
| | 13 | 1040000 | 6820000 | 2680000 | 40000 | 36900 | 3160000 |
| 9-17-94 | 5 | 380000 | 149000 | 141000 | 47000 | 655000 | 10000 |
| | 8 | 12160 | 21400 | 40800 | 10200 | 21300 | 2490 |
| | 13 | 20800 | 66900 | 25100 | 48800 | 51600 | 15700 |
| 10-10-94 | 5 | 145000 | 102000 | 2550000 | 22900 | 29000 | 202000 |
| | 8 | 4600 | 450000 | 9560 | 329000 | 40700 | 24500 |
| | 13 | 19500 | 10 | 6930 | 475000 | 200000 | 35000 |
| 10-23-94 | 5 | 1960000 | 15000000 | 5000000 | 2470000 | 185000000 | 195000 |
| | 8 | 14800 | 200000 | 32900 | 60700 | 2240000 | 48000 |
| | 13 | 2300000 | 5750000 | 1950000 | 44100 | 2800000 | 1660000 |
| 11-5-94 | 5 | 44700 | 167000 | 4900 | 81300 | 39400 | 37500 |
| | 8 | 14000 | 440000 | 7700 | 11000 | 25500 | 26600 |
| | 13 | 37600 | 2330000 | 7090 | 2310000 | 37500 | 11100000 |

* NB is nutrient broth

Table 1.6b. Most-probable-numbers data for 1994 DNRA bacteria

| TSB* Media | | | | | | | |
|---|---|---|---|---|---|---|---|
| all units in per gram oven dried soil | | | | | | | |
| | | Upland | Lowland | Upland | Lowland | Upland | Lowland |
| Date | Wetland | Site 1 | | Site 2 | | Site 3 | |
| 4-16-94 | 5 | 11000 | 107 | | 81.1 | | |
| | 8 | 2760 | 16000 | | 7250 | | |
| | 13 | 13100 | 43100 | | 19900 | | |
| 5-7-94 | 5 | 25700 | 18000 | | 1880 | | |
| | 8 | 30500 | 7660 | | 120 | | |
| | 13 | 17800 | 3900 | | 143000 | | |
| 6-5-94 | 5 | 149000 | 1580000 | 92600 | 456000 | 1340000 | 211000 |
| | 8 | 424000 | 1850000 | 32800 | 44000 | 20500 | 41000 |
| | 13 | 56800 | 770000 | 1150000 | 646000 | 207000 | 238000 |
| 6-19-94 | 5 | 23900 | 85.8 | 12800 | 619 | 291 | 44400 |
| | 8 | 9880 | 110000 | 20700 | 286 | 53200 | 554 |
| | 13 | 1102.1 | 29411.765 | 3815.03 | 5520.5 | 475.03 | 252.66 |
| 7-2-94 | 5 | 200000000 | 18000000 | 30700000 | 14800000 | 660000 | 3850000 |
| | 8 | 881000 | 31400 | 38000 | 1970000 | 385000 | 6000 |
| | 13 | 194000 | 6820000 | 386000 | 46200 | 807000 | 99100 |
| 7-22-94 | 5 | 6660000 | 1320000 | 3610000 | 8830000 | 5720000 | 5550000 |
| | 8 | 20500000 | 5380000 | 25300000 | 31700000 | 10700000 | 73600 |
| | 13 | 1530000 | 11000000 | 35800 | 4000000 | 1610000 | 25900 |
| 9-17-94 | 5 | 10000000 | 1290000 | 60900 | 218000 | 218000 | 43800 |
| | 8 | 59600 | 757000 | 1560000 | 81700 | 163000 | 67900 |
| | 13 | 420000 | 1340000 | 586000 | 325000 | 353000 | 110000 |
| 10-10-94 | 5 | 547000 | 363000 | 3660000 | 310000 | 39000 | 312000 |
| | 8 | 30000 | 7150000 | 8980 | 1000000 | 263000 | 317000 |
| | 13 | 109000 | 34000 | 55000 | 89300 | 65000 | 320000 |
| 10-23-94 | 5 | 323000 | 54300 | 3060000 | 78500 | 10600000 | 86400 |
| | 8 | 972000 | 2330000 | 3160000 | 2790000 | 4600000 | 262000 |
| | 13 | 15000000 | 8050000 | 1160000 | 1300000 | 3600000 | 8170000 |
| 11-5-94 | 5 | 246000 | 361000 | 26200 | 1650000 | 76300 | 504000 |
| | 8 | 19800 | 9330000 | 23700 | 40900 | 568000 | 16200 |
| | 13 | 37600 | 4520000 | 39500 | 4480000 | 1520000 | 6880000 |

* TSB is Tryptic soy broth

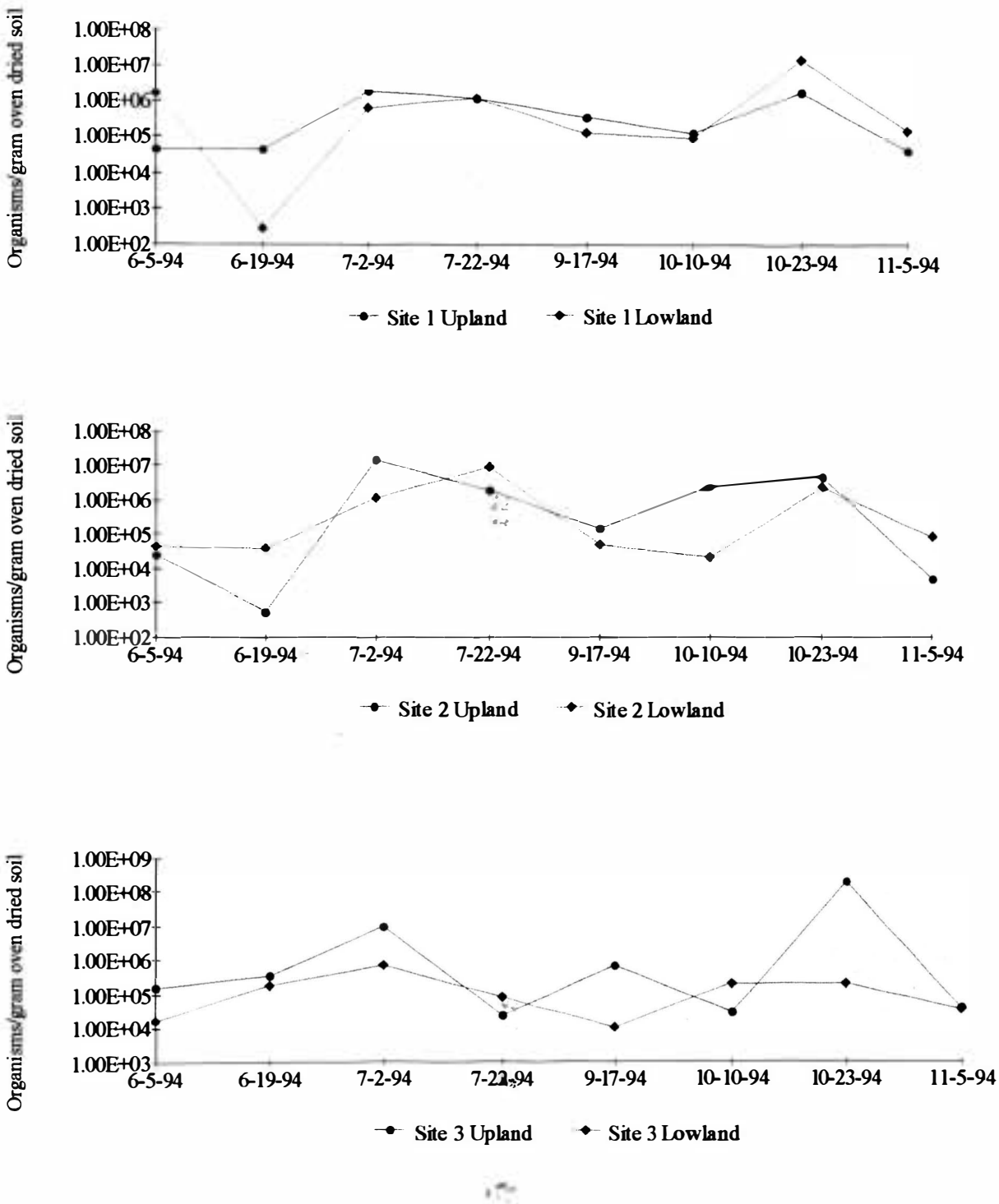



Figure 1.14. Most-probable-numbers for wetland 5 denitrifying bacteria during the 1994 sampling season.

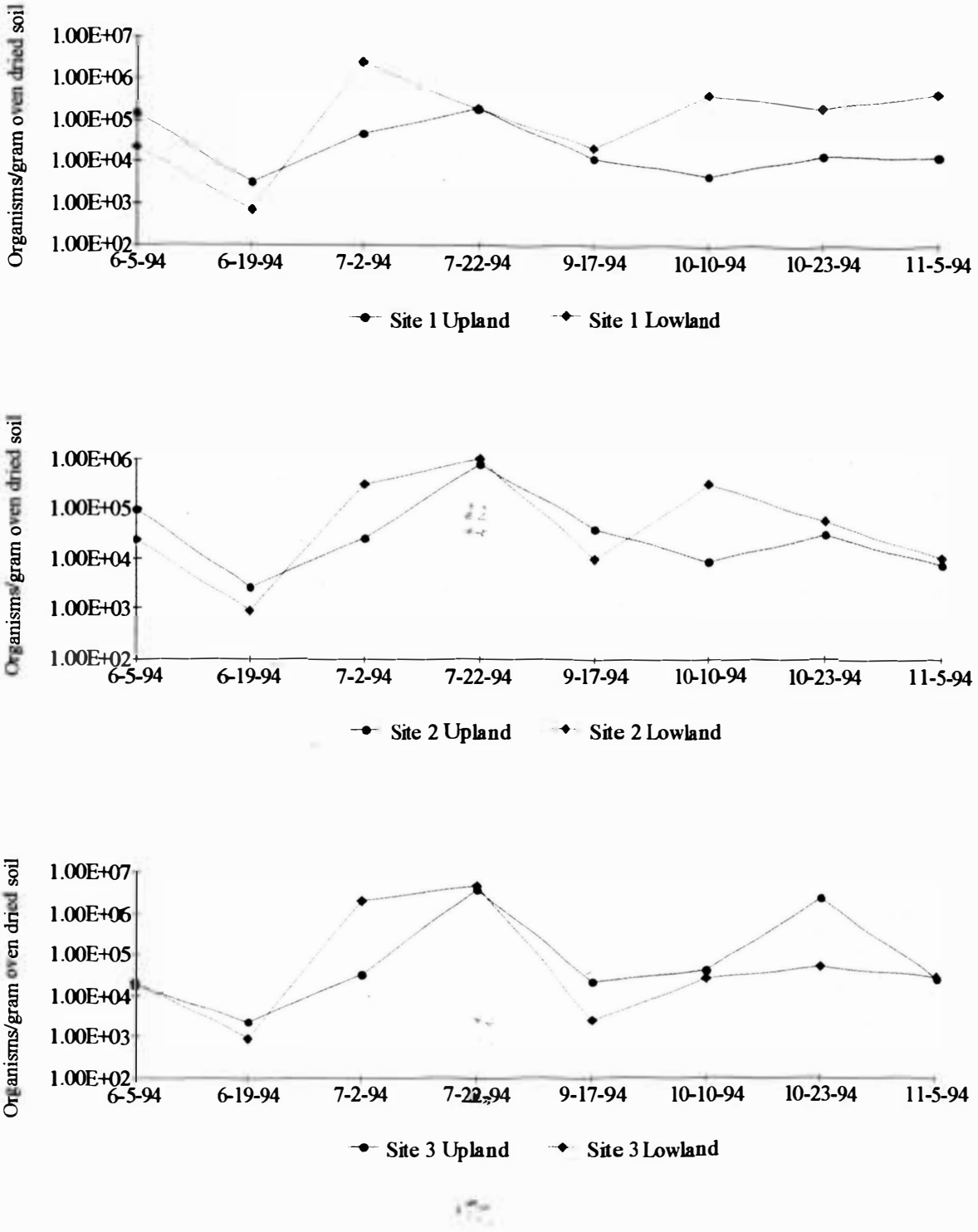



Figure 1.15. Most-probable-numbers for wetland 8 denitrifying bacteria during the 1994 sampling season.

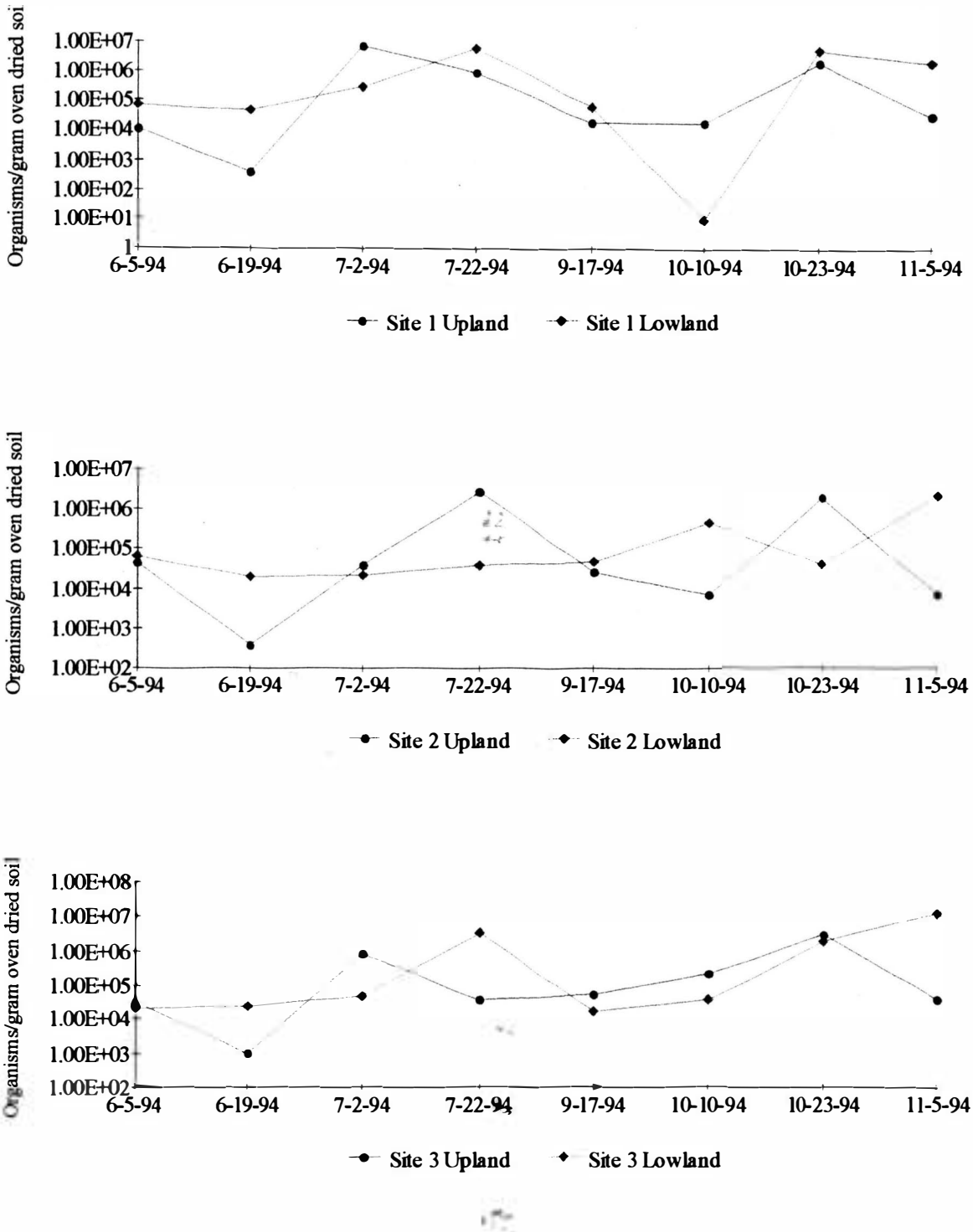
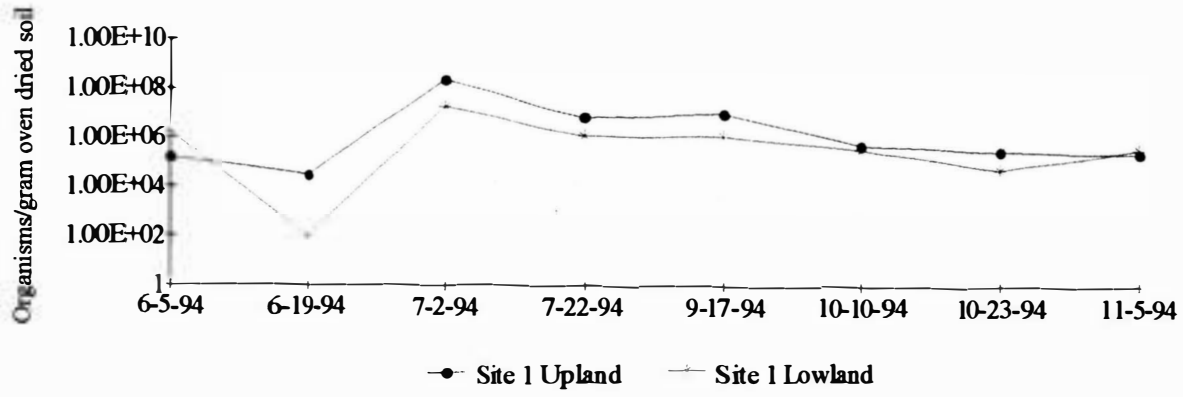
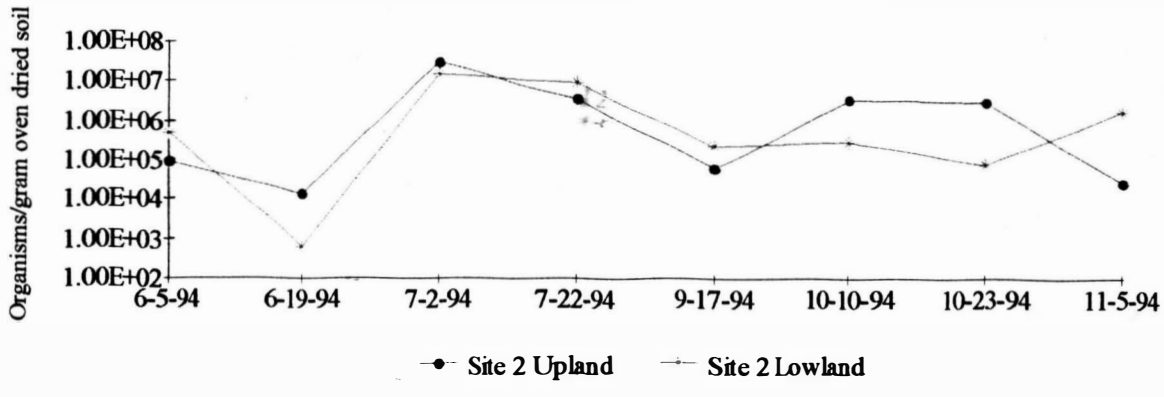



Figure 1.16. Most-probable-numbers for wetland 13 denitrifying bacteria during the 1994 sampling season.



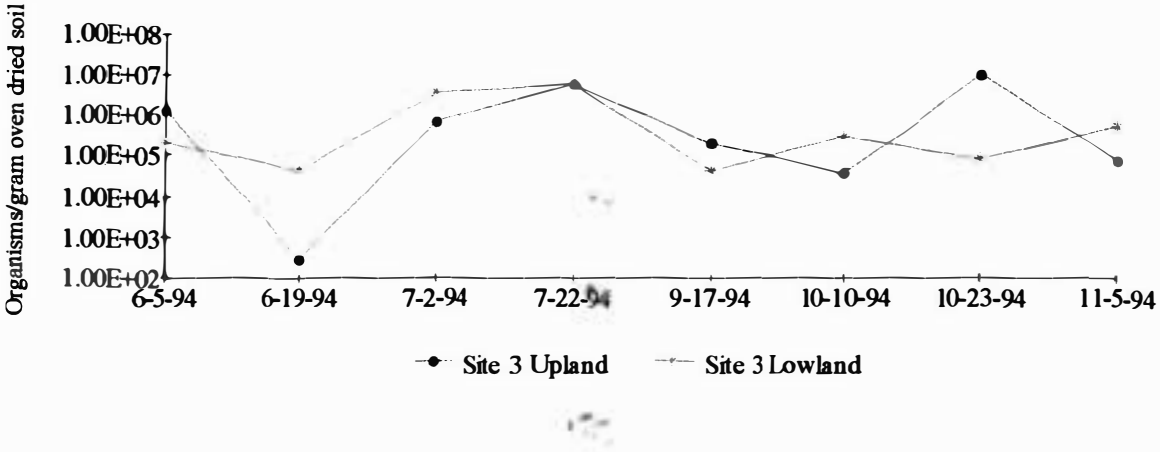




Figure 1.17. Most-probable-numbers for wetland 5 DNRA bacteria during the 1994 sampling season.

