WATER RESOURCES research center

Publication No. 55

EFFECT OF OXIDATION OF SOIL ORGANIC MATTER ON WATER QUALITY: ROLE OF NITRIFICATION AND DENITRIFICATION

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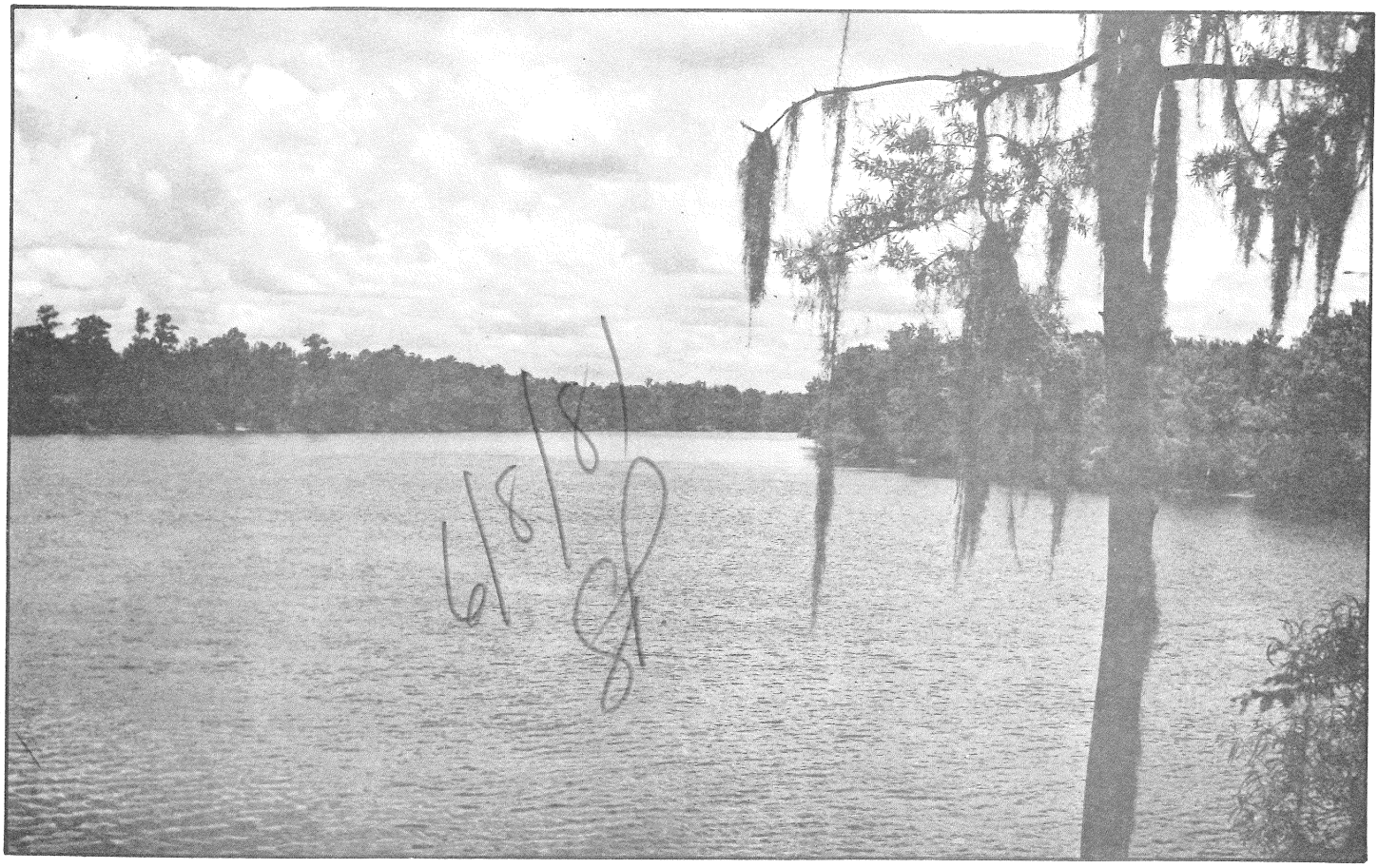

UNIVERSITY OF FLORIDA

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RESEARCH PROJECT TECHNICAL COMPLETION REPORT

OWRT Project Number B-041-FLA

Matching Grant Agreement Number
14-34-0001-9063

Report Submitted April, 1981

The work upon which this report is based was supported in part by funds provided by the United States Department of the Interior, Office of Water Research and Technology as Authorized under the Water Resources Research Act of 1964 as amended.

ACKNOWLEDGMENTS

Appreciation is gratefully extended to the Office of Water Research and Technology, United States Department of the Interior, for providing financial support of this project. The helpful comments of Dr. W. H. Morgan (former Director) and Dr. James Heaney (present Director) of the Florida Water Resources Research Center about administration of the project are greatly appreciated. Thanks are also due Ms. Mary Robinson for accounting assistance. Matching financial support from the Center for Environmental programs, and the Institute of Food and Agricultural Sciences, University of Florida is gratefully acknowledged.

The excellent technical help of Ms. Elsa Garcia, Ms. Elizabeth Hill, Ms. Clara Bedsole, Ms. B. Carol Hall, Mr. Gary Dresser, and Mr. William Reve is greatly appreciated.

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ABSTRACT

Mineralization of soil organic matter of drained Histosols has a significant effect on drainage waters. Thus, the microbiological transformations of nitrogen in Pahokee muck, a drained Histosol, were examined in order to quantify production of mineral nitrogen in the soil via mineralization of the soil organic matter and the subsequent nitrification, and denitrification of this nitrogen, and to observe the effect of the physical and chemical environment on these reactions. Highest potential denitrification rates were detected in cultivated fields. These rates, 7.21 and 7.27 μg N/cm^3/day for soils cropped to sugarcane (Saccharum spp. L) or St. Augustinegrass (Stenotapharum secundatum (Walt) Kuntz) respectively, were about 3-fold that of a soil from a fallow field. Sufficient denitrification capacity was detected to allow for denitrication of all the nitrogen mineralized from the soil organic matter. Nitrite accumulated in low concentrations in the soils as a result of denitrification. Denitrification was limited by carbon in uncultivated soils, but not in the soils from the sugarcane or grass fields.

Nitrous oxide evolution from the soil was inhibited by high nitrate levels in drained, cultivated and uncultivated Pahokee muck but not in flooded soils and ditch sediments. During flooding, the nitrous oxide evolution from the soil declined to nearly zero, denitrification capacity and denitrifier populations increased, while soil nitrate levels decreased about 80 percent. Ammonium levels increased about 5-fold.

Large populations of heterotrophic nitrifiers were found in Pahokee muck. These populations correlated with soil moisture and aerobic bacterial population densities. No such relationship was found for autotrophic nitrifiers. In soil that had been heated to kill the autotrophic nitrifiers but not the heterotrophs then amended with sodium acetate and/or ammonium sulfate, no nitrification was detected. Ammonium was oxidized to nitrate in control soil containing both autotrophic and heterotrophic nitrifiers. These data suggest that autotrophic nitrifiers were the sole population responsible for nitrification in Pahokee muck.

INTRODUCTION

While transformations of native soil organic matter in mineral soils may be contributory to water quality problems, the major practices of concern are the agronomic, industrial and urban uses of the soil imposed by the presence of man. That is, greater pollution problems are generally created by contamination of runoff waters due to anthropogenic causes than can be associated with the natural biological processes associated with the soil ecosystem. Such is not the case with drained organic soils (Histosols). These soils are composed of greater than 20 to 30 percent soil organic matter (the percent depends upon the clay content) and frequently may contain as much as 95 percent soil organic matter (McCollum et al. 1976; Farnham and Finney, 1965). For example, the organic soils of the Everglades Agricultural Area of south Florida contain greater than 80 percent colloidal organic matter (Terry and Tate, 1980a). Water quality problems are derived from the fact that this soil component is biodegradable. The soil organic matter accumulated in part due to the high moisture conditions existant prior to drainage. Once the protective effect of this water is removed, the organic matter is again available for catabolism by the soil microbial community. This mineralization of the soil organic matter results in the production of mineral nitrogen and phosphorus which may reach regional waterways. Recent studies have shown that of the 1400 kg N/ha/ year mineralized in the Everglades Agricultural Area (Tate, 1976; Terry, 1980), 12 to 40 kg/ha/ year reaches regional drainage waters (Florida Sugar Cane League, 1978). The problems associated with the mineralization of the nitrogen and phosphorus also result from water movement necessary for the development of this soil type. Organic soils are formed under water-logged conditions, i.e. they are generally swamps or marshes. Thus, the environment is water-saturated. This saturation results from water drainage from soils of higher elevation surrounding the swamp or accumulation of large quantities of rainwater or both. Thus, it is necessary to remove waters from the soil to maintain proper soil conditions for development. This water relocation combined with the production of potential water pollutants leads to regional water quality problems.

Thus, this project was initiated to study the microbiological processes occurring in Histosols of the Everglades Agricultural Area involved with the production of the mineral nitrogen forms which may lead to deterioration of regional waters. Specific processes examined were nitrification and denitrification. Environmental conditions limiting these processes as well as accumulation of their products, ammonium, nitrite and nitrate, within the soil matrix were also studied. Specific research objectives were as follows:

i) To identify and quantitate the nitrogenous products of subsidence of organic soils and to observe the movement of these products into regional waterways.

ii) To determine the microbial processes producing the nitrogenous compounds and to examine how the effect of the physical and

chemical properties of the organic soils and waters affected the level of this production.

iii) To determine the rates of denitrification in organic soils and drainage waters; and

iv) to evaluate environmental factors such as the effect of soluble organic carbon content on the rate of denitrification.

As will be indicated subsequently, the nitrogen problems investigated herein and the subsidence of the Histosols which precipitates these problems are common to all drained Histosols. Since the research presented involves soils predominantly of the Everglades Agricultural Area, much of the literature review which follows will contain examples of properties of the soils typical of that region. These data will be supplemented where appropriate with studies conducted on Histosols of other regions.

CHAPTER I

LITERATURE REVIEW

SUBSIDENCE OF ORGANIC SOILS

For all soils, subsidence is defined as the loss of surface elevation. In mineral soils, this loss generally results from compaction of the soil mass or collapse of the soil structure due to water or oil removal from the substructure or dissolution of the substructure by water movement. This type of subsidence is exemplified by the formation of sink holes in the limestone formations of north Florida and the loss of elevation around Long Beach, California due to removal of oil from the underlying substrata. Such processes also may be involved in the subsidence of organic soils (Histosols), but, because of the fact that these soils may be composed predominantly of colloidal organic matter, subsidence results primarily from the loss of soil mass. This loss of soil mass results from the total oxidation of the soil organic matter to gaseous and water soluble products by the soil microbial community. This loss of soil is substantial. Subsidence rates of Histosols of southern Quebec have been measured at 2.07 cm/year (Millette, 1976), of western Netherlands at 1.75 cm/year (Schothorst, 1976), and of the California Delta at 7.6 cm/year (Weir, 1950). The rate in California was elevated due to the frequent burning of the surface layers of the soil for pest control. Because of the high organic matter content of Histosols, they do ignite. These subsidence rates compare to a mean rate of 3 cm/year in the Florida Everglades Agricultural Area.

Many factors combine to yield subsidence of organic soils. These include loss of buoyancy due to water removal, compaction, shrinkage due to drying, water and wind erosion and microbial oxidation of the soil organic matter (Stephens, 1969). Following the initial drainage of the soil, compaction, loss of buoyancy, and shrinkage due to drying are the major contributors to the subsidence rate, but later, microbial oxidation of the soil organic matter becomes the major cause of the subsidence. Wind and water erosion are significant factors with small deposits of Histosols, but with proper conservation practices the effects of these factors can be substantially reduced. In regions of Histosols as large as the Everglades Agricultural Area, wind and water erosion are insignificant since soil lost from one field is transported to the adjacent land. Another factor that apparently has little effect on this rate is the type of development following draining. Once the soils are drained, the subsidence rate appears to be independent of the soil use (Neller, 1944). That is, with the same water table, regardless of the use of the soils, they will subside at the same rate.

The extent of the role of microbes in the oxidation of soil organic matter of the Histosols of the Everglades Agricultural Area was demonstrated by Volk (1972). He found that microbial oxidation of the colloidal soil organic matter accounted for 58 to 73 percent of the loss of

soil observed in the field. These data were produced by preparing natural soil columns of the various soil types of the Everglades Agricultural Area and measuring the carbon dioxide evolved which resulted from the oxidation of the soil organic matter. The proportion of the subsidence rate attributable to the microbial community does vary with the climate of the region of the soil deposit. This results from the fact that all biological metabolic reactions are temperature dependent. This relationship with temperature is described by the Q_{10} value, i.e. the proportional increase in reaction rate with each 10°C increase in temperature. A Q_{10} of about 2 has been estimated for the microbial oxidation of soil organic matter of Histosols (Stephens and Stewart, 1976). Thus, in the colder regions of the USSR where extensive deposits of Histosols are found (Farnham and Finney, 1965), biological processes are retarded and physical causes of subsidence assume major importance (Stephens and Stewart, 1976) whereas in the subtropical regions of south Florida, the biological processes are of primary importance.

Following draining of the soil, the physical and chemical changes in the soil structure are extensive. While flooded, plant debris which settles on the soil surface is only partially degraded by the microbial community. This protection of plant structure results in part from the oxygen limiting conditions created by the high moisture levels. Therefore, much of the original plant structure is retained. This material is referred to as peat. Following draining when the protective effect of high moisture is removed, as the microbes oxidize the various cellular components of the plant remains, cellular structure is lost. The product is an amorphous organic material called muck. The soils characteristic of the Everglades Agricultural Area are muck soils. This loss of structure is accompanied by an increased oxidation state of the organic components. Volk and Schnitzer (1973) demonstrated increased carboxy, phenolic hydroxyls, quinones and ketonic groups in soils with the highest subsidence rate. Decreases in aliphatic structures and alcoholic hydroxyl groups were also noted. These changes are representative of the production of a more oxidized muck product.

Recent studies have involved elucidation of the ecology of the microbes associated with the oxidation of this soil organic matter. In contrast to mineral soils where the majority of the microbial activity is confined to the surface few centimeters, in Histosols oxidation of the soil organic matter occurs throughout the soil profile above the water table. This has been demonstrated with ^{14}C-labeled substrates (Tate, 1979a), increased ash contents of the soil above the water table (Broadbent, 1960) and the distribution of ferric iron within the soil profile (Ellis and Morris, 1945). These studies demonstrated the loss of soil organic matter, the oxidation of various soil organic matter components and aerobic microbial activity throughout the soil profile. A decline was observed in aerobic microbial activities deeper in the soil profile, but microbial oxidation of carbon at the 60-70 cm depth of Pahokee muck of the Everglades Agricultural Area was still 25 to 75 percent of that of the surface soil. The rate variation depended upon the degree of water saturation of the subsurface soil layers. Further studies with ^{14}C-labeled substrates (Tate, 1980) revealed little change

in the nature of the carbon substrate oxidized by the microbial community occurred within the soil profile above the water table. For example, aromatic ring catabolism comprised a similar proportion of the overall microbial respiration throughout the soil profile above the water table. As would be expected, oxidation of these structures was sensitive to the oxygen depletion that occurred deep in the soil profile. This resulted from the fact that molecular oxygen is directly incor porated into the aromatic ring in the early steps of its degradation (Gibson, 1968).

Aside from the standard conservation practices to manage wind and water erosion (Davis and Lucas, 1959), most methods to minimize the subsidence rate of Histosols involve limitation of the microbial activity. The first discovered and the most effective means of limiting this process is through prudent water table control. This simply involves returning a portion of the soil within the profile to the water-saturated conditions existent prior to draining. Several workers (Harris et al., 1962; Stephens, 1969) have shown that the subsidence rate is linearly proportional to the depth of the water table. That is, the deeper the water table within the soil profile, the greater will be the subsidence rate. Maximum benefit of water saturation is realized with flooded soils.

Both with drained soils and flooded soils of the Everglades Agricultural Area, water has been demonstrated to be a major controller of microbial activity. Microbial biomass, dehydrogenase activity, aerobic bacterial populations and aromatic ring oxidation all correlated with soil moisture levels of drained, surface Pahokee muck (Tate and Terry, 1980a). Increased soil moisture resulted in greater microbial activity. A practical effect of this relationship was found during a study of the effect of amendment of Pahokee muck with municipal sewage effluent. Microbial activity was increased in the soils receiving sewage effluent, but this augmented activity did not result from the nutrient content of the effluent. Instead, the change in microbial activity stemmed from the increased soil moisture level (Tate and Terry, 1980b). The increases in aerobic microbial activity with soil moisture occurred until the soil became saturated. At that point, aerobic carbon oxidation ceased due to the exclusion of oxygen. Due to the mixing of the water above a flooded field and the diffussion of oxygen within the water layer, oxygen is apparently available for aerobic carbon oxidation in the soil surface layers. Thus, some aerobic carbon oxidation occurs. This was observed by Knipling et al. (1970) when they noted carbon dioxide evolution from flooded soil and concluded that soil decomposition continued under high moisture conditions. Tate (1979b) investigated this problem with ^{14}C-labeled substrates and found that carbon metabolism did continue in the flooded soil, but that the nature of the substrate oxidized by the microbes changed. Aromatic ring oxidation declined 90 percent whereas oxidation of readily degradable carbon sources such as glucose and amino acids only decreased approximately 50 percent. Thus, since aromatic ring containing compounds comprise the majority of the organic matter of Histosols, subsidence was essentially stopped. This decline in the subsidence rate occurred during the

initial three days of flooding.

Other means of mitigating soil subsidence involve the admixture of clay or copper with the soil. Mathur et al. (1979) reported a decline in soil extracellular enzymic activity in Histosols containing high levels of copper. These soils also exhibited diminished respiration and subsidence rates. Practical problems could be anticipated with this means of subsidence control as a result of the toxicity of copper. As long as sufficient soil organic matter remained in the soil profile, the copper should be retained within the soil matrix, but since the subsidence continues, albeit at a reduced rate, in the presence of the copper, a point should be reached where insufficient organic material exists to retain the excess copper. Thus, the copper added to retard the soil subsidence and thus, serve as a protector of regional drainage water quality could itself threaten the water quality at a later date.

Clay minerals have also been shown to retard microbial oxidation of organic materials. To test the feasibility of using these minerals to inhibit soil subsidence, Pessi (1960) mixed clay at the rate of 300 m^3 per ha with an organic soil. After 35 years of cultivation, the clayed soil subsidence was an average of 12.5 cm greater than soil not receiving clay. The increased subsidence rate was attributed to increased temperatures resulting from the greater heat adsorption properties of the clayed soil. Thus, admixture of clay with the organic soil at concentrations less than those necessary to convert the soil to a mineral soil appears to be ineffective in subsidence control.

These data lead to the conclusion that subsidence and the associated water quality problems are an unavoidable fact of the development of this soil type. The only practical means of mitigating subsidence is water table manipulation. The water table must be maintained at the highest level possible for maximal economic benefit from utilization of the land.

