IMPACT OF AGRICULTURAL PRACTICES

ON THE SOIL ENVIRONMENT

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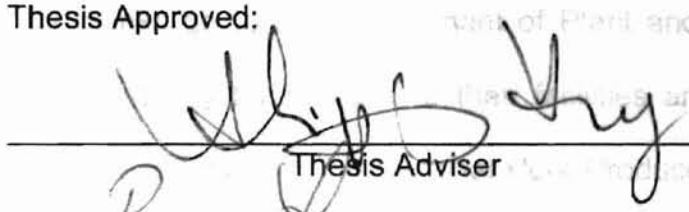

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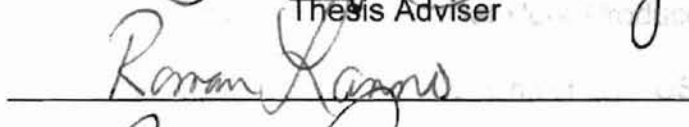

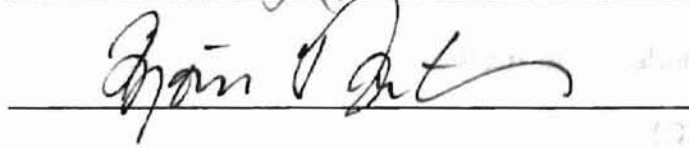

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Appendix

FORMAT OF THESIS

This thesis is presented in a combination of the Soil Biology & Biochemistry and Journal of Environmental Quality styles and formats, as outlined by the Oklahoma Sate University graduate college style manual. The use of this format allows the independent chapters to be suitable for submission to scientific journals. Each paper is complete in itself with an abstract, introduction, materials and methods, results, discussion, and reference section.

Chapter I

INTRODUCTION

To meet the world's increasing demand for food, agricultural productions are forced to use more chemicals to meet the demand while remaining economically competitive. These chemicals range from nutrient fertilizers such as phosphorous to disease controlling fungicides such as Panogen (Reigart and Roberts, 1999). Accumulation of these chemicals in soil may pose a threat to the environment because these chemicals may reach drinking water or cause eutrophication in lakes and rivers through surface run-off and/or leaching to ground water (Carpenter et al., 1998; Klassen, 1988; Stern et al., 1959). It is, therefore, essential to fully understand the fate of agricultural chemicals in soil following application, as well as develop environmentally sound methods for pest control.

One of such concerns is the accumulation of pesticides in the environment. In one year alone, 612 million pounds of pesticide are applied to crops and there is still $4 billion annual loss of U.S. crops caused by plant pathogens alone (Delvo and Lin, 1997; Lumsden and Papavizas, 1988). With 90 percent of all pesticides being suspected carcinogens, alternative methods of pest control need to be developed to decrease environmental threat and increase crop protection from pests (Tangley, 1987). The use of biological control is possibly such an alternative. Biocontrol is the use of beneficial organisms to reduce the number of or eliminate pest organisms. One such organism that has demonstrated potential as a biocontrol agent is *Trichoderma spp*.

It has long been recognized that suppression of plant pathogens in soil has been associated with the presence of *Trichoderma* spp. (Chet, 1987; Chet and Baker, 1981; Liu and Baker, 1980). The mechanisms behind their antagonistic actions are not fully understood. Evidence suggests that chitinase enzymes are involved (Lorito et al., 1994). Purified β-glucosaminidase, one of the three chitinases, from *Trichoderma* spp. demonstrated antifungal activities against several plant fungal pathogens (Lorito et al., 1994). Therefore, β-glucosaminidase in soil may serve as suppressing agents against plant pathogenic fungi. To explore this possibility, an assay method is needed to detect and quantify activities of β-glucosaminidase in soil.

Another concern is land application of animal waste, the most economical disposal method. Unfortunately, numerous reports indicated that repeated application of animal manure leads to accumulation of phosphorus in soil exceeding crop response range (Lemunyon and Gilbert, 1993; Pasek et al., 1998, Reed et al., 1998). Approximately 8.23×10^7 kg of P enters the environment in the form of animal manure annually in the United States (Wodzinski and Ullah, 1996). The excess P in soil may be transported to adjacent water bodies and cause eutrophication (Carpenter et al., 1998). Thus, several states are calling for regulating manure land application based on P level in the soil and adjacent water bodies (McFarland et al., 1998). Stringent regulation on animal waste disposal will affect large-scale animal production that may jeopardize animal industries in the United States.

Microorganisms play intricate roles in the transformation of chemicals. Thus, the fate of applied chemicals in soil is closely associated with microbial activity and community structure in the specified environment. It is, therefore, of interest to assess how soil treatment and management systems affect soil biochemical properties and microbial activity and community structure so that their long-term impact on the environmental quality can be evaluated.

Therefore, the objectives of this study are: (1) to develop an assay method for detection and quantification of β-glucosaminidase activity in soil, (2) to assess soil microbial activity and activities of enzymes involved in P cycling in soils amended with animal manure; (3) to determine microbial community structure in soils under different management systems.

Chapter II

LITERATURE REVIEW

Modern agricultural practices

The world population has increased from 900 million to 6.1 billion in the past two hundred years (United States Bureau of the Census, 2001; McEvedy and Jones, 1978). This has forced agriculture to improve production practices in order to meet the increasing demand for food. Farms in the United States have gone from small self-sufficient entities to corporations with an estimated 49.5 million tons of wheat being produced, 35 million cattle and 97 million hogs slaughtered, and 436 million chickens being raised per year (California Agricultural Statistics Services, 2000; USDA, 2000).

Increased production has occurred as a result of several agricultural revolutions. The first American agricultural revolution was a change from manpower to animal power that continued from early agriculture until the early 1900's. The next revolution, which was completed during the 1940's, was a shift from animal power to mechanized power. This revolution involved a continuation of the development of laborsaving technologies that enabled even greater substitutions of machinery and power for labor resources. The result of these two revolutions was a decrease from as many as 300 to as few as 6 labor-hours required to produce 2724 kg of wheat (USDA, 1990).

In 1960, the Green Revolution was started as a result of the development of high-yielding dwarf wheat by Norman Borlaug. By increasing the yield potential of crops, total wheat production in some areas increased by as much as 98% in two years (Easterbrook, 1997). Also during the 1960's, new chemical pesticides, such as benzimidazole fungicides, were introduced. Pesticide use increased from 106 million kg in 1964 to a high of 278 million kg in 1982 as farmers began using more chemicals to reduce loss of crops to pests (Delvo and Lin, 1997).

The recent mechanization of livestock production has resulted in concentrated animal feeding operations. Animal feeding operations are defined by the United States Soil Conservation Service (1992) as lots or facilities where animals are confined for 45 days or more a year and vegetation is not present. By mechanizing animal feeding operations, the standardization of production is accomplished which allows for more efficient operations. In Western Oklahoma, for instance, each employee at a nursery center of a major pork producer is able to manage 3,000 pigs, and a meat processing plant typically employs less than 2,000 people and is able to process 1,000 pigs per hour. The waste generated from swines is accumulated in lagoons and applied to soil nearby. This raises questions associated with modern agricultural practices.

Environmental protection is one of the concerns surrounding some of these modern agricultural practices. Fungicide applications to control plant pathogens and waste disposal from concentrated animal feeding operations have become issues. Agricultural chemicals, such as fungicides, have been regulated

under the Toxic Substances Control Act and the Federal Insecticide, Fungicide, and Rodenticide Act due to their potential for ecological and human risks (Rand and Zeeman, 1998). Animal feeding operations can adversely impact air, soil, and water quality. Final regulations were passed in 1976 classifying some concentrated animal feeding operations as point sources of pollution due to threats of decreased water quality from the large amounts of animal waste they release (United States Soil Conservation Service, 1992).

Control of plant pathogens in relation to environmental pollution

Plant pathogens are a group of microorganisms that are able to infect and inflict damage on host plants (Sylvia et al., 1998). When an organism damages crops, that organism is considered a crop pest species. Organisms can become crop pest species due to an altered environment that allows the pest species to out-compete others or by transportation to new environments void of predators, parasites and diseases.

Since all organisms are subject to physical and biological pressure, these factors, as well as the genetics of a species, determine the abundance of that organism in a given environment (Stern et al., 1959). Human activities may alter the environment to create conditions that promote the growth of certain populations (Ullyett, 1951). For example, alfalfa butterfly, *Colias philodice eurytheme* Boisduval, populations increased when alfalfa, *Medicago sativa* L. was introduced to California in 1850 (Smith and Allen, 1954). When the

Australian cottony cushion scale, *Icarya purchasi* Maskell, was transported to California, it thrived and eventually became a pest species in citrus production (Smith, 1959). Over the past several centuries, humans have been manipulating selection pressures by changing the environment to meet food and space requirements (Thomas, 1956). As a result, crop pest species numbers have increased (Stern et al., 1959).

There are an estimated 50 thousand crop pests (USDA, 1960). These pests cause a 35 to 42% worldwide decrease in food and fiber production annually (Hokkanen and Pimentel, 1989; Pimentel, 1991). It is imperative to find economical methods to combat crop loss from pests.