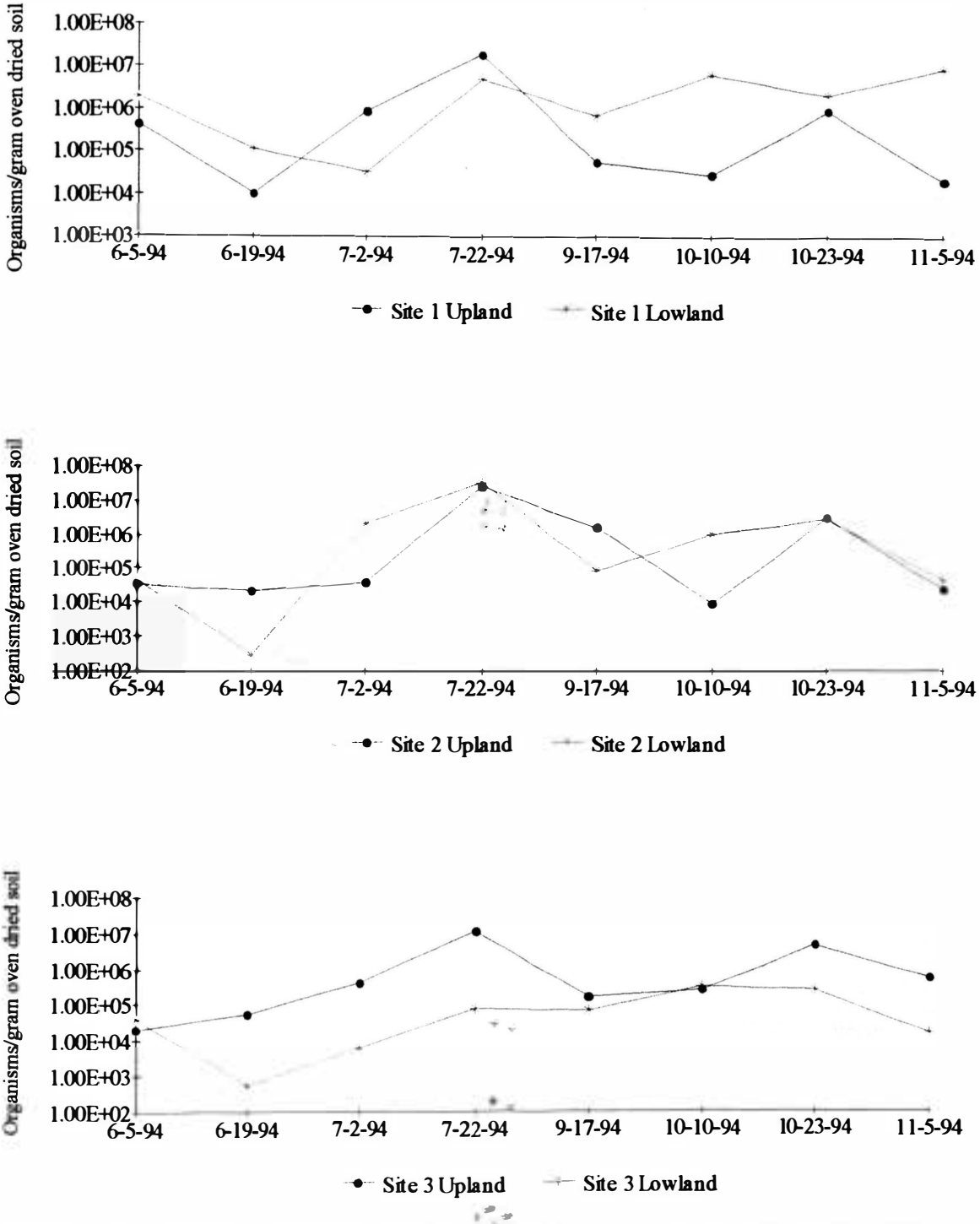



Figure 1.18. Most-probable-numbers for wetland 8 DNRA bacteria during the 1994 sampling season.

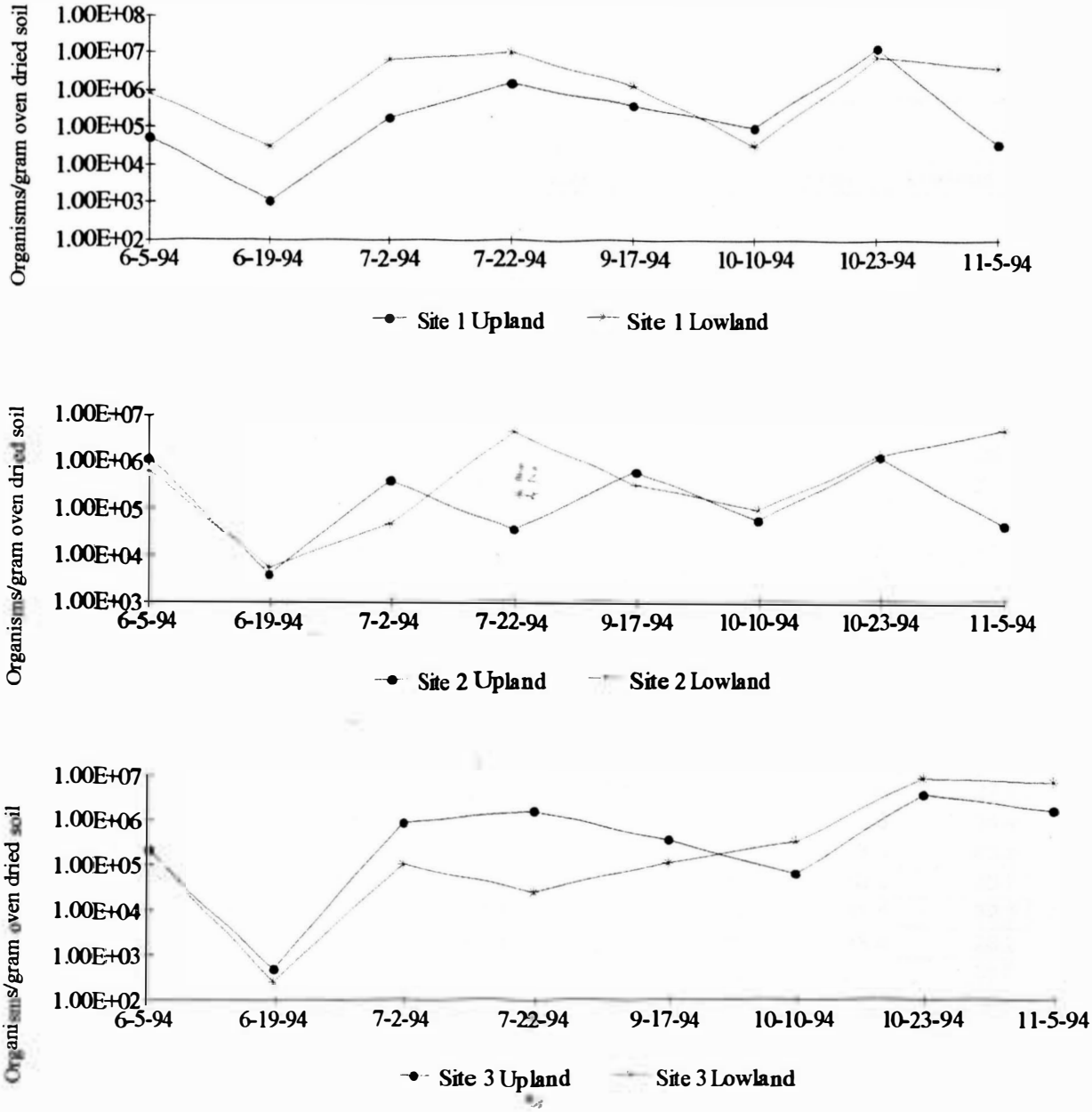



Figure 1.19. Most-probable-numbers for wetland 13 DNRA bacteria during the 1994 sampling season.

Table 1.7. Percent water content at time of analysis for soil samples used in 1994 most-probable-numbers (MPN) analyses.

| | 2nd Year (1994) | | | | | | |
|---|---|---|---|---|---|---|---|
| Date | Wetland | Site 1 | | Site 2 | | Site 3 | |
| | | Upland | Lowland | Upland | Lowland | Upland | Lowland |
| 4-16-94 | 5 | 13.5 | 24.5 | | 21.1 | | |
| | 8 | 16.7 | 31.2 | | 32.4 | | |
| | 13 | 16.2 | 44.3 | | 29.6 | | |
| 5-7-94 | 5 | 14.3 | 27.9 | | 25.7 | | |
| | 8 | 21.3 | 36 | | 20.7 | | |
| | 13 | 21.6 | 28.2 | | 35.7 | | |
| 6-5-94 | 5 | 132 | 30.4 | 14.7 | 23.3 | 18.1 | 19.6 |
| | 8 | 17.5 | 24.5 | 17.7 | 25.3 | 16.9 | 1.5 |
| | 13 | 13.8 | 30.3 | 20.3 | 28.8 | 17.8 | 28.5 |
| 6-19-94 | 5 | 35.9 | 24.9 | 14.3 | 27.3 | 21 | 21.2 |
| | 8 | 23.1 | 30.7 | 18 | 30.1 | 21.1 | 27.8 |
| | 13 | 13.8 | 25.2 | 13.5 | 36.6 | 17.9 | 24.8 |
| 7-2-94 | 5 | 10.7 | 33.4 | 9 | 35.8 | 18.8 | 27.3 |
| | 8 | 10.3 | 26.8 | 13.3 | 29 | 14.3 | 40.3 |
| | 13 | 7.4 | 28.1 | 14.5 | 30.8 | 13.3 | 30.4 |
| 7-22-94 | 5 | 8.8 | 28.8 | 8.5 | 20.7 | 24.8 | 40.5 |
| | 8 | 12 | 34.9 | 12.9 | 30.6 | 11.2 | 37.5 |
| | 13 | 8.6 | 28.1 | 30.8 | 21.8 | 13.2 | 30.4 |
| 9-17-94 | 5 | 8.3 | 26.1 | 8.1 | 28.3 | 17.5 | 24.8 |
| | 8 | 17.8 | 39.2 | 16.6 | 40 | 20 | 27.8 |
| | 13 | 18.4 | 31.2 | 16.4 | 32.4 | 20.6 | 36.4 |
| 10-10-94 | 5 | 10.5 | 10.5 | 25.9 | 10 | 3.5 | 45.5 |
| | 8 | 13.2 | 31.5 | 4.4 | 30.3 | 16.5 | 30.7 |
| | 13 | 12.7 | 23.5 | 12 | 30.6 | 15.4 | 32.2 |
| 10-23-94 | 5 | 13.3 | 13.4 | 28.2 | 31.2 | 13.4 | 28.2 |
| | 8 | 18.7 | 31.2 | 24.1 | 39 | 24.1 | 31.2 |
| | 13 | 21.8 | 39.1 | 18 | 29.7 | 22.7 | 33.9 |
| 11-5-94 | 5 | 10.6 | 22.5 | 8.3 | 21.3 | 18.8 | 30.6 |
| | 8 | 14.5 | 25 | 11.7 | 31.5 | 13.8 | 13.6 |

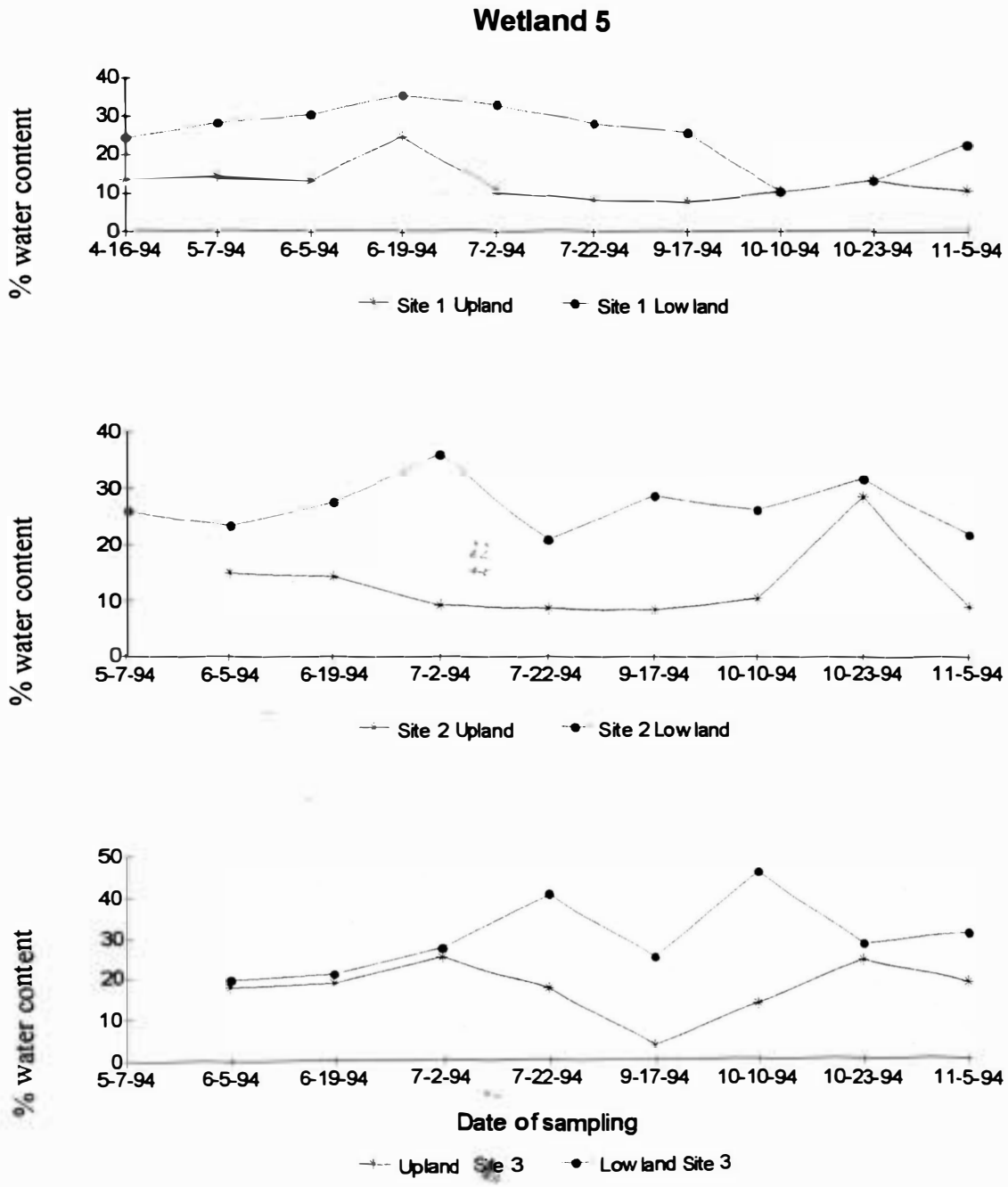



Figure 1.20. Percent water content of wetland 5 site 1, 2, and 3 soil samples during the 1994 sampling season.

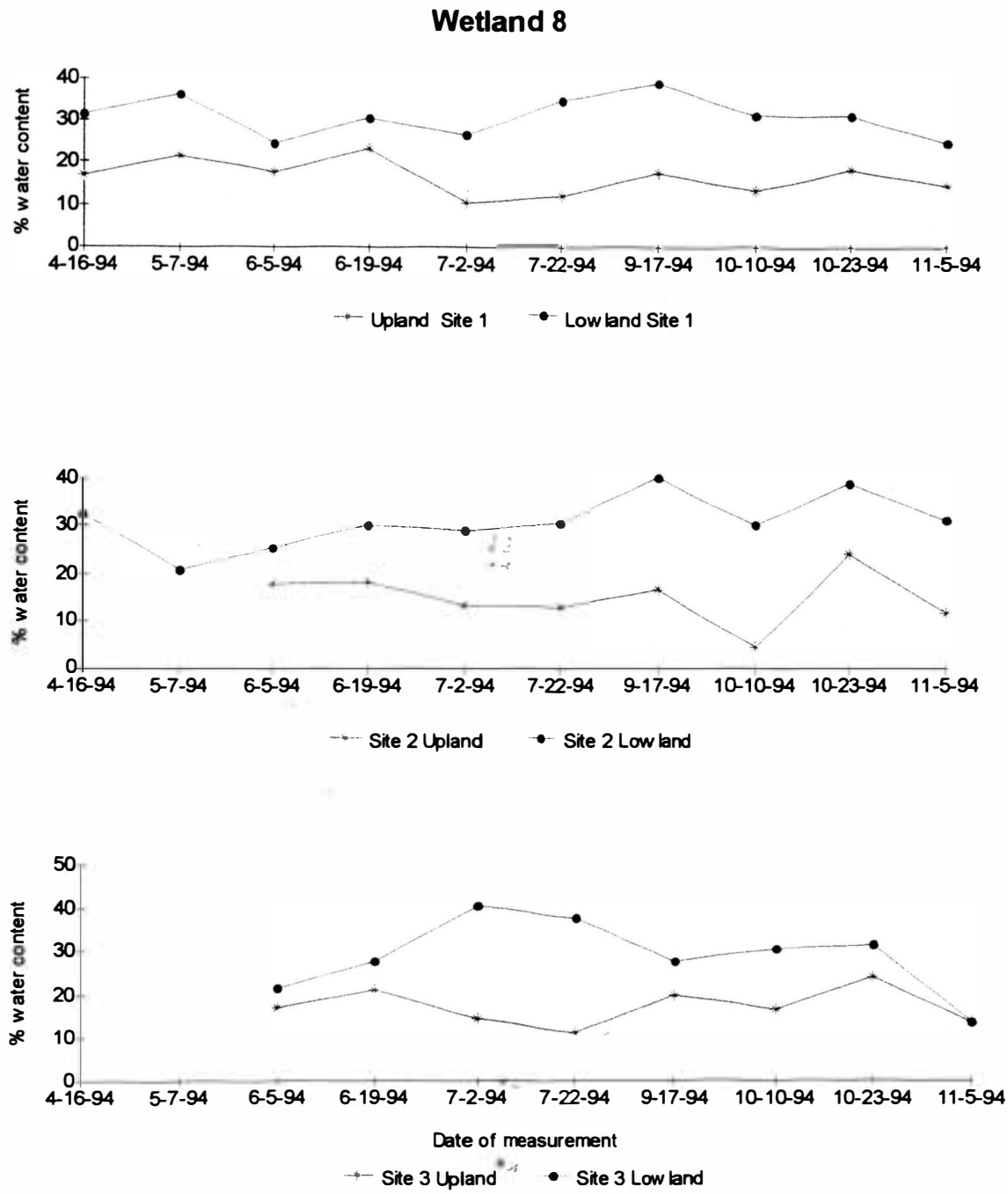



Figure 1.21. Percent water content of wetland 8 site 1, 2, and 3 soil samples during the 1994 sampling season.

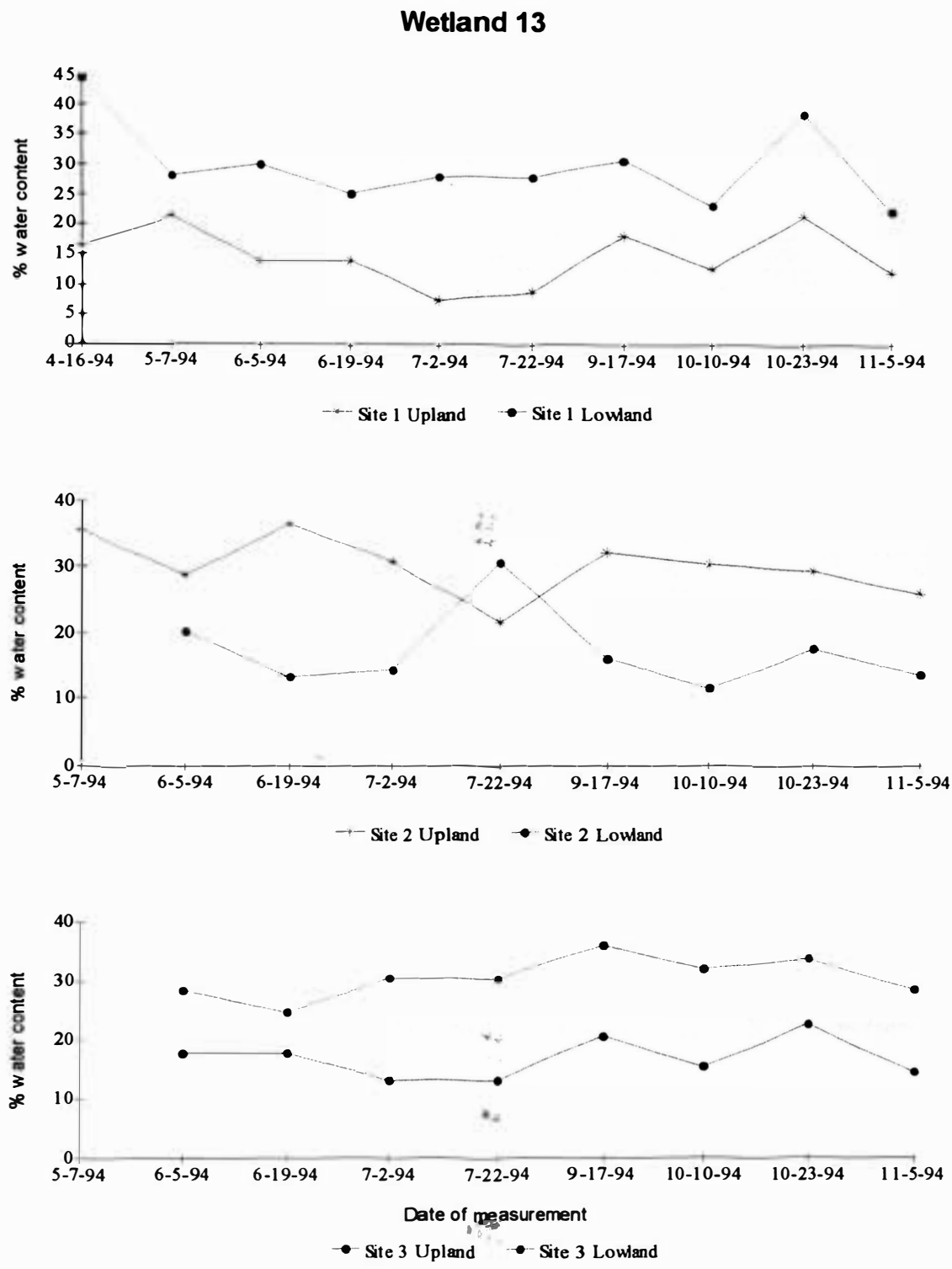



Figure 1.22. Percent water content of wetland 13 site 1, 2, and 3 soil samples during the 1994 sampling season.