NITROGEN TRANSFORMATIONS DURING SUBSIDENCE OF HISTOSOLS

Nitrogen is converted from the relatively biologically inert atmospheric form, dinitrogen, through plant and animal biomass and back to the atmosphere via the nitrogen cycle. This cycle may be depicted as commencing with the conversion of dinitrogen to ammonium. This process is termed nitrogen fixation. Flooded organic soils are sinks for this fixed nitrogen in that the nitrogen which is fixed is trapped in the plant debris which accumulates in the ecosystem. Once these soils are drained, all the steps of the nitrogen cycle, except fixation, occur. The soils are too nitrogen 'rich' for nitrogen fixation to occur to any great extent. As soon as the soils are drained, mineralization (conversion of the organic compounds to inorganic forms) of the accumulated biomass commences. The initial inorganic nitrogen product of this mineralization is ammonium. Some of the ammonium reaches drainage

waters, a portion is incorporated into plant biomass, but most is oxidized to nitrate and nitrite by the soil autotrophic nitrifying bacteria. Some nitrous oxide may be released from the soil due to nitrification (Blackmer et al., 1980). The amount and significance of this nitrous oxide production during nitrification is unknown. Following oxidation of the ammonium to nitrate and nitrite, the nitrogen cycle is completed by the reduction of the oxidized nitrogen forms to dinitrogen and nitrous oxide. The latter process is termed denitrification. Pollution problems are encountered with the nitrate and nitrous oxide. Since nitrate is more mobile in the soil than is ammonium, and thus, is more readily leached with the drainage waters, it may pose a greater threat to the quality of regional drainage waters. The magnitude of this process is demonstrated by the nitrogen transformations of the Everglades Agricultural Area. In this region, approximately 1400 kg N/ha/year is mineralized (Tate, 1976; Terry, 1980). Between 12 and 40 kg N/ha/year reaches the drainage waters (Florida Sugar Cane League, 1978). This nitrogen found in the drainage waters is distributed about equally between ammonium and nitrate. These data indicate that mineralization, denitrification and nitrification are active processes in these soils and that most of the nitrogen mineralized is either incorporated into the crop biomass or lost to the atmosphere through denitrification. Neller (1944) demonstrated that although a large proportion of the nitrate in the surface layers of Everglades muck soils was used by the crop, the majority remained in the soil. Thus, denitrification appears to be the major mechanism for nitrate removal from Everglades muck soils. Similar problems with nitrogen in drainage waters of Histosols have been detected with the muck soils of New York (Duxbury and Peverly, 1978) and in the Hula Valley of Israel (Avnimelech, 1971). Another potential environmental problem associated with nitrogen transformations in Histosols involves nitrous oxide evolution from the soil. This gas may be produced during both nitrification and denitrification. There is concern that increased atmospheric nitrous oxide levels may lead to reduction of atmospheric ozone (Cast, 1976).

NITRIFICATION

Nitrification, a process that assumes a central role in the nitrogen cycle, is defined as the biological conversion of reduced nitrogen to a more oxidized form. Classically, this has meant the oxidation of ammonium to nitrite and/or nitrate, but because of the currently ill defined role of the heterotrophic nitrifier in nitrogen oxidation in the environment, the definition has been modified to included the potentiality of oxidation of reduced organic nitrogen containing compounds, such as amines, to nitrate and/or nitrite, a reaction that the heterotrophic nitrifiers have been shown to catalyze in pure culture (Alexander, 1965). Nitrification is pivotal in the nitrogen cycle in that it produces nitrite and nitrate which are substrates for denitrification, the process that returns the biologically fixed nitrogen to the atmosphere.

The overall chemical transformation that occurs during nitrifica-

tion is as follows:

$$NH_4^+ + 2O_2 \text{ ----------> } NO_3^- + H_2O + 2H^+$$

This reaction is accomplished in soil and water predominantly by the autotrophic nitrifiers. These bacteria are referred to as autotrophic because they use inorganic carbon and nitrogen sources for growth and energy. This biological oxidation of ammonium occurs in two steps. In the first, the reduced nitrogen is oxidized to nitrite, whereas with the second, the oxidation is completed to nitrate. These reactions are accomplished by different groups of soil bacteria and yield different amounts of energy for microbial growth. Ammonium oxidation to nitrite occurs via the following reaction:

$$NH_4^+ + 1.5\ O_2 \text{ ----------> } NO_2^- + 2H^+ + H_2O$$

This reaction is catalyzed by bacteria of the genera Nitrosomonas, Nitrosospira, Nitrosolobus, Nitrosococcus, Nitrosocystis and Nitrosoglea. The free energy (ΔF) of the reaction is between -65.2 and -84 kcal per mole of ammonium oxidized (Gibbs and Schiff, 1960). The ammonium oxidation is completed by the reaction

$$NO_2^- + 0.5\ O_2 \text{ ----------> } NO_3^-$$

The free energy is about -17.5 to -10 kcal per mole (Gibbs and Schiff, 1960). The nitrite oxidizing bacteria belong to the general Nitrobacter and Nitrocystis. Since these reactions are both energy yielding, the autotrophic nitrifiers gain energy for growth, maintenance and waste metabolism. Although the relative proportions of energy involved in each of the three catagories is unknown, it is presumed that the majority of the energy is metabolized for cellular maintenance and waste metabolism (McLaren, 1971). Nitrous oxide may also be evolved during nitrification. Blackmer and Bremner (1970) have shown that nitrous oxide can be produced in sterile soil inoculated with ammonium oxidizing bacteria and receiving ammonium. The amount of nitrous oxide produced increased with the ammonium concentration and was sensitive to inhibition of nitrification by nitrapyrin and acetylene.

Several environmental variables may be suggested as potential controllers of nitrification rate in soils. These include substrate supply, temperature, soil aeration and moisture status, and soil pH. As shall be shown below, soil moisture and aeration status provides the major limitation for nitrification in organic soils of south Florida. The two substrates that could be limiting to the nitrifier are carbon dioxide, which it uses for synthesis of cellular components, and reduced nitrogen, which supplies energy. Since carbon dioxide is relatively abundant in the soil atmosphere as the result of the respiration of the microbial community and the plant roots, the supply of ammonium and nitrite must be the limiting substrates. Large quantities of reduced nitrogen are required for the growth and maintenance of nitrifying bacteria. With Nitrosomonas 35 units of nitrogen per unit of carbon dioxide assimilated

are necessary, whereas with Nitrobacter 100 units of nitrogen per unit of carbon assimilated are required (Alexander, 1965). Greater quantities are needed in the latter reaction as a result of the reduced energy yield of nitrite oxidation. These energy requirements can be compared to heterotrophic bacteria, which gain their energy through oxidation of carbonaceous substrates. These bacteria generally require 2 to 10 units of carbon for energy per carbon assimilated. Neither ammonium or nitrite would be expected to be limiting to the autotrophic nitrifier in muck soils of the Everglades Agricultural Area in that large quantities of ammonium are produced by the mineralization of the soil organic matter (Tate, 1976; Terry, 1980) and, of course, nitrite would be formed via the nitrification of a portion of this ammonium.

Soil temperature and pH are also variables which could limit nitrification in many soil types, but would be expected to be ineffective in controlling the nitrification rate of Everglades muck soils. Nitrification is a mesophilic reaction; that is, the optimum temperature for the reaction lies between 30 and 35°C. Nitrification rarely occurs above 40°C (there are no thermophilic nitrifiers that have as yet been discovered), whereas some ammonium oxidation can occur as low as 2°C (Alexander, 1965). As a result of the moderate climate of south Florida and the mediating effect of the high soil moisture characteristic of the muck soils on soil temperature variation, the soil temperature of the Everglades Agricultural Area is generally within the range for optimum nitrification to occur. Similar conclusions are reached by examination of the soil pH. The optimum pH for nitrification is between 7 and 9. The process is limited under acidic conditions and at alkaline pH values due to ammonia toxicity to ammonium oxidizers (Alexander, 1965). The near neutral pH of the Everglades muck soils are quite conducive for nitrification.

Thus, aeration and soil moisture status are the prime remaining limiting factors which could control the nitrification rate of Everglades muck soils. These factors are discussed together in that they are intimately related. As the soil moisture increases, the soil pores fill with water; thus, the availability of oxygen declines. Nitrification is an obligately aerobic process. Any process which limits soil oxygen levels limits the nitrification rate. Thus, under flooded conditions, nitrification would be expected to be oxygen limited in Everglades muck.

The effect of soil moisture and pH on nitrification in muck soils can be exemplified by noting changes in the population densities of the bacteria involved with changes in newly drained peat soils. Hirlihy (1973) examined the variation of nitrifier populations of drained, cultivated, uncultivated and undrained peats. In the surface soils of the undrained peat, as measured by the most-probable-number method, no Nitrosomonas and 1.3×10^3 Nitrobacter were detected per g peat. As the peats were drained and the soils became aerobic, the Nitrosomonas and Nitrobacter population densities increased to 2.3×10^6 and 2.4×10^8 per g peat, respectively. He also demonstrated how pH can be

limiting to nitrification of a peat soil when he compared the nitrifier populations of drained uncultivated wood fens with soils that had been drained, limed, and fertilized to provide for good crop growth. An approximate 10-fold increase in the nitrifiers was noted in the cultivated soil. Mishustin et al. (1974) noted similar changes following draining of a peat soil. The changes in the latter study were marked in the top 15 cm of the soil profile, where the maximum effect of the increased aeration would be expected. The nitrifiers provide a good marker for studies of the effect of increased soil aeration in that they are obligately aerobic and do not grow unless they can nitrify.

HETEROTROPHIC NITRIFICATION

Traditionally, autotrophic nitrifiers have been and still are considered to be the major catalysts for nitrogen oxidation in soils and waters, but recent studies have led to questions concerning the potential role of the heterotrophic bacteria in this process. Eylar and Schmidt (1959) isolated 978 heterotrophic organisms from 12 actively nitrifying soils. Each was tested for the capability to produce nitrite or nitrate when grown in glucose-peptone broth. About 7 percent of the bacteria yielded greater than 0.2 μg nitrite-N/ml whereas about 2 percent formed greater than 0.5 μg nitrite-N/ml. Of the bacterial cultures that were positive, none yielded greater than 2 μg nitrite-N/ml culture broth. The fungal isolates were the most active nitrogen oxidizers. Those fungi which oxidized nitrogen yielded between 5 and 45 μg nitrite-N/ml. Most of the active fungi were members of the species Aspergillus flavus. Doxtander and Alexander (1966) also examined nitrification by a variety of heterotrophic soil bacteria, actinomycetes and fungi. Ability to produce nitrite from a variety of reduced nitrogen sources including amines, amides, N-alkylhydroxylamines and aromatic nitro compounds was investigated. The amount of nitrite produced ranged from a few μg/ml culture fluid up to 190 μg/ml. The nitrite yield varied with the substrate. No isolate produced nitrate. Other heterotrophic nitrifiers have been isolated from soil by Odu and Adeoye (1970), Verstraete and Alexander (1973), Gowda et al. (1976) and Tate (1977). These studies lead to the conclusion that heterotrophic microbes with the ability to nitrify are reasonably common in soil and water ecosystems.

Problems are encountered when attempts are made to assign a role for these organisms in the nitrogen cycle. Whereas autotrophic bacteria acquire energy from the nitrogen oxidation and therefore their presence in an environmental sample definitely indicates function in nitrification, such is not the case with the heterotrophs. These latter organisms do not require the oxidation of ammonium for growth (Marshall and Alexander, 1962; Obaton et al., 1968). Thus, large populations of heterotrophic nitrifiers could exist in a soil or water sample as the result of growth by an alternate pathway and not through the oxidation of nitrogen. Also, in culture, nitrite and nitrate production occurs after the active growth phase of the heterotrophic nitrifiers (Obaton

et al., 1968; Alexander et al., 1960). Even with these difficulties, presumptive evidence for potential function of these organisms in nitrification was recently supplied by Verstraete and Alexander (1973). They detected hydroxylamine, 1-nitrosoethanol, nitrite and nitrate (all products of heterotrophic nitrification) in samples of sewage, river water, lake water and soils amended with ammonium and acetate. The carbon source was necessary for production of the oxidized nitrogen forms. Since no carbon source would be necessary for the autotrophic nitrifiers, this implies function of the heterotrophs in nitrification in these ecosystems.

Tate (1977) found high populations of arthrobacter in cultivated and fallow Pahokee muck of the Everglades Agricultural Area which were capable of producing nitrate and/or nitrite from reduced nitrogenous compounds. Amendment of the soils with N-serve (2-chloro-6-(trichloromethyl)pyridine) prevented growth of Nitrosomonas but not the heterotrophic nitrifiers and nitrate production in the presence of the inhibitor was not prevented. These data suggest that a portion of the nitrate could have been produced by the heterotrophic nitrifiers.

The existence of heterotrophic nitrifiers in the microbial world is interesting but the occurrence of large populations of these organisms in soils and waters is intriguing. These organisms would have the capability in the organic soils of oxidizing the nitrogen of the native soil organic matter directly to nitrate without the intervention of the autotrophs. Thus, the heterotrophs could participate in the subsidence of the soil and produce a potential water pollutant. Further studies to delineate the role of these organisms in the muck soils of the Everglades Agricultural Area will be reported herein.

DENITRIFICATION

Denitrification, the process which completes the nitrogen cycle as it has been described herein, results in the reduction of nitrate and/or nitrite to nitrous oxide and dinitrogen. The reaction sequence is as follows (Payne, 1973):

$$NO_3^- \text{ -----> } NO_2^- \text{ -----> } NO \text{ -----> } N_2O \text{ -----> } N_2$$

The major product is dinitrogen, but some of the reduced nitrogen escapes from the ecosystem as nitrous oxide. The proportion of the nitrogen evolved as nitrous oxide ranges from 0 to 100 percent (Cast, 1976). High nitrous oxide production is favored generally by conditions less conducive for denitrification. These are low temperature, low pH, and marginal Eh (Cast, 1976).

Since fixed nitrogen is an essential plant nutrient, past studies have concentrated on means of reducing denitrification in soil so that return of fixed nitrogen to the atmosphere would not result in the reduction of nitrogen availability for crop production. Currently, an op-

posing view is combined with this idea in that denitrification also results in the removal of excess nitrogen from soils before it can be leached into regional waterways. Thus, the excess fixed nitrogen can be removed from the ecosystem before it can serve as a nutrient for noxious algae production during lake eutrophication. This is exemplified by the nitrogen transformations of Pahokee muck of the Everglades Agricultural Area. In these soils, the majority of the nitrogen mineralized is denitrified (Terry and Tate, 1980a).

Although some chemical reactions can result in nitrate reduction to dinitrogen, denitrification is predominantly a biological process in soils and waters. Nitrate and/or nitrite is reduced to dinitrogen and nitrous oxide by a group of bacteria termed denitrifiers. Gamble et al. (1977) examined the species diversity of denitrifiers isolated from 19 soils, 9 fresh water lake sediments and oxidized poultry manure. The major group of bacteria isolated was representative of Pseudomonas fluorescens biotype II. The second most prominent were members of the genus Alkaligenes. None of the isolates resembled the most studied denitrifier species, P. denitrificans, P. perfectimonas and Paracoccus denitrificans. While denitrifying, these bacteria use oxidized nitrogen as an electron acceptor. Energy and carbon for growth and cell maintenance are provided by the oxidation of soluble carbon, such as glucose and methanol. Thus, these denitrifiers are heterotrophs. The organisms are versatile in their electron transport capabilities in that in aerobic atmospheres, oxygen serves as the final electron acceptor, whereas when oxygen becomes limiting, nitrate becomes the preferred electron acceptor. As a result of this dual capability of electron transfer, the presence of denitrifiers in an ecosystem does not imply active denitrification or serve as a measure of denitrification capacity. The organisms could have grown as the result of reduction of oxygen instead of nitrogen.

In past studies, as a result of the heterotrophic capacity of denitrifiers, it has been difficult to study the ecology of these bacteria. New methods recently developed now provide tools for the elucidation of the relationships of these interesting bacteria with their environment. Basically, the major discovery allowing these studies is that acetylene inhibits the reduction of nitrous oxide to dinitrogen (Yoshinari et al., 1977). Thus, this easily quantifiable compound, nitrous oxide, serves as a measure of the total nitrogen denitrified. It is difficult, if not impossible, to quantitate the small amount of dinitrogen produced through denitrification against the high background of dinitrogen in the atmosphere. Previous techniques for estimating denitrification in soil have involved either use of ^{15}N or estimating denitrification rates by measuring the amount of nitrogen unaccounted for when all other nitrogen forms in the sample had been quantified. These techniques are laborious, costly and, in the latter case, imprecise. Thus, use of the acetylene block technique has the advantage of being precise, inexpensive and facile. The presence of acetylene in the atmosphere of the sample does not appear to affect the overall denitrification rate (Smith et al., 1978). Also, due to the kinetics of induction of denitrification capability in the incubated samples, denitrifi-

cation rates measured during the initial four hours of incubation reflect the natural denitrification capacity of the freshly collected sample (Smith and Tiedje, 1979). This technique has been incorporated into the studies reported herein.

Since denitrification is a biological reaction, factors controlling its rate in soil would be similar to those previously discussed for nitrification. These are substrate concentration, pH, temperature and soil aeration. These have been recently reviewed by Focht and Verstraete (1977), and thus, will not be delved into here. Basically, the organic soils of the Everglades Agricultural Area, as was demonstrated for nitrification, are ideally suited for denitrification. Large quantities of nitrate are present for nitrogen source (Tate, 1976) and the soil is composed of greater than 44 percent carbon (Terry and Tate, 1980a). This quantity of carbon suggests that this nutrient may not be limiting to denitrification in Pahokee muck, but closer examination of the form of the carbon suggests that this may not be the case. The majority of the carbon of this well humified muck soil is found in humic substances. Although these substrates are degraded slowly by the microbial community, as is attested by the occurrence of subsidence, they are poor substrates for the denitrifier. Thus, it is reasonable to anticipate that soluble carbon may also limit denitrification in Histosols as occurs in various mineral soils (Burford and Bremner, 1975; Smid and Beauchamp, 1976; Stanford et al., 1975). The neutral pH and moderate temperature of Pahokee muck soils are quite conducive for the denitrifier.

CHAPTER II

DENITRIFICATION IN CULTIVATED HISTOSOLS

Because large concentrations of nitrate are denitrified in Histosols, and because aerobic decomposition of the soil organic matter occurs throughout the soil profile above the water table (Tate, 1979a), the objective of this study was to measure the potential denitrification rates of organic soils of the Everglades Agricultural Area and the effect of the denitrifier populations, crop, and position in the soil profile on this rate.

MATERIALS AND METHODS

The soil used herein was Pahokee muck (a Lithic Medisaprist), collected at the Agricultural Research and Education Center, Belle Glade, FL. Composite surface (0 to 10 cm) samples were collected monthly from adjacent, 5-ha fallow (bare), St. Augustinegrass (Stenotaphrum secundatum (Walt) Kuntz) and sugarcane (Saccharum spp. L.) fields. Subsurface (60 to 70 cm) samples from the fallow field were obtained by preparing a trench (2 x 1 m) and collecting composite samples. Composited samples consisted of a minimum of 10 approximately 100-g soil samples, selected from dispersed areas of the fields. Soil samples were collected by mixing a portion of the surface layer of the soil before selecting 100 g. Cropped soils were sampled equally between and within rows to minimize row effects. Samples were placed in sterile plastic bags and transported to the laboratory for analysis (transit time less than 5 min). Assays were performed immediately to obviate further storage.

Several properties of the Pahokee muck are presented in Table 1. Soil pH was measured on a 2:1 water:moist soil mixture. Total nitrogen was determined by the method of Nelson and Sommers (1972), and total carbon was assessed by dry combustion (Leco Carbon Analyzer, Leco Corp., St. Joseph, MI). Ash was estimated by heating oven-dry samples at 550°C overnight.

Potential denitrification rates were estimated with two methods: (i) a modification of the method of Terry and Nelson (1975), or (ii) measuring nitrous oxide accumulation from nitrate-amended soil in the presence of acetylene (Yoshinari et al., 1977). With the method of Terry and Nelson (1975), moist soil samples, having a weight equivalent of 1.0 g oven-dry solids, were dispensed into 50-ml polypropylene centrifuge tubes. The samples were amended with 10 ml of 50-μg/ml nitrate-N (as potassium nitrate) solution. The tubes were then capped and incubated at 30°C for 4 days. The depth of the water over the soil was about 2 cm. At the end of the incubation, inorganic nitrogen was extracted from the samples with 25 ml of 2 M potassium chloride. The ammonium-N, nitrite-N and nitrate-N contents of the potassium chloride extracts were determined by steam distillation (Bremner and Keeney, 1965). To assess the denitri-

Table 1. Properties of soils used in this investigation.