Chemical control of plant pathogens

Control of plant pathogens is aimed at reducing the damage or loss from plant diseases. A common method of controlling plant diseases is the use of fungicides. Fungicides, by definition, kill fungi or inhibit fungal growth. Many common chemicals used for controlling fungi, however, are fungistatic (Maloy, 1993). A fungistat is a chemical that prevents the development of the fungi and therefore prevents spore germination. Chemicals that destroy bacteria are referred to as bactericides. However, fungistats and bactericides are often grouped together and referred to as fungicides (Maloy, 1993). Fungicides, herbicides (chemicals that destroy weeds), and insecticides (chemicals that destroy insects) are grouped together and referred to as pesticides (chemicals

that destroy pests). Pesticide is a more general term that describes chemicals used to reduce damage or loss in crop production.

Along with the increase in pesticide use has come a realization of the problems associated with chemical control of pests. Repetitiously applying pesticides to minimize rapid resurgence of pests can result in increasing numbers of individuals surviving the treatment (Holloway and Young, 1943). It was documented as early as 1916 that species can gain a resistance to pesticides through selection (Dekker, 1976; Klassen, 1988; Metcalf, 1955). Furthermore, pesticides have resulted in heavy metal toxicity and are suspected as carcinogens, thus, improper application may result in pesticide levels harmful to non-target species including humans (Hallberg, 1987; Klassen, 1988; Stern et al., 1959; Tangley, 1987). Another problem with pesticides is a lack of specificity for target organisms. Lack of pesticide specificity can result in outbreaks of pests, other than those against which control was originally directed, as a result of the interference of the pesticide on biological control (Bartlett and Ortega, 1952; DeBach, 1964, 1974; Hau and Beute, 1983; Klassen, 1988). The problems associated with pesticides are interrelated. Pest resistance to pesticides results in a need for larger doses or more frequent pesticide applications. Increased pesticide use alters the ecosystem and may result in secondary pest outbreaks, requiring additional pesticide application. The result is a crop with pesticide residues exceeding tolerance limits at harvest time (Stern et al., 1959).

Specific concern over the safety of fungicides has been a major concern since the 1970's. During this time, the potential for mercury poisoning in humans

was demonstrated and all fungicides containing alkylmercury had their registration suspended by the United States Department of Agriculture (Reigart and Roberts, 1999). While the use of fungicides containing some harmful substance, such as cadmium, are prohibited, other fungicides known to cause adverse health affects in humans, such as panogen, metam-sodium, and ziram, are still in use (Reigart and Roberts, 1999).

With the threat to human health caused by fungicides, and an estimated $4 billion annual loss to U.S. crops caused by soilborne plant pathogens, a safe and effective replacement control system for soilborne plant pathogens needs to be developed (Lumsden and Papavizas, 1988).

Alternative methods for controlling plant pathogens

There are several alternative methods to chemical fungicides currently used in controlling plant pathogens, including biological control and disease resistant crops created through plant breeding or genetic manipulation.

Biological control. Biological control can be defined ecologically or as an action involving man, in which case it is considered the field of biological control. Biological control, from an ecological viewpoint, is the action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence (DeBach, 1964). The field of biological control is "the study, importation, augmentation, and conservation of beneficial organisms for the regulation of population densities of other organisms" (DeBach, 1964). In short, it is "the conscious use of living organisms

(biocontrol agents) to restrict the population sizes of unwanted organisms (target pests)" (Hokkanen, 1997).

The field of biological control dates back 8,000 years to the farming practices of the Sumerians on the Tigris and Euphrates River flood plains. The Sumerians utilized annual deposits of soil that were relatively free from pathogens and intermixed the planting of crops to further reduce the likelihood of pathogens (Grigg, 1974). During the 17th and 18th centuries, tree wounds were treated using concoctions of cow dung or mud to prevent fungal infections (DeBach, 1964). In 1908, it was discovered at Durham University in England that the activity of a plant pathogen could be inhibited by an accumulation of its own metabolic products (Hokkanen, 1997).

Although biological control had been used by farmers for thousands of years, the term biological control was first used by H.S. Smith in 1919 when he described insect control by natural enemies (DeBach, 1964). Later, Sanford (1926) suggested that the control of potato scab by green manure was a result of saprophytic bacteria that thrived on fresh organic matter. Biological control of potato scab by inoculating soil with saprophytes was further confirmed by Millard and Taylor (1927). A series of papers written by R. Weingling on the parasitism of *Trichoderma viride* on other soil fungi (Weindling, 1932, 1934, 1937, 1941; Weindling and Emerson, 1936; Weindling and Fawcett, 1936) were the basis for many further studies on the control of plant pathogens. Bliss (1951) was among the first to publish an account of integrated pest management, which is a combination of chemical and biological control mechanisms acting together. He

reported that the pathogen *Armillaria mellea* in soil survived treatment of fungicide, but was killed where *T. viride* was present. Garrett (1957) explained the findings of Bliss (1951) as a result of a two-step action against *A. mellea*. First, the fungicide directly killed some of the pathogenic fungi. Then, *T. viride*, whose population was dramatically increased in fumigated soils, preyed upon the remaining fungi and reduced crop damage from the plant pathogen.

Plant pathogens may be suppressed biologically through several mechanisms including pathogen-suppressive soils, cross protection and induced resistance, competition, antibiosis, hypovirulence, predation, and parasitism.

A pathogen-suppressive soil is a soil in which a pathogen does not establish or persist, establishes but causes little disease, or establishes and causes disease for a short while, but then has no adverse effects (Mehrotra et al., 1997). Currently, suppressive soils are known against the plant pathogens *Blumeria. graminis* var. *tritici*, *Fusarium oxysporum*, *F. solani* f. sp. *phaseioli*, *Phytophthora cinnamomi*, *Pythium* spp., and *Streptomyces scabies* (Mehrotra et al., 1997). While the mechanisms in pathogen-suppressive soils are not fully understood, it has been suggested that they are linked to montmorillonite clay which favors the development of antagonistic microflora capable of reducing the activity of the pathogens (Mehrotra et al., 1997).

Biological control mechanisms that take place primarily in the targeted plant include cross protection and induced resistance. Cross protection is a process by which a first organism invades the host plant and then fights off invasions by the pathogenic fungi (Mehrotra et al., 1997). This has been shown

effective at controlling fusarium wilt (Baker et al., 1987). Induced resistance occurs when the first organism that invades a plant induces a response from the host plant inhibiting a second organism from invading the plant (Mehrotra et al., 1997). This has been shown to occur in cucumber, watermelon, and muskmelon plants (Mehrotra et al., 1997).

Competition is a mechanism of biological control whereby the interactions of two organisms trying to attain the same resource such as space or nutrients result in a decrease in the survivability of one or both organisms (Mehrotra et al., 1997). Most species prefer to avoid competition (Odum, 1953). Therefore, by providing an environment that enhances the competitive advantage to nonpathogenic organisms, plant pathogens may be outcompeted (Mehrotra et al., 1997).

Some organisms have evolved mechanisms to gain competitive advantage, such as production of substances that are toxic to their competitors. This phenomenon is termed antibiosis. The antagonistic metabolic products are many, including antibiotic compounds and some extracellular enzymes (Mehrotra et al., 1997; Maloy, 1993). The control of Armillaria root rot by *T. viride*, damping-off disease by *Pseudomonas fluorescens*, and crown gall by *Agrobacterium radiobacter* are examples of antibiosis (Maloy, 1993).

Biological suppression of plant pathogens using a transmittable virus resulting in neutralization of the ability of pathogens to cause disease is known as hypovirulence (Cook and Baker, 1983). Hypovirulence was first discovered in chestnut blight fungus in Europe (Cook and Baker, 1983; Maloy, 1993).

Hypovirulent strains carry an agent, possibly a mycovirus, which they transmit to virulent strains through hyphal connections. Once infected, the disease causing potential of the once virulent strain is neutralized (Maloy, 1993).

The use of predation, the utilization of an organism for food by another, to control plant pathogens is another mechanism for biological control. Some genera of fungi are capable of trapping plant pathogenic nematodes using finger-like constricting loops, sticky knobs, or nets of hyphae (Maloy, 1993). Once the nematode is trapped, fungal hyphae penetrate the nematode body and extract nutrients (Maloy, 1993). Little success has occurred in enhancing predation by this group of fungi as they are poor competitors, intolerant of desiccation, and easily lysed (Maloy, 1993).

Similar to predation is parasitism. In parasitism, however, the action is subtler and the parasite does not intentionally kill the host (Maloy, 1993). Parasitism among fungi is known as mycoparasitism (Mehrotra et al., 1997). A hyperparasite is an organism that is parasitic on an organism that is also a parasite (Maloy, 1993). Many hyperparasitic mycoparasites, including *Cladosporium spongiosum*, *Verticillium psalliotae*, *Pythium nunn*, and *Trichoderma harzianum*, are being considered for use in biological control (Kim and Nik, 1983; Lifshitz et al., 1984; Mathur and Mukerji, 1981; McLean and Stewart, 2000; Mehrotra et al., 1997). Information on biocontrol using hyperparasites is still incomplete, but some hyperparasites, such as *T. harzianum*, are commercially available for use.