# Appendices

## B

## Chapter 2 Research Results Data

| | | 30 min | 60 min | 90 min | 105 min | 120 min |
|---|---|---|---|---|---|---|
| Wetland 5 | Treatment 1 | 26325 | 39078 | 48645 | 58589 | 73076 |
| | Treatment 2 | 15412 | 23719 | 49142 | 19758 | 63143 |
| | Treatment 3 | 24129 | 36173 | 41496 | 26836 | 46594 |
| | Treatment 4 | 7632 | 31579 | 0 | 9657 | 0 |
| | Treatment 5 | 20169 | 7803 | 0 | 0 | 15556 |
| Wetland 8 | Treatment 1 | 0 | 0 | 0 | 0 | 0 |
| | Treatment 2 | 7674 | 21700 | 32740 | 37260 | 45345 |
| | Treatment 3 | 5730 | 15076 | 21716 | 25534 | 23280 |
| | Treatment 4 | 15274 | 24348 | 29224 | 77713 | 27606 |
| | Treatment 5 | 3354 | 3912 | 7469 | 0 | 5901 |
| Wetland 13 | Treatment 1 | 9500 | 31768 | 36557 | 53128 | 64600 |
| | Treatment 2 | 5270 | 13374 | 23350 | 27453 | 47719 |
| | Treatment 3 | 5015 | 17090 | 28940 | 27949 | 31938 |
| | Treatment 4 | 2520 | 3138 | 5912 | 13049 | 1560 |
| | Treatment 5 | 2089 | 3766 | 5444 | 0 | 0 |

Treatment 1: Glucose, $KNO_3$, no chloramphenicol
Treatment 2: $KNO_3$ and chloramphenicol
Treatment 3: Glucose, $KNO_3$, chloramphenicol
Treatment 4: Glucose and chloramphenicol
Treatment 5: (Control) only chloramphenicol

Table 2.5. Modified Phase I data given as integrator units of $N_2O$ in mv/sec.

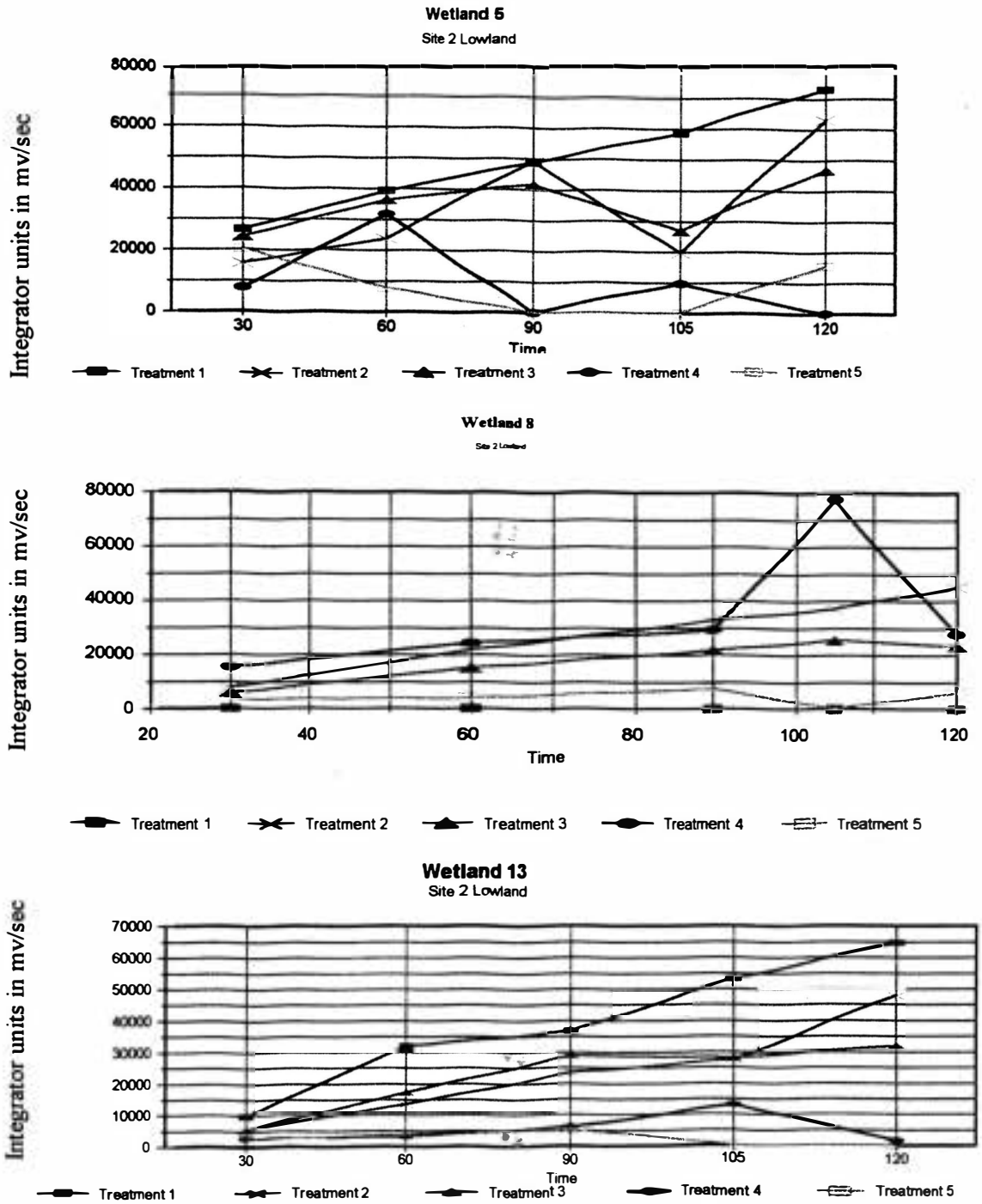



Figure 2.7. $N_2O$ production from modified Phase I assays

- Treatment 1. Glucose, $KNO_3$, no chloramphenicol
- Treatment 2. $KNO_3$ and chloramphenicol
- Treatment 3. Glucose, $KNO_3$, chloramphenicol
- Treatment 4. Glucose and chloramphenicol
- Treatment 5. (Control) only chloramphenicol

| REPORT OF ANALYSIS | | | | | |
|---|---|---|---|---|---|
| Wetland | Flask Amendments | Flask # | Glucose (ppm) | Ammonia mg/L (ppm) | Nitrate Nitrogen mg/L (ppm) |
| 13 | Soil plus | 1 | 111.00 | 0.30 | 5.83 |
| | $KNO_3$ and Glucose | 2 | 113.00 | < 0.10 | 6.71 |
| 8 | | 1 | 130.00 | 0.44 | 4.58 |
| | | 2 | 122.00 | 0.22 | 8.72 |
| 5 | | 1 | 124.00 | 0.26 | 5.26 |
| | | 2 | 122.00 | 0.23 | 5.79 |
| 13 | Soil plus | 3 | 3.80 | 0.43 | 11.90 |
| | $KNO_3$ and | 4 | 6.90 | 0.19 | 13.20 |
| 8 | Chloramphenicol | 3 | 0.00 | 0.50 | 12.20 |
| | | 4 | 0.00 | 0.66 | 12.50 |
| 5 | | 3 | 0.00 | 0.17 | 11.80 |
| | | 4 | 0.00 | 0.20 | 12.60 |
| 13 | Soil plus | 5 | 122.00 | 0.28 | 10.40 |
| | $KNO_3$, Glucose, and | 6 | 118.00 | 0.44 | 8.67 |
| 8 | Chloramphenicol | 5 | 126.00 | 0.53 | 11.90 |
| | | 6 | 141.00 | 0.58 | 20.10 |
| 5 | | 5 | 134.00 | 0.17 | 9.83 |
| | | 6 | 129.00 | 0.13 | 10.10 |
| 13 | Soil plus | 7 | 126.00 | 0.35 | 3.58 |
| | Glucose and | 8 | 131.00 | 0.17 | 3.38 |
| 8 | Chloramphenicol | 7 | 108.00 | 0.42 | 4.27 |
| | | 8 | 119.00 | 0.60 | 4.61 |
| 5 | | 7 | 147.00 | < 0.10 | 3.95 |
| | | 8 | 120.00 | < 0.10 | 3.44 |
| 13 | Soil plus | 9 | 5.30 | 0.11 | 3.30 |
| 8 | Chloramphenicol | 9 | 0.00 | 0.33 | 4.37 |
| 5 | | 9 | 0.00 | < 0.10 | 3.66 |
| | Control Blank No Soil Glucose, $KNO_3$, Chloramphenicol | | 211.00 | | 23.00 |

Table 2.6. Chemical analyses for modified Phase I assays

| | | | 10/10/93 | 10/23/93 | 4/16/94 | 5/7/94 | 6/5/94 | 6/19/94 | 7/22/94 | 9/17/94 | 10/10/94 | 10/23/94 | 11/5/94 | 6/15/95 | 9/24/95 | 10/20/96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WETLAND 13 (CON) (Wolles) | site 1 | upland | 17.60 | 13.80 | 16.20 | 21.01 | 27.63 | 20.65 | 27.88 | 18.27 | 19.47 | 22.03 | 19.99 | 21.88 | 21.84 | |
| | | lowland | 39.88 | 35.68 | 44.28 | 32.54 | 31.71 | 16.89 | | 31.14 | 39.61 | 38.00 | 30.53 | 39.17 | 37.09 | |
| | site 2 | upland | | | | | 27.63 | 19.63 | 21.80 | 16.70 | 20.88 | 18.67 | 19.44 | 23.08 | 20.80 | |
| | | lowland | | | 29.60 | 21.60 | 35.16 | 37.80 | 33.60 | 32.53 | 32.72 | 34.79 | 36.52 | 36.08 | 42.89 | |
| | site 3 | upland | | | | | 28.33 | 19.57 | 14.2 | 20.65 | 22.37 | 23.19 | 24.52 | 27.33 | 23.52 | |
| | | lowland | | | | | 38.68 | 31.43 | 13.5 | 36.16 | 34.15 | 33.47 | 31.71 | 36.57 | 42.53 | |
| | | Top 1-3" | | | | | | | | | | | | | 18.96 | 52.54 |
| | | (combined) | | | | | | | | | | | | | | 36.4 |
| WETLAND 5 (ORG) (Johnson) | site 1 | upland | 11.80 | 13.40 | 13.48 | 32.18 | 27.92 | 17.89 | 8.80 | 15.31 | 17.28 | 19.24 | 16.63 | 23.77 | 16.79 | |
| | | lowland | 25.00 | 28.20 | 27.88 | 37.84 | 28.09 | 29.05 | | 29.88 | 31.75 | 32.56 | 27.28 | 38.11 | 30.64 | |
| | site 2 | upland | | | | | 24.80 | 17.87 | 8.50 | 14.81 | 16.51 | 18.59 | 16.01 | 28.33 | 22.64 | |
| | | lowland | | | 25.68 | 27.60 | 31.63 | 30.27 | 20.70 | 32.81 | 29.32 | 30.33 | 26.96 | 38.68 | 34.41 | |
| | site 3 | upland | | | | | 23.93 | 25.12 | | 25.68 | 23.96 | 27.21 | 24.79 | 27.92 | 27.63 | |
| | | lowland | | | | | 35.37 | 24.45 | | 34.39 | 37.15 | 31.80 | 34.03 | 28.09 | 37.17 | |
| | | Top 1-3" | | | | | | | | | | | | | 19.96 | 42.99 |
| | | (combined) | | | | | | | | | | | | | | 36.6 |
| WETLAND 8 (TNT) (Jonke) | site 1 | upland | 14.60 | 18.68 | 16.68 | 21.28 | 23.96 | 22.44 | 7.60 | 17.60 | 20.71 | 21.53 | 19.27 | 23.17 | 21.96 | |
| | | lowland | 35.00 | 31.20 | 32.40 | 35.92 | 34.76 | 35.16 | 34.88 | 38.65 | 36.29 | 36.99 | 38.05 | 39.21 | 38.32 | |
| | site 2 | upland | | | | | 23.64 | 20.45 | | 16.56 | 20.36 | 21.52 | 20.09 | 25.19 | 20.89 | |
| | | lowland | | | 31.20 | 20.68 | 34.85 | 30.27 | | 37.55 | 37.57 | 39.75 | 36.52 | 41.29 | 48.37 | |
| | site 3 | upland | | | | | 25.64 | 24.12 | 28.80 | 19.87 | 23.45 | 24.12 | 22.03 | 37.80 | 23.83 | |
| | | lowland | | | | | 33.09 | 36.00 | 11.20 | 28.08 | 29.83 | 29.35 | 31.04 | 27.89 | 35.65 | |

Table 2.7. Semi-permanent wetland soil percent water content

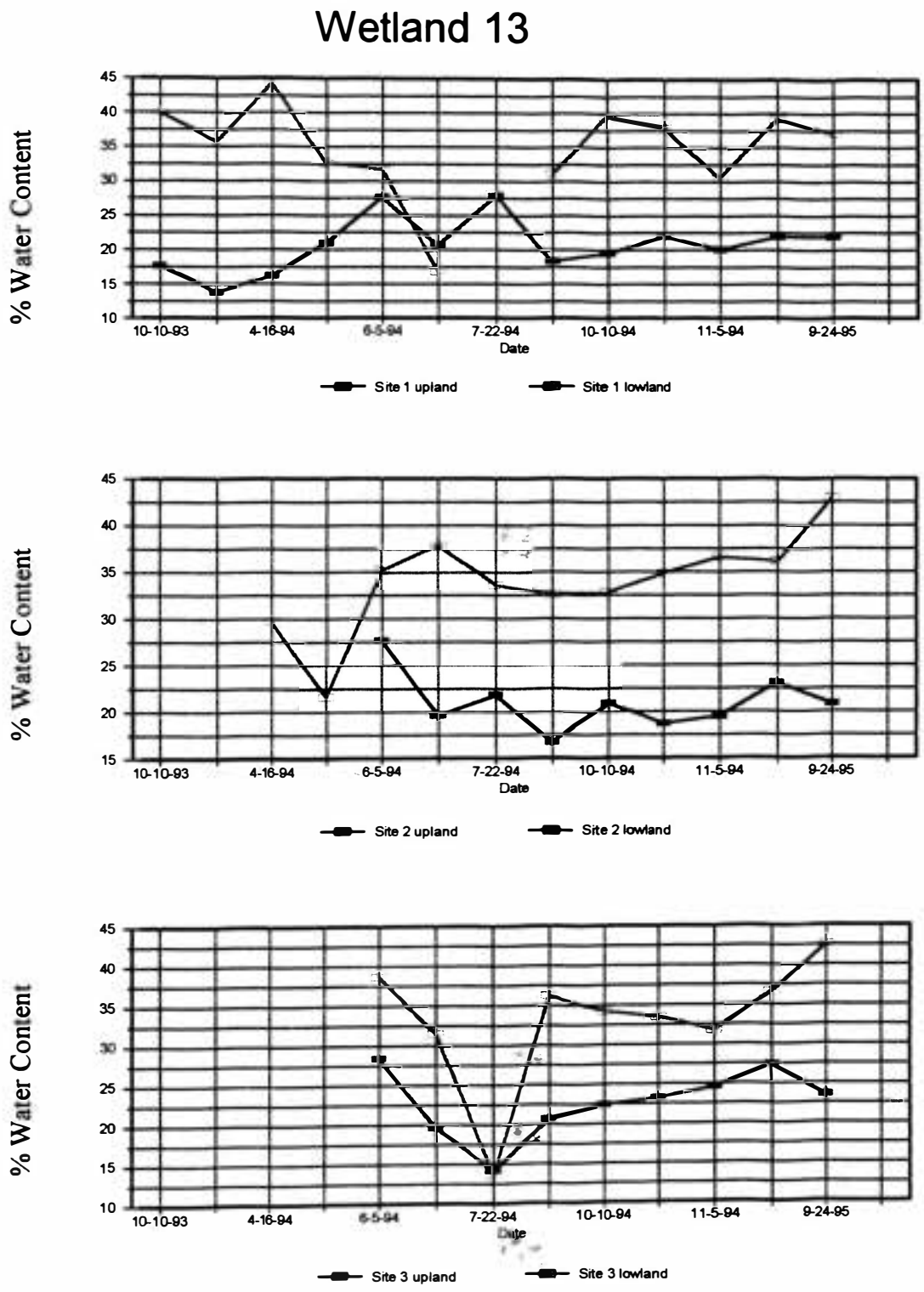



Figure 2.8. Semi-permanent wetland 13, percent water content

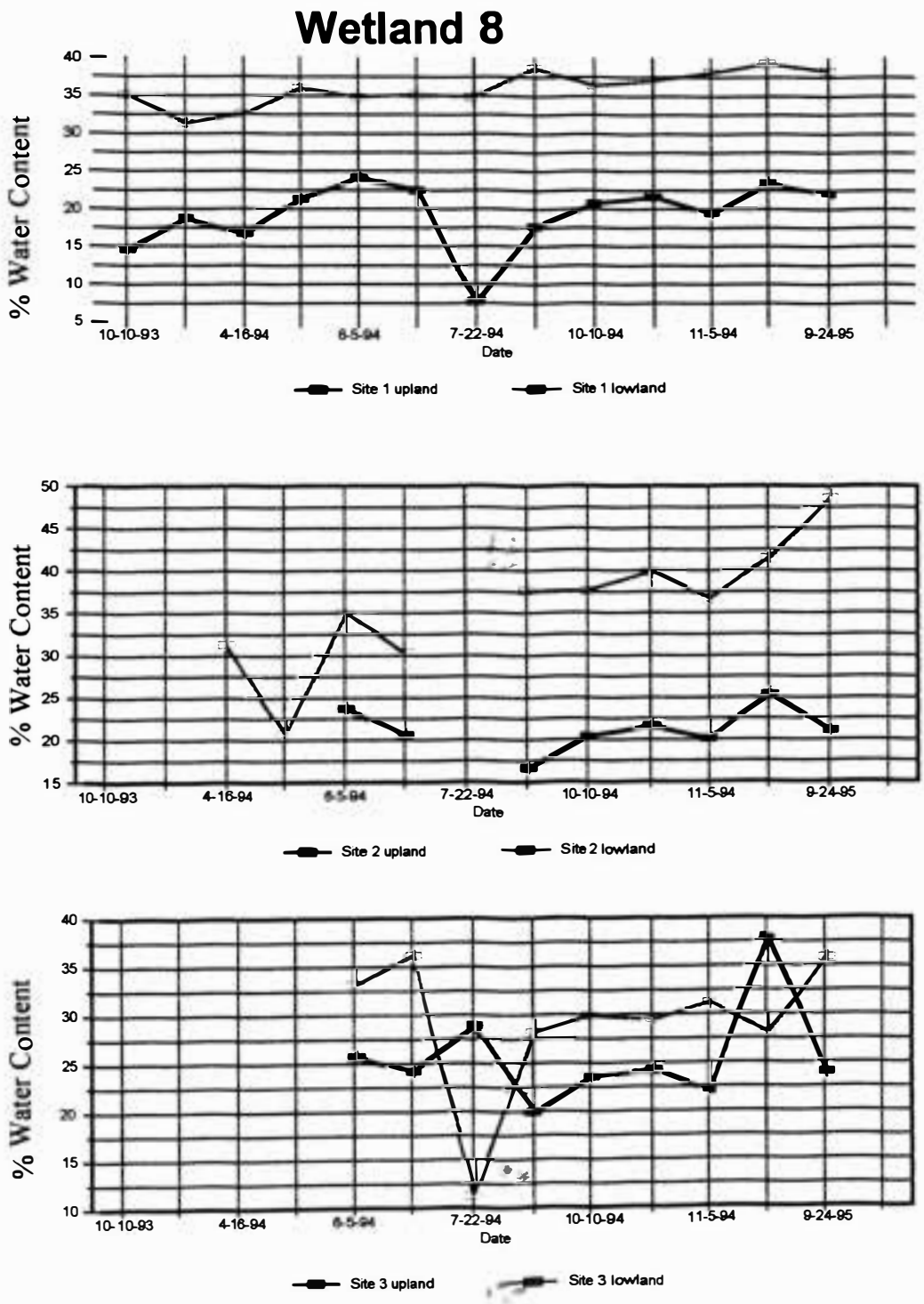



Figure 2.9. Semi-permanent wetland 8, percent water content.