Soil	pH	Bulk density	Ash	Total C	Total N
		g/cm^3	----------% dry wt.----------		
Fallow field (0-10 cm)	5.9	0.34	16.0	44.8	1.97
Fallow field (60-70 cm)	6.3	0.17	16.2	48.5	2.06
Cane field (0-10 cm)	5.7	0.35	16.0	51.2	1.96
Grass field (0-10 cm)	5.8	0.34	15.1	47.6	2.13

fication rate in the presence of acetylene, 10-g samples of field-moist soil were added to 60-ml serum bottles and amended with 10 ml 50 µg/ml nitrate-N solution. The bottles were capped with septum stoppers and twice evacuated and flushed with helium. A portion of the helium was removed by syringe and replaced with acetylene to bring the headspace concentration to 0.1 atm acetylene. The samples were incubated at 30°C on a rotary shaker for a maximum of 48 hours. Samples were removed from the headspace and analyzed for nitrous oxide. Nitrous oxide was detected with a Hewlett Packard 5840A gas chromatograph with a thermal conductivity detector. Nitrous oxide was separated on a Porapak Q column (0.003 x 3 m). The oven, injector, and detector temperatures were 35, 200, and 210°C, respectively. The carrier gas (helium) flow rate was 20 ml/min. Headspace concentrations of nitrous oxide were corrected for nitrous oxide dissolved in the aqueous phase.

Denitrification rates in terms of µg/cm^3 soil/day were calculated by the following equation:

$$\text{Rate} = (N_0 - N_t) \cdot \text{bulk density/days}$$

where N_0 is the µg/g (nitrite + nitrate)-N at the start of the incubation, and N_t is the µg/g (nitrite + nitrate)-N at the end of the incubation. During the incubation, concentrations of nitrate were sufficient to provide zero-order reaction kinetics. Measurement of the nitrous oxide-N produced in the presence of acetylene versus the nitrate-N lost from the soil sample indicated that immobilization of nitrate during the incubation period was negligible.

Denitrifiers were assayed by the method of Focht and Joseph (1973). Dilutions for microbial analysis were prepared in 0.1 percent sodium chloride.

All assays were performed in triplicate. Statistical analyses of the data were performed as described by Steel and Torrie (1960).

RESULTS

Potential denitrification rates of soils collected from fallow, sugarcane, and grass fields were estimated at monthly intervals from July 1977 through August 1978 by the flooded-soil technique (Table 2). The mean rates grouped into two classes. Samples from both depths of the fallow field exhibited low denitrification potentials, whereas the rates in the cropped soils were threefold to sevenfold greater. Although mean rates of 2.02 and 0.99 µg N/cm^3/day were recorded for the 0-to 10-and 60-to 70-cm depths of the fallow field, respectively, the differences were not significant. Cropping of the soil resulted in an increase in the potential to approximately 7 µg N/cm^3/day. The variability of the rate during the sample period was exhibited by the range of rates detected. The maximum denitrification rate of 37.7 µg N/cm^3/day was detected with soil from the sugarcane field collected on 2 March 1978, 2 weeks after

Table 2. Potential denitrification rate in Pahokee muck.

Soil Use	Flooded Soil Method		Acetylene Method	
	$\bar{x}$	range	$\bar{x}$	range
Fallow field (0-10 cm)	2.02B*	ND**-6.87	16.2C	10.2-21.1
Fallow field (60-70 cm)	0.99B	ND-5.05	6.1C	1.4-9.6
Sugarcane field (0-10 cm)	7.21A	0.74-37.17	38.1B	29.6-52.0
Grass field (0-10 cm)	7.27A	1.69-12.03	65.6A	52.4-78.5

* Values within a column followed by the same letter are not significantly different at the 95% level (Duncan's Multiple Range Test).

** Not detectable.

the crop had been harvested. The value was threefold greater than any observed with the other soil types and with other rates recorded from soil of the sugarcane field. The elevated denitrification rate at that collection time could be explained in part by the litter of decaying plant residue on the soil surface, which, together with the dying roots of the crop, provided additional carbon for the microbial activities, including denitrification.

Measuring denitrification potential by nitrous oxide production yielded higher values than were found with the flooded-soil technique, but the relationship between the cropped and uncropped soils was the same (Table 2). For this study, soils were collected 2 March 1978, 31 March 1978, 1 June 1978, and 21 August 1978. The sample dates were selected to overlap with those used in the flooded soil assay. In all cases, the range of values obtained was narrower and the rates were sevenfold to eightfold greater than the values measured by the flooded-soil technique. This difference in potential measurements by the two methods resulted from the different composition of the headspace gas in the incubation vessels of the two procedures.

A helpful tool in discerning the relationship between denitrification and nitrification in soil samples is measurement of the quantities of various inorganic nitrogen forms present in the soil samples. Accordingly, the inorganic nitrogen content of the surface soils from the fallow, St. Augustinegrass, and sugarcane fields, as well as of the 60- and 70-cm depth of the fallow field, was measured at monthly intervals from July 1977 through August 1978. The mean concentrations of ammonium-N, nitrite-N and nitrate-N are presented in Table 3. Although the mean ammonium levels in the soils varied from 8.9 to 12.8 μg N/g dry soil, because of the variability of the concentration of this nutrient, no significant variation relating to field use was detected. Utilization of the nitrate by the crop was demonstrated by the diminished concentrations of nitrate in the cropped soils versus the surface soils of the fallow field. Apparently, the denitrifiers were limited at the 60- to 70-cm depth of the fallow field, in that not only was denitrification potential decreased at that depth, but the nitrate concentrations were elevated. Some limitation of denitrification was suggested by the occurrence of nitrite in the soil samples. This was particularly evident in the 60- to 70-cm samples, where an average of approximatley 60 μg nitrite-N/g dry soil was detected.

Noting that the nitrate concentration deep in the soil profile increased after a rainfall, and that aerobic activity at the 60- to 70-cm depth was diminished from that found in the surface soil samples (Tate, 1979), we can conclude that at least a portion of the nitrate present in the soil samples from the 60- to 70-cm depth of the fallow soil resulted from leaching of nitrate through the soil profile. This was evident with data collected during August and September 1977. During this period, a total of 460 mm of rain fell. The mean nitrate-N concentration of the fallow surface (0 to 10 cm) soil was reduced from 335 to 58 μg/g. Much of this nitrate was leached to the lower horizons of the profile, in that

Table 3. Variation of inorganic nitrogen in Pahokee muck measured at monthly intervals (July 1977 - Aug. 1978).

Soil Use	NH_4^+	NO_2^-	NO_3^-
	μg N/g dry muck		
Fallow (0-10 cm)	8.5 ± 1.6A*	15.2 ± 4.5B	112 ± 23B
Fallow (60-70 cm)	12.8 ± 2.6A	59.6 ± 17.4A	311 ± 52A
Sugarcane	8.9 ± 3.1A	5.9 ± 1.2B	38.4 ± 13.1C
Grass	9.0 ± 1.9A	5.4 ± 1.9B	56.7 ± 9.7BC

* Values within a column followed by the same letter are not significantly different at the 95% level (Duncan's Multiple Range Test).

the nitrate-N concentrations of the 60- to 70-cm depth increase from 62 to 508 µg/g.

The denitrifying bacteria in these soils were estimated at each sample time (Table 4). These bacteria were only 20 percent as numerous at the 60- to 70-cm depth of the fallow field as in the surface samples. Variation in the population density of denitrifiers among the three surface soil samples was insignificant. A linear correlation technique was used to determine if the number of denitrifiers in the surface samples related to the denitrification rate. The correlation coefficient indicated no significant relationship.

DISCUSSION

These data reveal the variation of the denitrification potential in different soils of the Everglades Agricultural Area. The data are useful in evaluating the relationships among soils exposed to various agronomic practices, but they provide limited estimates of the actual denitrification rate in the field. All methods of estimating denitrification rates in the laboratory suffer from the artificial conditions imposed by the techniques used. Since denitrification is a biological process that is extremely sensitive to oxygen concentration, slight variations in the methods of incubation of the samples will have considerable effect upon the values obtained. To get a better estimate of the relationship of potential denitrification rates among different soil uses, two methods of estimating the potential were selected for these studies. As would be expected, different rates were found with each method. The flooded soil was incubated under an atmosphere of air, whereas the headspace of the vessels used for the acetylene method were purged with helium. Thus, the denitrifiers in the latter procedure would be functioning under less oxygen stress than those found in the flooded soil. Hence, greater denitrification rates were recorded with the acetylene method. The question thus arises: which method best estimates the actual denitrification rates in the field. Several reasons to support each method can be proposed, but because soils in the field do not exist in a helium environment, the flooded soil more likely approximates the state in the field. In reality, the only valid method for determining the denitrification rate in the field is to measure the rate in the field, keeping intervention by the experimentor to a minimum. The values recorded here merely provide information about the range of difference amongst various fields and serve as a guide in designing future experiments to measure denitrification rates in situ. Accomplishment of the latter objective must await development of new techniques.

Table 4. The variation in most-probable-number of denitrifying bacteria in organic soils collected over a one year period.

Source of Soil	Average	Range
	----------MPN/cm^3 x 10^3----------	
Fallow (0-10 cm)	101A*	1.7-526
Fallow (60-70 cm)	5B	0.03-30
Sugarcane Field	153A	13-485
Grass Field	129A	13-620

* Values followed by the same letter are not significantly different at the 95% level (Alexander, 1965).

SUMMARY

Potential denitrification rates of a cultivated, drained Everglades Histosol, Pahokee muck, were examined to determine their variation with crop and position in the soil profile. The average rates, as measured with a flooded soil technique in soil samples collected at monthly intervals between July 1977 and August 1978 from a fallow field at two depths, 0- to 10-cm and 60- to 70-cm, were 2.02 and 0.99 $\mu g\ N/cm^3/day$, respectively. As a comparison, the mean rates in soils cropped to sugarcane (*Saccharum* spp. L.) and St. Augustinegrass (*Stenotaphrum secundatum* (Walt) Kuntz), similarly collected and assayed, were 7.21 and 7.27 $\mu g\ N/cm^3/day$, respectively. Although the relationship among the rates measured from the different soils remained the same, higher values were obtained if nitrous oxide production in nitrate-amended soil in the presence of acetylene was used to estimate denitrification rate. The differences between the two values resulted from variation in the atmosphere of the incubation vessels. The increased denitrification rates of the cropped soil likely reflected the greater availability of organic carbon and increased microbial activity in them. No correlation between the number of denitrifying bacteria and the denitrification rate was observed.

CHAPTER III

NITRITE PRODUCTION IN PAHOKEE MUCK

The objectives of this phase of the project were i) to document the occurrence of nitrite in Pahokee muck, ii) to determine the frequency of detection of significant levels of nitrite in the soil, and iii) to elucidate the source of the nitrite.

MATERIALS AND METHODS

The soil used in this study was Pahokee muck collected at the Agricultural Research and Education Center, Belle Glade, FL. Soil samples were prepared from the surface (0-10 cm) of a fallow field, and fields cropped to St. Augustinegrass or sugarcane. In addition, the 60- to 70-cm depth of the fallow field was sampled as previously described (Terry and Tate 1980a). The soil properties were as listed in Table 1.

Initial studies involved comparison of the relationship between soil nitrite concentrations and ammonium, nitrate and moisture in freshly collected soil samples. For this, samples were collected 3 to 4 times weekly between January and September, 1980. This sample period provided the greatest environmental variation allowed by the moderate climate of south Florida. All nitrogen concentrations were calculated as $\mu g\ N/cm^3$ soil so as to allow a meaningful comparison of soils with varying bulk densities. Also, data previously collected (Terry and Tate 1980a) were recalculated on a volume basis. Inorganic nitrogen was extracted from the soil samples with 2 M potassium chloride. The ammonium-N, nitrite-N and nitrate-N were determined in the potassium chloride extracts by steam distillation and titration (Bremner and Kenney 1965). Moisture was measured by drying soil samples at 100°C to a constant weight. All assays were performed in triplicate.

To determine the effect of carbon and/or nitrate amendments and aeration status on nitrite production, soils from the St. Augustinegrass field and the fallow field were dispensed 5 g moist soil/test tube (16 x 150 mm) or 10 g/milk dilution bottle. The soil was amended with 0.1 ml 500 µg/ml nitrate-N (as potassium nitrate) or 2500 µg/ml nitrate-N/g dry soil and/or 0.1 ml 0.5 M glucose/g dry soil. The soil in bottles was incubated aerobically whereas that in tubes was incubated anaerobically in GasPak systems (BBL). All samples were incubated at 30°C in the dark. At intervals, samples were extracted with 2 M potassium chloride and the inorganic nitrogen analyzed as indicated above.

RESULTS

Less nitrite was detected in the surface soils from all fields during the 1980 portion of the study than was found during the 1977-78

sample period. Nitrite ranged from 0 to 5.10 and 0 to 21.8 μg nitrite-N/cm^3 in the surface soils from the 1980 and 1977-78 periods, respectively. In 1980, 83 percent (n_2 = 125) of the surface soil samples contained 0 to 0.5 μg nitrite-N/cm^3 (Fig. 1) with 33 percent containing no detectable nitrite. This compares to 1977-78 when 44 percent and 33 percent (n = 39) ranged from 0 to 2 and 2 to 4 μg nitrite-N/cm^3, respectively, and 23 percent of the samples contained no nitrite (Fig. 2). This variation between the two sample periods was reflected by the mean nitrite concentrations in the soils under different cultivation practices. In 1980, 0.74, 0.38 and 0.36 μg nitrite-N/cm^3 were detected in soil from the fallow, grass and sugarcane fields, respectively, as compared to mean concentrations of 5.17, 1.90 and 2.58 μg nitrite-N/cm^3 from fallow, grass, and sugarcane fields, respectively, in 1977-78. Due to the range of nitrite levels measured in these samples, the differences between soil uses for each sample period were not statistically significant. The highest nitrite concentrations were found at the 60- to 70-cm depth of the fallow field during the 1977-78 study. In these samples, nitrite ranged from 0 to 31.8 μg nitrite-N/cm^3 with a mean concentration of 10.4 μg nitrite-N/cm^3. This concentration was significantly greater than was detected in the surface soils. Only 15 percent of the samples contained no nitrite.

Nitrate and ammonium concentrations also varied between the two sample periods (Fig. 1 and 2). To see how this variation related to differences in nitrite concentration and to note effect of differences in concentrations of these nitrite precursors would have upon the nitrite levels in the soil, correlation of nitrite with ammonium, nitrate and soil moisture was calculated (Table 5). In most cases, nitrite correlated at the 99 percent level with soil nitrate. No significant correlation was recorded for grass soil samples or with soils from the sugarcane field collected in 1980. Since nitrate is mobile in the soil profile and is also assimilated by the growing plant, the lack of correlation in these cases likely resulted from the active crop growth or leaching of the nitrate from the soil profile. In the soil samples from the 60-70 cm depth of the fallow field, nitrite also correlated with soil moisture. Greatest nitrite concentrations were found in soils with the highest moisture. Multiple linear regression analysis of the data demonstrated that nitrite was related to nitrate and soil moisture in soils from the 60-70 cm depth by the following equation:

$$[NO_2^-] = 0.186[NO_3^-] + 0.361(\%H_2O) - 29.98$$

$$(R^2 = 0.46)$$

In 1977-78, nitrite levels were significantly related to soil moisture and nitrate for the pooled data by the following equation:

$$[NO_2^-] = 0.097[NO_3^-] + 0.143(\%H_2O) - 5.52$$

$$(R^2 = 0.39)$$

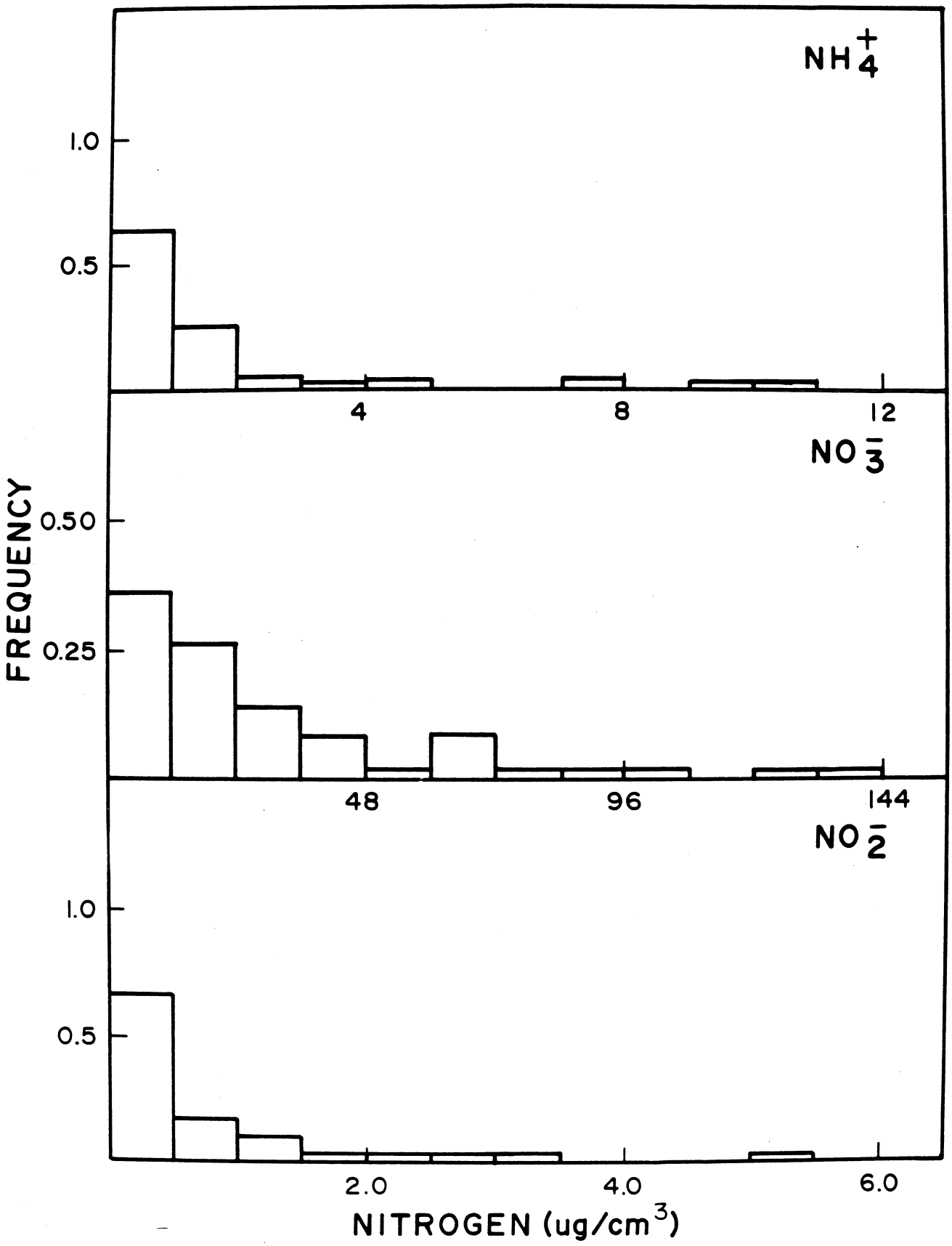

Fig. 1. Frequency of occurrence of nitrite, nitrate and ammonium in Pahokee muck between January and September, 1980. (n = 125)