Commercially available biological control agents are available for controlling plant diseases such as damping-off, wood decay, fusarium wilt, and annosus root rot. However, for biological control to be most efficient, chemical fungicides may also need to be applied as part of an integrated pest management plan (Maloy, 1993). As biological control technologies are being perfected, methods for further reducing fungicide use are continuing to be developed.

Disease resistant crops. In addition to biological control, the development of crop lines resistant to infections are another alternative disease control method against soilborne plant pathogens (Lumsden and Papavizas, 1988). The resistance of crops to disease may be increased by breeding resistant strains or through the creation of genetically manipulated crops. To create a resistant strain through breeding, the mechanism responsible for preventing the pathogen from causing damage must already be present in the species and the mechanism must be a heritable trait. Plants conferring resistance are selected and bred until a variety is developed that is resistant to the pathogen. Crop resistance has been shown to prevent many diseases, such as maize streak virus in maize, stripe rust resistance in barley, and hay molding caused by saprophytic fungi in alfalfa (Chen and Line, 1999; Kim, 1989; Kimbeng et al. 2000).

Transgenic crops are created by inserting the gene, or genes, responsible for resistance in one organism into a crop. This has been done to protect wheat from stinking smut disease using genes from maize, to protect carrots from

mildew using a human gene, to protect sweet corn and cotton from lepidopteran larvae using genes from *Bacillus thuringiensis* (*Bt*), and many more (Burkness et al., 2001; Clausen et al., 2000; Takaichi and Oeda, 2000).

With over 2 million acres of *Bt* cotton grown in the U.S., it is one of the most widely used transgenic crops. Crops with *Bt* genes inserted into their genome produce proteins toxic to several insects, including tobacco budworm and bollworm. These two insects decrease agricultural profits by $300 million per year in the U.S. alone (Greb, 2001). At least nine insects have gained resistance to *Bt* toxins (Greb, 2001). To delay further resistance, federal regulators require *Bt* cotton growers to plant at least 5% of their crop to non-*Bt* cotton. Farmers are reluctant to comply with these regulations and minimally care for the non-*Bt* crop because of its decreased production resulting from insect pests compared to the genetically modified varieties.

Although agriculturists have accepted genetically improved crops for controlling plant pathogens, a major limitation is that resistance is not available for all pathogens. Furthermore, concerns as to the safety of transgenic crops has resulted in three U.S. agencies, the EPA, FDA, and APHIS/USDA, imposing regulations on their creation and release to the environment. Several countries have imposed regulations to prevent genetically altered crops from being imported due to potential environmental and health risks.

To prevent environmental and human health problems, it is necessary to fully evaluate the potential environmental risks involved in creating disease resistant transgenic crops, as well as other advances in agricultural practices.

Concentrated animal feeding operations, land application of animal manure, and related environmental concerns

In addition to concerns of chemical accumulation in the environment, waste disposal from concentrated animal feeding operations is provoking further debate among regulators as to the safety of modern agriculture.

Prior to concentrated animal feeding operations, most animal feed was grown on the same farm where the animals were raised. When animals ate the crop, not all nutrients were digested. Hogs, for instance, may only adsorb 60 % of the P they are fed (Hamilton et al., 1997). The remaining P, along with N and K were returned to the field they came from in the form of manure. This process of recycling nutrients from a crop to an animal and then back to the original field the crop was grown on is the livestock-crop cycle (Gasser, 1987). An increase in the efficiency of fertilizer production during World War II resulted in an increase in the average United States annual commercial fertilizer use from 6.6 million tons during the 1930's to 22.3 million tons during the 1950's (USDA, 1990). Modern agriculture requires less reliance on manure as fertilizer. The livestock-crop cycle has been broken as more separation between crop and livestock production has been made possible.

Since the 1980's, a rapid increase in the number of concentrated animal feeding operations has taken place (Matson et al., 1997). During this time, nationwide hog production has grown by 18% and the number of producers has

fallen by 72% (Glover, 1996). Large-scale animal feeding operations are capable of marketing over 10,000 hogs every six months and each hog can generate up to 9.4 pounds of manure per day (Hamilton et al., 1997; Stanton, 1996). In Oklahoma, swine populations have increased by 761% from 1990 to 1998 and almost 9 million tons of manure from cattle, poultry, horse, and swine are being produced in confined animal feeding operations per year (Chapin and Boulind, 1999; Johnson et al., 2000). The manure produced at animal feeding facilities, especially from cattle and hogs, is stored in lagoons or pits (Jenner, 1998). The animal waste in pits and lagoons is commonly in the form of a slurry which is difficult to handle and expensive to transport (Gasser, 1987).

The disposal and treatment of animal waste has caused concerns about the environmental impact of animal feeding operations. Only 25% of feed inputs in swine and 17% of feed inputs in cattle leave the animal feeding operation in the animal. Sixty to 80% of nutrients, as well as salts, pharmaceuticals, and other compounds fed to the animal, remain on-site in waste (McFarland et al., 1998).

Animal waste can contain up to 2530 mg L^{-1} total N and 329 mg L^{-1} total P (Ham and DeSutter, 2000). Due to the amount of N and P in manure, leakage from lagoons, or land applying the waste at levels exceeding crop nutrient needs, may possibly contaminate groundwater resulting in nitrate poisoning or contaminate surface water resulting in eutrophication.

Weil et al. (1990) reported that fertilization of crops with poultry manure resulted in increased NO_3-N levels in groundwater and may have been a

contributing factor in the contamination of the Columbian Aquifer. If high levels of NO_3-N are present in drinking water, a blood disorder in infants, known as blue baby syndrome (methemoglobinemia) can result. This threat has resulted in the development of National Primary Drinking Water Standards for Maximum Contaminant Levels, which are enforceable by law, of 10 mg L^{-1} for nitrate and 1 mg L^{-1} nitrite (U.S. EPA, 2001).

Unlike NO_3-N, excessive P in water is not known to cause any direct harm to humans. However, excess P may cause environmental problems. Applications of manure to crops at rates exceeding crop response to P may result in eutrophication (Parry, 1998). Eutrophication is a description of the effects on a water body as it becomes enriched with nutrients, resulting in an algal bloom. As the algae decompose, the water becomes devoid of oxygen and can result in fish kills and unpalatable drinking water. Furthermore, consumption of cyanobacterial blooms, or the toxins released when these blooms die, can kill livestock and be hazardous to humans (Lawton and Codd, 1991).

Aside from possible N and P contamination of water, additional environmental and human health concerns are also associated with concentrated animal feeding operations. Animal waste lagoons can contain fecal coliforms, fecal streptococcus, and hepatitis E virus that are all potentially harmful to humans and animals (Karetnyi et al., 1999; Krapac et al., 1998; Peterson et al., 2000). Antibiotics, such as tetracycline and sulfonamides, have been found in lagoon waste and are believed to have led to the development of antibiotic-resistant bacteria (Karetnyi et al., 1999; Meyer et al., 1999). Hormones, such as

testosterone and 17β-estradiol, which has been linked to reproductive harm and cancer, have been detected in springs and traced back to animal feeding operations (Barber et al., 1999; Peterson et al., 2000).

Due to the large amounts of manure produced, difficulty in handling manure, high costs of transporting manure, and the presence of pharmaceuticals and animal pathogens in animal waste, animal waste producers have come to deal with animal manure as a waste product (National Research Council, 1993).

In summary, as humans have developed agricultural practices to meet the increasing amount of food needed to support society, repercussions have occurred which are not only damaging to the environment, but also to man. By understanding the mechanisms that result in these problems, better management practices can be developed to allow for maximum agricultural production with minimal negative impacts.

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Chapter III

DETECTION, QUANTIFICATION AND CHARACTERIZATION OF β-GLUCOSAMINIDASE ACTIVITY IN SOIL

Abstract

A simple and sensitive method was developed to detect and quantify *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) activity in soil. This enzyme is also listed as β-hexosaminidase (EC 3.2.1.52) in Enzyme Nomenclature. The optimum pH and temperature for the enzyme were approximately pH 5.5 and 63°C, respectively. The K_m and V_{max} values were calculated from three linear transformations of the Michaelis-Menten equation. The K_m values of the enzymatic reaction in the two soils tested ranged from 0.56 to 1.48 mM and the V_{max} values ranged from 29 to 40 mg *p*-nitrophenol released kg^{-1} soil h^{-1}. The activation energy (E_a) for the enzymatic reaction was about 58 kJ mol^{-1} for soils tested. The Q_{10} values ranged from 1.35 to 2.50 at temperatures ranging from 10 to 60°C. With the exception of field-moist Renfrow soil, neither chloroform fumigation nor toluene pretreatment of soil samples affected the activity of β-glucosaminidase significantly. The activity of this enzyme in field-moist Renfrow soil increased about 20% upon fumigation or toluene treatment. Autoclaving the soils reduced β-glucosaminidase activity by 58% in the air-dried soils and 96% in the field-moist soils. Air-drying of field-moist soil samples reduced β-glucosaminidase activity by 12% and 22% in Renfrow and Teller soil,

respectively. Our results suggest that activity of β-glucosaminidase is mostly due to extracellular enzymes.