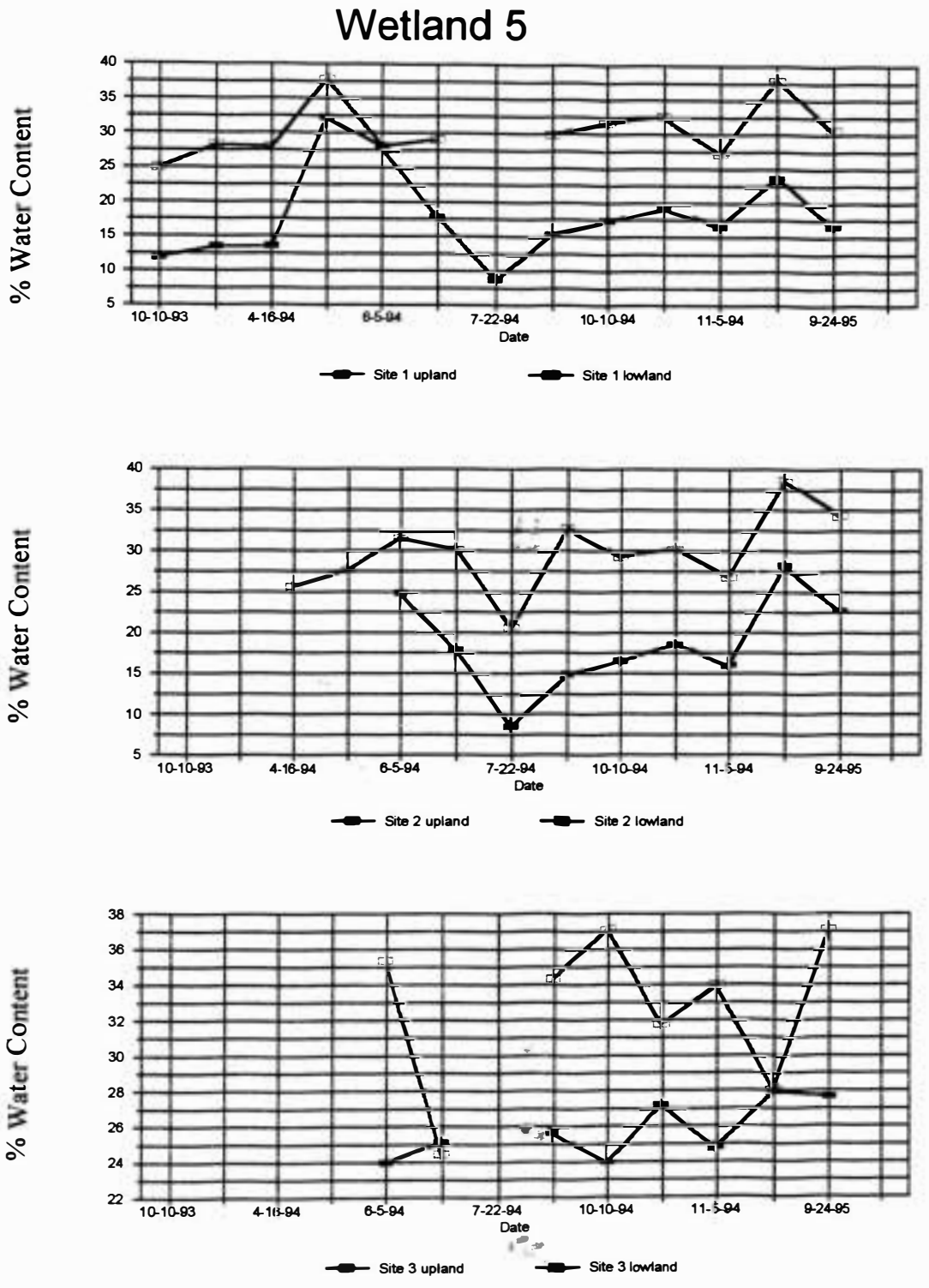



Figure 2.10. Semi-permanent wetland 5, percent water content.

| | | | 10-10-93 | 10-23-93 | 4-16-94 | 5-7-94 | 6-5-94 | 6-19-94 | 7-22-94 | 9-17-94 | 10-10-94 | 10-23-94 | 11-5-94 | 6-15-95 | 9-24-95 | 6-5-96 | 10-20-96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WETLAND 13 (CON) (Wolles) | site 1 | upland | 0.028 | 0.055 | 0.018 | 0.085 | 0.066 | 0.005 | | 0.030 | 0.017 | 0.041 | 0.024 | 0.135 | 0.056 | 0.063 | |
| | | lowland | 0.539 | 0.405 | 0.104 | 0.072 | 0.043 | 0.068 | | 0.053 | 0.086 | 0.171 | 0.018 | 0.122 | 0.122 | 0.169 | |
| | site 2 | upland | | | | | 0.382 | 0.048 | 0.051 | 0.014 | 0.037 | 0.000 | 0.004 | 0.013 | 0.001 | | |
| | | lowland | | | 0.020 | 0.029 | 0.348 | 0.227 | 0.031 | 0.019 | 0.062 | 0.021 | | 0.048 | 0.010 | | |
| | site 3 | upland | | | | | 0.118 | 0.013 | 0.001 | 0.051 | 0.028 | 0.050 | 0.045 | 0.077 | 0.056 | 0.062 | |
| | | lowland | | | | | 0.152 | 0.020 | 0.014 | 0.274 | 0.101 | 0.094 | 0.135 | 0.105 | 0.326 | 0.006 | |
| | | Top 1-3" (combined) | | | | | | | | | | | | | | 5.628 | 0.547 |
| | | | | | | | | | | | | | | | | | 0.148 |
| WETLAND 5 (ORG) (Johnson) | site 1 | upland | 0.953 | 0.052 | 0.029 | 0.055 | 0.027 | 0.048 | 0.075 | 0.043 | 0.001 | 0.043 | 0.016 | 0.069 | 0.048 | 0.029 | |
| | | lowland | 0.797 | 1.338 | 0.436 | 0.125 | 0.030 | 0.048 | 0.183 | 0.039 | 0.187 | 0.192 | 0.017 | 0.360 | 0.085 | 0.228 | |
| | site 2 | upland | | | | | 0.018 | 0.054 | 0.057 | 0.031 | 0.049 | 0.025 | 0.035 | 0.055 | 0.101 | 0.610 | |
| | | lowland | | | 0.032 | 0.074 | 0.070 | 0.111 | 0.029 | 0.047 | 0.078 | 0.045 | 0.047 | 0.157 | 0.182 | 0.291 | |
| | site 3 | upland | | | | | 0.032 | 0.709 | | 0.058 | 0.032 | 0.051 | 0.028 | 0.067 | 0.244 | 0.211 | |
| | | lowland | | | | | 0.348 | 0.051 | | 0.072 | 0.077 | 0.033 | 0.018 | 0.084 | 0.199 | 0.387 | |
| | | | | | | | | | | | | | | | | 2.511 | 0.236 |
| | | | | | | | | | | | | | | | | | 0.038 |
| WETLAND 8 (TNT) (Jonke) | site 1 | upland | 0.318 | 0.282 | 0.037 | 0.175 | 0.012 | 0.023 | 0.001 | 0.010 | 0.073 | 0.030 | 0.090 | 0.018 | 0.001 | | |
| | | lowland | 4.036 | 3.129 | 0.727 | 0.125 | 0.028 | 0.076 | 0.014 | 0.255 | 0.210 | 0.088 | 0.107 | 0.040 | 0.020 | | |
| | site 2 | upland | | | | | 0.033 | 0.055 | | 0.013 | 0.030 | 0.063 | 0.035 | 0.0437 | 0.528 | | |
| | | lowland | | | 0.060 | 0.116 | 0.185 | 0.167 | | 0.086 | 0.047 | 0.111 | 0.031 | 0.089 | 0.178 | | |
| | site 3 | upland | | | | | 0.018 | 0.044 | 0.016 | 0.015 | 0.088 | 0.029 | 0.096 | 0.174 | 0.051 | 0.244 | |
| | | lowland | | | | | 0.025 | 0.590 | 0.006 | 0.020 | 0.064 | 0.013 | 0.050 | 0.025 | 0.310 | 0.652 | |

Table 2.8. Semi-permanent wetland regular phase I denitrification rates (expressed as nanomoles $N_2O$/ gram oven dried soil / minute).

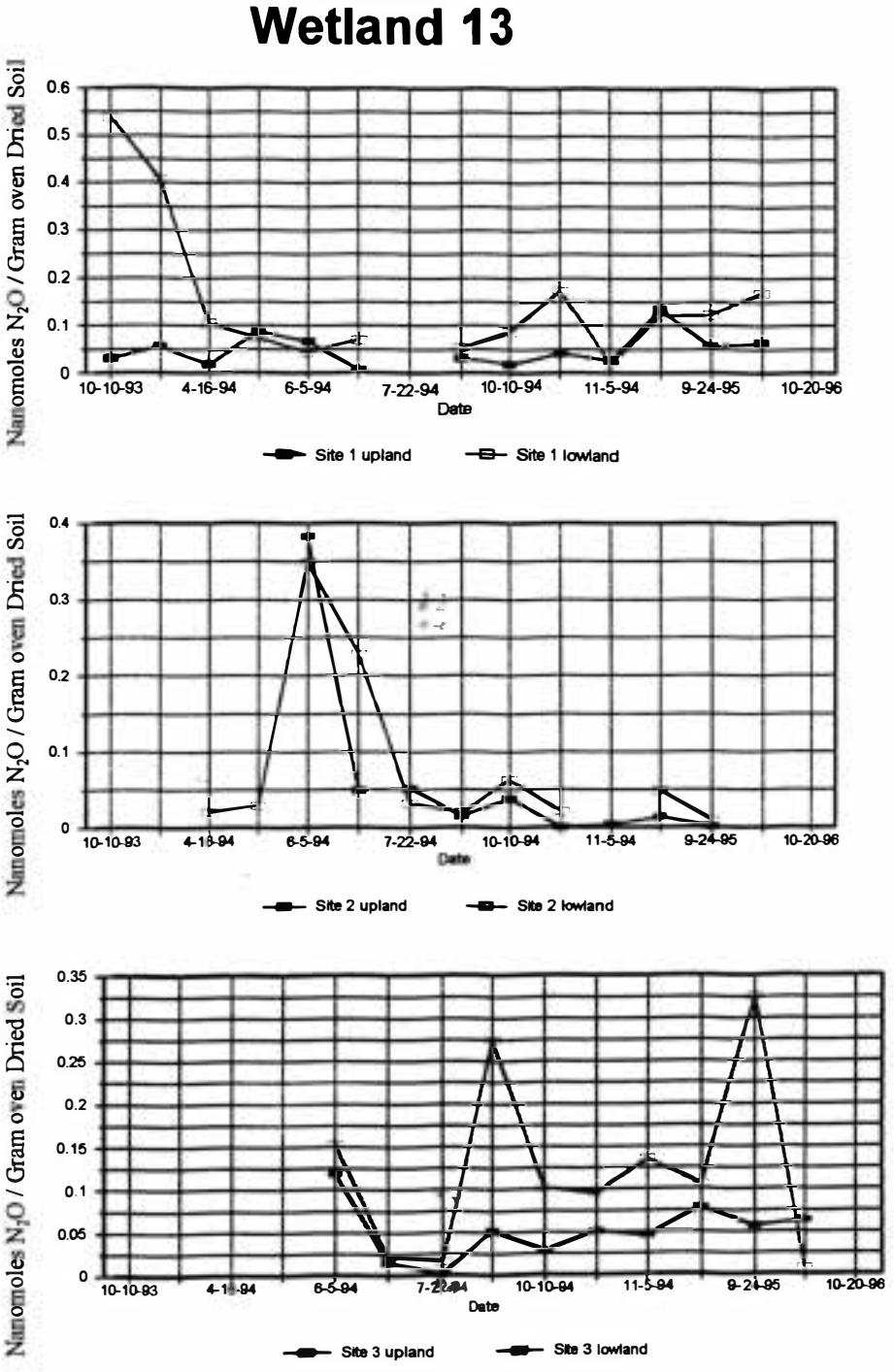



Figure 2.11. Wetland 13, regular phase I rates.

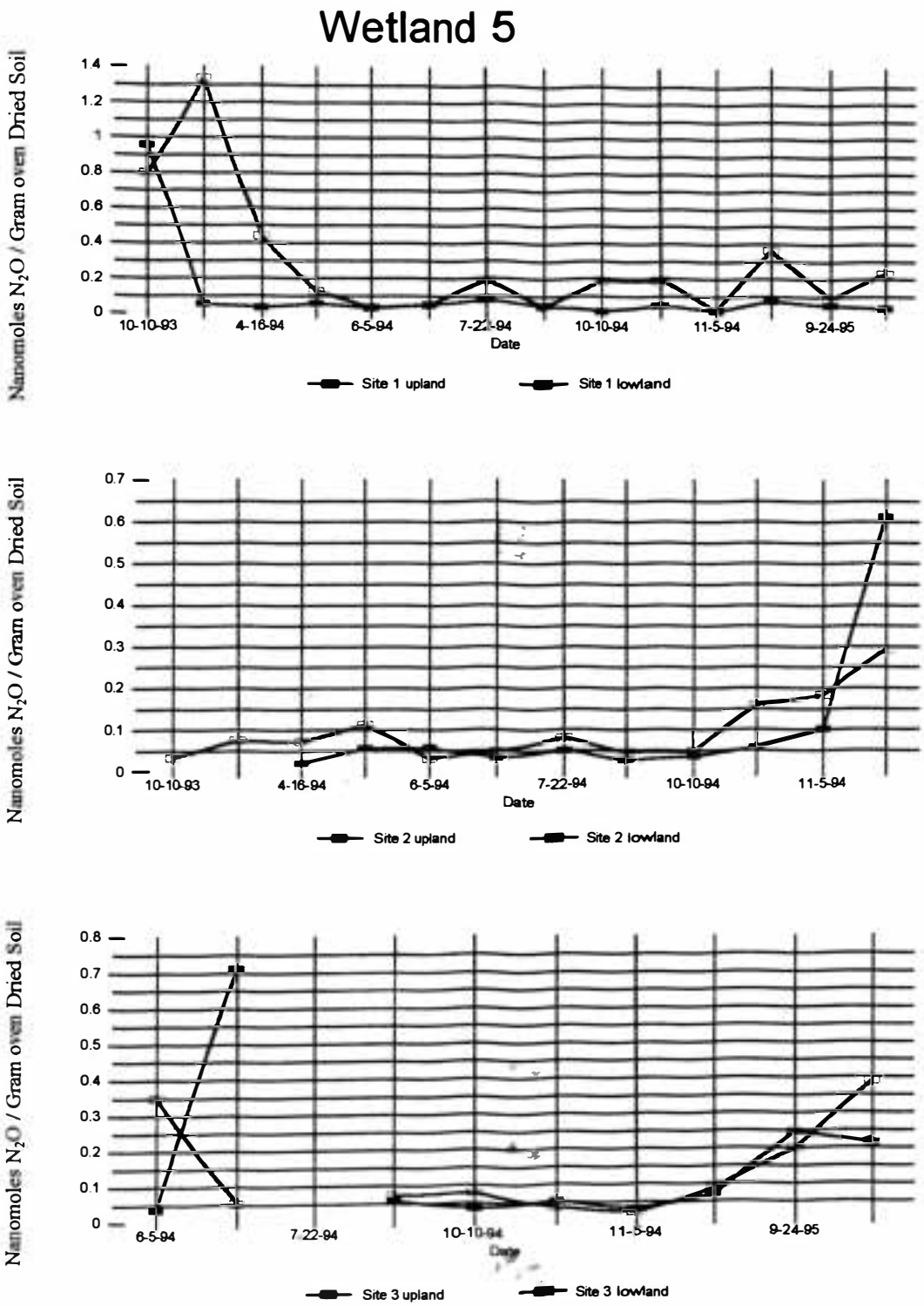



Figure 2.12. Wetland 5, regular phase I rates.

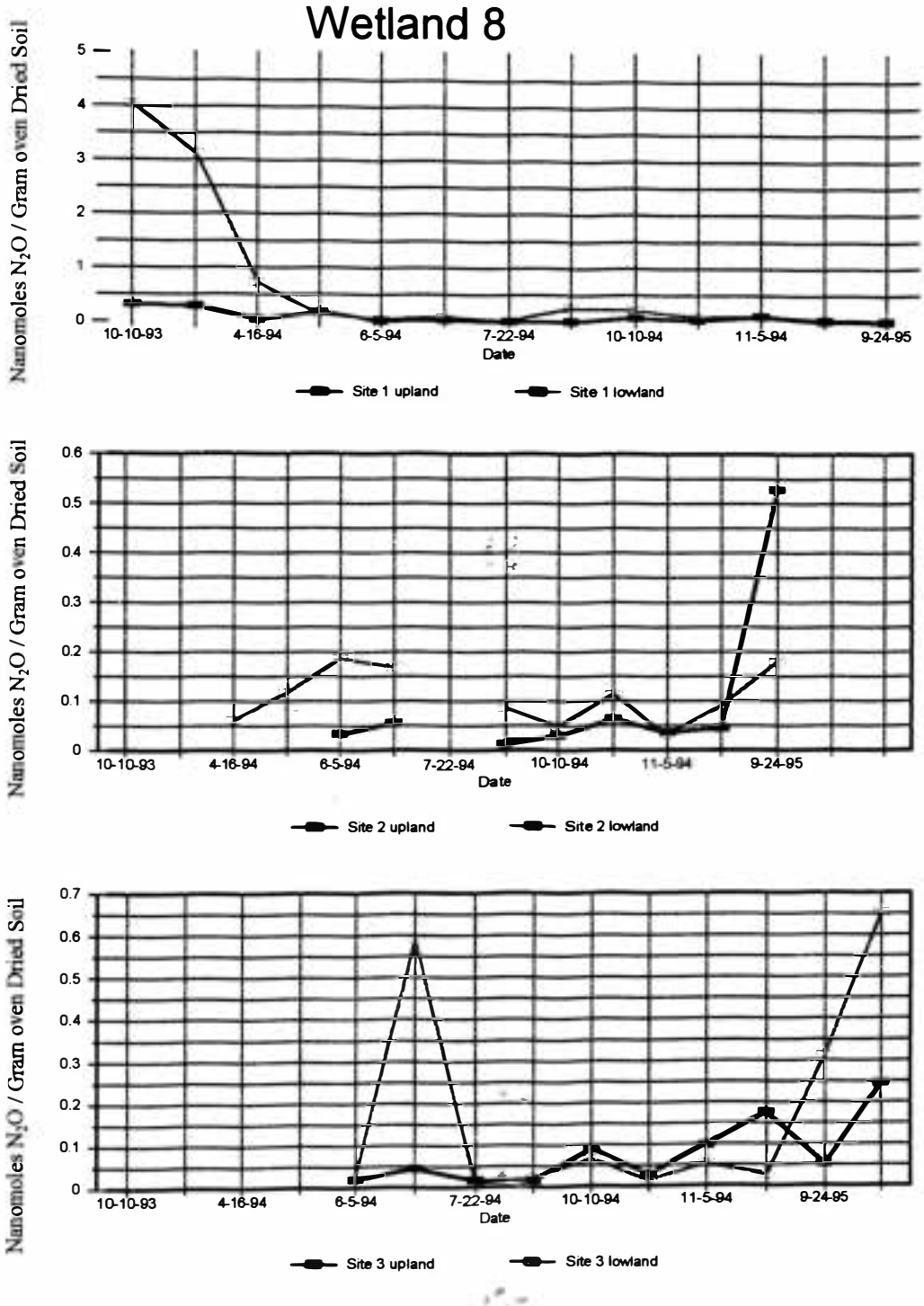



Figure 2.13. Wetland 8, regular phase I rates.

# Appendices
# C
## Chapter 3 Research Results Data

| Set 1 | 0 hours | 4.5 hours | 19.5 hours | 24 hours | 28 hours |
|---|---|---|---|---|---|
| 0-5 cm | -1.6996 | 3.7041 | -0.2086 | -1.6996 | -1.6996 |
| 5-10 cm | -0.9921 | -1.0217 | -0.9921 | -0.9921 | -0.9921 |
| 10-15 cm | -0.7701 | 2.1788 | 2.0887 | 1.0554 | -0.7701 |
| Set 2 | | | | | |
| 0-5 cm | -1.3443 | -0.0247 | -1.3443 | -1.3443 | -1.3443 |
| 5-10 cm | -0.7847 | -0.7613 | -0.7847 | -0.7847 | -0.7847 |
| 10-15 cm | -0.6091 | -0.5777 | -0.6091 | 3.1097 | 1.3846 |
| Set 3 | | | | | |
| 0-5 cm | -42.218 | -42.218 | -42.218 | -42.218 | -42.218 |
| 5-10 cm | -24.6446 | -25.2853 | -24.6446 | -24.6446 | -24.6446 |
| 10-15 cm | -18.0995 | -19.1284 | -19.1284 | -19.1284 | -19.1284 |
| Set 4 | | | | | |
| 0-5 cm | -22.0834 | -21.7529 | -21.0646 | -22.3677 | -19.0989 |
| 5-10 cm | -12.9252 | -0.494 | 17.0886 | -0.0013 | 16.6511 |
| 10-15 cm | -10.0133 | 14.5625 | 73.5869 | 303.3026 | 297.6659 |
| Set 6 | | | | | |
| 0-5 cm | -42.3303 | -42.3303 | -39.9485 | -36.4554 | -40.1779 |
| 5-10 cm | -24.7102 | -19.2859 | -20.0699 | -24.7102 | -17.3466 |
| 10-15 cm | -9.725 | -14.08 | -8.2045 | -17.1128 | -12.8578 |
| Set 7 | | | | | |
| 0-5 cm | -16.2723 | -16.2723 | 6809.6446 | 318.9801 | -16.2723 |
| 5-10 cm | -9.4989 | 23.6586 | 14.0683 | 129.0784 | -9.4989 |
| 10-15 cm | 11.3871 | 36.63 | -7.3728 | 361.6733 | -4.9427 |
| Set 8 | | | | | |
| 0-5 cm | -26.9809 | -26.9809 | 5.8014 | 108.772 | -25.887 |
| 5-10 cm | -15.75 | 28.4846 | 33.0229 | 200.2394 | -15.3383 |
| 10-15 cm | 14.568 | 72.9907 | -11.5864 | 295.3281 | -8.1954 |

| Set 5 | 0 | 30 | 60 | 90 | 105 |
|---|---|---|---|---|---|
| 0-5 cm | -21.3492 | -17.1686 | -18.5152 | 7.4667 | -9.2148 |
| 5-10 cm | -12.4625 | 15.6181 | 16.766 | 49.809 | 89.4618 |
| 10-15 cm | -9.676 | -9.673 | -6.0847 | -4.1604 | 0.4603 |

**Table 3.1.** Natural denitrification potentials in nanomoles $N_2O$/gram oven dried soil (sets 1-4; 6-8) and natural denitrification assay comparison in nanomoles $N_2O$ per gram oven dried soil (set 5).