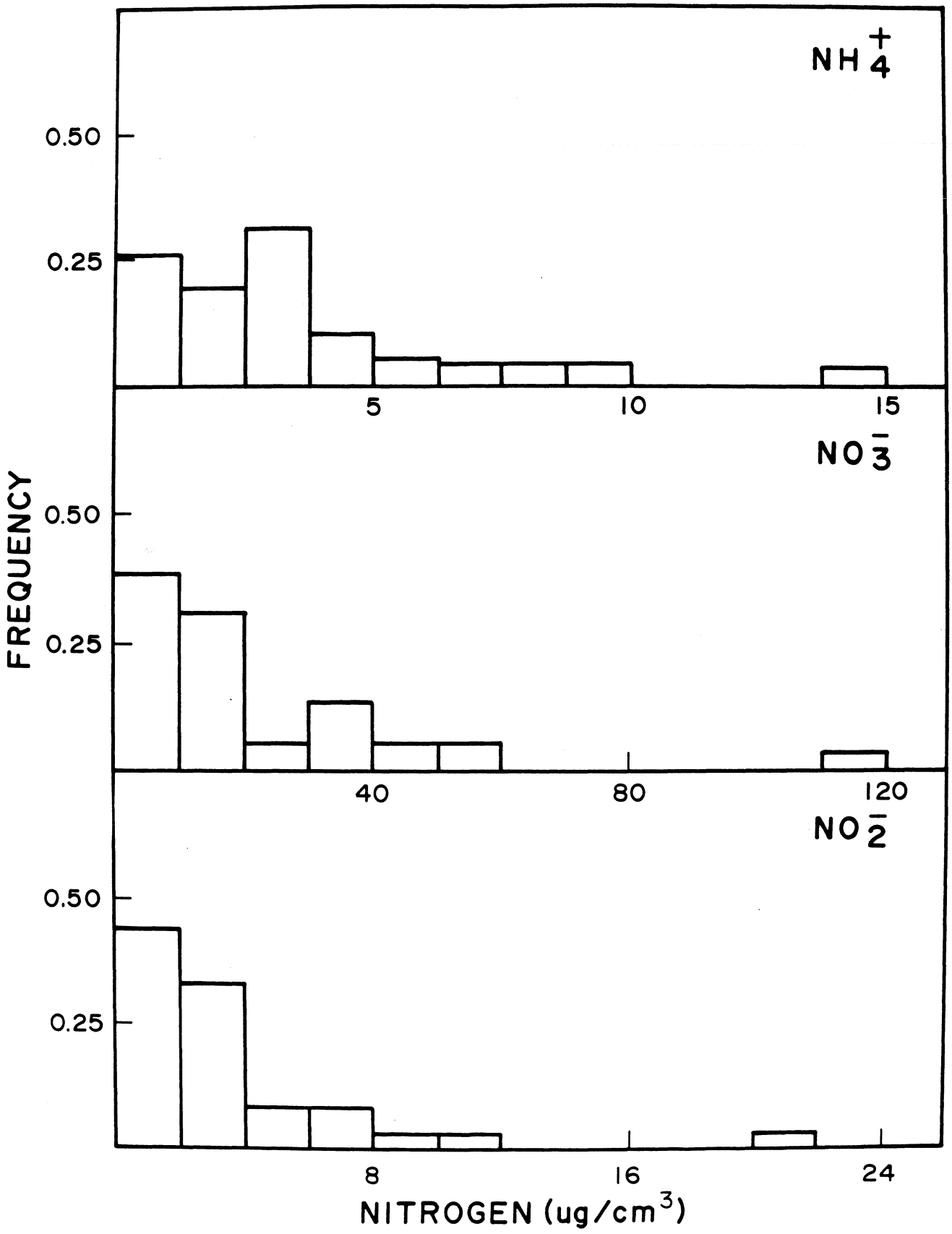

Fig. 2. Frequency of occurrence of nitrite, nitrate and ammonium in Pahokee muck between July 1977 and August 1978. (n = 39)

Table 5. Correlation of soil nitrite levels with nitrate and ammonium concentrations and soil moisture.[a]

Soil	Sample Period	Correlation Coefficient (r)		
		$NO_2^- \times NO_3^-$	$NO_2^- \times NH_4^+$	$NO_2^- \times H_2O$
Fallow (0-10 cm)	1980	0.63**	-0.02NS	-0.27NS
	1977-78	0.18NS	0.14NS	0.04NS
Grass (0-10 cm)	1980	0.22NS	0.01NS	-0.08NS
	1977-78	0.15NS	-0.33NS	0.24NS
Cane (0-10 cm)	1980	-0.01NS	-0.07NS	-0.04NS
	1977-78	0.74**	0.32NS	0.05NS
All Surface	1980	0.53**	0.06NS	-0.16NS
	1977-78	0.39**	0.08NS	-0.22NS
Fallow (60-70 cm)	1977-78	0.59**	0.11NS	0.30**

[a] ** = significant at 99% level; NS, not significant.

In both examples, the effect of soil moisture on nitrite levels was about two-fold that of nitrate. The multiple coefficient of determination values indicate that 39 and 46 percent of the variability in the nitrite concentrations for the composited soil data of 1977-78 and those for the 60- to 70-cm depth, respectively, was accounted for by nitrate and soil moisture. In the samples which correlated with nitrate only, between 30 and 50 percent of the variability was related to soil nitrate levels. No significant correlation between nitrite and ammonium was found for any sample type.

Since nitrate is the direct nitrite precursor in denitrification and high soil moisture results in the anaerobic conditions necessary for denitrification to occur, these data suggest that denitrification is the source of the nitrite found in Pahokee muck. To test this hypothesis, freshly collected soil from the fallow field was dispensed and amended as indicated above. The unamended soil contained approximately 105 μg nitrate-N/cm^3 whereas nitrate amended samples contained about 140 and 300 μg nitrate-N/cm^3 (Table 6). Under anaerobic conditions, transient nitrite accumulations were detected (Fig. 3). Maximum nitrite levels were detected in those samples with the highest nitrate levels. Glucose amendment resulted in the nitrite peak appearing earlier in the incubation period (Fig. 3 and Table 6). No nitrite was detected in unamended samples incubated aerobically, the condition most conducive for nitrification and nitrogen mineralization. The only nitrite detected in the aerobic samples was in those receiving both nitrate and glucose. Glucose amendment could have caused oxygen limiting conditions thereby stimulating denitrification. In the anaerobic samples, ammonium increased from 3.35 ± 0.12 to 7.51 ± 0.11 μg ammonium-N/cm^3 whereas under aerobic conditions ammonium was depleted. Similar results were obtained with soil from the grass field (data not shown).

Nitrate concentrations declined in all samples in which nitrite was detected. The percent of the missing nitrate at the time of maximum nitrite accumulation which could be accounted for as nitrite ranged from 4.1 to 39.2 (Table 6). Maximum percentages were found in those soils which contained the greatest initial nitrate levels.

DISCUSSION

The occurrence of significant quantities of nitrite in Pahokee muck, a soil with a pH of approximately 6 which received no nitrogenous fertilizer, was shown herein. Previous observations of nitrite accumulation in soil have included studies with mineral soils fertilized with anhydrous ammonia or urea and having a pH of greater than 7.2 (Alexander, 1965; Clark et al., 1960; Soulides and Clark, 1951). Under these conditions, nitrite accumulation resulted from ammonia inhibition of nitrification. Denitrification has also been shown to result in nitrite accumulation in mineral soils (Cooper and Smith, 1963; Cady and Bartholomew, 1960). The failure of the nitrite levels to correlate with soil ammonium concentrations in the present study and the stimulation of nitrite ac-

Table 6. Nitrite production in soil amended with nitrate and/or glucose or left unamended.

Soil Atmosphere	Initial NO_3^- ($\mu g\ N/cm^3$)	Exogenous Carbon	Maximum NO_2^- ($\mu g\ N/cm^3$)	Day of Maximum NO_2^-	% NO_3^- Lost as NO_2^-
Anaerobic	105 ± 0.5	None	16.4 ± 0.4[A]	3	18.9
	145 ± 1.0	None	32.2 ± 2.0	3	29.4
	299 ± 1.5	None	57.7 ± 0.8	4	27.9
	104 ± 0.7	Glucose	4.2 ± 0.6	3	4.1
	143 ± 1.1	Glucose	27.9 ± 0.5	1	25.6
	297 ± 1.3	Glucose	51.2 ± 2.3	1	39.2
Aerobic	91 ± 0.2	None	0	-	--
	119 ± 3.7	None	0	-	--
	260 ± 1.0	None	0	-	--
	93 ± 0.5	Glucose	0	-	--
	127 ± 0.6	Glucose	9.5 ± 0.9	1	23.2
	265 ± 1.2	Glucose	6.4 ± 0.9	1	16.9

[A] Mean ± Standard Error (n = 3).

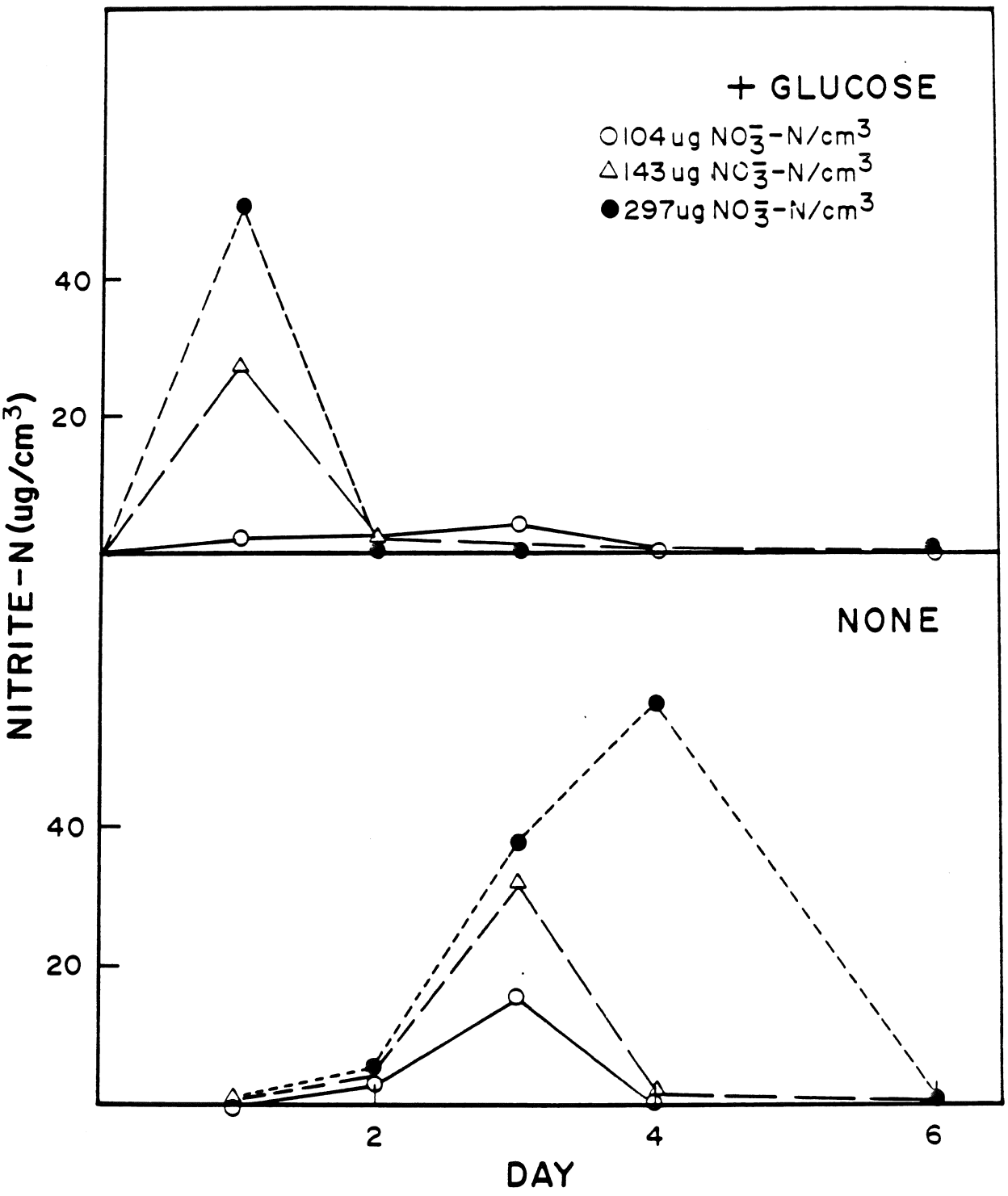

Fig. 3. Nitrite accumulation in Pahokee muck collected from a grass field, amended with nitrate and/or glucose and incubated under anaerobic conditions.

cumulation in soil samples from the 60- to 70-cm depth by soil moisture, a situation that would be inhibitory to the obligately aerobic process of nitrification, suggests that denitrification is the source of nitrite in Pahokee muck. This conclusion was supported by the correlation of nitrite levels with soil nitrate. The correlations were complicated by the mobility of nitrate under field conditions, but support for nitrate reduction being the source of the nitrite was also provided by A) the demonstration of nitrite production in soil incubated in the laboratory under conditions conducive to denitrification and not nitrification and mineralization, B) the stimulation of this process by glucose amendment, and C) the fact that nitrate levels declined in samples accumulating nitrite. Nitrite is formed by the reduction of nitrate during denitrification and during assimilatory reduction of nitrate to ammonium. Ammonium concentrations in the laboratory study increased by about 3 μg ammonium-N/cm^3 in those samples incubated anaerobically. However, nearly 20-fold that level of nitrite accumulated. Thus, more nitrate was trapped as nitrite than reduced to ammonium. The more logical conclusion is that the nitrite originated from denitrification in that 30- to 60-fold more nitrogen was lost from the ecosystem through denitrification than was found as ammonium.

The ecological significance of the low levels of nitrite detected in the Pahokee muck samples from the Everglades Agricultural Area is unknown. Reports of nitrite accumulation in native soils due to denitrification are reasonably uncommon. Although it is most likely that the majority of the nitrite accumulated would be further reduced via denitrification, the possibility exists that nitrite could react with secondary amines in the soil to form carcinogenic N-nitrosamines. Further research is needed to evaluate the importance of the latter reaction in the ecosystem.

SUMMARY

Nitrite production in Pahokee muck, a drained Histosol, collected in the Everglades Agricultural Area was examined. Soil nitrite levels ranged from 0 to 21.8 and 0 to 5.10 μg nitrite-N/cm^3 in surface (0 to 10 cm) soils in 1977-78 and 1980, respectively. In 1980, 83 percent (n = 125) of the samples contained less than 0.5 μg nitrite-N/cm^3 with 33 percent containing no detectable nitrite. No effect of crop on mean nitrite levels was detected. Nitrite concentrations generally correlated with nitrate and, in some cases, soil moisture, but not ammonium. In laboratory studies, maximum nitrite accumulated in muck samples amended with nitrate plus a carbon source and incubated anaerobically. No nitrite was detected in unamended samples incubated aerobically. These data suggest denitrification as the source of nitrite in Pahokee muck.

CHAPTER IV

EFFECT OF NITRATE ON NITROUS OXIDE REDUCTION

This study was commenced with the objective of determining the effect of nitrate concentration on nitrous oxide reduction in organic soils and sediments and to observe the environmental parameters limiting this inhibition. It was of particular interest to observe the effect of nitrate on denitrification products in Pahokee muck in that nitrate is typically found in high concentrations in these drained, cultivated Histosols (Terry and Tate, 1980a). Thus, any adaptation of the microbial community to persistent high nitrate levels would be readily observed.

MATERIALS AND METHODS

Surface (0- to 15-cm) samples of Pahokee muck were collected from adjacent fallow (bare), St. Augustinegrass, and sugarcane fields at the Agricultural Research and Education Center, Belle Glade, Florida. Soil samples were also collected from a vegetable field at the same location which had been flooded for 22 days. Sediment samples were collected with an Ekman dredge from a drainage ditch adjacent to the fallow field and from the Hillsboro Canal near Belle Glade. The depth of water above the vegetable field, ditch sediment and canal sediment was 0.3, 0.5, and 5.0 m, respectively. Samples were transported to the laboratory (transit time ca. 5 min.) and sieved (2 mm for drained soils and 4 mm for flooded soil and sediments). The properties of the soils and sediments are listed in Table 7. Sediment pH was determined by glass electrode in stirred, wet sediment. Soil pH was measured on a 2:1 water:moist soil mixture. Approximately 200 g portions of the samples were air-dried for subsequent chemical analysis. Total nitrogen was determined by the method of Nelson and Sommers (1972) and total carbon was measured by dry combustion (Leco Carbon Analyzer, Leco Corp., St. Joseph, MI). Ash was estimated by heating oven-dry samples at 550°C overnight. Values are reported on an oven-dry soil basis.

Incubation experiments were begun immediately after sample collection to avoid the effects of storage on denitrification rates and products. The acetylene inhibition method (Yoshinari et al., 1977) was used to measure denitrification. Ten gram (wet wt.) samples of soil or sediment were placed in 60 ml serum bottles and treated with 10 ml potassium nitrate solution ranging in concentration from 10 to 200 mg N/liter. The bottles were sealed with septum stoppers, connected to a manifold system and evacuated and purged 3 times with argon or an argon-10 percent acetylene mixture. The samples were incubated up to 48 hr on a rotary shaker (200 rpm) at 25°C. At the end of incubation, samples were frozen in a dry ice-methanol mixture. Since nitrous oxide dissolved in the water was not equilibrated with the headspace gases, the samples were submerged in a 0°C water bath for 24 hr to allow equilibration. The low temperature minimized denitrification during the equilibration period.

Table 7. Properties of soils and sediments.

Type of sample	pH	Bulk density	Ash	Total C	Total N
		g/cm^3	% dry wt.		
Pahokee muck	5.9	0.34	16.0	44.8	1.97
Drainage ditch sediment	7.0	0.26	19.5	43.7	2.07
Hillsboro Canal sediment	7.1	0.19	57.2	23.4	1.08

A Hewlett Packard 5840A gas chromatograph with a ^{63}Ni electron capture detector was used to measure nitrous oxide. Samples (0.1 ml) of the headspace gas were removed by syringe and nitrous oxide separated on a Porapak Q column (0.003 x 3.0 m). The oven, injector, and detector temperatures were 45, 200, and 300°C, respectively. The carrier gas (5% methane in argon) flow rate was 30 ml/min. Headspace concentrations of nitrous oxide were corrected for nitrous oxide dissolved in the aqueous phase. Total denitrification was determined by nitrous oxide production in acetylene amended samples and is reported in terms of μg nitrous oxide-N/cm^3 of soil or sediment (Duxbury and Tate, 1981). Nitrous oxide production was measured in samples without acetylene. The ratio of dinitrogen to nitrous oxide-N was determined by subtracting nitrous oxide produced in the absence of acetylene from nitrous oxide produced in the presence of acetylene and dividing by nitrous oxide produced in the absence of acetylene.

Samples were extracted with 2M potassium chloride and ammonium-N and nitrate-N were determined by steam distillation (Bremner and Keeney, 1965). The concentration of nitrate-N initially present (native + added) and present at the end of incubation are presented on the basis of mg/ liter of interstitial water. Nitrate levels were the same in samples with and without added acetylene. Incubation experiments were conducted in triplicate and all replications were within 5 percent of the mean.

RESULTS AND DISCUSSION

By comparing nitrous oxide production in the presence and absence of acetylene both total nitrogen denitrified and that portion of the reduced nitrate which remains as nitrous oxide can be determined (Smith et al., 1978; Yoshinari et al., 1977). Accumulation of nitrous oxide under these conditions in soil and sediment samples is presented in Table 8. In Pahokee muck with an initial nitrate concentration of 22 mg N/liter, nearly all of the nitrate reduced was found as nitrous oxide. After the nitrate levels decreased to 1 mg/liter, the inhibition of nitrous oxide reduction was relieved. This reduction of nitrous oxide resulted in an increase in the dinitrogen/nitrous oxide ratio from 0.2 to approximately 407. Reduction of nitrous oxide was inhibited throughout the 48 hour incubation period in samples of Pahokee muck with initial nitrate-N concentrations of 72 mg/liter. In this case nitrate-N levels remained above 8 mg/liter. By contrast, nitrous oxide was readily reduced in sediments with initial nitrate-N concentrations as high as 90 mg/liter. During the incubation, the nitrate-N content was reduced from 90 to 39 mg/liter, yet less than 10 percent of the gaseous denitrification products were in the form of nitrous oxide. Note that at nitrate-N levels of 40 mg/liter in Pahokee muck, nearly 100 percent of the nitrate reduced accumulated as nitrous oxide.

The pH of Pahokee muck was approximately one unit lower than the sediments (Table 7). The lower pH may have affected nitrous oxide reduction in Pahokee muck. Blackmer and Bremner (1978) found that in

Table 8. The effect of nitrate concentration on N_2O produced in the presence and absence of acetylene.