Keywords: Chitinase, β-Glucosaminidase, enzyme kinetics, method of assay

Introduction

N–acetyl-β-D-glucosaminidase (EC 3.2.1.30), sometimes referred to as NAGase, is an enzyme that hydrolyzes *N*–acetyl-β-D-glucosamine (NAG) residues from the terminal non-reducing ends of chitooligosaccharides (Bielka et al., 1984). The substrates for this enzyme include chitobiose and higher analogs and glycoproteins. In humans, the same enzyme is found in lysosomes and also cleaves the amino sugar *N*–acetyl-β-D-galactosamine. It is therefore also listed as β-hexosaminidase (EC 3.2.1.52) in Enzyme Nomenclature (Bielka et al., 1984). A specific *N*–acetyl-β-D-glucosaminidase was also identified in the cytosol of animal cells with a neutral pH optimum and unknown biological function (Braidman et al., 1974). This enzyme is one of the three chitinases that degrade chitin (Tronsmo and Harman, 1993). Chitin, which consists of NAG residues in β-1,4 linkages, is the second most abundant biopolymer on earth (Stryer, 1988). It is not surprising that chitinolytic enzymes are widely distributed in nature. They are found in bacteria, fungi, plants and invertebrates such as protozoans, arachnids, insects, crustaceans and nematodes (Trudel and Asselin, 1989), as well as in humans (Neufeld, 1989).

The importance of this enzyme in biological systems has long been recognized. Recently, scientists have begun to explore the role of this enzyme in microorganisms, plants and invertebrates. Activities of β-glucosaminidase may be involved in N-acquiring activities of microorganisms (Sinsabaugh and Moorhead, 1995). The activities of this enzyme were also highly correlated with fungal biomass and were proposed to be used as a semi-quantitative indicator of soil fungal biomass (Miller et al., 1998). As a major structural component in insects and fungal cell walls, chitin is an important transient pool of organic C and N in the soil (Wood et al., 1994). Thus, β-glucosaminidase may play an important role in both C and N cycling in soil.

Activities of β-glucosaminidase might also be involved in biological control of plant pathogens. Among the numerous β-glucosaminidases that have been isolated and characterized, one was from the biocontrol fungus *Trichoderma harzianum* P1 (Lorito et al., 1994). Purified β-glucosaminidase from *Trichoderma* spp. demonstrated antifungal activities against several plant fungal pathogens (Lorito et al., 1994). It has long been recognized that suppression of plant pathogens in soil has been associated with the presence of *Trichoderma* spp. (Liu and Baker, 1980; Chet and Baker, 1981; Chet, 1987). Therefore, β-glucosaminidase in soil may suppress plant pathogenic fungi. To explore this possibility, an assay method is needed to detect and quantify activities of β-glucosaminidase in soil.

The limited information reported on β-glucosaminidase activity in soil was derived using methods developed by Tronsmo and Harman (1993) for use in

pure fungal culture extracts (Naseby and Lynch, 1997, 1998; Miller et al., 1998). These methods were not evaluated for use in soil systems. The method used by Miller et al. (1998) involved quantification of the fluorogenic end product, methylumbelliferyl. It is known that methods available to quantify this compound in soil extracts exhibit quenching effects and the fluorescence is unstable over time (Freeman et al., 1995). The method used by Naseby and Lynch (1997, 1998) involved incubating the reaction mixture at 37°C for 24 h. Long-term incubations increase the risk of microbial growth and autohydrolysis of the substrate during the assay. Although short-term incubations were engaged to study β-glucosaminidase activity in soils by some other researchers (Martens et al., 1992; Serra-Wittling et al., 1995), these methods, however, have not been evaluated thoroughly.

We evaluated the factors and conditions involved in the enzymatic reaction and developed a method for assaying β-glucosaminidase activity in soil. We also characterized its activity with respect to kinetic parameters (K_m and V_{max}), activation energy (E_a), and temperature coefficient (Q_{10}). In addition, the effects of soil treatments, including air-drying, autoclaving, toluene and chloroform, on β-glucosaminidase activity were evaluated.

Materials and methods

Soils

Surface soil samples (0-15 cm) were taken from two locations in Oklahoma, USA. These two soils are typical Oklahoma soils that are under

continuous production of wheat which is a major Oklahoma crop. Both soils are classified as Kastanozems in the FAO system. Renfrow is a silty clay loam and Teller is a fine sandy loam. The properties of the soils are reported in Table 1. Soils were ground, sieved, and each was divided into two parts. One part was air-dried and stored at room temperature, and the other was kept field-moist and stored at 4°C. The pH values were determined by using a combination glass electrode (soil:water ratio = 1:2.5), the organic C and total N by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Sulphur Analyzer (Schepers et al., 1989), and the particle size distribution by a pipette analysis (Kilmer and Alexander 1949).

Reagents

Acetate buffer (100 mM, pH 5.5): Prepared by dissolving 13.6 g sodium acetate trihydrate in about 800 ml of double deionized (DD) water. The solution was titrated to pH 5.5 with 99% glacial acetic acid, and the volume was adjusted to 1 l.

Modified Universal Buffer (MUB, 5X stock solution): Prepared by dissolving 12.1 g tris(hydroxymethyl)aminomethane (THAM), 11.6 g maleic acid, 14.0 g citric acid, and 6.3 g boric acid (H_3BO_3) in 488 ml of 1 M sodium hydroxide and adjusting to a final volume of 1 l with DD water. The 5X stock solution was stored at 4°C. This solution was titrated to the desired pH and diluted five times by volume with DD water before use.

Table 1. Properties of the soils used

Soil						
Series	Subgroup	pH*	Organic C	Total N	Clay	Sand
			----- g kg^{-1} -----		---- % ----	
Renfrow	Udertic Paleustolls	6.53	11.4	1.16	30.0	17.5
Teller	Udic Argiustolls	5.86	7.2	0.67	12.5	55.0

*Soil : Water ratio = 1:2.5.

ρ-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNNAG, 10 mM): Prepared by dissolving 0.342 g ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminidine (N-9376, Sigma Chem. Co., St. Louis, MO.) in 100 ml of acetate buffer (0.1 M, pH 5.5). The solution was stored at 4°C.

Calcium chloride ($CaCl_2$, 0.5 M): Prepared by dissolving 36.75 g $CaCl_2.2H_2O$ in about 400 ml of DD water, and adjusting to a final volume of 500ml with DD water.

Sodium hydroxide (NaOH, 0.5 M): Prepared by dissolving 10 g of NaOH in about 400 ml of DD water, and adjusting to a final volume of 500 ml with DD water.

Standard ρ-nitrophenol solution: Prepared by dissolving 1.000 g of ρ-nitrophenol (Sigma Chem. Co., St. Louis, MO.) in ca. 800 ml of DD water, and adjusting the final volume to 1 l with DD water. The solution was stored at 4°C.

Procedure

Unless otherwise indicated, soil β-glucosaminidase activity was assayed by placing 1.0 g of soil into a 50-ml Erlenmeyer flask, and then adding 4 ml of 0.1M acetate buffer (pH 5.5) and 1 ml of 10 mM ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminide solution. The slurries were mixed thoroughly, stoppered, and placed in an incubator at 37°C. After 1 h of incubation, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added to stop the reaction. The samples were swirled and filtered through Whatman no. 2v filter paper. The colour intensity of the filtrate was measured at 405 nm with a spectrophotometer. Controls were

performed with the substrate being added after the reactions were stopped. Additional controls were performed by following the procedure described but without addition of soil to the reaction mixtures. The controls were designed to allow for subtraction of the soil background colour and any trace amount of *p*–nitrophenol produced by chemical hydrolysis of pNNAG during the incubation. The *p*–nitrophenol contents of the filtrates were then calculated by comparing the results to a standard curve for *p*–nitrophenol developed as described by Tabatabai and Bremner (1969).

The above procedure was modified to test for the effects of different buffer pH and substrate concentrations on *β*-glucosaminidase activity and to determine K_m and V_{max} values. MUB buffers were adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, or 10.0 to be used to evaluate the pH effect. Substrate concentrations of 0, 0.5, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 10 mM were tested. The effect of reaction time was evaluated by having the samples reacting for 30, 60, 90,120 or 150 min. The effect of varying the amount of soil was determined by using 0.5, 1.0, 1.5, 2.0 or 2.5 g of soil.

The temperature dependence of *β*-glucosaminidase activity was evaluated by reacting the samples at temperatures of 10, 20, 30, 40, 50, 60, 70, or 80 °C using the assay procedures described above. The E_a and Q_{10} values were then calculated.

The effects of autoclaving, toluene, chloroform fumigation and air-drying the soil on enzyme activity were also tested. Autoclaving was performed by placing 50-ml Erlenmeyer flasks, that each containing 1 g of soil, in an autoclave

for 20 min at 0.14 MPa and 121°C. Toluene was applied to soil samples (0.2 ml for 1 g soil) as a pretreatment for 30 min prior to the enzyme assay. The chloroform fumigation was performed by the method described by Horwath and Paul (1994). Briefly, 1.0 g of soil sample in a 50-ml Erlenmeyer flask was placed in a vacuum desiccator along with a beaker containing 50 ml of ethanol-free chloroform and antibumping granules. The desiccator was evacuated until the chloroform boiled vigorously for 15 s and then the desiccator was opened until it was filled with air. This was repeated three times to facilitate the distribution of the chloroform throughout the soil. Then, the desiccator was evacuated for the fourth time, allowing chloroform to boil for 2 min before closing the valve on the desiccator. The desiccator was then placed in the dark at room temperature for 24 h. Following fumigation, chloroform was removed under the hood and the desiccator and soil samples were evacuated several times to remove residual chloroform.