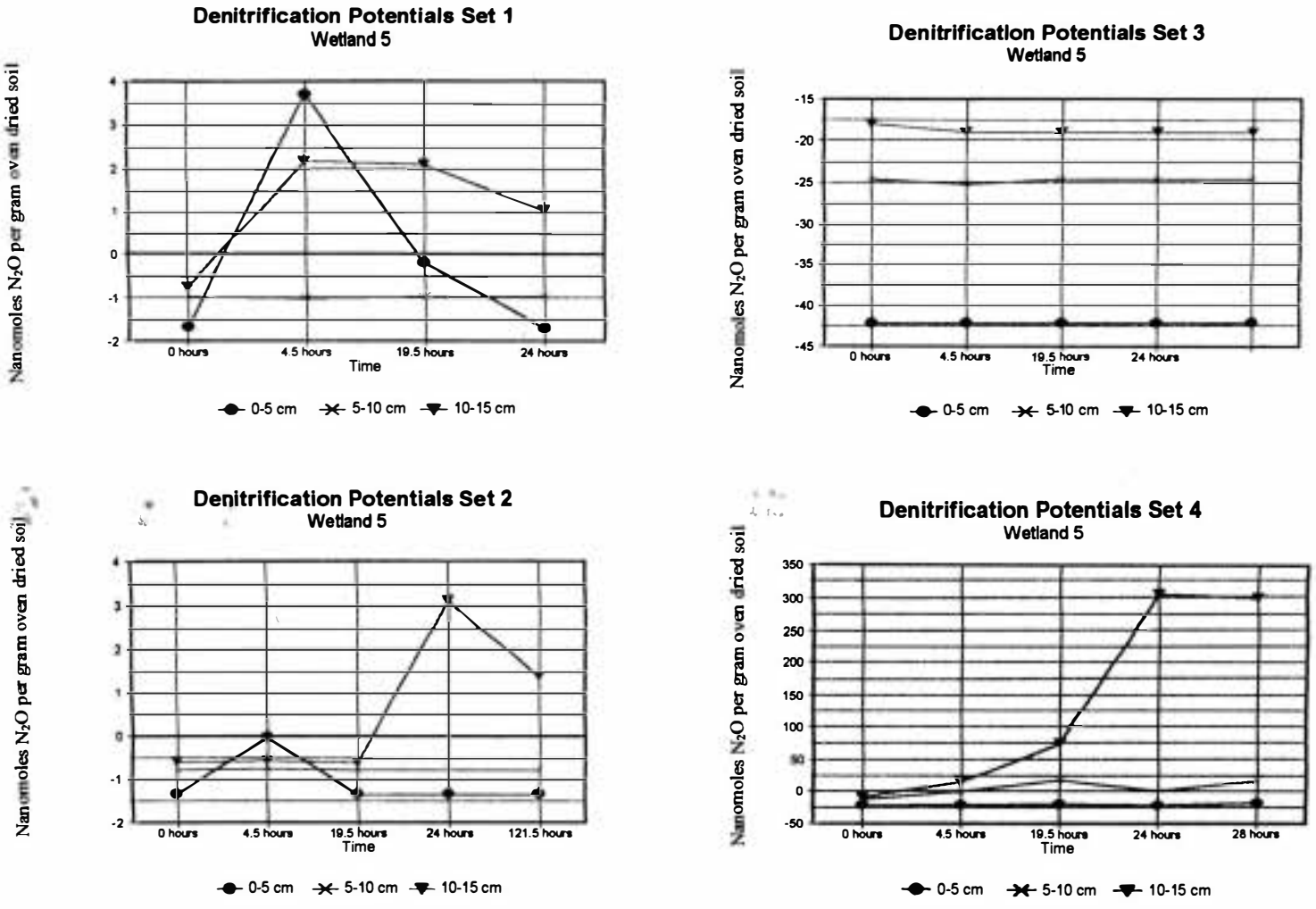



Figure 3.23. Wetland 5 denitrification potentials, sets 1-4

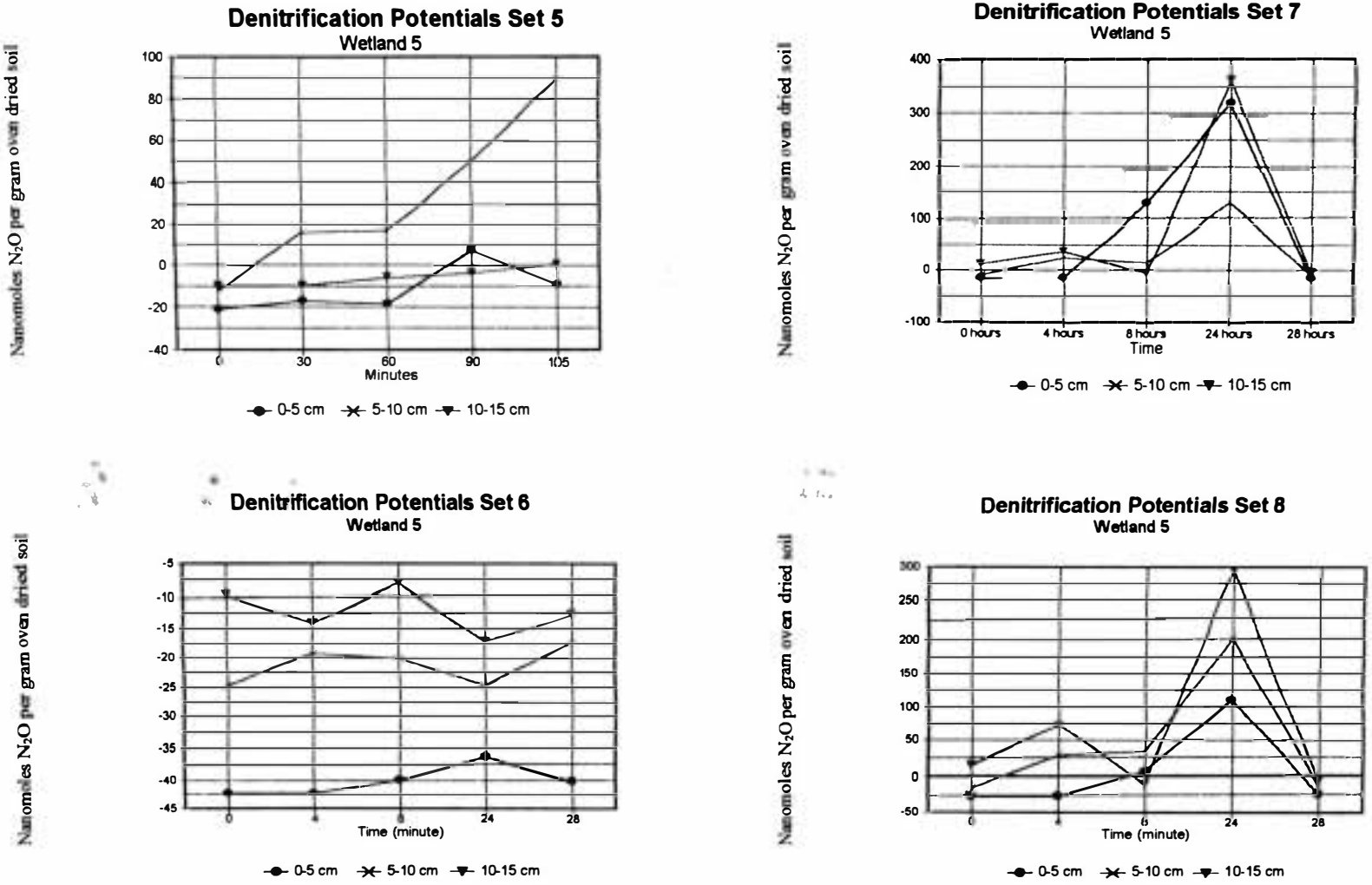



Figure 3.24. Wetland 5 denitrification potentials, sets 5-8.

| | | 0-5 cm | 5-10 cm | 10-15 cm |
|---|---|---|---|---|
| Set 1 | 7-7-98 | 0.01 | 0.02 | 0.29 |
| Set 2 | 7-7-98 | 0.29 | 0.01 | 0.14 |
| Set 3 | 7-7-98 | 0.00 | 0.02 | 0.29 |
| Set 4 | 7-7-98 | 0.06 | 0.81 | 11.22 |
| Set 6 | 7-7-98 | 20.81 | 14.78 | 28.19 |
| Set 7 | 10-15-98 | 14.71 | 5.69 | 15.50 |
| Set 8 | 10-15-98 | 6.03 | 8.95 | 11.88 |
| Set 5 | 7-7-98 | 0.19 | 0.85 | 0.09 |

Table 3.2. Natural denitrification potential rates in nanomoles $N_2O$/gram oven dried soil/hour and set 5 results in nanomoles $N_2O$/gram oven dried soil/minute.

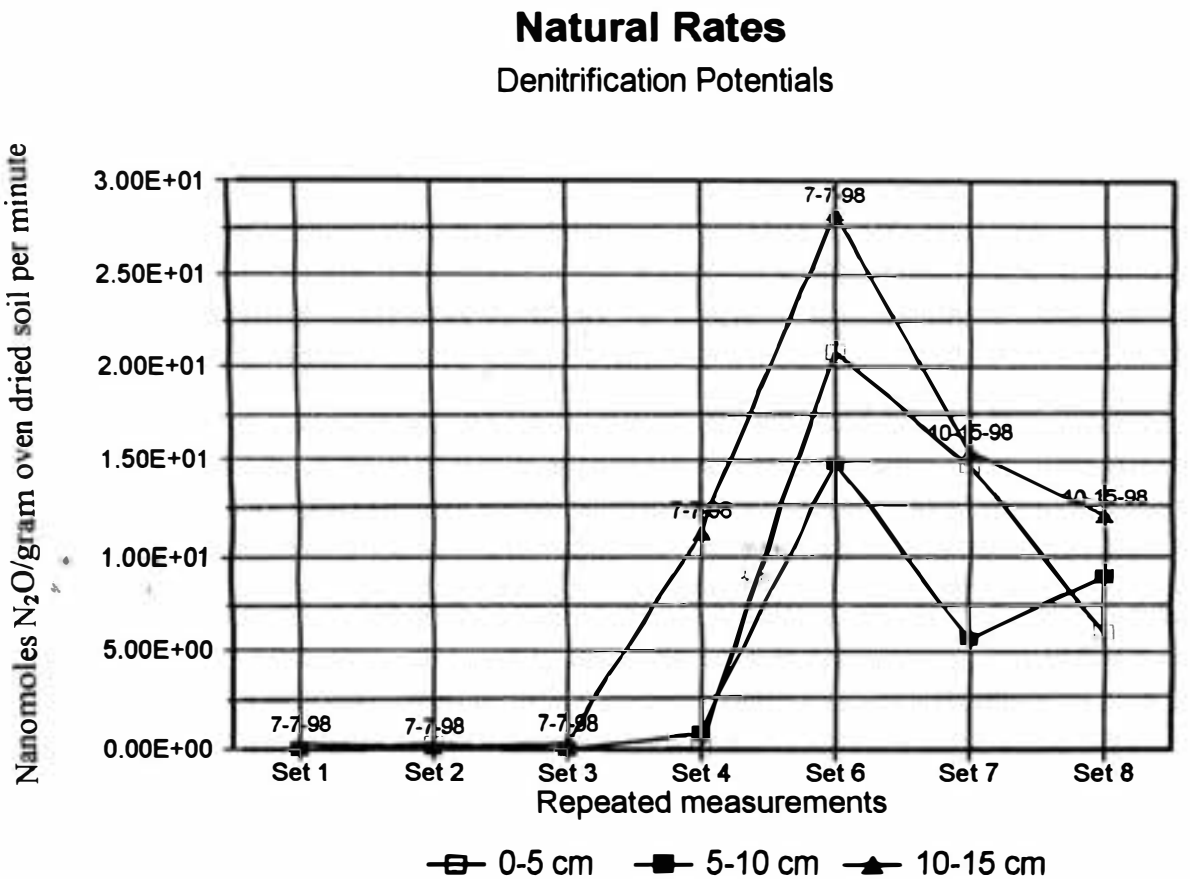



Figure 3.25. Natural denitrification potential rates in nanomoles $N_2O$/gram oven dried soil/hour.

| Chloramphenicol Concentration | 0 | 30 | 60 | 90 | 105 | 120 |
|---|---|---|---|---|---|---|
| 0 g/l | 2.6925 | 4.1592 | 8.0972 | 15.932 | 21.2612 | 24.2811 |
| 0.05 g/l | 2.6925 | 3.8975 | 8.0594 | 11.7748 | 9.7563 | 8.9926 |
| 0.075 g/l | 2.6925 | 3.697 | 8.3376 | 9.1293 | 14.4607 | 17.4769 |
| 0.1 g/l | -1.4236 | 1.7947 | 6.082 | 9.8795 | 12.1559 | -1.4236 |
| 0.15 g/l | -1.4236 | 2.0163 | 6.7906 | 8.9392 | 7.4226 | -1.4236 |
| 0.25 g/l | -1.4236 | 1.8119 | 5.9189 | 9.9988 | 58.7966 | -1.4236 |
| 0.50 g/l | -3.689 | -1.4412 | 2.5329 | 7.7157 | 9.1502 | 12.9252 |
| 0.750 g/l | -3.6989 | -1.8179 | 2.5329 | 6.0381 | 5.9317 | 0.0616 |
| 1.00 g/l | -3.6989 | -0.1886 | 4.2788 | 5.5558 | 7.0122 | 10.2717 |

Table 3.3. 1998 and 1999 Phase I date with varying chloramphenicol concentrations. Phase I results are expressed as nanomoles $N_2O$/gram oven dried soil.

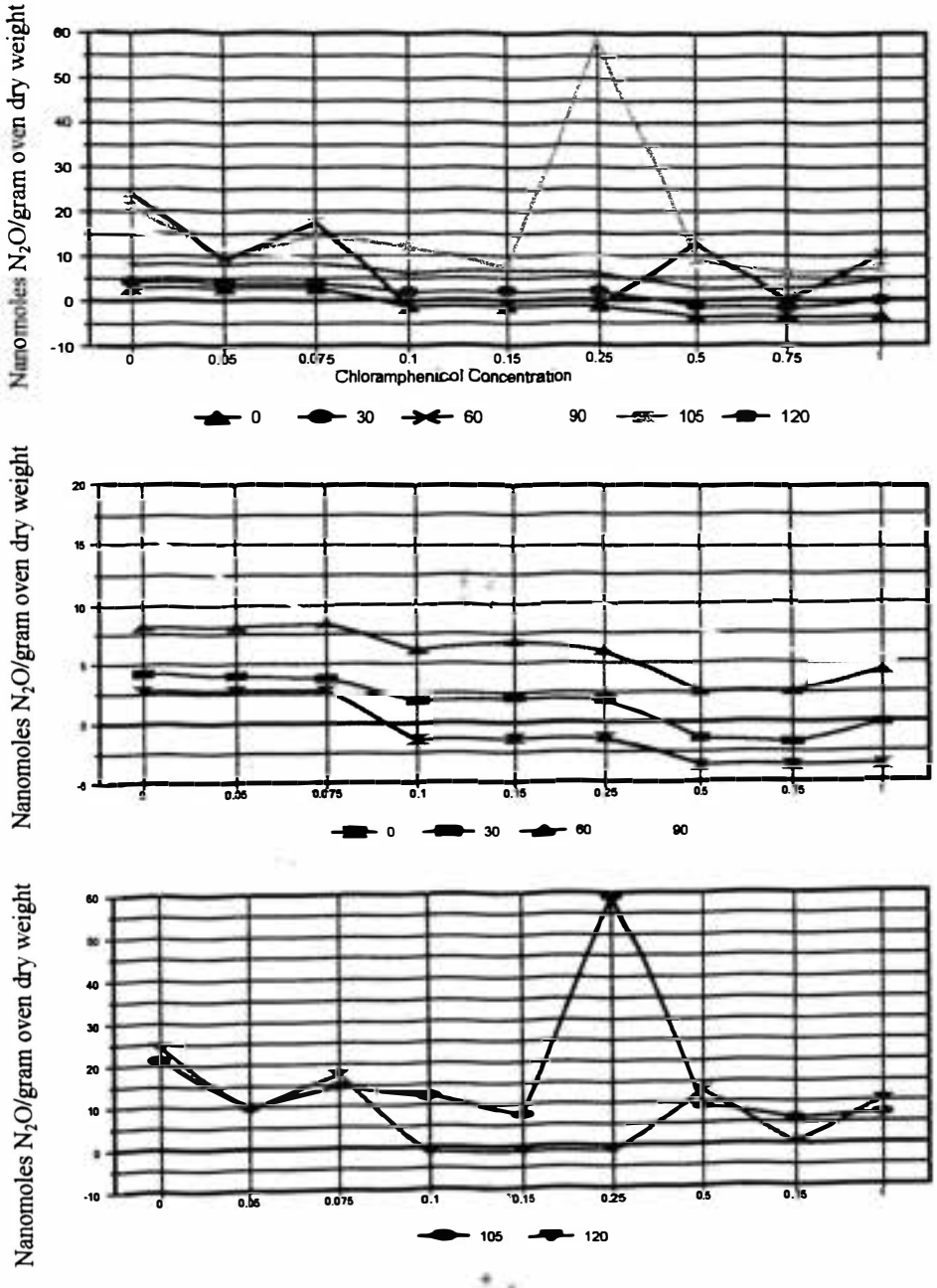



Figure 3.26. $N_2O$ per minute per chloramphenicol concentration.

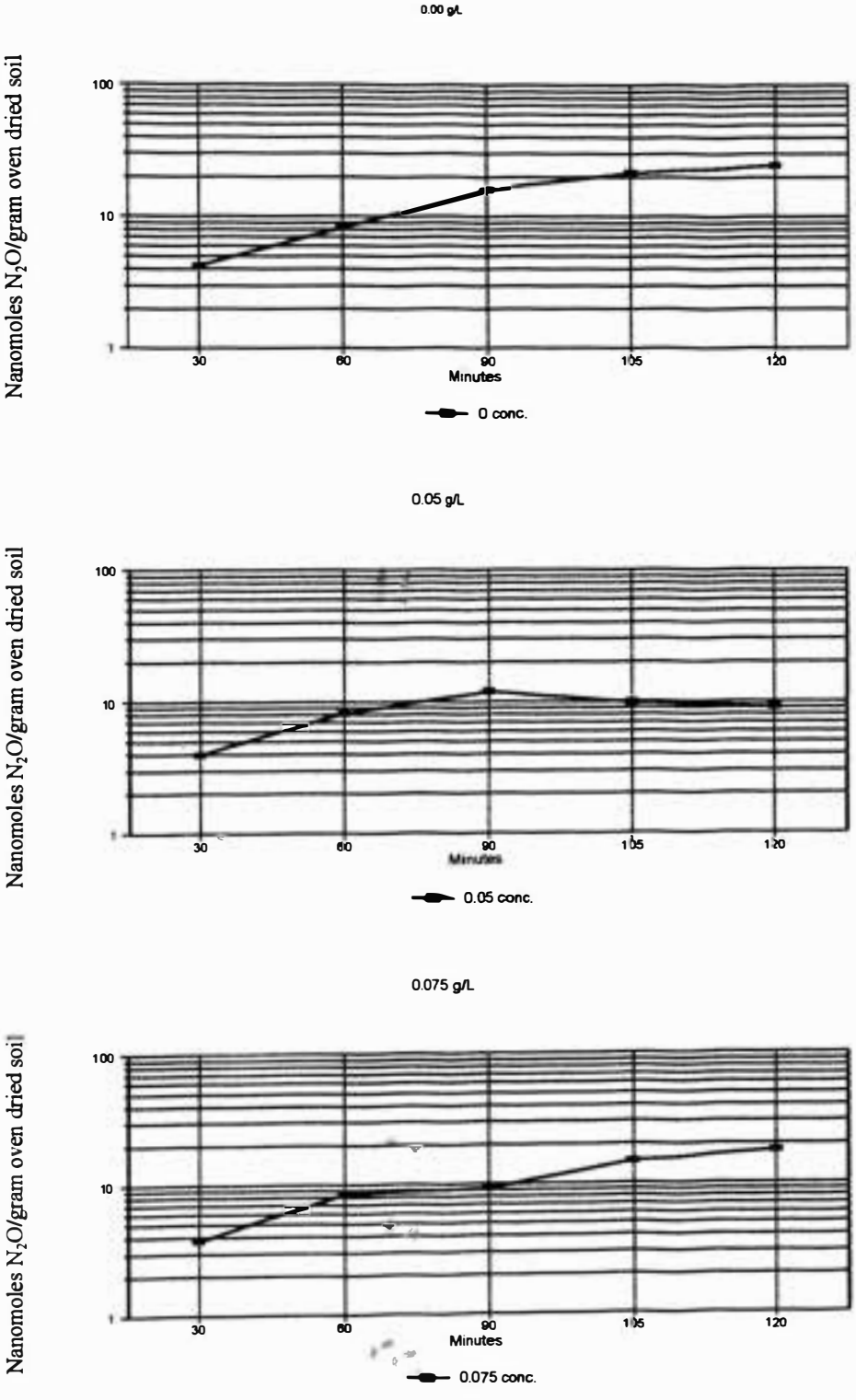



Figure 3.27a. Varying 1998 chloramphenicol concentrations, for Phase I, wetland 5 lowland.