Sediment or Soil	Interstitial NO_3^--N		N_2O-N evolved					
			6 hours			24 hours		
	Initial	Final	$+C_2H_2$	$-C_2H_2$		$+C_2H_2$	$-C_2H_2$	
	--- mg/liter[#] ---		---------- µg N/cm^3 soil or sediment ----------					
Pahokee muck	22	< 1	6.0	5.0	(0.2a)[†][c][±]	31.0	23.8	(0.3a)[c]
	43	< 1	5.3	6.1	(<0.1b)	30.5	28.2	(0.1b)
	72	8	5.5	5.6	(<0.1b)	29.9	26.8	(0.1b)
Drainage ditch	23	< 1	13.7	<0.1	(282b)[a]	62.9	4.0	(15c)[a]
	48	< 1	15.4	<0.1	(306c)	70.9	1.2	(60a)
	90	39	16.7	<0.1	(279b)	71.3	2.6	(26b)
Hillsboro Canal	23	< 1	9.3	0.4	(23)[b]	30.3	3.4	(7.9)[b]

[#] To convert NO_3^--N from mg/liter to µg N/cm^3 soil multiply values for Pahokee muck, drainage ditch, and Hillsboro Canal by 1.36, 3.77, and 2.33, respectively.

[†] Values in parentheses are the ratio of N_2 to N_2O-N. Ratios for a single soil or sediment at each incubation period followed by the same letter are not significantly different at the 95% level (Duncan's Multiple Range Test).

[±] Ratios at each incubation period followed by the same letter in brackets are not significantly different at the 95% level (Duncan's Multiple Range Test).

mineral soils with a pH range of 5.7 to 8.2 the inhibitory effect of nitrate on reduction of nitrous oxide to dinitrogen by soil microorganisms increased with decreasing soil pH. To determine the effect of pH on nitrous oxide reduction in flooded Pahokee muck, soil samples were collected from a vegetable field flooded for 22 days. The addition of nitrate-N (initial concentration 111 mg/liter) to the flooded soil had almost no inhibitory effect on nitrous oxide reduction (Table 8). Ratios of dinitrogen to nitrous oxide ranged from 29 to 221 over 24 hours of incubation. It is interesting that after a period of flooding the effects of nitrate and pH on the inhibition of nitrous oxide reduction were overcome.

Smith and Tiedje (1979) have shown that denitrification rates measured during the first 4 hours of incubation of freshly collected soil samples can be attributed to pre-existing denitrification enzymes. That is, the denitrification rate measured early in the incubation period provides an indication of the denitrification potential of the soil sample in situ. Thus, surface samples of cropped and fallow Pahokee muck were collected in July 1979 and incubated in the laboratory to determine the effect of crop on denitrification and the dinitrogen/nitrous oxide-N ratio. Conditions for denitrification should have been optimum at this sampling time due to the high rainfall and rapid crop growth. The denitrification rate was greater in sugarcane and St. Augustinegrass field soils than in the fallow soil (Table 9). In a previous study of potential denitrification rates in Pahokee muck, this relationship was attributed to greater availability of organic carbon in the cropped soils (Terry and Tate, 1980a). In these freshly collected samples of Pahokee muck, not only was the denitrification rate greater in the cropped soils but the dinitrogen/nitrous oxide-N ratios were increased. At 4 hours of incubation 17, 27, and 40 percent of the gaseous denitrification products were in the form of dinitrogen in fallow, sugarcane and St. Augustinegrass field samples, respectively.

Although nitrate inhibition of nitrous oxide reduction does not appear to be a problem with soils which have been flooded for extended periods, this inhibitory effect may be an important factor in the production of nitrous oxide from drained soils which are subjected to periodic denitrification events such as following rainfall or irrigation. It is interesting that the apparent inhibition of nitrous oxide by the lower soil pH of the Pahokee muck was relieved by flooding.

Table 9. The effect of nitrate on N_2O reduction in fallow, cropped, and flooded Pahokee muck.

Cropping Practice	Interstitial NO_3^--N		N_2O-N evolved								
			4 hours			8 hours			20 hours		
	Initial	Final	$+C_2H_2$	$-C_2H_2$		$+C_2H_2$	$-C_2H_2$		$+C_2H_2$	$-C_2H_2$	
	---mg/liter[#]---		----------μg N/cm^3 soil----------								
Fallow	80	76	1.2	1.0	(0.17c)[†]	4.9	3.5	(0.40b)	10.2	7.2	(0.41b)
Cane	121	106	3.0	2.2	(0.36b)	5.4	4.3	(0.25c)	14.9	13.0	(0.15c)
Grass	58	32	8.4	5.0	(0.68a)	23.9	15.7	(0.52a)	38.0	23.8	(0.59a)
Vegetable (flooded 22 days)	111	86	3.0	<0.1	(29)	7.8	0.1	(77)	17.8	0.2	(88)

[#] To convert NO_3^--N from mg/liter to μg N/cm^3 soil multiply values for fallow, cane, grass, and vegetable fields by 1.54, 1.54, 1.73, and 1.97, respectively.

[†] Values in parentheses are the ratio of N_2 to N_2O-N. Ratios for each incubation period followed by the same letter are not significantly different at the 95% level (Duncan's Multiple Range Test).

SUMMARY

Inhibition of nitrous oxide reduction by nitrate in cultivated Pahokee muck was determined by measuring nitrous oxide accumulation in soil samples incubated in the presence and absence of acetylene. At nitrate-N levels of 40 mg/liter or greater, nearly 100 percent of the gaseous denitrification products were nitrous oxide in Pahokee muck. Reduction of the accumulated nitrous oxide proceeded rapidly after the nitrate was exhausted from the soil. In comparison, nitrous oxide was readily reduced in ditch sediment samples in the presence of comparable concentrations of nitrate. With nitrate-N concentrations in the ditch sediments ranging from 90.1 to 39.3 mg/liter, less than 10 percent of the gaseous denitrification products were nitrous oxide. Similarly, nitrate levels in soil samples from a vegetable field which had been flooded 22 days had negligible effect on nitrous oxide reduction. These data demonstrate that the inhibitory effect of nitrate on nitrous oxide reduction was less in sediments and in organic soils that had been subjected to prolonged flooding than in the cultivated soils.

CHAPTER V

NITRIFICATION AND DENITRIFICATION IN FLOODED SOIL

In regions of organic soils, an agricultural practice that could further reduce the nitrate content of the soil and, subsequently, of the drainage waters is flooding of the fields between harvests. This would not only reduce levels of soil inorganic nitrogen by retarding mineralization of the soil organic matter (Tate, 1979b), but it would also result in a stimulation of denitrification (Raveh and Avnimelech, 1973). Raveh and Avnimelech (1973) examined nitrate levels in a flooded muck at weekly intervals following flooding and noted an approximate 70 percent decline in the soil nitrate concentrations. This study was undertaken to determine if this residual nitrate results simply from the blockage of denitrification by limitations of soluble carbon, as proposed by Raveh and Avnimelech (1973), or if both denitrification and nitrification are occurring simultaneously in the surface layers of the flooded muck, as would be observed in sediments (Patrick and Reddy, 1976), but at a reduced rate over that which occurred in the soil prior to flooding. To accomplish this (i) the changes and microbial populations catabolizing nitrogen in the flooded muck were examined, (ii) levels of inorganic soil nitrogen were determined and (iii) variations in the denitrification rate were estimated.

MATERIALS AND METHODS

The soil used herein was Pahokee muck collected at AREC, Belle Glade, Florida, from a field that had been cropped to sweet corn (*Zea mays* L. *rugosa*). The properties of the Pahokee muck were presented in Table 1. Samples were collected from the flooded field with an Ekman dredge as previously described (Tate, 1979b). In the first year of the study, flooding commenced 14 June 1977, and the field was drained 25 July 1977. The water was maintained at 30 cm above the soil surface. The duration of the flooding for the second year was from 25 June 1978 through 18 July 1978. The depth of flooding was approximately equivalent for the two trials.

Samples for microbial analysis were diluted in 0.1 percent (wt/vol) sodium chloride. Autotrophic nitrifiers were detected by the most-probable-number (MPN) method of Alexander and Clark (1965), except that a 35-day incubation period was used. Denitrifiers were enumerated by the MPN method of Focht and Joseph (1973). Denitrification rates were estimated by a modification of the method of Terry and Nelson (1975). This technique is described under the Materials and Methods Section of Chapter II. Inorganic nitrogen was extracted from the samples with 2 M potassium chloride and assayed by steam distillation as described by Bremner and Keeney (1965). Denitrification rates were calculated as described in

Chapter II. All assays were performed in triplicate.

RESULTS

Changes in the denitrification capacities of the flooded soil were reflected by an increased denitrification rate, augmented populations of denitrifying bacteria, and a decreased concentration of nitrate-N in the muck samples. Immediately upon flooding, the denitrification rates increased (Fig. 4). Some variation was noted in this parameter between 1977 and 1978, but the basic patterns of change were the same. The maximum rate was detected in 1977 when the rate approached 18 μg N/cm^3 muck/day. The rates were more variable in 1978, but a maximum of about 13 μg N/cm^3/day was observed. The denitrifying bacterial population increased parallel to the increase in denitrification potential (Table 10). These data were collected from the 1978 sample period. Approximately a 15-fold increase in the denitrifiers was observed during the first 2 days of flooding. This increased population was maintained for at least 10 days before the population began to decline. Two periods of decline in the denitrifier population were observed. One occurred between 10 and 18 days of flooding, and the other occurred about 13 days after the soil was drained. The former likely represents depletion of the nitrogenous substrate; the latter is representative of the more aerobic conditions of the drained field.

Changes in the oxygen status of the field and the increase denitrification rate were reflected by the variation in the ammonium and nitrate contents of the soil (Fig. 5). Nitrate-N concentrations decreased from 50.5 μg/g soil prior to flooding to 12 μ g/g within 3 days of flooding. This decreased concentration was maintained until the field was drained, when again the nitrate concentration reached the pre-flood levels. At no time during the study did the nitrate concentration reach zero. Ammonium-N concentrations increased during the study. Preflood concentrations of 4 μg/g soil were augmented to about 20 μg/g soil during the flooding. Upon drainage of the field, the ammonium-N concentration returned to 4 μg/g. Similar variations in the nitrate and ammonium concentrations were observed during the 1977 season.

No statistically significant effect of flooding on the nitrifier populations was recorded during the 21-day flood period of 1978 (Table 11). Although Nitrosomonas populations ranged from 4.7×10^4 to 1.1×10^5 bacteria/cm^3 muck, no statistically significant differences in this population were recorded. Similar results were found with the Nitrobacter population, except that a slight increase was noted 5 days after flooding.

DISCUSSION

The data reported here demonstrate that flooding of the nitrate-laden muck soils is an effective means of controlling soil nitrate levels. Soil nitrate concentrations were decreased approximately 80 percent

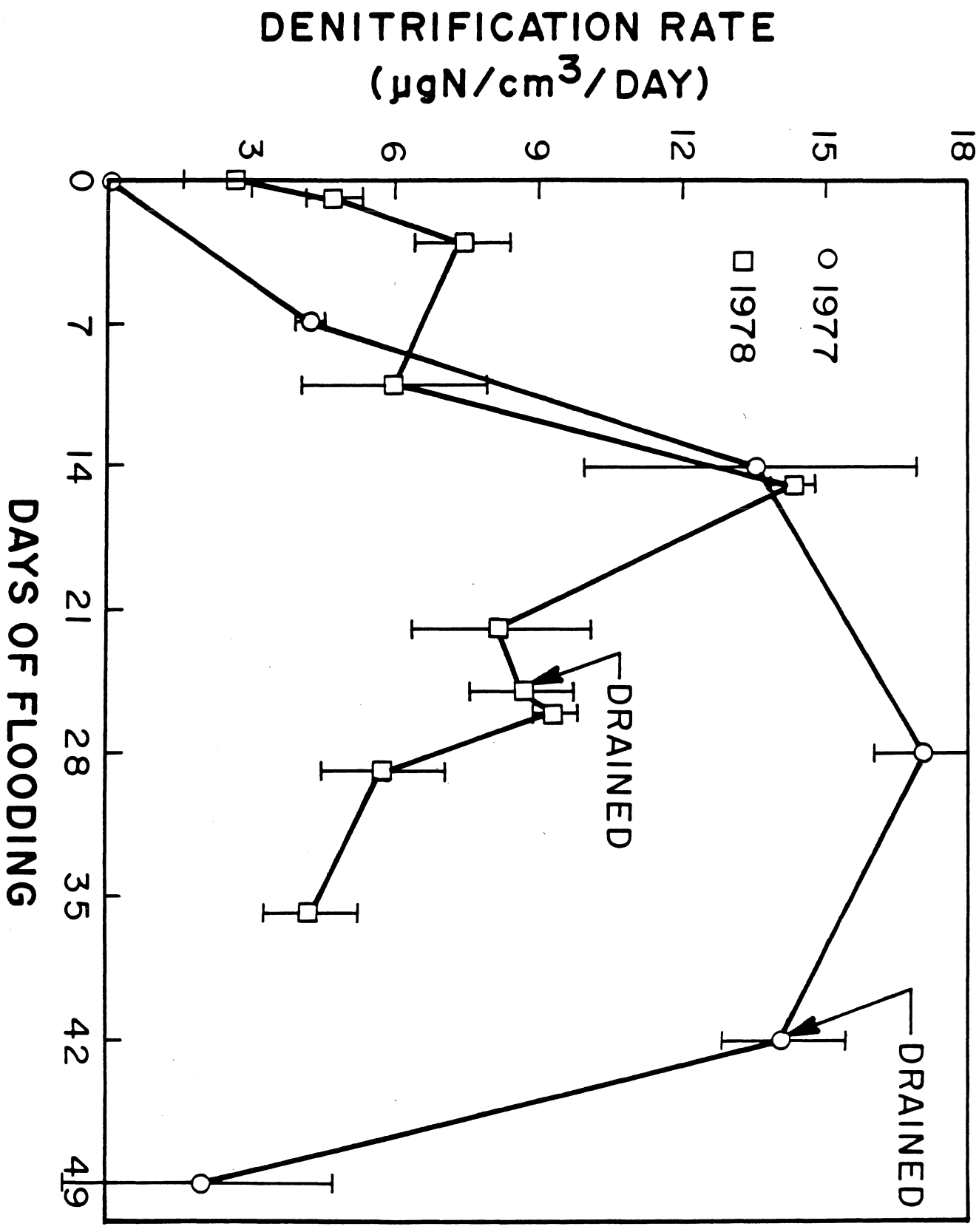

Fig. 4. Changes in potential denitrification rate during flooding of Pahokee muck (1977, 1978).

Table 10. Variation in MPN of denitrifying bacteria in Pahokee muck during flooding. The field was drained on day 21.

Day	Denitrifiers/cm^3 muck
0	7.4×10^4 c*
2	1.0×10^6 ab
3	8.5×10^5 ab
10	2.1×10^6 a
18	1.3×10^5 c
26	2.7×10^5 bc
29	9.1×10^4 c
36	2.0×10^4 d

* Values followed by the same letter are not significantly different at the 0.05 level (Alexander, 1965).

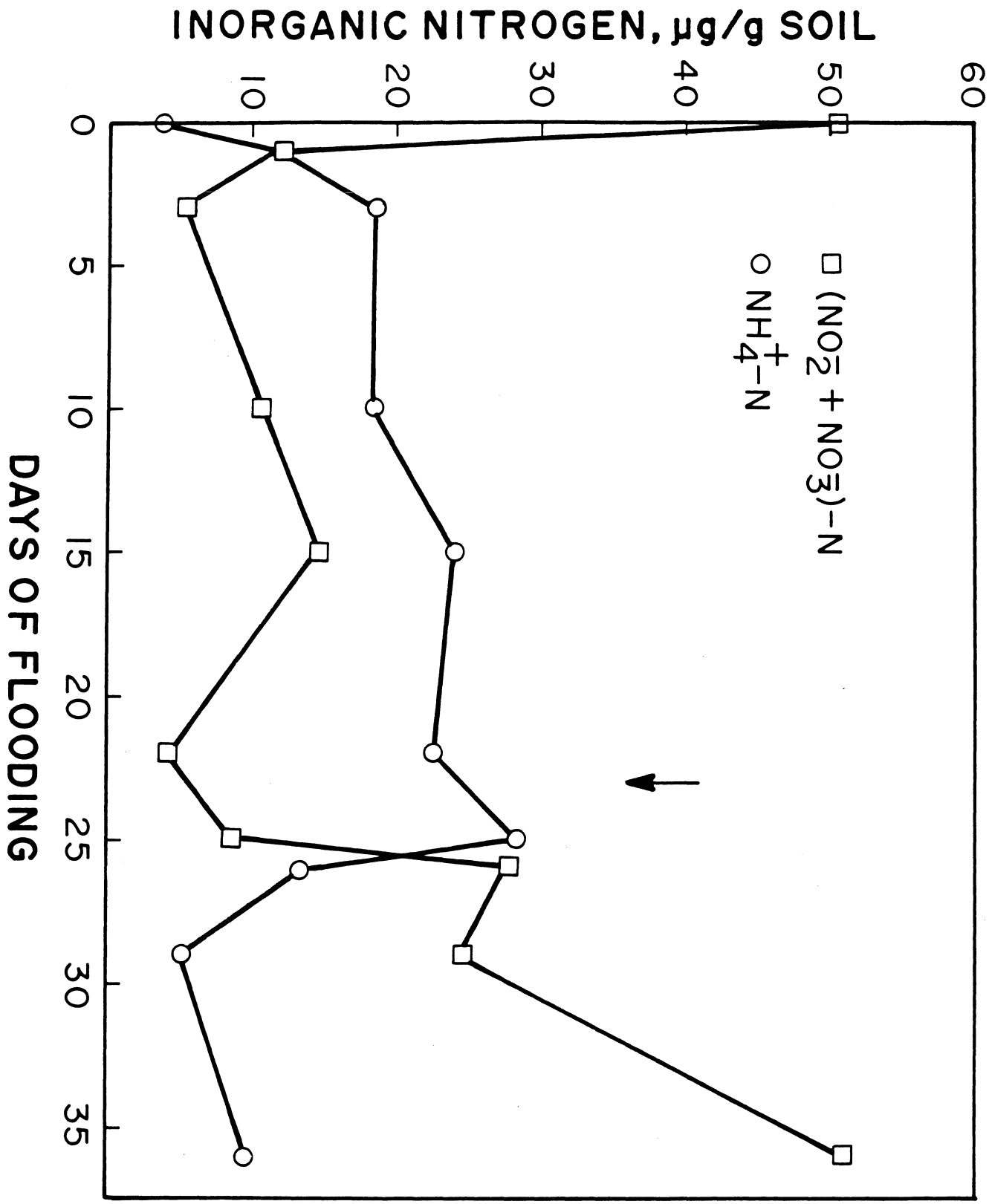

Fig. 5. Variation in nitrate and ammonium concentrations in muck during flooding (1977). The arrow indicates the end of the flood period.

Table 11. Variation in MPN of nitrifiers in Pahokee muck during flooding.

Day	Nitrobacter	Nitrosomonas
0	2.8×10^5 b	5.4×10^4 a
1	7.1×10^5 ab	8.8×10^4 a
5	1.1×10^6 a	1.1×10^5 a
10	2.2×10^5 b	1.1×10^5 a
17	2.3×10^5 b	4.7×10^4 a

[a] Values followed by the same letter are not significantly different at the 0.05 level (Alexander, 1965).

during the first few days of flooding. Although the soils were flooded for 3 to 4 weeks, no further gain in nitrate removal was found after the first 3 days. The residual nitrate in the surface layers of the soil profile could result from limitation of denitrification by a diminished source of soluble carbon, as was suggested by Raveh and Avnimelech (1973), or from a combined inhibition of both nitrification and denitrification. There was sufficient oxygen present in the surface of the soil profile for aerobic mineralization of the soil organic matter to occur, albeit at a reduced rate (Tate, 1979b). The accumulation of ammonium during flooding supports this observation. Because all the nitrate was not reduced via denitrification, the quantity of soluble carbon produced by this mineralization of the soil organic matter apparently was insufficient to provide substrate for the complete denitrification of the nitrate present. Although the accumulation of ammonium indicates an inhibition of nitrification in the flooded soil, the failure of the nitrifier population to decline significantly during flooding indicates that some nitrification could still be occurring. These data suggest that the nitrogen transformations in flooded muck, as in sediments (Patrick and Reddy, 1976), exist in a dynamic state, where nitrate production is equivalent to the amount of denitrification allowed by the limited soluble carbon produced by the soil organic matter oxidation.