Data analysis

All results are expressed on soil dry weight basis. Moisture was determined after drying at 105°C for 48 h. Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was done using the least significant difference (LSD) test. All results reported are averages of duplicate assays and analyses.

Results

Method of assay

Activities of β-glucosaminidase showed a relatively broad pH optimum in Renfrow soil ranging from 4.5 to 6, but activity peaked around pH 5.5 for both soils tested (Fig. 1). The activity of β-glucosaminidase increased with increasing substrate concentration up to about 8 mM in both soils (Fig. 2). Substrate concentration of 10 mM would ensure substrate saturation for a sufficient reaction time to conduct the measurements. This substrate concentration was thus chosen to be used for the method developed. Under the conditions selected and tested, β-glucosaminidase activity in soil was linear with increasing incubation time or amount of soil used (Figs. 3 and 4). Activity of β-glucosaminidase in soil increased with increasing reaction temperature and peaked between 60° to 65°C (Fig. 5). It is important to note that the substrate is stable when the incubation temperature is below 40°C (appendix). Autohydrolysis of the substrate was observed starting around 40°C and increased considerably afterward. Therefore, the temperature effect curves were obtained with double controls. One was performed with the substrate being added after incubation and stopping the reaction, and the other by following the same procedure but without addition of soil in the reaction mixture. These values were subtracted from the activity values reported. Thus, 37°C was used for the reaction temperature for the subsequent experiments in this study. These results suggested that the method is sensitive for detection of β-glucosaminidase using 1 g of soil and 1 h

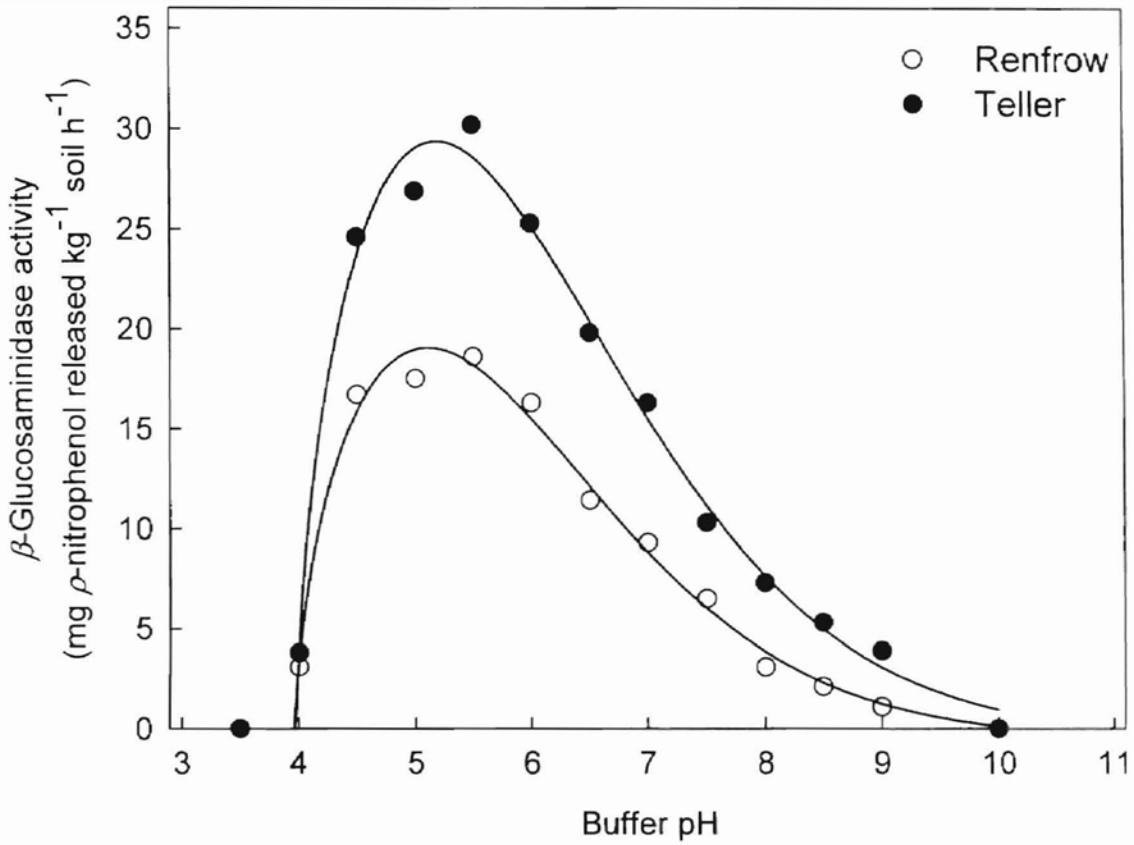


Figure 1. Effect of buffer pH on release of ρ-nitrophenol in assay of β-glucosaminidase activity in soils tested.

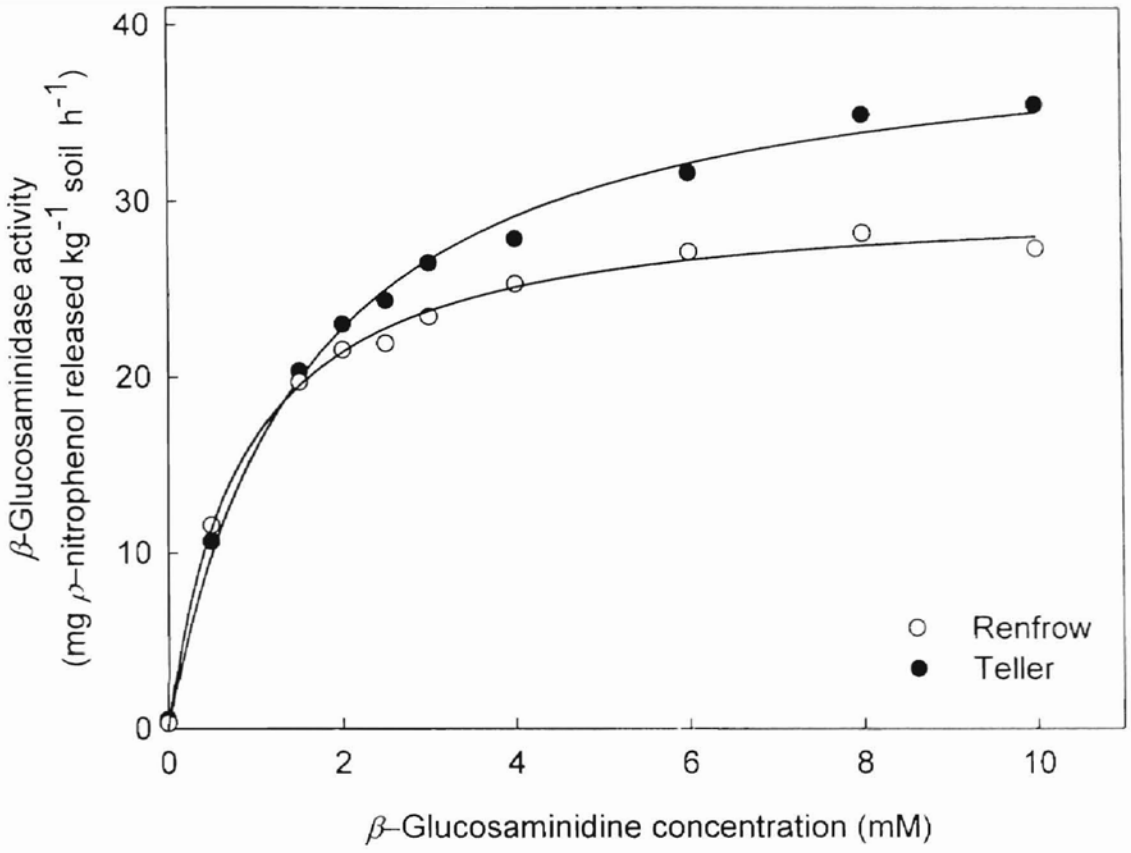


Figure 2. Effect of substrate concentration on release of ρ-nitrophenol in assay of β-glucosaminidase activity in soils tested.