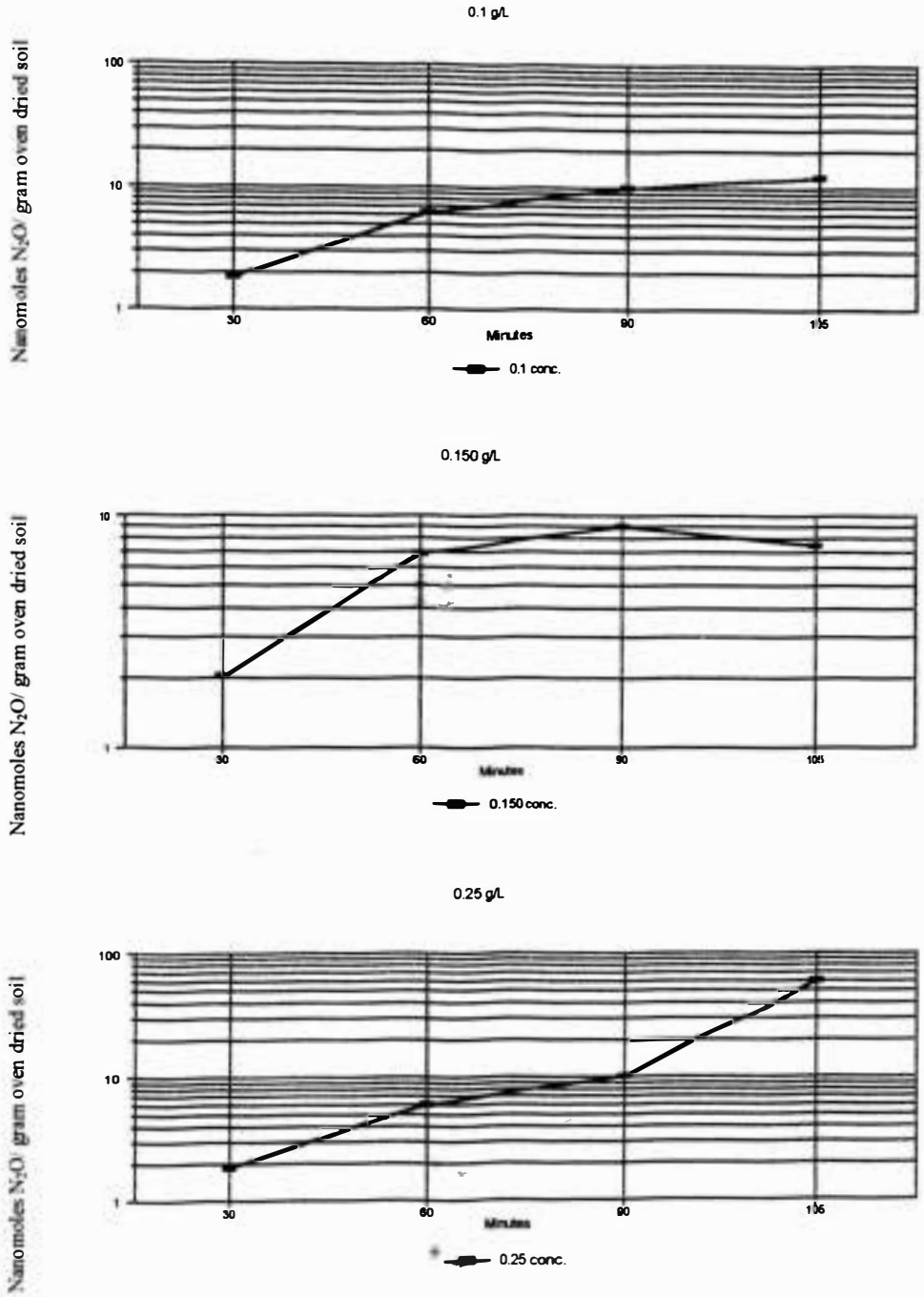



Figure 3.27b. Varying 1998 chloramphenicol concentrations, for Phase I, wetland 5 lowland.

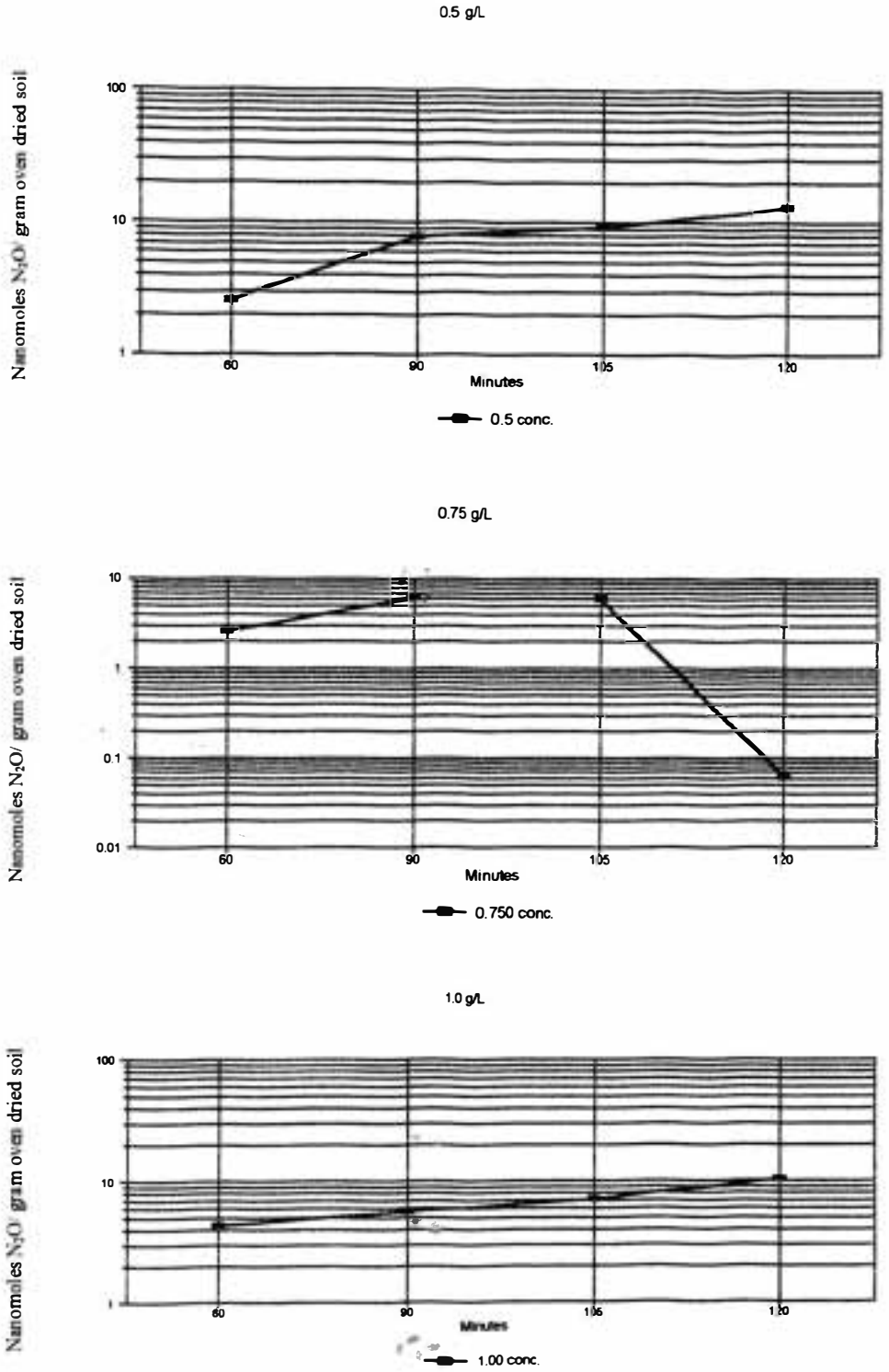



Figure 3.27c. Varying 1998 chloramphenicol concentrations, for Phase I, wetland 5 Lowland.

| Concentration (g/l) | Rate in Nanomoles/g oven dried soil |
|---|---|
| 1 | 0.1097 |
| 0.75 | 0.1150 |
| 0.5 | 0.1206 |
| 0.25 | 0.1265 |
| 0.15 | 0.1326 |
| 0.1 | 0.1391 |
| 0.075 | 0.1458 |
| 0.05 | 0.1529 |
| 0 | 0.1604 |

Table 3.4. 1998 and 1999 Phase I rates for varying chloramphenicol concentrations. Units are in nanomoles $N_2O$ per gram oven dried soil.

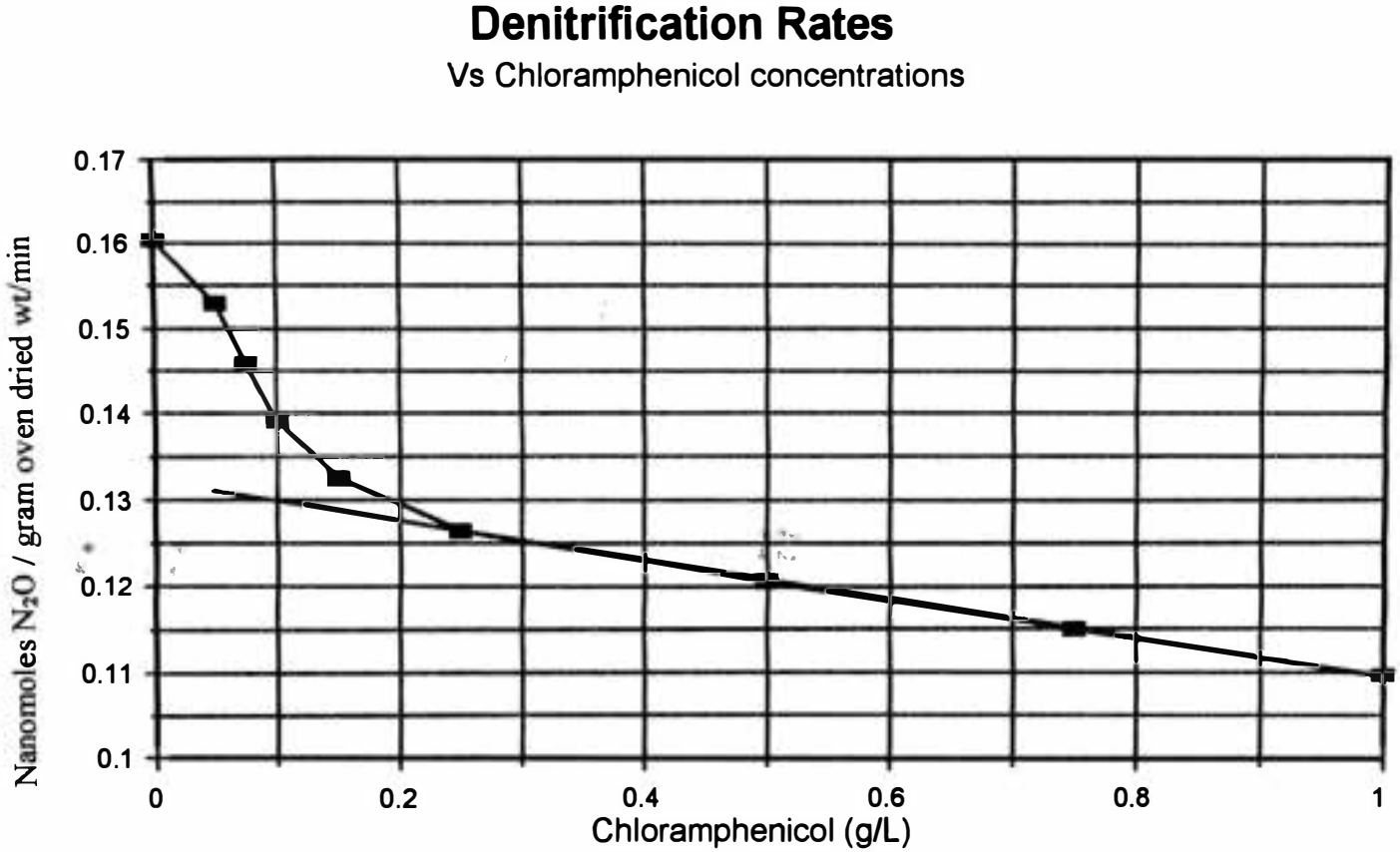
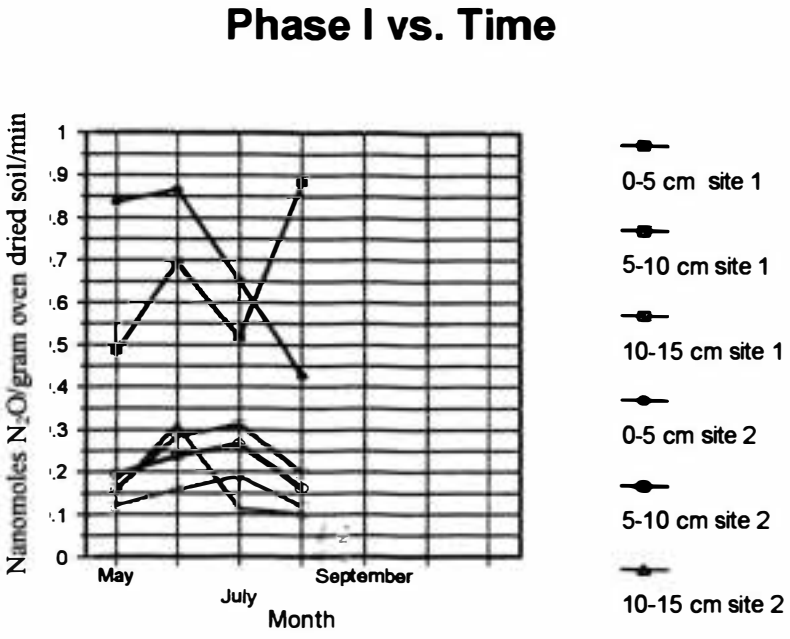



Figure 3.28. 1998 Denitrification rates vs. chloramphenicol concentrations in g/L.

| Position | Depth | Date | | | | |
|---|---|---|---|---|---|---|
| | | 5-21-98 | 6-24-98 | 7-7-98 | 10-2-98 | 10-15-98 |
| Site 1A | 0-5 cm | 0.4468 | 1.5620 | 0.4468 | 0.0561 | 9.5074 |
| | 5-10 cm | 0.0994 | 0.5174 | 0.0994 | 0.0730 | 5.5868 |
| | 10-15 cm | 0.0803 | 0.1753 | 0.0803 | 0.0547 | 5.0633 |
| Site 1B | 0-5 cm | 0.7117 | 0.1373 | 0.7147 | 0.0110 | 106.2060 |
| | 5-10 cm | 0.2862 | 0.1218 | 0.2142 | 0.3601 | 35.3828 |
| | 10-15 cm | 0.1575 | 0.1318 | 0.1790 | 0.0640 | 31.5431 |
| Site 1C | 0-5 cm | 0.2925 | 0.3691 | 0.2925 | 0.4018 | 1.3185 |
| | 5-10 cm | 0.0951 | 0.2150 | 0.0951 | 0.6726 | 0.5871 |
| | 10-15 cm | 0.1121 | 0.1634 | 0.1121 | 0.4840 | 0.5692 |
| Site 2A | 0-5 cm | 1.1397 | 0.6525 | 1.1398 | 2.1886 | 96.8257 |
| | 5-10 cm | 0.1805 | 0.1478 | 0.1805 | 1.6489 | 24.8144 |
| | 10-15 cm | 0.1577 | 0.1455 | 0.1577 | 0.8475 | 10.7306 |
| Site 2B | 0-5 cm | 0.5675 | 1.7549 | 0.3783 | 2.6097 | 241.8404 |
| | 5-10 cm | 0.4123 | 0.4003 | 0.0987 | 0.3068 | 53.1215 |
| | 10-15 cm | 0.3086 | 0.5186 | 0.0995 | 1.1223 | 24.0256 |
| Site 2C | 0-5 cm | 0.6525 | 0.6363 | 0.6525 | 5.7063 | 12.3000 |
| | 5-10 cm | 0.1478 | 0.2199 | 0.1478 | 2.3519 | 4.05916 |
| | 10-15 cm | 0.1455 | 0.2740 | 0.1455 | 2.1621 | 1.3143 |

Table 3.5. Denitrification rates by depth and site for 1998 research.



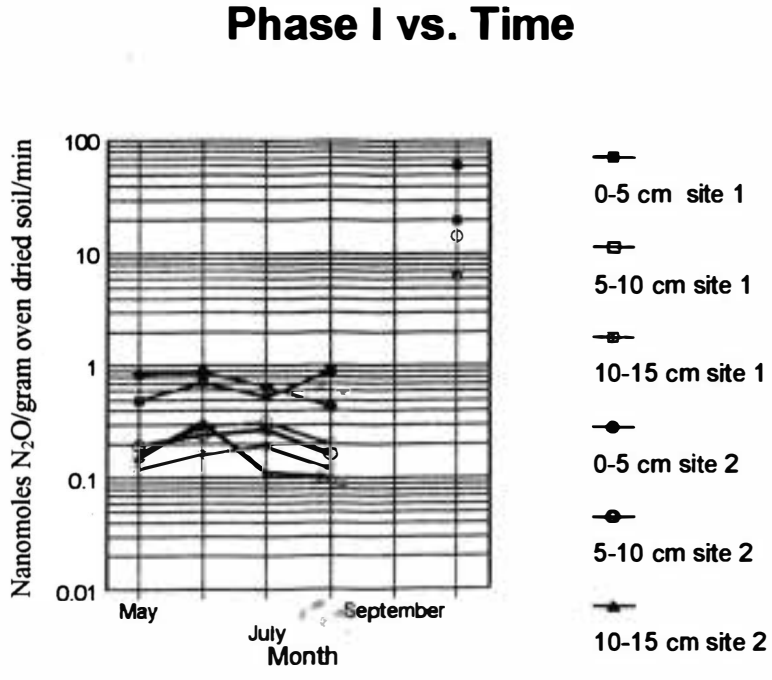
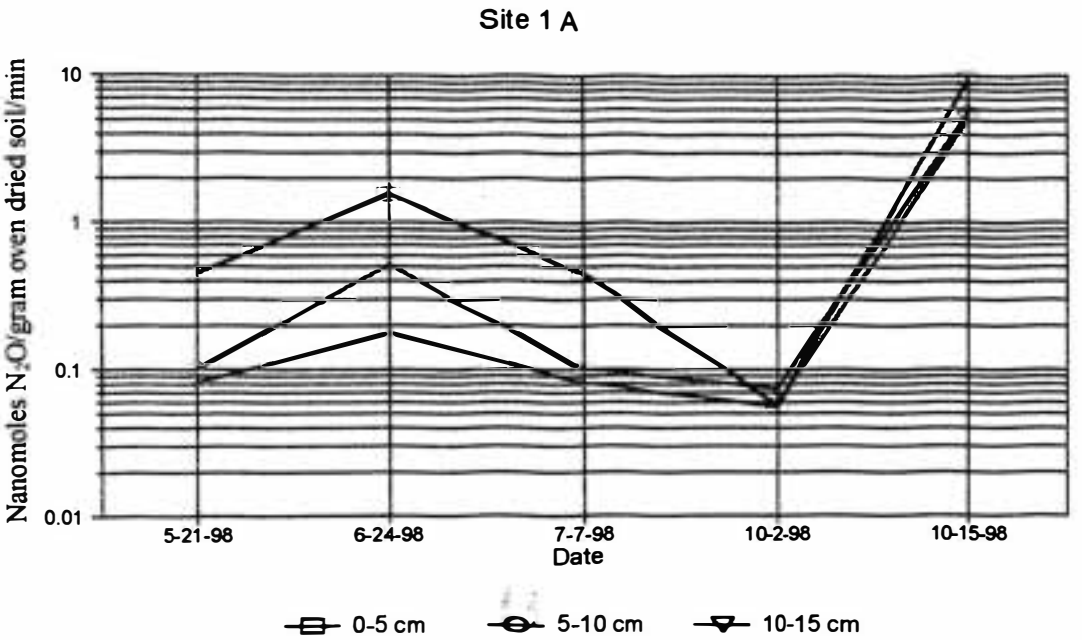




Figure 3.29. 1998 denitrification rates vs. time in nanomoles $N_2O$/gram oven dried soil/min.



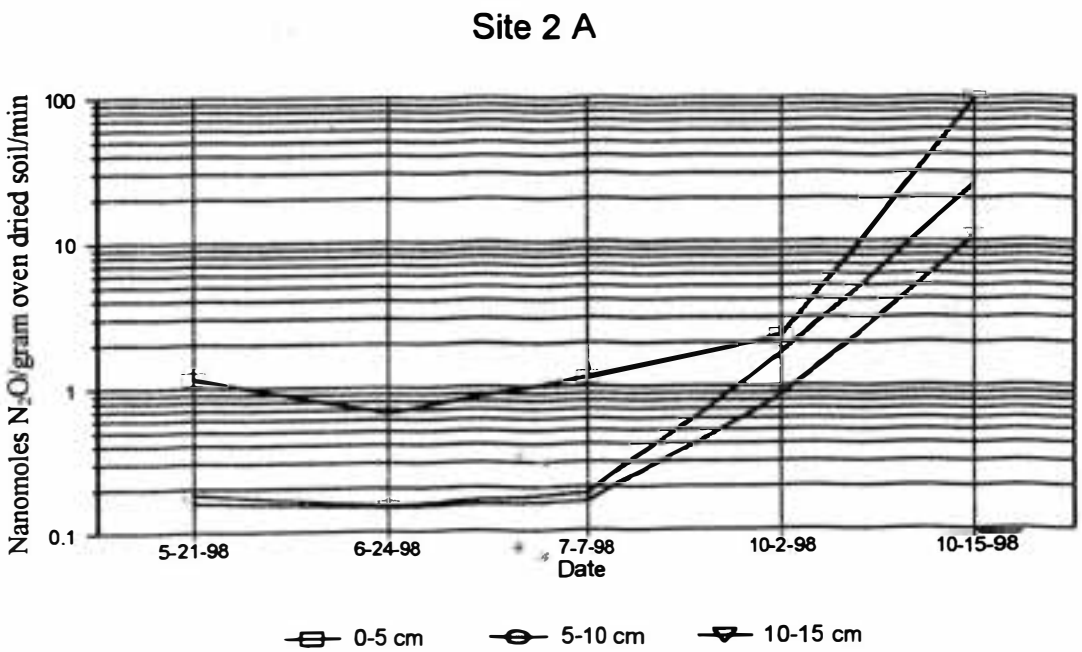




Figure 3.30. Phase I rates for site 1A and site 2A.