We can conclude from this study that the incorporation of a flooded period into agronomic practices on muck soils is an efficient means of removing nitrate from the soil. The primary decline in soil nitrate was observed in the first few days of flooding, which demonstrates that, for water quality purposes, extended flood periods are not necessary.

SUMMARY

Variation in potential denitrification rate, denitrifying bacteria, nitrifiers, and inorganic nitrogen was measured in Pahokee muck during flooding. The denitrification rate increased immediately upon flooding. A maximum rate of 18 μg N/cm^3/day was measured. Denitrifying bacteria increased 15-fold during the first 2 days of flooding. The population was maintained at the higher level for at least 10 days before it began to decline. Nitrate concentrations decreased about 80 percent during the first 3 days of flooding. No further change was detected until the field was drained. The nitrate level then increased to the preflood levels. Ammonium concentrations increase fivefold during flooding. These data indicate that flooding of Pahokee muck is a good method of nitrate removal from the soil and, subsequently, from drainage waters.

CHAPTER VI

NITROUS OXIDE EMISSIONS FROM FLOODED SOIL

Emissions of nitrous oxide from soil can be affected by flooding. In a recent laboratory study of nitrate inhibition of nitrous oxide reduction in organic soils, it was observed that the major gaseous denitrification product in samples of drained soil was nitrous oxide; however, the major product in samples of flooded organic soil and ditch sediment was dinitrogen (Terry and Tate, 1980c). Also, Denmead et al. (1979) reported that during the flooding of a rice field, emissions of nitrous oxide-N decreased rapidly from a maximum of 328 g/ha/day on the second day of flooding to non-detectable levels after 12 days. Emissions of nitrous oxide were not measured prior to flooding. Since a significant portion of the drained, cultivated organic soils of south Florida are flooded for at least a portion of the year, this study was commenced to elucidate further change in nitrous oxide emissions, denitrification rates and dinitrogen/nitrous oxide-N ratios throughout the flood period.

MATERIALS AND METHODS

Nitrous oxide fluxes were measured in a field that had been cropped to sweet corn (*Zea mays* L. *rugosa*) located at the Agricultural Research and Education Center, Belle Glade, FL. The soil was Pahokee muck. Some properties of Pahokee muck at four depths in the profile are listed in Table 12. Soil pH was measured on a 1:2 soil:water mixture by glass electrode. Ash was determined by heating oven-dry samples of soil at 550°C overnight. Total carbon was estimated by dry combustion (Leco Carbon Analyzer) and total N by the method of Nelson and Sommers (1972).

Prior to flooding of the field, a pier was constructed from the adjoining road into the field a distance of 12 m to aid in sample collection. The support poles for the pier were placed in the soil such that the soil in the area from which samples would be collected was not disturbed. Flooding of the field commenced 16 June 1979, and lasted until 26 July 1979. The depth of water was maintained at 30 cm above the soil surface.

Nitrous Oxide Flux Measurements

Prior to flooding, four aluminum rings 78 cm in diameter and 15 cm high were placed in the soil to a depth of 10 cm. Aluminum chambers 76 cm in diameter and 17.8 cm high were constructed to fit inside the rings. For flux measurements, the chambers were sealed to the rings with 10 cm wide rubber bands cut from innertubes. The chambers were fitted with a 0.64 cm i.d. x 6 cm high vent to prevent pressure differences from de-

Table 12. Properties of Pahokee muck at various depths.

Depth	pH	Bulk density	Ash	Total C	Total N
cm		g/cm^3	----------%		----------
0 to 15	5.6	0.34	16.0	42.9	2.30
30 to 35	5.5	0.22	16.0	44.6	2.11
60 to 65	5.8	0.17	16.2	46.3	2.16
90 to 95	6.6	0.23	43.7	27.3	1.47

veloping between the inside and outside of the chambers, and a septum port to facilitate sampling by syringe. Ten-ml gas samples were withdrawn from the chambers at 10 min. intervals for up to 30 min. with disposable glass syringes. The needle hubs were sealed to the syringes with high vacuum grease and gas was sealed inside the syringes by inserting the needles into rubber stoppers. At the 1 ppm (parts per million Vol/Vol) level, less than 1 percent of the nitrous oxide was lost from syringes sealed in this manner and stored for 24 hours. All samples were analyzed within that period. As the field was flooded the aluminum rings were removed from the soil and bricks were placed on the soil surface to support the rings. The rings extended 5 cm above and 10 cm below the water surface. Brackets inside the rings supported the bottom of the chambers at the water surface.

A Hewlett Packard 5840A gas chromatograph with a ^{63}Ni electron capture detector was used to measure nitrous oxide. The gas chromatograph was equipped with a gas sampling valve and a 1 ml sampling loop. Nitrous oxide was separated on a Porapak Q column (0.003 x 3.0 m). The oven and detector temperatures were 50 and 330°C, respectively. The carrier gas (5% methane in argon) flow rate was 30 ml/min. The gas chromatograph was recalibrated at 1 hour intervals with a standard gas. Nitrous oxide gas standards ranged from 0.249 to 1.050 ppm in air (Scott Environmental Technology Inc., Plumsteadville, PA). Standards above 1 ppm were prepared by adding aliquots of a 539 ppm nitrous oxide standard to air in capped serum bottles. Nitrous oxide flux was computed by multiplying the concentration increase per unit time by the height of the soil chamber.

Determination of Dissolved Nitrous Oxide

At each sampling time, flood water samples (125 ml) were added to 250 ml glass bottles. Water samples were collected from the drainage canal, which was the source of flood water for the field. The bottles were sealed with mininert septum valves (Precision Sampling Corp., Baton Rouge, LA) and placed, inverted, in a 0°C water bath for 24 hours. During that time, nitrous oxide dissolved in the water equilibrated with the headspace gas. Samples (5 ml) of the headspace gas were removed from the bottles by syringe and injected into the gas sampling loop of the gas chromatograph. The concentration of nitrous oxide in the water samples (N_2O_{dis}) was calculated using the following relationship, which corrects for ambient nitrous oxide present in the bottle headspace:

$$N_2O_{dis} = [(N_2O_h - N_2O_a)\, H_{vol} + \alpha \cdot N_2O_h\, (W_{vol})]/W_{vol}$$

where N_2O_h is the concentration (ppm) of nitrous oxide in the headspace after equilibration, N_2O_a is the ambient concentration at the time of sample preparation, H_{vol} is the headspace volume (125 ml), W_{vol} is the volume of water (125 ml), and α is the Bensen coefficient (1.3 for water at 0°C). For equal headspace and water volumes, the relationship reduces to:

$$N_2O_{dis} = 2.3N_2O_h - N_2O_a$$

Values for dissolved nitrous oxide are presented in terms of ppm (vol/vol) and represent the mean of 4 replicates. The percent of saturation of flood water with nitrous oxide was based on the water temperature and the ambient concentration of the nitrous oxide above the water at the time of sampling. In this case, the appropriate Bunsen coefficient for the water temperature was used.

Incubation Experiments

Soil samples were collected, transported to the laboratory (transit time ca. 5 min.) and sieved (4 mm). Since the soil samples were immediately dispensed for analysis, no storage was necessary. Ten-g (wet wt.) samples of soil were placed in 60 ml serum bottles and 10 ml of a 200 mg/liter nitrate-N solution (as potassium nitrate) was added. The bottles were sealed with septum stoppers, connected to a manifold system and evacuated and purged 3 times with argon or an argon + 10% acetyelene mixture. The samples were then incubated for 4 hours on a rotary shaker (200 rpm) at 25°C. The products of denitrification in the samples were determined by the acetylene inhibition method of Terry and Tate (1980c). Headspace concentrations of nitrous oxide were corrected for nitrous oxide dissolved in the aqueous phase. Total denitrification was determined by nitrous oxide production in acetylene amended samples and is reported in terms of μg nitrous oxide-N/cm^3 of soil. Nitrous oxide production was measured in samples without acetylene. The ratio of dinitrogen to nitrous oxide-N was determined by subtracting nitrous oxide produced in the absence of acetylene from nitrous oxide produced in the presence of acetylene and dividing by nitrous oxide produced in the absence of acetylene. Following incubation, samples were extracted with 2M potassium chloride and ammonium-N, nitrite-N and nitrate-N were determined by steam distillation (Bremner and Keeney, 1965). Incubation experiments were conducted in triplicate and replications were within 5 percent of the mean.

RESULTS AND DISCUSSION

Previous studies have demonstrated the efficient reduction of the soil nitrate during flooding of Histosols (Terry and Tate, 1980b; Raveh and Avnimelech, 1973). Similar changes in the soil nitrate-N and ammonium-N concentrations were detected during this study. Nitrate-N decreased from 68 μg/cm^3 soil prior to flooding to < 0.1 μg/cm^3 within 5 days after flooding (Table 13). The nitrate levels remained low until the field was drained when again the nitrate-N increased. Pre-flood ammonium-N concentrations of 0.1 μg/cm^3 increased to 23 μg/cm^3 during flooding. Upon drainage of the field, ammonium-N concentrations approached the pre-flood levels.

Nitrous oxide fluxes from the field, two days prior to flooding, ranged from 23 to 175 g N/ha/day (Table 13). After flooding, nitrous oxide approximated zero. One day after the field was drained, the flux was still below 1 g N/ha/day; however, 3 days after drainage the nitrous oxide flux

Table 13. The effect of flooding on inorganic nitrogen, the N_2O flux from the field and N_2O dissolved in flood water.

Date	NH_4^+-N	$(NO_2^- + NO_3^-)$-N	N_2O-N flux	Dissolved N_2O	N_2O saturation
	-----$\mu g/cm^3$ soil-----		g/ha/day	ppm	%
13 June	0.1	56.1	22.9 ± 5.1	--[1]	--
14	0.1	68.2	174.4 ± 98.4	--	--
16	---------- Flooding began ----------				
18	3.5	1.8	0.4 ± 0.4	0.10	68
19	2.6	1.1	-0.3 ± 0.4	0.12	76
21	0.1	0.1	-0.4 ± 0.1	0.08	56
25	1.7	0.1	0.5 ± 0.1	0.10	62
6 July	14.3	0.5	-2.0 ± 1.6	0.37	167
10	23.1	0.1	-0.4 ± 0.4	0.05	29
23	1.0	0.1	0.2 ± 0.3	0.22	135
26	---------- Flooding ended ----------				
27	14.8	2.5	0.2 ± 1.0	--	--
31	3.7	5.0	1122.0 ± 296.7	--	--
2 Aug.	4.5	10.3	512.7 ± 44.2	--	--

[1] Values not determined.

was in excess of 1,000 g N/ha/day.

The negative flux values in Table 13 indicate nitrous oxide consumption from the atmosphere, but because of the statistical variation in the data, it was impossible to determine if the flooded soil was a source or sink for atmospheric nitrous oxide. A suggested answer to this question was provided by measuring the concentrations of nitrous oxide dissolved in the flood water and determination of the percent of saturation (Table 13). Nitrous oxide dissolved in the water ranged from 29 to 167 percent of saturation. On 6 July and 23 July the flood water was supersaturated with nitrous oxide. This increased nitrous oxide level could have resulted from the fact that drainage water from adjacent fields was pumped into the flooded field to maintain the water level. During July, nitrous oxide dissolved in the drainage water from these drained, cultivated fields ranged from 27 to 0.35 ppm. Thus, the nitrous oxide saturation of flood water on 6 July and 23 July likely resulted from the use of this drainage water. During most of the flood period, nitrous oxide dissolved in the flood water was below saturation. The low levels of nitrous oxide dissolved in the water and the minimal fluxes from the water suggest that the field may have acted as a sink for nitrous oxide.

Laboratory incubation experiments were conducted to determine the effect of flooding on denitrification rates and the ratio of gaseous denitrification products. A 4-hour incubation period was chosen so that the products of native enzymes could be measured (Smith and Tiedje, 1979). Thus, the effects of laboratory incubation on enzymatic activity would be minimized. The production of nitrous oxide in samples incubated with and without acetylene and the dinitrogen/nitrous oxide-N ratios are listed in Table 3. The production of nitrous oxide in acetylene amended samples provided a measure of potentail denitrification rates. Prior to flooding, the potential denitrification rate was 16.2 μg N/cm^3/day and the dinitrogen/nitrous oxide ratio was less than 1.0 indicating that the major product of denitrification was nitrous oxide. The potential denitrification rate in soil samples collected after flooding increased to a maximum of 31.2 μg N/cm^3/day. The denitrification rate remained high until several days after drainage. The dinitrogen/nitrous oxide-N ratio increased to 29 as the soil was flooded. This indicated that the nitrous oxide reducing activity of the soil microflora increased after flooding and the major product of denitrification in the flooded soil was dinitrogen. These data confirm the findings of Terry and Tate (1980c) that the ratio of dinitrogen to nitrous oxide-N production is greater in sediments and flooded soils than in drained soils and indicate that this increased nitrous oxide reducing activity extends throughout the flood period. The dinitrogen/nitrous oxide-N ratio decreased after the field was drained (Table 3).

A major determinant on the dinitrogen/nitrous oxide-N ratio is the level of soil nitrate and nitrite. Blackmer and Bremner (1979) demonstrated that small amounts of nitrate (5 μg nitrate-N/g soil) stimulated the reduction of nitrous oxide to dinitrogen under anaerobic conditions by the microflora of several soils, whereas higher NO_3^- concentrations (50 to 2000 μg nitrate-N/g soil) inhibited this reduction (Blackmer and Bremner

Table 14. The effect of flooding on gaseous denitrification products in incubated soil samples.

Date	N_2O-N produced		
	$+C_2H_2$	$-C_2H_2$	N_2/N_2O-N
	------- $\mu g/cm^3/day$ -------		
13 June	15.6	8.4	0.9
14	16.2	10.8	0.5
16	----------Flooding began----------		
18	14.4	4.8	2.0
19	31.2	4.2	6.4
21	25.2	1.2	20.0
25	23.4	2.4	8.5
9 July	18.0	0.6	29.0
26	----------Flooding ended----------		
31	29.4	2.4	11.3
20 Aug.	10.8	2.4	3.5

1978). Firestone et al. (1979) recently demonstrated that nitrite at concentrations < 0.75 mg/liter inhibited nitrous oxide reduction in soil slurries. All incubations in the present study were conducted at nitrate-N levels of 100 mg/liter. Nitrate concentrations were reduced by less than 5 percent during the short incubation period and nitrite-N concentrations ranged from 1.0 to 3.0 mg/liter. The major gaseous product of denitrification in the samples collected prior to flooding was nitrous oxide, due possibly to the inhibitory effect of nitrate and nitrite on nitrous oxide reduction (Table 14). The major product of denitrification in soil samples collected after flooding was dinitrogen. Firestone and Tiedje (1979) have presented evidence that increases in nitrous oxide reducing activity in soils after a period of anaerobiosis resulted from enzyme synthesis. Thus, it is possible that nitrate and nitrite inhibited synthesis of nitrous oxide reductase in the drained soil; however, nitrous oxide was readily reduced in the presence of nitrate and nitrite in the flooded soils due to the presence of nitrous oxide reductase synthesized upon flooding of the soil.

The low nitrous oxide fluxes from the flooded field were likely due not only to low nitrate levels but also to increased nitrous oxide reducing activity of the soil microflora. Therefore, as suggested by Denmead et al. (1979), flooded soils may contribute less nitrous oxide to the atmosphere than is commonly assumed. It should be recognized, however, that a flooded soil is a unique environment which favors reduction of nitrous oxide to dinitrogen over escape of nitrous oxide to the atmosphere. Denitrification under non-flooded conditions may well lead to substantial emissions of nitrous oxide because the soil microflora have less ability to reduce nitrous oxide and diffusion of nitrous oxide from the soil is more rapid.

SUMMARY

To determine the effect of flooding of Pahokee muck (a drained, cultivated Histosol) on nitrous oxide emissions from the soil, field nitrous oxide fluxes and nitrous oxide produced in soil samples incubated under controlled laboratory conditions were measured. During the first five days of flooding of a field which had previously been cropped to sweet corn (*Zea mays* L. *rugosa*), the nitrate-N levels declined from 68 to < 0.1 μg N/cm^3. Similarly, the nitrous oxide flux decreased from 174 g N/ha/day prior to flooding to approximately 0 following flooding. Comparison of the flux values and the nitrous oxide concentrations in the flood water suggested that the flooded field may have acted as a sink for atmospheric nitrous oxide. In laboratory incubated samples of the pre-flood and flooded soils, the potential denitrification rates increased from 16.2 μg N/cm^3/day prior to flooding to 31.2 μg N/cm^3/day after flooding. Whereas nitrous oxide was the major product of denitrification in soil samples collected prior to flooding, very little nitrous oxide accumulated in samples collected from the flooded field. Nitrous oxide was readily reduced in the flooded soil in the presence of nitrate-N and nitrite-N concentrations of 100 and 3 mg/liter, respectively.

CHAPTER VII

EFFECT OF EXOGENOUS CARBON ON DENITRIFICATION IN PAHOKEE MUCK

To acquire a better understanding of those factors limiting denitrification in Pahokee muck, this study was commenced with the objective of determining the effect of various carbon amendments on the denitrification rate of Pahokee muck and that proportion of the nitrate reduced which is released as nitrous oxide. Denitrification rates were measured and carbon amendments were made in a manner to minimize induction of new metabolic activities, i.e. the effect of amendment on indigenous microbial activity was determined.

MATERIALS AND METHODS

The soil used in this study was Pahokee muck collected from the surface (0-10 cm) of a fallow field and a field cropped to St. Augustinegrass located at the Agricultural Research and Education Center, Belle Glade, FL. Generally, freshly collected soil was sieved to pass 2-mm, placed in a plastic bag and stored at 4°C without drying. The soil was not stored longer than 2 weeks. No significant change in the denitrification rate of samples stored in this manner was detected. The initial study of denitrification rate was conducted with soil collected from the grass field and stored 2 months at 25°C. Soil properties are presented in Table 1.

Denitrification capacity was measured as previously described (Terry and Tate, 1980). For this, soil was dispensed into 10 g/60 ml serum bottle and 8 or 9 ml of water (depending upon the other amendments) were added. The samples were incubated 16 hours at 25°C, then 1 ml of 500 µg/ml nitrate-N (as potassium nitrate) was added. For samples receiving carbon amendments, the quantity of carbon added was amended to the samples in 1.0 ml water so that the total volume of water in the samples was always 10 ml. The bottles were sealed with septum stoppers, connected to a manifold system and evacuated and purged 3 times with argon or an argon-10 percent acetylene mixture. The samples were then incubated on a rotary shaker (200 rpm) at 25°C until the reaction was stopped by freezing the samples in dry ice/methanol. The samples were then equilibrated 24 hours at 0°C prior to analysis of the nitrous oxide. This allowed the nitrous oxide to reach equilibrium between the aqueous and gaseous phases. Headspace nitrous oxide was corrected for nitrous oxide dissolved in the aqueous phase. Total denitrification was determined by nitrous oxide production in acetylene amended samples and is reported in terms of µg nitrous oxide-N/cm^3soil/hour. Nitrous oxide production was measured in samples without acetylene. Following incubation, the samples were extracted with 2M potassium chloride (nitrate + nitrite)-N concentration was determined by steam distillation (Bremner and Keeney, 1965). All experiments were conducted in triplicate.

Nitrous oxide was assayed as previously described (Terry and Tate, 1980c). A Hewlett Packard 5840A gas chromatograph with a ^{63}Ni electron capture detector was used. Nitrous oxide was separated on a Porapak Q column (0.003 x 3.0 m). The oven and detector temperatures were 50 and 330°C, respectively. The carrier gas (5% methane in argon) flow rate was 30 ml/min. The gas chromatograph was calibrated with standard gases (Air Products, Miami, FL) within the concentration ranges detected in the samples.

Denitrifying bacteria were enumerated by the most-probable-number method of Focht and Joseph (1973). Soil dilutions were prepared in 0.1 percent (wt/vol) sodium chloride.