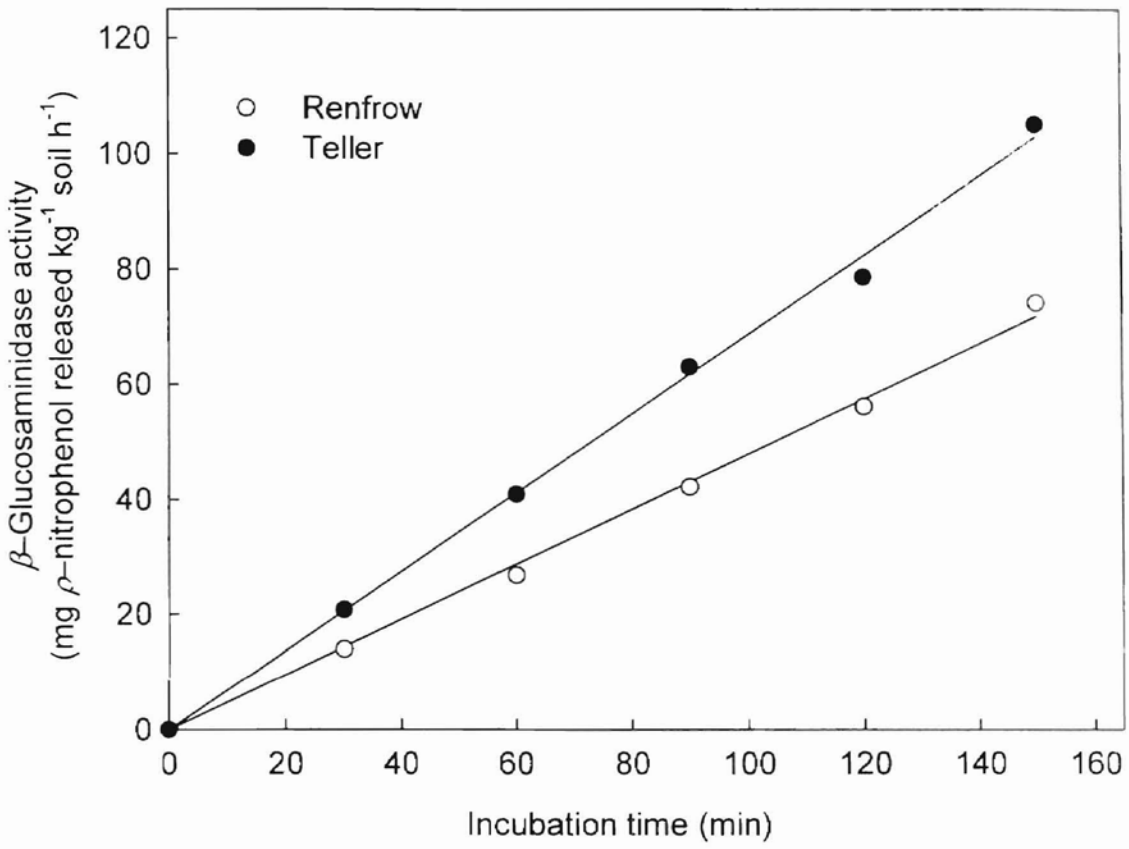


Figure 3. Effect of incubation time on release of ρ-nitrophenol in assay of β-glucosaminidase activity in soils tested.

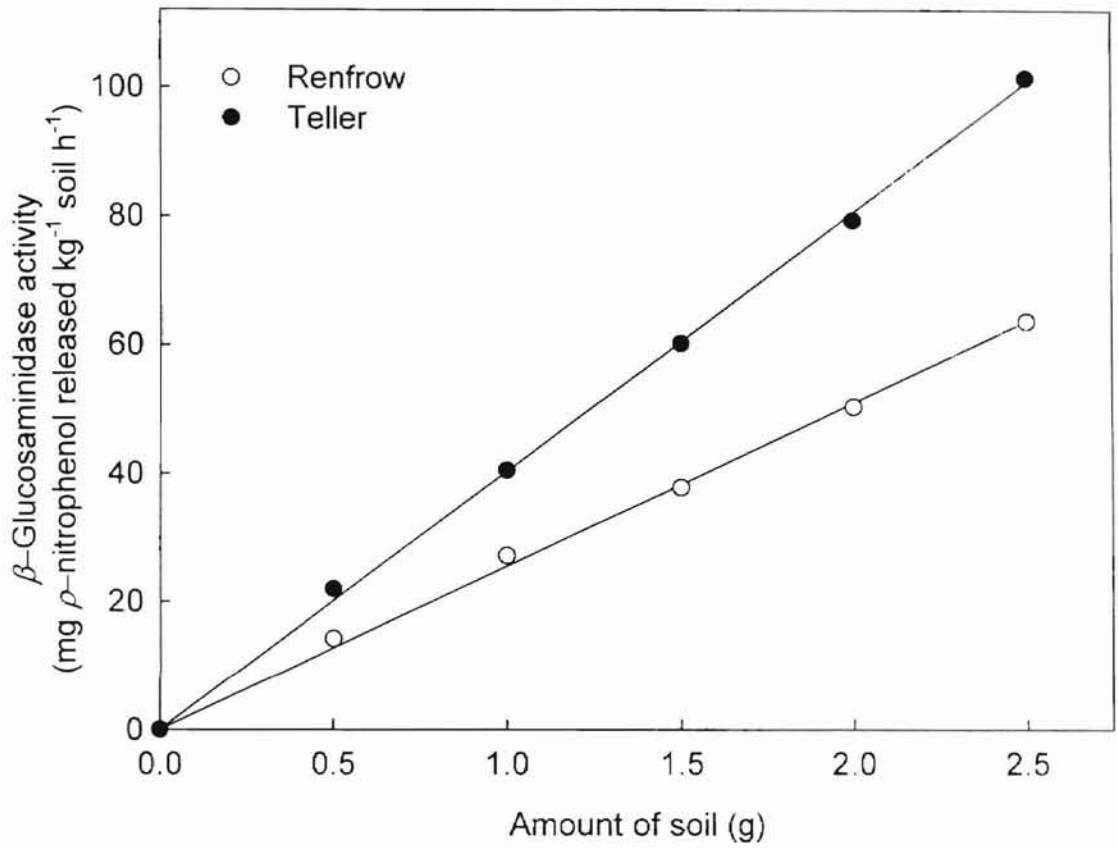


Figure 4. Effect of amount of soil on release of ρ-nitrophenol in assay of β-glucosaminidase activity in soils tested.

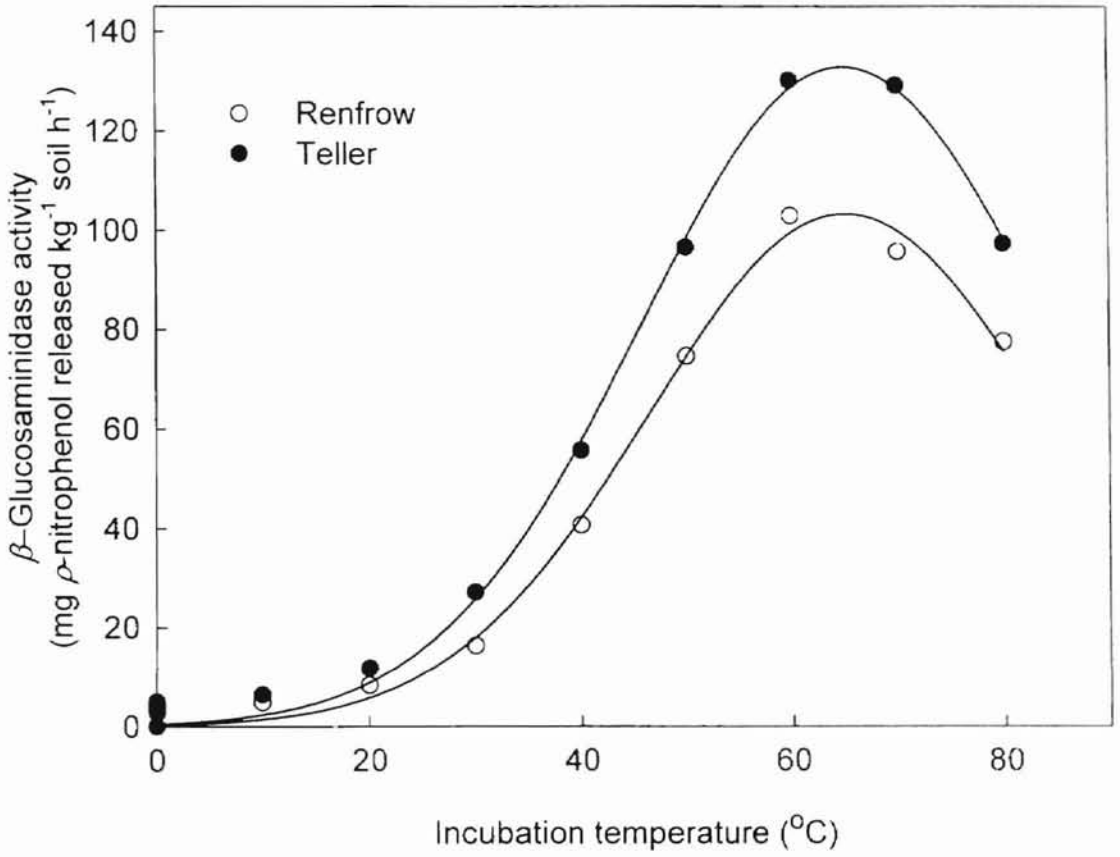


Figure 5. Effect of incubation temperature on release of ρ-nitrophenol in assay of β-glucosaminidase activity in soils tested.

of reaction time at 37°C. Therefore, these conditions were chosen for the assay of β-glucosaminidase activity in soil. The assay method developed is not only sensitive but also precise with coefficient of variance (CV) values less than 6% (Table 2).