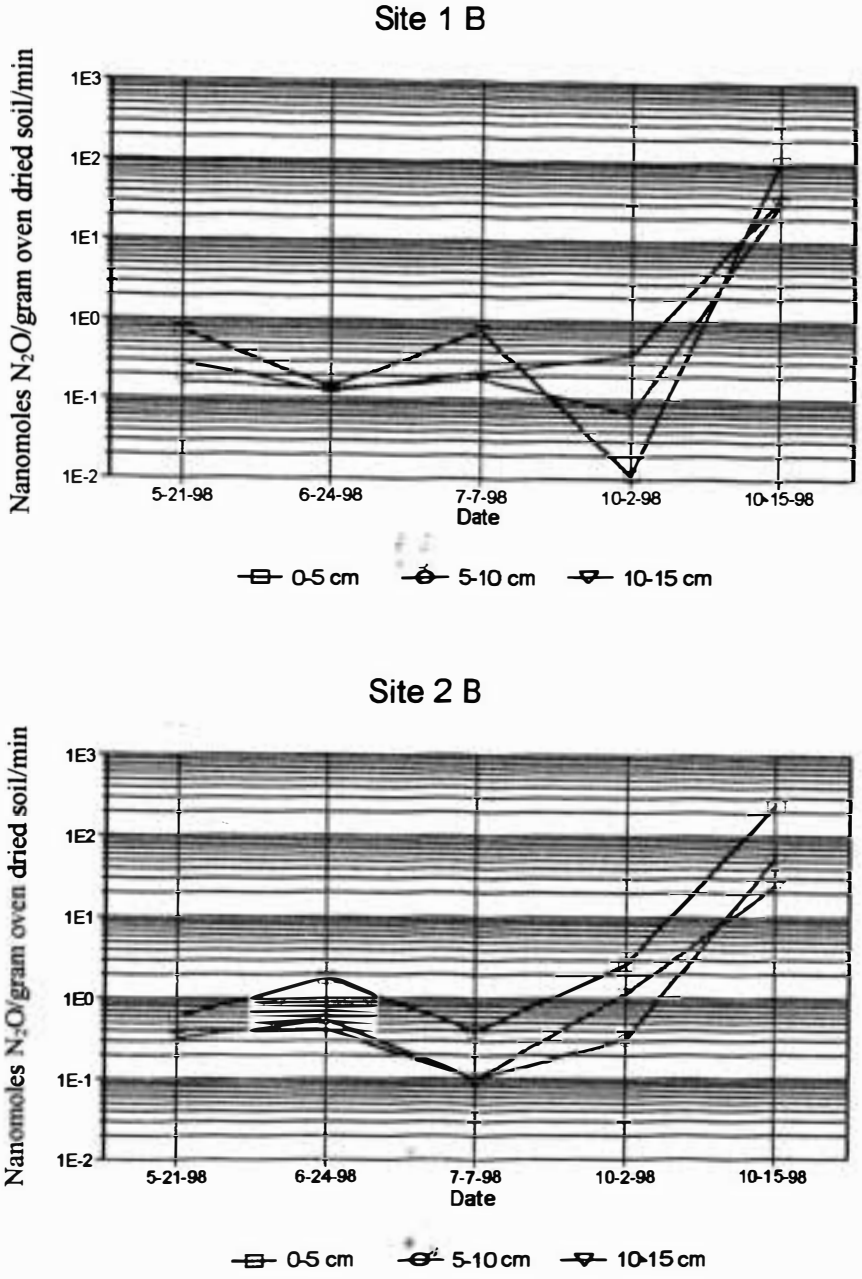



Figure 3.31. Phase I rates for site 1B and site 2B.

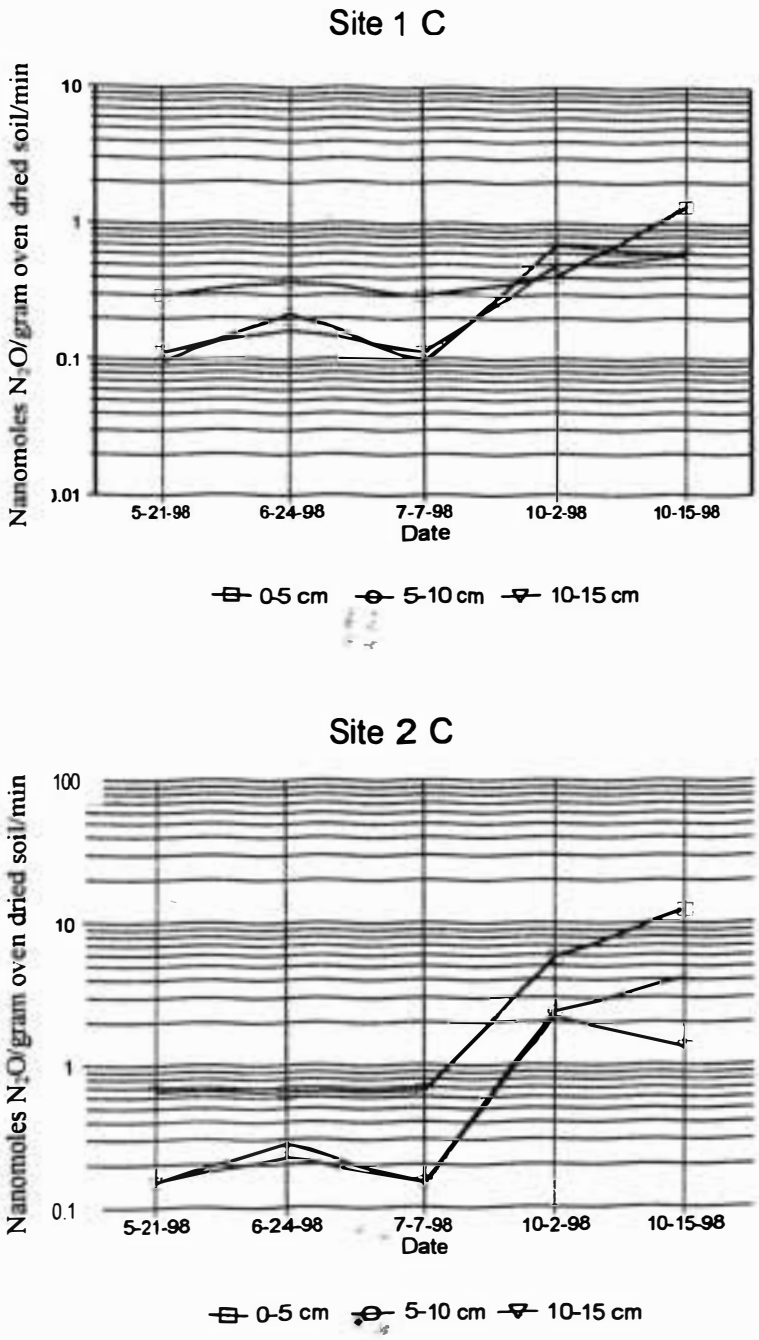
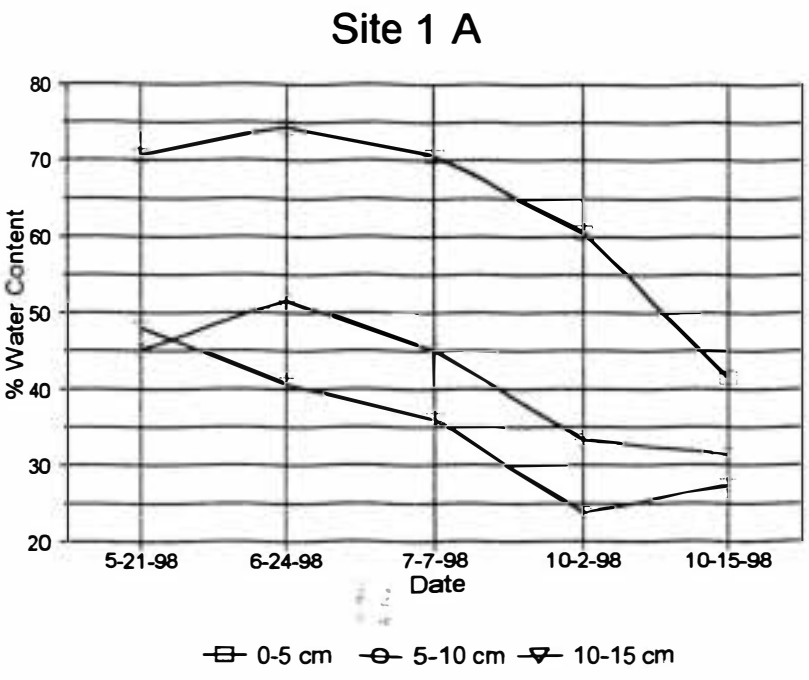



Figure 3.32. Phase I rates for site 1C and site 2C.

| Position | Depth | Date | | | | |
|---|---|---|---|---|---|---|
| | | 5-21-98 | 6-24-98 | 7-7-98 | 10-2-98 | 10-15-98 |
| Site 1A | 0-5 cm | 70.56 | 74.28 | 70.56 | 60.57 | 41.52 |
| | 5-10 cm | 45.00 | 51.60 | 45.00 | 33.32 | 31.36 |
| | 10-15 cm | 47.96 | 40.56 | 35.98 | 23.68 | 27.28 |
| Site 1B | 0-5 cm | 62.57 | 63.87 | 65.32 | 48.84 | 53.02 |
| | 5-10 cm | 42.23 | 50.12 | 43.56 | 31.26 | 27.16 |
| | 10-15 cm | 35.84 | 39.94 | 33.04 | 24.32 | 20.80 |
| Site 1C | 0-5 cm | 48.28 | 61.54 | 65.32 | 48.84 | 38.08 |
| | 5-10 cm | 38.24 | 41.88 | 42.36 | 31.26 | 24.70 |
| | 10-15 cm | 37.06 | 36.32 | 37.62 | 24.32 | 22.18 |
| Site 2A | 0-5 cm | 76.00 | 58.88 | 46.02 | 49.82 | 56.84 |
| | 5-10 cm | 47.26 | 38.52 | 40.28 | 26.40 | 34.76 |
| | 10-15 cm | 41.80 | 34.74 | 37.46 | 20.42 | 30.30 |
| Site 2B | 0-5 cm | 55.13 | 77.14 | 56.08 | 72.10 | 66.44 |
| | 5-10 cm | 35.80 | 48.38 | 39.34 | 63.07 | 41.72 |
| | 10-15 cm | 34.00 | 43.47 | 36.74 | 44.38 | 30.54 |
| Site 2C | 0-5 cm | 56.52 | 62.94 | 64.20 | 66.00 | 57.60 |
| | 5-10 cm | 44.68 | 42.22 | 42.72 | 52.56 | 40.04 |
| | 10-15 cm | 37.72 | 38.16 | 36.80 | 43.96 | 25.04 |

Table 3.6. Gravimetric percent water content by depth and site for 1998 research.



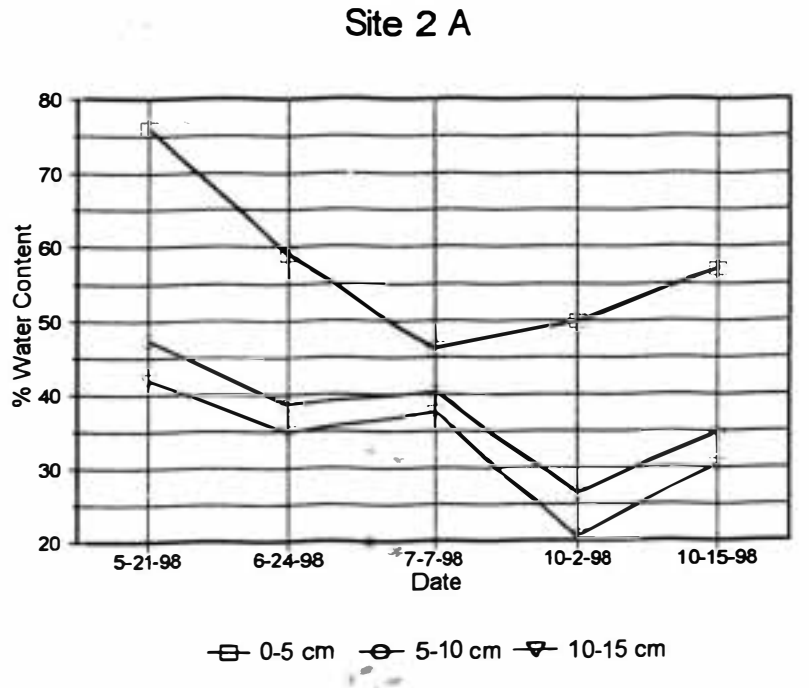




Figure 3.33. Gravimetric percent water content for site 1A and site 2A

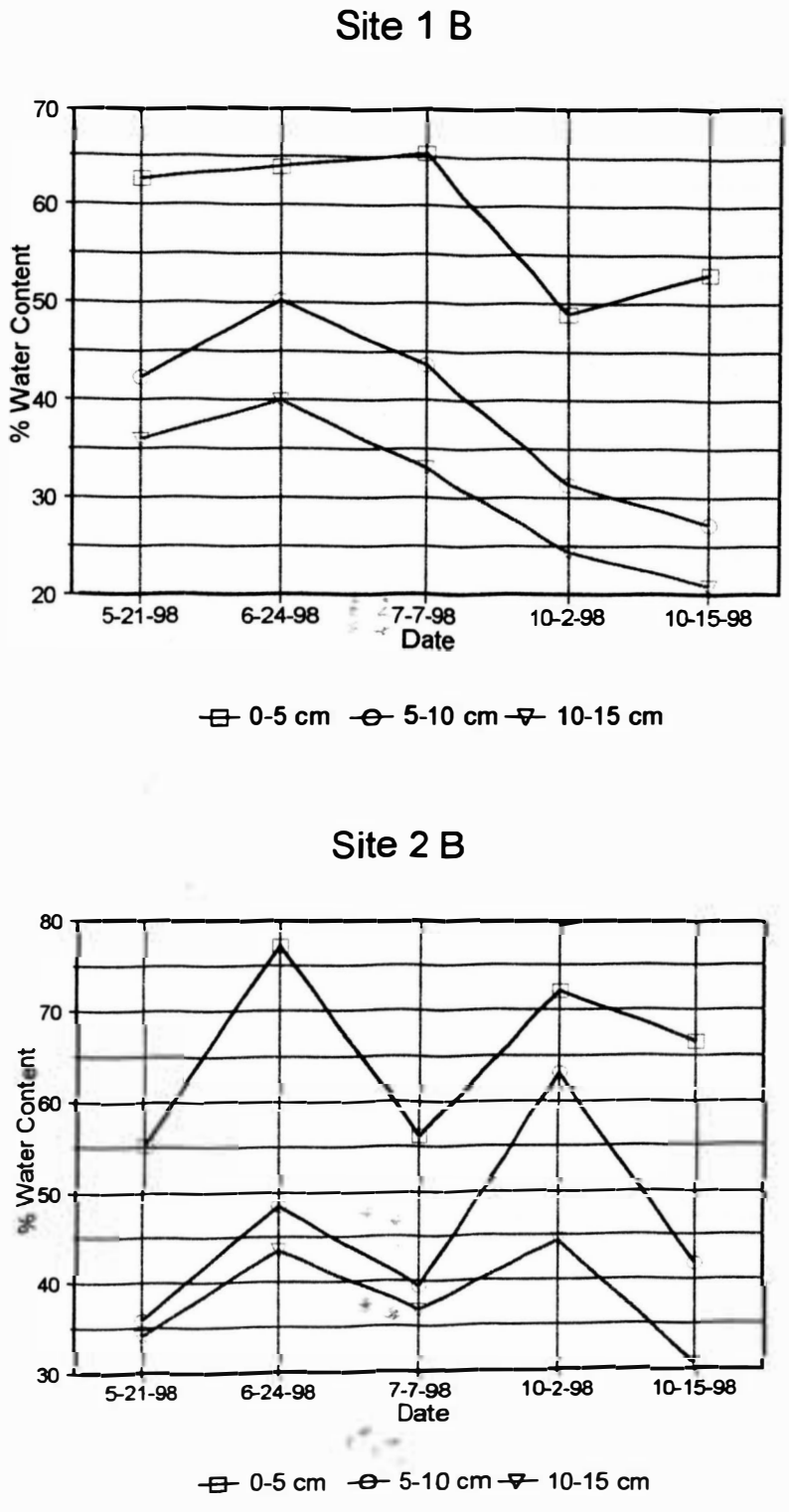
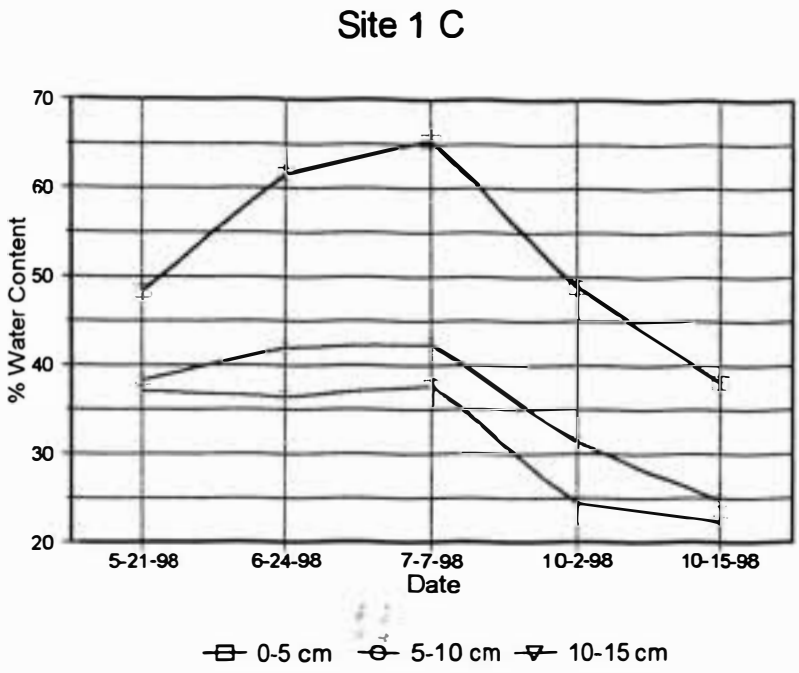



Figure 3.34 Gravimetric percent Water Content for site 1B and site 2B.



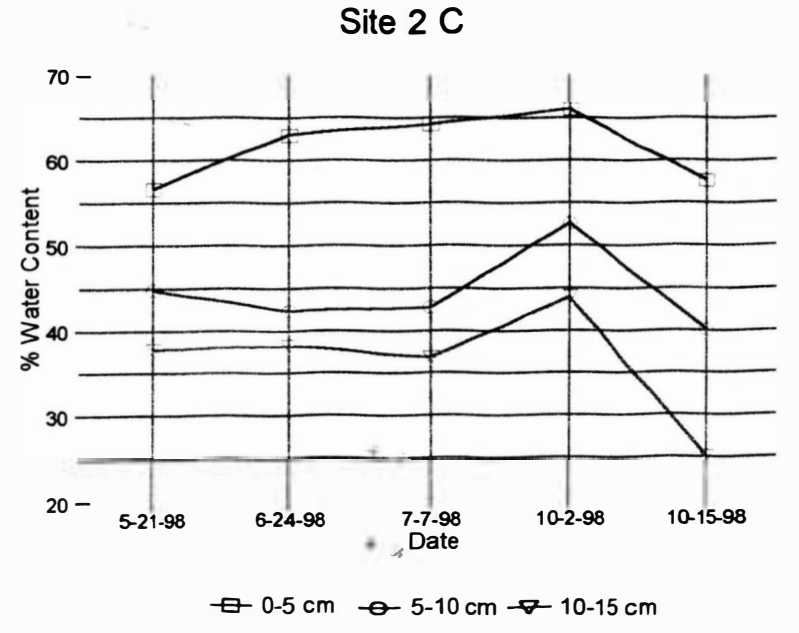




Figure 3.35. Gravimetric percent water content for site 1C and 2C.

| Position | Depth | Date | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 5-21 | 6-24 | 7-7 | 7-22 | 8-11 | 10-2 | 10-15 |
| Site 1 A | 0-5 cm | 17 | 5 | 15 | 13 | 10 | 19 | 8 |
| | 5-10 cm | 12 | 8 | 13 | 14 | 6 | 8 | 7 |
| | 10-15 cm | 11 | 3 | 11 | 3 | 2 | 7 | 7 |
| Site 1 B | 0-5 cm | 12 | 7 | 12 | 10 | 11 | 18 | 11 |
| | 5-10 cm | 11 | 6 | 10 | 10 | 5 | 8 | 10 |
| | 10-15 cm | 10 | 6 | 9 | 11 | 9 | 8 | 10 |
| Site 1 C | 0-5 cm | 14 | 8 | 11 | 13 | 13.7 | 12 | 10 |
| | 5-10 cm | 12 | 6 | 11 | 5 | 6 | 10 | 10 |
| | 10-15 cm | 13 | 7 | 2 | 1 | 5.3 | 8 | 10 |
| Site 2 A | 0-5 cm | 17 | 6 | 12 | 10 | 5 | 14 | 11 |
| | 5-10 cm | 16 | 3 | 10 | 2 | 2 | 14 | 10 |
| | 10-15 cm | 17 | 6 | 11 | 1 | 3 | 7 | 10 |
| Site 2 B | 0-5 cm | 18 | 8 | 11 | 10 | 23 | 26 | 19 |
| | 5-10 cm | 17 | 7 | 9 | 10 | 12 | 10 | 10 |
| | 10-15 cm | 16 | 8 | 10 | 10 | 6 | 10 | 10 |
| Site 2 C | 0-5 cm | 14 | 7 | 11 | 11 | 26 | 18 | 11 |
| | 5-10 cm | 11 | 8 | 9 | 10 | 8 | 1 | 10 |
| | 10- 15 cm | 20 | 7 | 9 | 9 | 5 | 10 | 10 |

Table 3.7. Wetland soil nitrate in parts per million by depth for 1998 soil samples.