RESULTS

Nitrous oxide production in unamended Pahokee muck or muck receiving 3.1 mg glucose-C/cm^3 and incubated under an argon/acetylene atmosphere was linear ($r = 0.99$ for both treatments) (Fig. 6). The soil used in this study was collected from the surface of the St. Augustinegrass field, sieved to 2-mm and stored in the laboratory at room temperature for 2 months. Both regression lines passed through the origin with slopes of 48.86 and 16.37 µg nitrous oxide-N/cm^3/hour for glucose amended and unamended samples, respectively. Thus, since nitrous oxide production in the presence of acetylene is reflective of the denitrification rate, glucose amendment of the samples resulted in approximately a 3-fold stimulation of this rate. This increased denitrification occurred in the absence of any lag in nitrous oxide production. No significant changes in the (nitrate + nitrite)-N occurred during the 3 hour incubation of the unamended samples. There was about a 12 percent decline in nitrate + nitrite in the glucose amended samples after 2 hours of incubation (35.8 ± 1.3 to 28.4 ± 1.8 µg N/cm^3). The denitrifier population remained at 3.4 ± 1.1 x 10^5 bacteria/cm^3 for both treatments throughout the incubation period. Because of the linearity of the nitrous oxide production and the constancy of nitrate concentration and denitrifier populations during the first hour of incubation, this period was used to measure denitrification rate in subsequent experiments. Denitrification rate was expressed as µg nitrous oxide-N/cm^3/hour.

Both the nature of the carbon source added to the soil and the duration of the pre-incubation period affected the denitrification rate (Table 15). With freshly collected soil from the grass field, a decline in the denitrification rate was recorded in all amended soil samples with a 16 hour pre-incubation. After 64 hours of pre-incubation, substrate amendment resulted, generally, in a stimulation of the denitrification rate. Succinate (3.1 mg C/cm^3) and acetate (0.31 and 3.1 mg C/cm^3) amendment resulted in a decline in the denitrification rate. It must be noted that, with all amendments, greater denitrification capacity was detected with the 16 hour samples than with the 64 hour samples. No change in the nitrate concentrations was detected in these samples. Pre-incubation had little effect on the stimulation due to substrate amend-

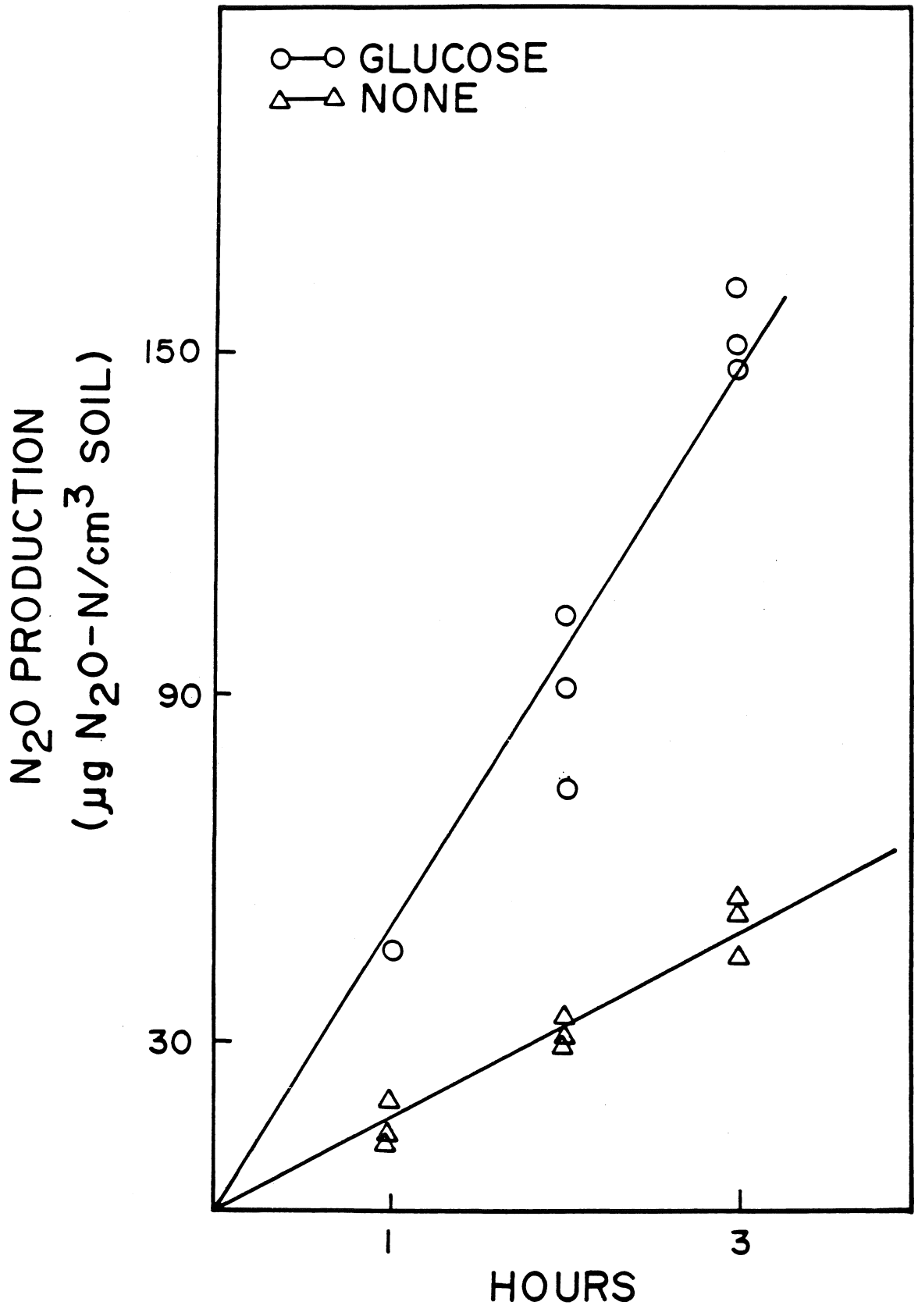

Fig. 6. Effect of addition of 3.1 µg glucose-C/cm^3 soil on the denitrification rate of Pahokee muck from a St. Augustinegrass field.

Table 15. Effect of pre-incubation time and carbon source on the denitrification rate of Pahokee muck collected from a grass field.[a]

Substrate	Conc.[b]	Denitrification Rate ($\mu g\ N_2O\text{-}N/cm^3/h$)	
		16 h[d]	64 h[d]
None	--	31.63a	12.03f
Glucose	0.31	24.08d	18.08d
	3.10	25.76c	22.54a
Succinate	0.31	27.93b	17.05de
	3.10	18.59f	0.37h
Glycerol	0.31	27.45b	20.21bc
	3.10	27.51b	19.27c
Acetate	0.31	12.16g	8.67g
	3.10	12.20g	1.02h
Amino Acids	0.10[c]	21.68e	16.42e
	1.00[c]	26.36c	21.23b

[a] Values within a single incubation time followed by a different letter are significantly different at the 95% level (Duncan's Multiple Range Test).

[b] Concentration, mg C/cm^3 muck.

[c] Final % amino acids (w/w).

[d] Hours of pre-incubation.

ment in freshly collected soil from the fallow field (Table 16). In this soil, all substrates, except succinate at 3.1 mg C/cm^3, were stimulatory.

The lack of stimulation of denitrification rate by glucose amendment in the soil from the grass field was not due to insufficient quantities being added (Table 17). With glucose amendments ranging from 0 to 4.7 mg C/cm^3, no stimulation of denitrification rates occurred. For comparison, with the same variation in glucose concentration denitrification rate increased in the soil from the fallow field from 1.49 to 8.19 μg N_2O-N/cm^3/hour. Succinate was inhibitory at all concentrations with the soil from the grass field (Table 18). But in the soil from the fallow field, amendments of 0.31 to 3.1 mg C/cm^3 were stimulatory. Addition of 4.7 mg succinate-C/cm^3 resulted in an 80 percent decline in the denitrification rate compared to that of the unamended muck.

Although some variation was observed in the proportion of nitrogen reduced which was released as nitrous oxide, no significant effect of substrate amendment on this value was observed (Table 19). These values were determined by measuring nitrous oxide in the presence and absence of acetylene. Thus, although the rate of denitrification was stimulated or inhibited by carbonaceous substrate amendment, the nitrous oxide component of the reduced nitrogen remained constant. This does not suggest that some change in this value would not be expected under conditions where new enzymic activity was induced by substrate amendment. In fact, it has been shown that amendment of mineral soils with a carbon source resulted in a reduction in the proportion of reduced nitrogen released as nitrous oxide (Nommik, 1956; Yoshinari et al., 1977). Terry and Tate (1980c), observed dinitrogen/nitrous oxide ratios of fallow and cropped Pahokee muck of 0.17 to 0.68, respectively. The difference between these values and those reported herein likely resulted from the inclusion of the pre-incubation period in this study. No pre-incubation was used by Terry and Tate.

DISCUSSION

Previous studies have demonstrated stimulation of denitrification rates in soil following incubation with a variety of carbonaceous substrates (Bowman and Focht, 1974; Stanford et al. 1975). The data reported herein show that with Pahokee muck collected from a fallow field, this process was increased immediately upon amendment of the soil with a diverse range of readily oxidizable carbon sources. This could result from either direct catabolism of the substrate by the denitrifying bacteria or a reduction of the oxygen tension of the soil environment by the heterotrophic bacterial population which in turn results in increased denitrification rates. This latter option is unlikely in that no effect of substrate amendment was observed when any carbon source was added to freshly collected soil from the grass field. This indicates that the evacuation and replacement of the atmosphere in the reaction vessels with argon or argon/acetylene and the pre-incubation of the samples for 16 hours allowed for the reduction of the oxygen tension to a level that was

Table 16. Effect of pre-incubation on denitrification rate in Pahokee muck collected from a fallow field.[a]

Substrate	Conc.[b]	Denitrification Rate ($\mu g\ N_2O\text{-}N/cm^3/h$)	
		16 h[e]	64 h[e]
None	--	1.49h	3.03g
Glucose	0.31	9.15c	11.29c
	3.10	7.74d	12.83b
Succinate	0.31	7.86d	9.66d
	3.10	1.13h	ND[c]
Glycerol	0.31	4.93f	7.90e
	3.10	3.59g	7.81e
Acetate	0.31	6.21e	11.42c
	3.10	6.24e	6.77f
Amino Acids	0.10[d]	10.60b	15.32a
	1.00[d]	12.16a	15.95a

[a] Values within a single incubation time followed by a different letter are significantly different at the 95% level (Duncan's Multiple Range Test).

[b] Concentration, mg C/cm^3 muck.

[c] None detected.

[d] Final % amino acids (w/w).

[e] Hours of pre-incubation.

Table 17. Effect of glucose concentration on denitrification rate.[a]

Soil	Concentration (mg C/cm^3)	Denitrification Rate (μg N_2O-N/cm^3/h)
Fallow	0.00	1.49d
	0.31	4.73c
	1.60	5.23c
	2.30	6.83b
	4.70	8.19a
Grass	0.00	31.61a
	0.31	22.15c
	1.60	27.29b
	3.10	23.43c
	4.70	21.33c

[a] Values for each soil followed by a different letter are significantly different at the 95% level (Duncan's Multiple Range Test).

Table 18. Effect of succinate concentration on denitrification rate.[a]

Soil	Concentration (mg C/cm^3)	Denitrification Rate (μg N_2O-N/cm^3/h)
Fallow	0.00	1.49d
	0.31	6.78a
	1.60	6.44a
	2.30	2.89b
	4.70	0.34e
	3.10	2.07c
Grass	0.00	31.61a
	0.31	29.38b
	1.60	16.80c
	2.30	8.77d
	3.10	5.07e
	4.70	0.79f

[a] Values for each soil followed by a different letter are significantly different at the 95 percent level (Duncan's Multiple Range Test).

Table 19. Effect of substrate amendment on the proportion of nitrogen reduced which is released as N_2O[a].

Soil	Amendment[b]	$N_2O/(N_2 + N_2O) \times 100$
Fallow	Glucose	0a
	Amino Acids[c]	18a
	Succinate	15a
	Glycerol	0a
	Acetate	15a
	None	0a
Grass	Glucose	77a
	Amino Acids[c]	68a
	Succinate	75a
	Glycerol	84a
	Acetate	72a
	None	67a

[a] Values for each soil followed by a different letter are significantly different at the 95% level (Duncan's Multiple Range Test).

[b] 0.31 mg C/cm^3 soil.

[c] Final % amino acids = 0.1% (w/w).

not inhibitory to the denitrifier. Thus, the more likely explanation of the data is that in the fallow field, where the only source of oxidizable carbon was the native soil organic matter, the microbial community was limited by the rate that this substrate was solubilized. This limitation was overcome by the carbon supplied by growth of grass upon the soil. For limitation of denitrification to be the result of carbon supply, nitrate and the population density of the denitrifiers must not be limiting. This is reasonable in Pahokee muck in that the soil generally contains high nitrate levels and large amounts of nitrate are denitrified annually (Terry and Tate, 1980a).

The explanation of the inhibition of denitrification by all carbon sources in the grass soil and by acetate and succinate in the soil from the fallow field is not readily apparent. No measurable change in nitrate concentration was observed during the study. But, nitrate could have been limiting at the microsites where the denitrifier and other heterotrophs were competing. This is the most apparent and logical explanation for the inhibition.

This study demonstrated that although the denitrifier exists in an environment composed of 48 percent organic carbon, in the fallow Pahokee muck, the organism is not existing in luxury. With the high nitrate concentrations characteristic of these soils, available carbon has become the limiting nutrient for the denitrifier.

SUMMARY

Amendment of soil samples from a fallow field of Pahokee muck with glucose, acetate, succinate, amino acids or glycerol resulted in an immediate stimulation of the denitrification rate. No stimulation was observed in freshly collected samples from a St. Augustinegrass field. Preincubation of the latter soil samples for 64 hours under conditions conducive to denitrification resulted in the denitrification rate being stimulated by exogenous carbon amendment. No immediate effect on that proportion of nitrogen reduced which was released as nitrous oxide was detected. These data suggest that denitrification is limited by soluble carbon in Pahokee muck from a fallow field.

CHAPTER VIII

HETEROTROPHIC AND AUTOTROPHIC NITRIFIERS IN PAHOKEE MUCK

The occurrence of large populations of heterotrophic nitrifiers in organic soils was recently demonstrated (Tate, 1977). These studies with ammonium and acetate amended Pahokee muck and with the nitrification inhibitor N-serve (2-chloro-6-(trichloromethyl)pyridine) suggested that the heterotrophs may produce some of the nitrate found in the organic soils. Because of the large populations of heterotrophic nitrifiers found in these soils and their suggested role in nitrification, it was considered that Pahokee muck would provide an ideal medium for further study of the function of these interesting organisms in a natural ecosystem. Thus, the effect of environmental parameters on the population densities of autotrophs and heterotrophs was determined to note if the two nitrifier populations varied similarly under like conditions and to document the extent of occurrence of heterotrophic nitrifiers in Histosols. Also, population changes and production of nitrate and nitrite in Pahokee muck treated to inactivate autotrophs, but not heterotrophs was examined.

MATERIALS AND METHODS

The soil used in this study was Pahokee muck collected at the Agricultural Research and Education Center at Belle Glade, FL. To determine variation in microbial activity, soil samples were collected as follows: surface 0- to 10-cm samples were composite samples from a 5 ha fallow (bare) field, an adjacent field of St. Augustinegrass and a nearby sugarcane field. Soils within the profile were collected by digging a 2 x 2 m trench in the fallow field to the indicated depth. Composite samples were collected at several depths. All soil samples were placed in sterile plastic bags and transported to the laboratory (transit time approximately 5 min) where they were diluted in water for microbial analysis.

Heterotrophic nitrifiers were assayed as previously described (Tate, 1977). For this, the appropriate soil dilutions were plated on soil extract agar by the spread plate technique. After 7 d incubation at 30°C, the numerically dominant colonies were selected and tested for nitrification capacity in a broth developed by Eylar and Schmidt (1959). Autotrophic nitrifiers were assayed by the method of Alexander and Clark (1965) except a 35 d incubation period was used in place of the 21 d period. Aerobic bacterial populations were estimated by plating appropriate soil dilutions on soil extract agar (Tate, 1977). Soil moisture was determined by drying samples at 110°C to a constant weight. Values are presented as percent of the wet weight of soil.

For those studies requiring amendment of the soil in the laboratory, fallow surface soil, which had been sieved to pass 2 mm, was dispensed

into 10 g/milk dilution bottle. To inactivate the autotrophic nitrifiers, the soil was heated at 100°C in an oven for 16 hr. After the soil had cooled to room temperature, the soil moisture was adjusted to approximately 60 percent and the soil was incubated 7 d at 25°C to allow the surviving populations to equilibrate. After 7 d, sterile sodium acetate and/or ammonium sulfate were added to give final concentrations of 1.0 percent (wt/wt) and 0.94 percent (wt/wt) (100 μg N/g soil), respectively. Microbial populations were assayed as indicated above. Ammonium and nitrate plus nitrite concentrations were estimated by titration following steam distillation of potassium chloride extracts by the method of Bremner and Keeney (1965). Nitrite was estimated in the extracts by the colorimetric method of Montgomery and Dymock (1962).

RESULTS

Initial studies involved examination of the variation of the population densities of both heterotrophic and autotrophic nitrifying populations and aerobic bacteria in Pahokee muck with time, crop and soil moisture. Heterotrophic nitrifier populations ranged from a minimum of 2.0×10^5 to a maximum of 3.9×10^6 bacteria/cm^3 muck in the fallow surface (0- to 10-cm) soil between December, 1976 and February, 1978 (Fig. 7). This population fluctuated in like manner as soil moisture and aerobic bacterial populations. When examined by linear regression analysis, the relationship of heterotrophic nitrifiers to soil moisture indicated that in the moisture range detected (40 to 60 percent), increased moisture stimulated the heterotrophic nitrifier population. The correlation of the two variables was statistically significant (Table 20). Within the soil profile, as the depth of soil increased, the number of heterotrophic nitrifiers/cm^3 soil declined (Fig. 8). With the soil profile samples, the moisture ranged from approximately 55 percent to saturation. At the higher moisture levels, the resultant exclusion of air from the soil caused an inhibition of aerobic microbial activity (Tate, 1979a). Hence, the negative correlation coefficient indicated that although there was a significant correlation, the two variables were related inversely. Significant variation in both autotrophic and heterotrophic nitrifiers was noted between the cropped soils and fallow soils (Table 21). In each of the environments examined, the heterotrophic nitrifiers varied in parallel to the aerobic bacterial populations. Linear regression analysis of the relationship between these populations indicated a correlation that was significant, in all cases, at the 0.99 level (Table 20).