Characterization with respect to K_m, V_{max}, E_a, and Q_{10} values

Three transformations of the Michaelis-Menten equation, Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee were performed and used to calculate the K_m and V_{max} values for this enzyme (Table 3). The K_m values range from 0.56 to 0.84 mM for the Renfrow soil and 1.14 to 1.48 mM for the Teller soil. The V_{max} values were about 30 mg ρ–nitrophenol kg^{-1} soil h^{-1} for the Renfrow soil and 39 mg ρ–nitrophenol kg^{-1} soil h^{-1} for the Teller soil. The Hanes-Woolf plots produced the lowest values for both soils while the Lineweaver-Burk plots had the highest values.

Q_{10} values were calculated from the temperature effect data. In the temperature range of 10° to 60°C, the Q_{10} values ranged from 1.38 to 2.5 for Renfrow, and 1.35 to 2.31 for Teller with averages of 1.88 and 1.86, respectively (Table 4). The highest Q_{10} values were found at temperature around 30° to 40°C, and the lowest around 50° to 60°C. The reaction obeyed the Arrhenius equation:

$$k = A \exp(-E_a/RT)$$

Table 2. Precision of the method

Soil	β-Glucosaminidase activity			
	Range[a]	Mean	SD[b]	CV(%)[c]
	------ mg ρ-nitrophenol kg^{-1} soil h^{-1} ------			
Renfrow	21.2 - 24.9	22.6	1.3	5.8
Teller	41.3 - 45.0	42.6	1.4	3.2

[a] Range of six replicated extractions and assays.

[b] SD, standard deviation.

[c] CV, coefficient of variation.

Table 3. K_m and V_{max} values of β–glucominidase in two soils calculated from three linear transformations of the Michaelis-Menten equation

Michaelis-Menten Transformation	Soil	K_m [a]	V_{max} [b]
Lineweaver-Burk plot	Renfrow	0.84	30.3
(1/*V* vs. 1/*S*)	Teller	1.48	39.8
Hanes-Woolf plot	Renfrow	0.56	29.1
(*S*/*V* vs. *S*)	Teller	1.14	38.8
Eadie-Hofstee plot	Renfrow	0.81	30.2
(*V* vs. *V*/*S*)	Teller	1.39	39.2

[a] ρ-Nitrophenyl-*N*-acetyl-β-D-glucosaminidine concentration (mM).

[b] mg ρ–nitrophenol released kg^{-1}soil h^{-1}.

Table 4. Temperature coefficients of β-D-glucosaminidase in soils

	Q_{10} for temperatures (°C) indicated[a]					
Soil	20	30	40	50	60	Average
Renfrow	1.73	1.96	2.50	1.84	1.38	1.88
Teller	1.83	2.31	2.06	1.73	1.35	1.86

[a]Q_{10} = Glucosaminidase activity at temperature given/Glucosaminidase activity at temperature given - 10°C

where k is the rate constant of the reaction, A is the Arrhenius constant, E_a is the Arrhenius activation energy, R is the gas constant, and T is the absolute temperature. The Arrhenius equation can be expressed in the log form as follows:

$$\ln k = (-E_a/R)(1/T) + \ln A$$

By plotting ln *k* vs 1/T, A and E_a values can be calculated from the intercept and slope of the linear relationship. The β-glucosaminidase reaction in the two soils tested obeyed the Arrenius equation from 10° to 50°C as indicated by the linear relationships (Fig. 6). The slopes of the lines were similar, indicating similar activation energy values for β-glucosaminidase in these soils. The activation energy values of the reaction catalyzed by β-glucosaminidase are 58.2 and 57.9 kJ mol^{-1} for Renfrow and Teller, respectively.

Effect of air-drying, autoclaving, toluene and chloroform fumigation

Air-drying reduced β-glucosaminidase activity by 12% for Renfrow and 22% for Teller. Chloroform fumigation and toluene treatments did not affect the enzyme activity significantly with the exception of field-moist Renfrow soil. The activity of β-glucosaminidase in field-moist Renfrow soil increased significantly by about 20% upon fumigation and toluene treatment (Fig. 7). Autoclaving the soil reduced enzyme activity considerably in both soils under field-moist or air-dried

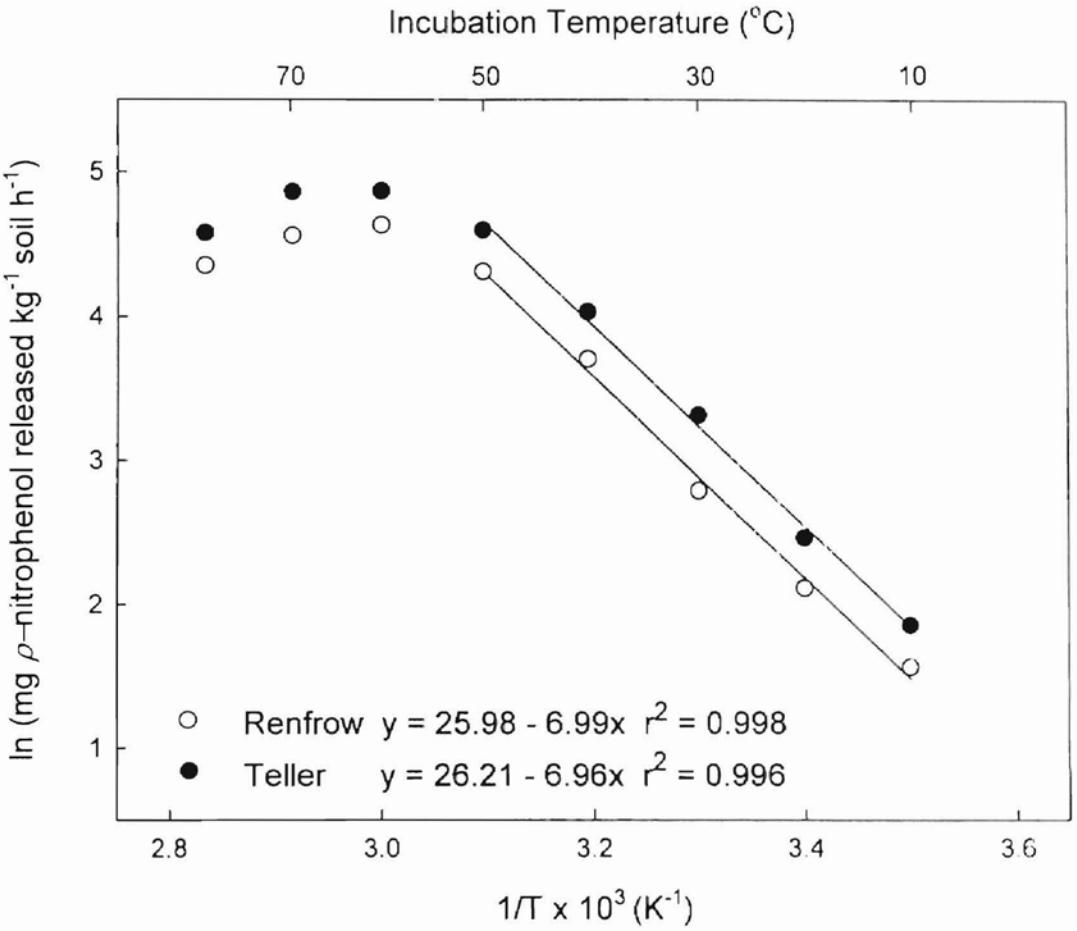
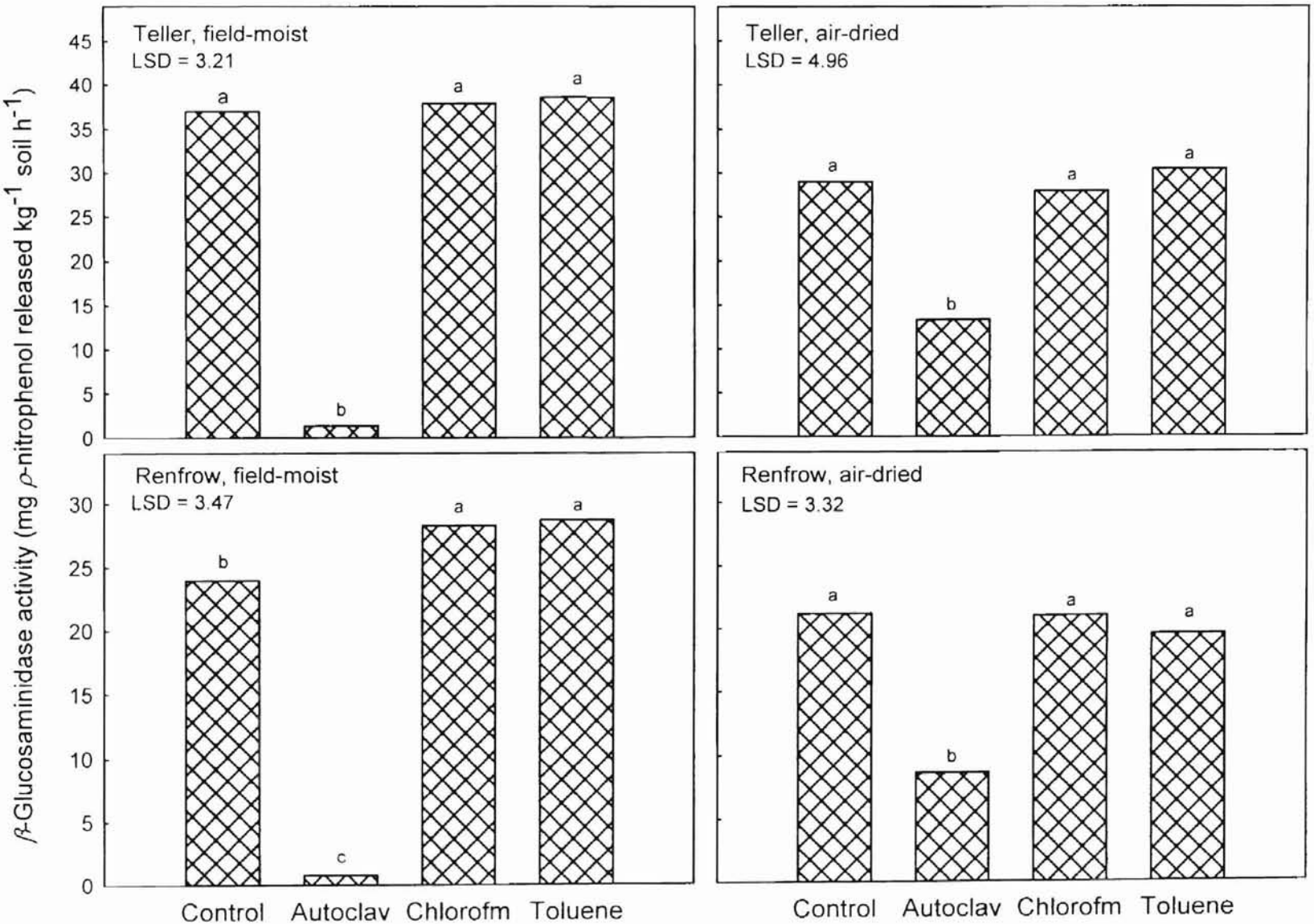