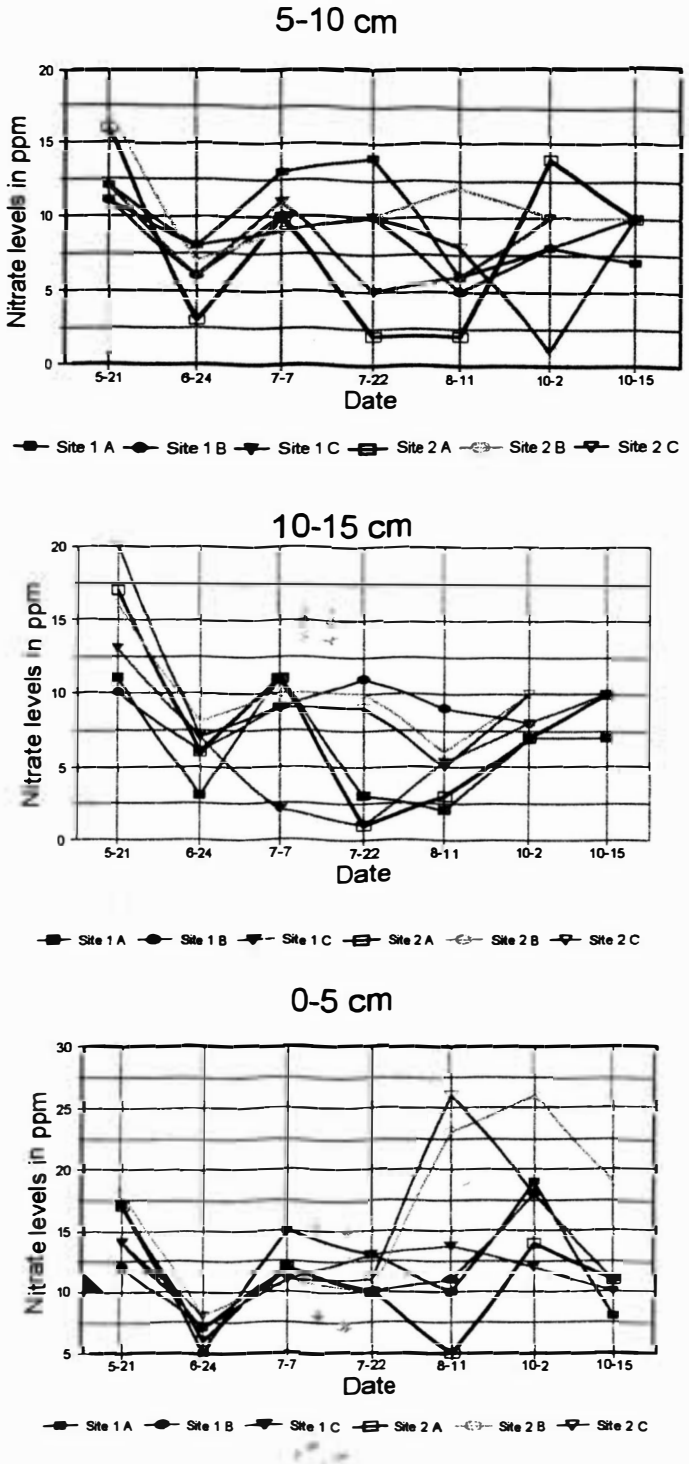



Figure 3.36. Wetland soil nitrate levels by depth.

| Position | Depth | Date | | |
|---|---|---|---|---|
| | | 5-21 | 8-11 | 10-15 |
| Site 1 A | 0-5cm | 14.5 | 13.9 | 8.1 |
| | 5-10 cm | 6.7 | 9.1 | 5.8 |
| | 10-15 cm | 4.9 | 5.2 | 5.6 |
| | | | | |
| Site 1 B | 0-5 cm | 8 | 13.6 | 10.2 |
| | 5-10 cm | 4.8 | 6.2 | 5.5 |
| | 10-15 cm | 4.6 | 4.6 | 4.8 |
| | | | | |
| Site 1 C | 0-5 cm | 8.6 | 13.7 | 9 |
| | 5-10 cm | 5.3 | 6 | 6.1 |
| | 10-15 cm | 4.7 | 5.3 | 5.6 |
| | | | | |
| Site 2 A | 0-5 cm | 14.3 | 7.4 | 11.2 |
| | 5-10 cm | 6 | 6.7 | 7.1 |
| | 10-15 cm | 4.8 | 6 | 5.6 |
| | | | | |
| Site 2 B | 0-5 cm | 11.4 | 9.6 | 14.9 |
| | 5-10 cm | 5.8 | 6.4 | 9.1 |
| | 10-15 cm | 4.1 | 4.4 | 5.5 |
| | | | | |
| Site 2 C | 0-5 cm | 7.5 | 9.4 | 11.5 |
| | 5-10 cm | 4.3 | 4.8 | 7.7 |
| | 10-15 cm | 4.3 | 3.9 | 5.8 |
| | | | | |

Table 3.98. Wetland soil percent organic matter by depth for 1998 soil samples.

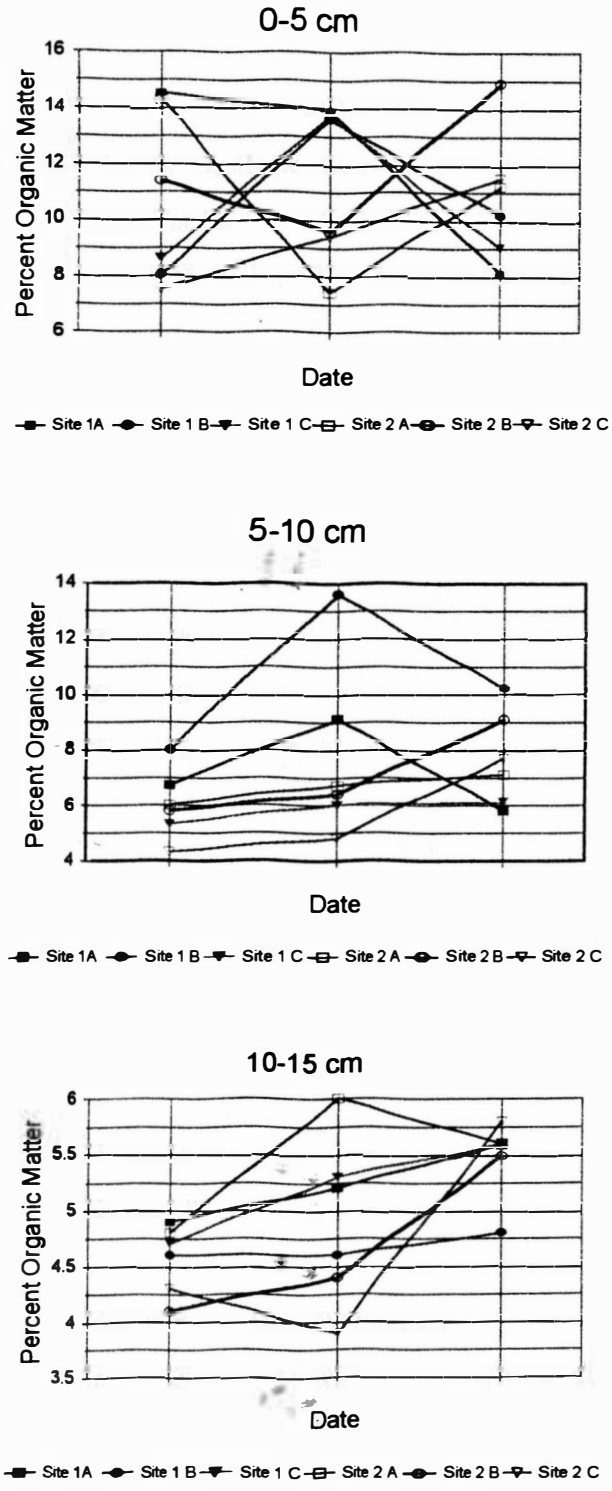



Figure 3.37. Wetland soil percent organic matter by depth.

| Position | Depth | Date | | |
|---|---|---|---|---|
| | | 5-21-98 | 8-11-98 | 10-15-98 |
| site1 A | | | | |
| | 0-5cm | 6.9 | 6.3 | 7.5 |
| | 5-10cm | 6.8 | 6.2 | 7.6 |
| | 10-15cm | 7 | 6 | 7.7 |
| site1 B | | | | |
| | 0-5cm | 6.9 | 6.3 | 7.6 |
| | 5-10cm | 7.3 | 6 | 7.7 |
| | 10-15cm | 7.5 | 6.4 | 7.7 |
| site1 C | | | | |
| | 0-5cm | 7.1 | 6.2 | 7.6 |
| | 5-10cm | 7.3 | 6.1 | 7.7 |
| | 10-15cm | 7.4 | 5.3 | 7.7 |
| site2 A | | | | |
| | 0-5cm | 7.2 | 6.2 | 7.6 |
| | 5-10cm | 7.2 | 6.5 | 7.6 |
| | 10-15cm | 7.2 | 6.3 | 7.8 |
| site2 B | | | | |
| | 0-5cm | 7.2 | 6.3 | 7.7 |
| | 5-10cm | 7.1 | 6.3 | 7.5 |
| | 10-15cm | 7.4 | 6.4 | 7.7 |
| site2 C | | | | |
| | 0-5cm | 7.1 | 6.2 | 7.6 |
| | 5-10cm | 7.2 | 6.4 | 7.6 |
| | 10-15cm | 7.4 | 6.7 | 7.8 |

Table 3.9. Wetland soil pH measurements by depth for 1998 soil samples.

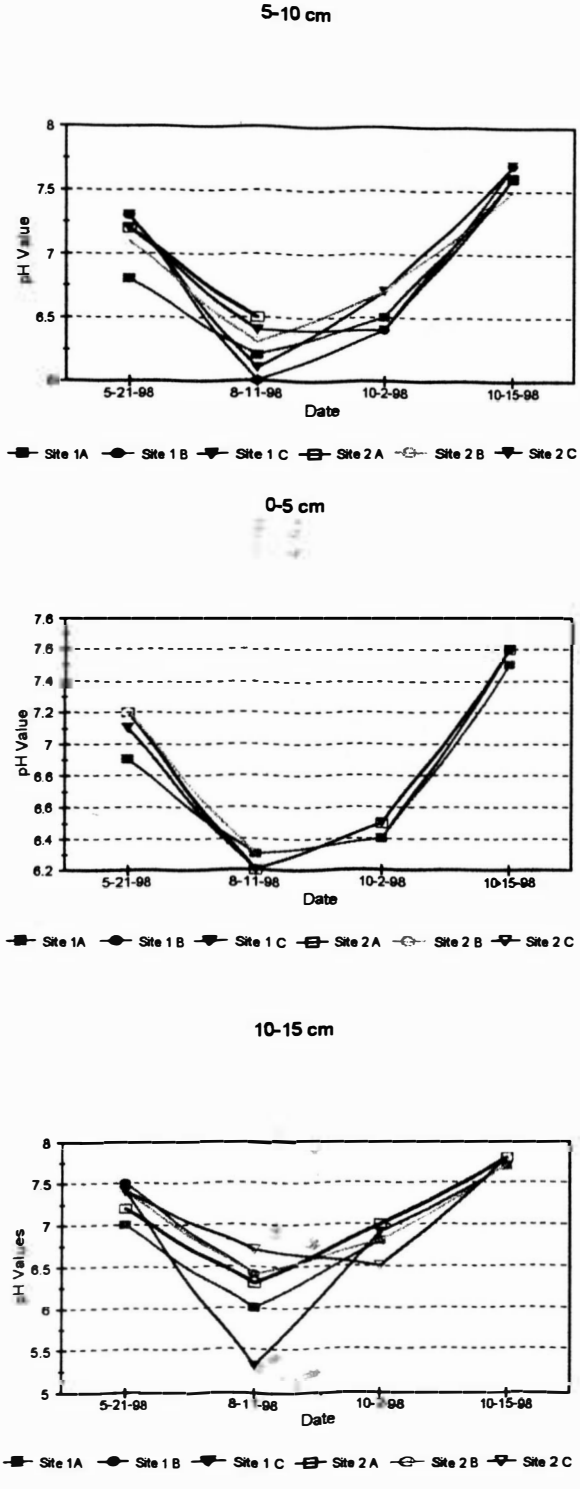



Figure 3.38. Wetland soil pH by depth.

| mmho/cm | Salts | | |
|---|---|---|---|
| | | | |
| site1 A | | site2 A | |
| 0-5 cm | 4 | 0-5 cm | 3.4 |
| 5-10 cm | 3.7 | 5-10 cm | 3.1 |
| 10-15 cm | 3.5 | 10-15 cm | 4 |
| site1 B | | site2 B | |
| 0-5 cm | 2.8 | 0-5 cm | 3.1 |
| 5-10 cm | 2.5 | 5-10 cm | 2.9 |
| 10-15 cm | 2 | 10-15 cm | 3.9 |
| site1 C | | site2 C | |
| 0-5 cm | 3.4 | 0-5 cm | 2.6 |
| 5-10 cm | 2.7 | 5-10 cm | 3.7 |
| 10-15 cm | 4.3 | 10-15 cm | 4.3 |

Table 3.10. Wetland soil salts content by depth for 1998 soil samples.

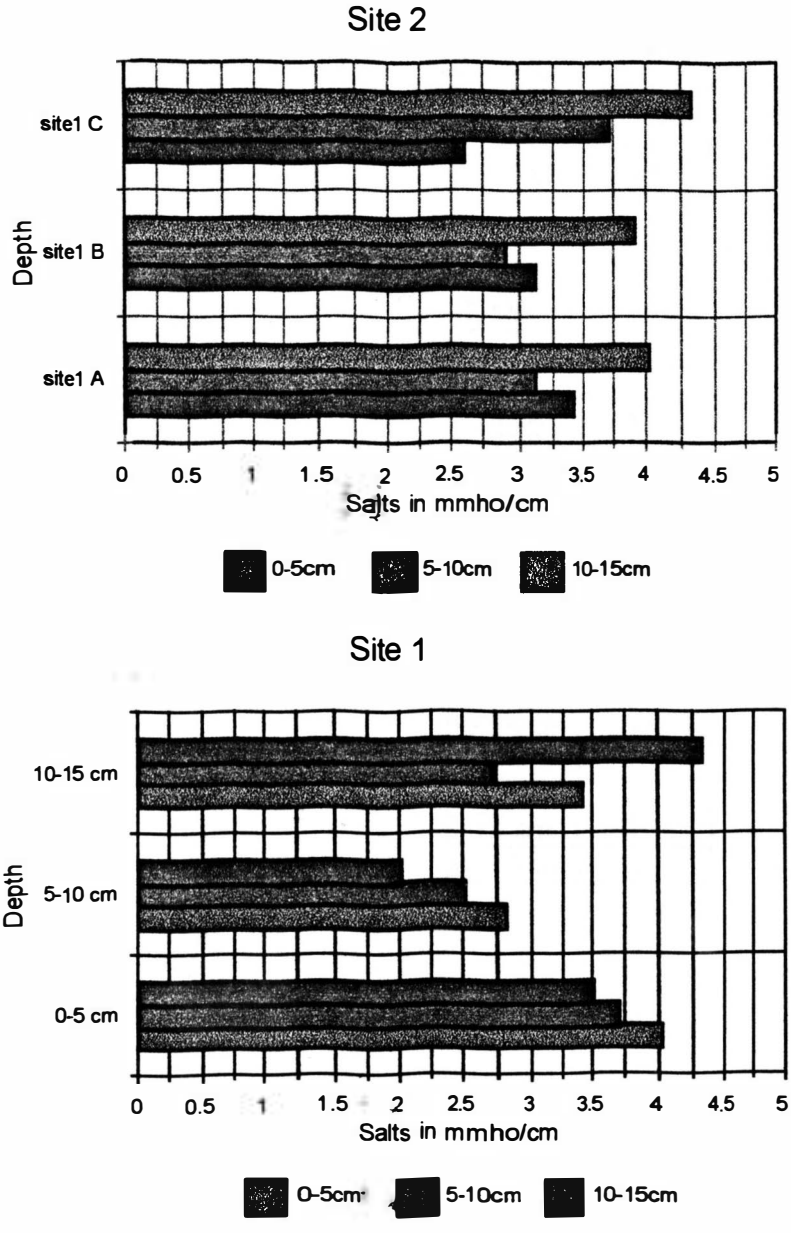



Figure 3.39. Wetland soil salts.

| Soil Phosphorus | | |
|---|---|---|
| site1 A | 0-5 cm | 39 |
| | 5-10 cm | 38 |
| | 10-15 cm | 37 |
| | | |
| site1 B | 0-5 cm | 45 |
| | 5-10 cm | 43 |
| | 10-15 cm | 45 |
| | | |
| site1 C | 0-5 cm | 43 |
| | 5-10 cm | 52 |
| | 10-15 cm | 47 |
| | | |
| site2 A | 0-5 cm | 38 |
| | 5-10 cm | 48 |
| | 10-15 cm | 52 |
| | | |
| site2 B | 0-5 cm | 42 |
| | 5-10 cm | 30 |
| | 10-15 cm | 32 |
| | | |
| site2 C | 0-5 cm | 31 |
| | 5-10 cm | 43 |
| | 10-15 cm | 37 |

| Soil Potassium | | |
|---|---|---|
| site1 A | 0-5 cm | 269 |
| | 5-10 cm | 338 |
| | 10-15 cm | 325 |
| | | |
| site1 B | 0-5 cm | 404 |
| | 5-10 cm | 325 |
| | 10-15 cm | 265 |
| | | |
| site1 C | 0-5 cm | 378 |
| | 5-10 cm | 370 |
| | 10-15 cm | 318 |
| | | |
| site2 A | 0-5 cm | 338 |
| | 5-10 cm | 410 |
| | 10-15 cm | 381 |
| | | |
| site2 B | 0-5 cm | 308 |
| | 5-10 cm | 358 |
| | 10-15 cm | 302 |
| | | |
| site2 C | 0-5 cm | 403 |
| | 5-10 cm | 387 |
| | 10-15 cm | 334 |

Table 3.12. Wetland soil potassium and phosphorus levels in parts per million by depth for 1998 soil samples.

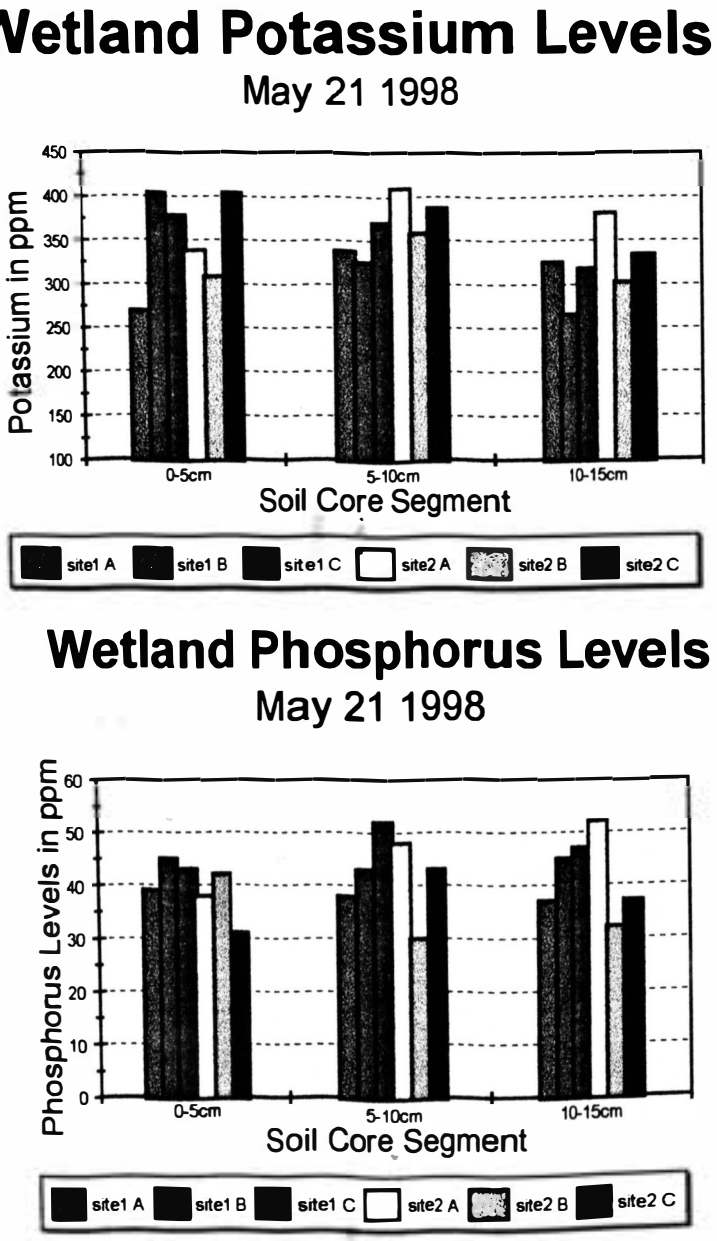



Figure 3.40. Wetland potassium and phosphorus levels.

# Appendices Finale