Changes in the population densities of the autotrophic nitrifiers appeared to follow the same pattern as did the heterotrophic organisms. For example, with the 8 January sample a 40-fold decrease in heterotrophic nitrifiers occurred between the surface and the 60 cm depth whereas, the ammonia oxidizer and nitrite oxidizer populations declined from 2.0×10^3 to 7.7×10^1/cm^3 and 1.8×10^4 to 1.5×10^2/cm^3, respectively (Table 22). Similar variations were recorded for the other sample periods. Comparison of these populations with soil moisture levels revealed no significant correlation (Table 20). This suggests that in opposition to the situation

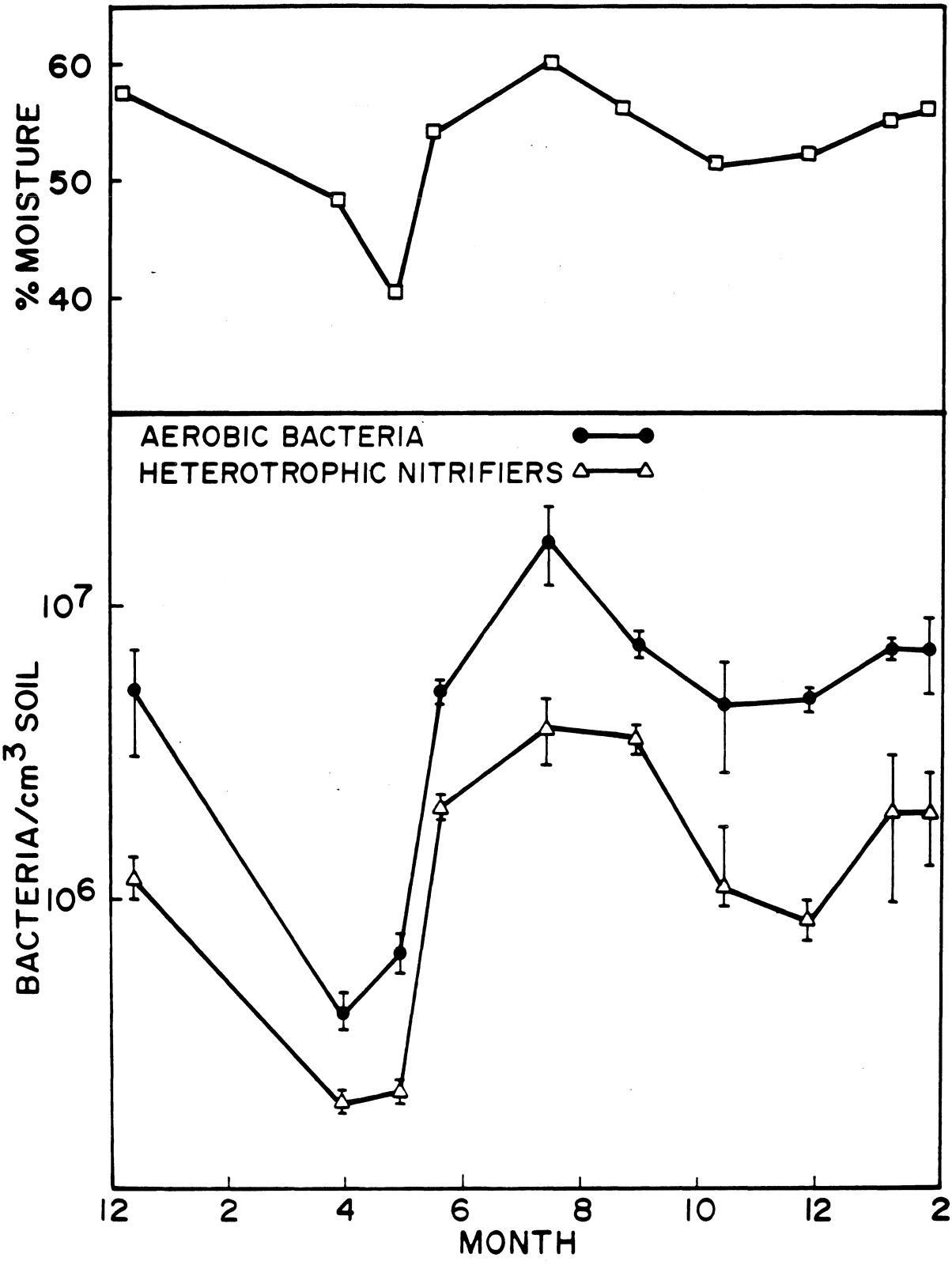

Fig. 7. Variation of aerobic bacteria, heterotrophic nitrifiers, and percent moisture in surface (0 to 10 cm) fallow Pahokee muck from December, 1976 to February, 1978.

Table 20. Comparison of the relationship between nitrifiers and soil moisture, and heterotrophic nitrifiers and aerobic bacteria by linear regression analysis.

Sample Type	x	y	Correlation Coefficient
Fallow Surface	Het. Nitr.[a]	H_2O	0.76**
	Nitrite Oxidizers	H_2O	0.16
	Ammonium Oxidizers	H_2O	0.34
	Het. Nitr.	Aerob. Bact.[b]	0.79**
Soil Profile	Het. Nitr.	H_2O	-0.53**
	Nitrite Oxidizers	H_2O	-0.25
	Ammonium Oxidizers	H_2O	0.21
	Het. Nitr.	Aerob. Bact.	0.91**
Surface Soils	Het. Nitr.	Aerob. Bact.	0.83**

** Correlation significant at the 0.99 level.

[a] Heterotrophic nitrifiers.

[b] Aerobic bacteria.

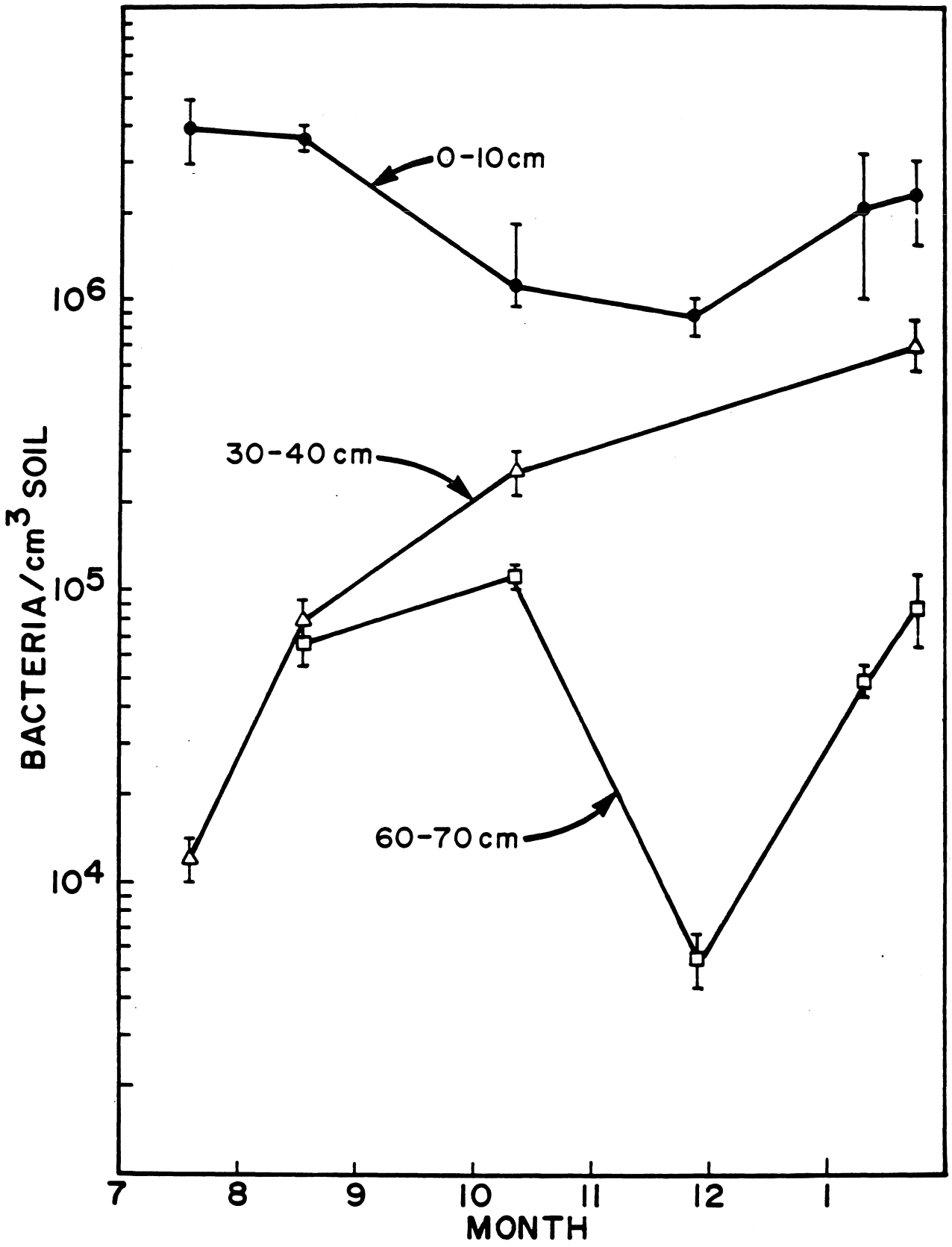

Fig. 8. Variation of heterotrophic nitrifiers with depth of muck from July, 1977 to February, 1978.

Table 21. Nitrifiers in fallow, cane and St. Augustinegrass surface (0-10 cm) soils.

Date	Soil	Ammonia Oxidizers	Nitrite Oxidizers	Heterotrophic Nitrifiers
		----------MPN/cm^3 soil----------		---bacteria/cm^3 soil---------
3-29-77	fallow	7.5×10^3 CD[a]	3.6×10^4 CD	$1.9 \pm 0.2 \times 10^5$[b]
	grass	9.4×10^3 CD	2.3×10^4 CD	$5.4 \pm 0.7 \times 10^5$
	cane	1.7×10^4 CD	3.9×10^4 CD	$9.4 \pm 1.7 \times 10^4$
5-17-77	fallow	1.0×10^4 CD	1.9×10^4 D	$2.1 \pm 0.07 \times 10^6$
	grass	7.4×10^4 A	3.3×10^4 CD	$1.6 \pm 0.3 \times 10^7$
	cane	4.9×10^4 AB	4.6×10^3 E	$2.2 \pm 0.4 \times 10^6$
1-18-78	fallow	4.2×10^4 AB	3.9×10^4 CD	$2.0 \pm 1.1 \times 10^6$
	grass	1.0×10^5 A	1.4×10^4 D	$1.5 \pm 0.3 \times 10^6$
	cane	4.9×10^4 AB	3.5×10^4 CD	$4.2 \pm 2.1 \times 10^6$
2-8-78	fallow	4.6×10^3 D	6.5×10^4 BC	$2.9 \pm 0.7 \times 10^6$
	grass	2.1×10^4 BC	6.4×10^5 B	$2.3 \pm 1.3 \times 10^6$
	cane	2.2×10^4 BC	1.4×10^5 A	$2.6 \pm 0.3 \times 10^6$

[a] MPN values within a population followed by a different letter are significantly different at the 95% level (Alexander, 1965).

[b] Population ± standard error of the mean (n = 3).

Table 22. Variation of autotrophic nitrifiers with depth of muck.

Date	Depth cm	% Moisture	Ammonia Oxidizers	Nitrite Oxidizers
			----------MPN/cm^3 soil----------	
12-20-77	0-10	55.6	6.8×10^3 A[a]	3.1×10^5 A
	30-40	ND[b]	ND	ND
	60-70	81.2	$<10^2$	$<10^2$
1-8-78	0-10	55.4	2.0×10^3 B	1.8×10^4 B
	30-40	ND	ND	ND
	60-70	85.5	7.7×10^1 D	1.5×10^2 D
2-8-78	0-10	57.5	2.1×10^3 A	2.2×10^4 B
	30-40	80.2	2.7×10^2 C	3.3×10^3 C
	60-70	80.3	2.6×10^1 D	5.2×10^2 C
4-14-78	0-10	48.3	7.1×10^2 BC	4.9×10^4 B
	30-40	81.2	4.6×10^1 D	1.5×10^3 C
	60-70	81.7	$<10^2$	6.0×10^1 D

[a] Values for a genus followed by a different letter are significantly different at the 95 percent level (Alexander, 1965).

[b] Not determined.

with the heterotrophic nitrifiers other factors than soil moisture were the primary controllers of the autotrophic population density. Thus, it appears that the two populations (autotrophic vs heterotrophic nitrifiers) were limited by different environmental parameters in Pahokee muck.

An ideal study of the role of heterotrophic nitrifiers would be to examine nitrogen oxidation by these organisms in the absence of autotrophic nitrifiers. No natural samples were found with one, but not the other, population. Thus, to create such a soil sample, fallow surface Pahokee muck was collected and heated as indicated above. The control soil was not heated. The heat treatment resulted in the reduction of the autotrophic nitrifier populations to undetectable levels while maintaining a population of approximately 1.3×10^6 heterotrophic nitrifiers/g wet soil (Table 23). No detailed speciation was conducted on the surviving heterotrophic nitrifiers, but it was noted that a minimum of 7 colonial types were preserved. During the incubation of the acetate and/or ammonium amended, heated soil, although a significant increase in heterotrophic nitrifiers occurred (Table 23), no significant increase in nitrate plus nitrite concentrations was observed (Fig. 9A and 9B). Nitrite was not detected in these samples. In the control soil which contained both autotrophic and heterotrophic nitrifiers, ammonium amendment resulted in increases in (nitrate + nitrate)-N levels from 14.3 to 181 μg/g wet soil (Fig. 9c). Approximately 48 μg/g nitrite-N was also produced in the latter samples. This nitrogen oxidation resulted in 800 and 600-fold increases in ammonia oxidizer and nitrite oxidizer populations, respectively (Table 23). These data suggest that the production of both the nitrate and nitrite in Pahokee muck resulted from the metabolism of the autotrophs.

DISCUSSION

This study documents the extensive occurrence of heterotrophic nitrifiers in Pahokee muck. Definitive evidence for the function of these nitrifiers in natural ecosystems is difficult, if not impossible, to obtain. While neither of the studies presented herein is a direct approach to the question, they do strongly indicate that if the heterotrophs are functional in the oxidation of nitrogen in Histosols their role is minimal. Comparison of the relationship of the population densities of nitrifiers to soil moisture indicated that this variable affected the two populations differently. In fact, the relationship of heterotrophic nitrifiers to aerobic bacterial population densities suggests that variation of the heterotrophic nitrifiers was directly related to processes that stimulate heterotrophs in general and not to factors controlling nitrification specifically. This hypothesis was supported by population changes in the artificial ecosystem created by heating the soil samples. Although large populations of heterotrophic nitrifiers survived the high temperatures, no nitrification occurred following amendment of the heated soil with acetate and/or ammonium, but the population of heterotrophic nitrifiers increased. These two studies combined suggest that the sole population involved in the production of oxidized nitrogen in Pahokee muck is the autotrophic nitrifier.

Table 23. Variation in bacterial populations/g moist soil in heated plus acetate and/or ammonium and freshly collected soil plus ammonium.

	Day	Aerobic Bacteria ($\times 10^6$)	Nitrifiers		
			Heterotrophic ($\times 10^5$)	Ammonia Oxidizers	Nitrite Oxidizers
Heated+N	0	8.4B[a]	13 B[a]	ND[c]	ND
	4	6.2B	19 AB	ND	ND
	7	9.2B	7.3C	ND	ND
	9	33 A	26 A	ND	ND
	11	--[b]	--	ND	ND
Heated+N+C	0	9.4A	14 B	ND	ND
	4	15 A	36 A	ND	ND
	7	17 A	41 A	ND	ND
	9	10 A	14 B	ND	ND
	11	--	--	ND	ND
Unheated+N	0	34 A	99 A	1.3×10^3B[d]	4.0×10^3D[d]
	4	13 C	65 B	3.0×10^5A	1.8×10^4C
	7	23 B	55 C	6.8×10^5A	6.6×10^4B
	9	7.3D	11 D	5.1×10^5A	6.4×10^4B
	11	--	--	8.4×10^5A	2.6×10^5A

[a] Values (aerobic bacteria or heterotrophic nitrifiers) followed by a different letter are significantly different at the 95% level (Duncan's New Multiple Range Test).

[b] Not determined.

[c] None detected.

[d] Values (Ammonia Oxidizers or Nitrite Oxidizers) followed by a different letter are significantly different at the 95% level (Alexander, 1965).

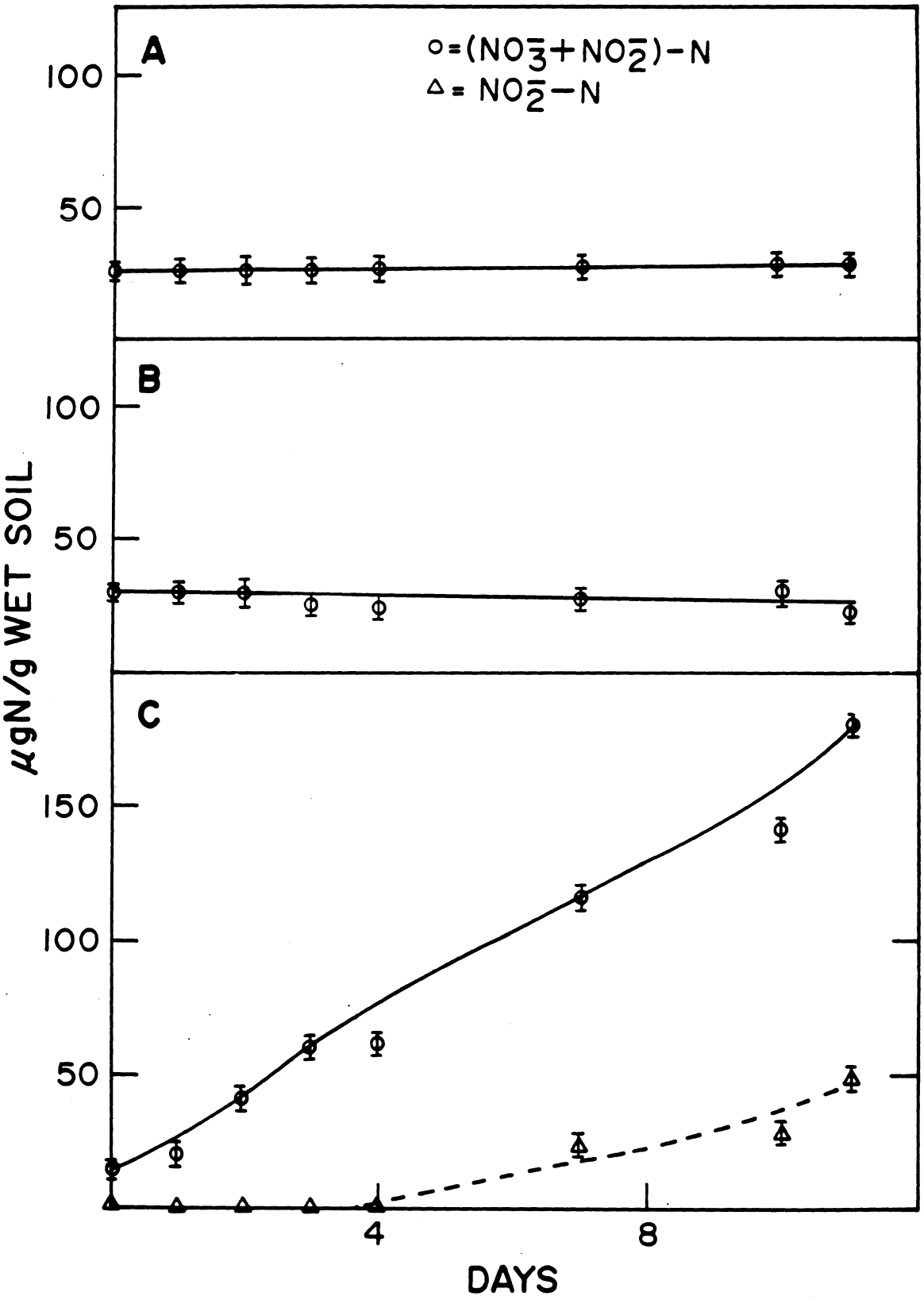

Fig. 9. Oxidation of ammonium in heat shocked Pahokee muck amended with sodium acetate and/or ammonium sulfate; and unheated Pahokee muck amended with ammonium sulfate. A: Heated soil plus ammonium sulfate; B: Heated soil plus sodium acetate and ammonium sulfate; C: Unheated soil plus ammonium sulfate.

SUMMARY

The extent of the occurrence of heterotrophic and autotrophic nitrifiers in Pahokee muck and the role of these organisms in the ecosystem were assessed by surveying their population densities under different field conditions and by observing the relationship of these populations with aerobic bacteria and soil moisture. Heterotrophic nitrifier populations varied from 2.0×10^5 to 3.8×10^6 bacteria/cm^3 muck in surface fallow Pahokee muck during the annual cycle. This population decreased 40-fold between the surface and the 60- to 70-cm depths of soil. Similar variations were noted with the autotrophic nitrifier populations. Significant correlations were found between heterotrophic nitrifiers and both soil moisture and aerobic bacteria. These relationships did not exist for the autotrophic nitrifiers. In soil that had been heated to kill the autotrophic nitrifiers, while preserving a population of the heterotrophs, and then amended with sodium acetate and/or ammonium sulfate, no nitrate or nitrite accumulated although significant increases in heterotrophic nitrifiers were detected. In unheated control soil, nitrate plus nitrite-N increased from 14.3 to 181 μg/g wet soil and 48 μg/g nitrate-N was produced. These data suggest that the autotrophic nitrifiers were the sole population responsible for nitrification in Pahokee muck.

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