Figure 6. Linear transformation plots of the Arrhenius equation for β-glucosaminidase activity in soils tested at temperatures ranging from 10 to 50°C.

Figure 7. Effect of air-drying, autoclaving, toluene and chloroform fumigation of soil samples on activity of β-glucosaminidase in soils. Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test.


Teller, field-moist
LSD = 3.21
Teller, air-dried
LSD = 4.96
Renfrow, field-moist
LSD = 3.47
Renfrow, air-dried
LSD = 3.32
β-Glucosaminidase activity (mg ρ-nitrophenol released kg-1 soil h-1)
Control
Autoclav
Chlorofm
Toluene

condition. This reduction was about 58% for air-dried soils and 96.5% for the field-moist soils.

Discussion

MUB buffer was used for determination of the enzyme pH optimum to avoid the effect of ionic strength and test effects of pH alone. For both soils, the optimal pH range for this enzyme was between 5 and 6. This is consistent with data reported by Rodriguez-Kabana et al. (1983) and Naseby and Lynch (1997) for β-glucosaminidase and chitinase activity in soils.

Microorganisms have been suggested to be the major sources of soil enzyme activities (Skujins, 1976). Purified β-glucosaminidase from microbial sources indicated optimum pH ranging from 4.0 to 7.5 (Chitlaru and Roseman, 1996; Amutha et al., 1998; Nogawa et al., 1998; Cifali and Dias Filho, 1999). The observed pH optimum for β-glucosaminidase activity in soils tested is likely an integrated total effect of β-glucosaminidase originated from all the sources related to the soil development and cultivation history.

The optimum pH value of around 5.5 for β-glucosaminidase is close to the pH optima of other enzymes involved in C transformation in soil. For instance, glucosidases and galactosidases are most active at pH 6 (Eivazi and Tabatabai, 1988), and cellulase has an optimum pH at 5 (Deng and Tabatabai, 1994). Activities of β-glucosaminidase might also be important for N transformations in acidic soils because most of the enzymes known to be involved in N

transformations in soil have pH optima in the alkaline pH range. Glutaminase has a pH optimum at 10 (Frankenberger and Tabatabai, 1991a), aspartase at 8.5 (Senwo and Tabatabai, 1996), asparaginase from 8 to 12 (Frankenberger and Tabatabai, 1991b), urease at 9 (Tabatabai and Bremner, 1972), and amidase at 8.5 (Frankenberger and Tabatabai, 1980).

Results obtained from variation of substrate concentrations for the assay of soil β-glucosaminidase activity followed the patterns expected from classical theory of enzyme kinetics (Tabatabai, 1994). The data obtained fit the Michaelis-Menten equation and indicated that 10 mM pNNAG chosen for the standard assay resulted in β-glucosaminidase activity at the maximum velocity and the reaction followed zero-order kinetics. The relationships between reaction time or amount of soil (enzyme concentration) and β-glucosaminidase activity were linear, suggesting that the amount of substrate chosen for the standard assay was not limiting when 0.5 to 2.5 g of soil were incubated with the substrate for up to 150 min.

Denaturation of the enzyme during incubation occurred around 65°C, suggesting that this enzyme in soil is fairly stable. The optimum temperature for this enzyme purified from microorganisms varied depending on the source, 37°C from *Tritrichomonas foetus* (Cifali and Dias Filho, 1999), 50 °C from *Trichoderma reesei* (Nogawa et al., 1998), and 70°C from the thermotolerant *Bacillus* sp. NCIM 5120 (Amutha et al., 1998). We chose 37°C for the standard assay procedure to be consistent with the temperature used in most other published procedures for determination of soil enzyme activity.

The K_m value of β-glucosaminidase purified from *Bacillus* spp. is 0.34 mM (Amutha et al., 1998), which is much lower than the K_m values found in the two soils studied. The difference in K_m and V_{max} values in the two soils tested is likely due to presence of different competitive and non-competitive inhibitors in soils. Potential enzyme inhibitors in soils include fertilizers, pesticides, municipal and industrial wastes that are added as parts of soil and crop management, and salts and trace elements added as impurities in lime, fertilizers, and animal wastes. The K_m and V_{max} values that were calculated from the three linear transformations varied because each transformation gives different weight to errors in the variables (Dowd and Riggs, 1965).

The Q_{10} values for this enzyme in the soils tested were around 2 or higher at temperatures ranging from 30° to 40°C, indicating that the reaction rates doubled for every 10°C increase in temperature. This suggests that a 1°C difference in the reaction temperature would result in approximately 10% variation among analyses. With the relatively high Q_{10} values around 37°C, it is critical to control the reaction temperature precisely to obtain reproducible results.

If a reaction obeys the Arrhenius equation, energy of activation, E_a, can be calculated and it is independent of temperature. The E_a value is approximately equal to the difference in energy between the reactants and the transition state (Levine, 1988). The E_a values of β-glucosaminidase in the two soils tested are almost identical; suggesting that sources of this enzyme may be similar in these soils. The E_a value of β-glucosaminidase that was isolated from *Bacillus* spp. was

reported to be 43.2 kJ mol^{-1} using the same substrate (Amutha et al., 1998), suggesting that *Bacillus* may not be the dominant source for this enzyme in these soils.

It is important to note that end product inhibition of the enzyme activity was observed from β-glucosaminidase isolated from the marine bacterium *Vibrio furnissii* (Chitlaru and Roseman, 1996). Thus, caution should be exercised for assaying samples with high β-glucosaminidase activity.

Activities of β-glucosaminidase in soils were mostly due to extracellular enzymes. This is evidenced by limited effects of chloroform fumigation or toluene treatment of the soil on activities of this enzyme. This is consistent with literature in that β-glucosaminidase produced by many other microorganisms is mostly extracelluar and was detected and isolated from culture filtrates of *Trichoderma harzianum* (Lorito et al., 1994), *Bacillus* spp. (Amutha, 1998), and *Trichoderma reesei* PC-3-7 (Nogawa et al., 1998).

Our results also indicated that this enzyme is fairly stable in the soil environment. Air-drying reduced its activity by less than 25%. Autoclaving of the air-dried soils reduced its activity by about 58%. The most significant effect was found in autoclaving field-moist soils, which reduced its activity by approximately 96%. These results suggest that more protected forms of β-glucosaminidase were established during air-drying process through interactions with clay minerals, organic matter and soil aggregates.

In conclusion, activity of β-glucosaminidase in soils can be detected and quantified with the simple colorimetric method developed in this study. It

appeared that β-glucosaminidase in the two soils tested originated from similar sources and demonstrated similar values of pH optimum and energy of activation.

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Chapter IV

EFFECT OF ANIMAL MANURE APPLICATION ON PHOSPHORUS TRANSFORMATION IN SOIL

Abstract

Repeated land application of animal manure at rates to satisfy N requirements for crops may increase soil P to levels that cause environmental concern. Biochemical and microbiological parameters are important factors dictating P transformations in soil. Studies were conducted to investigate effects of animal manure application on activities of enzymes involved in P cycling in soils under a long-term continuous wheat experiment. Treatments included manure, P, NP, NPK, and NPK plus lime. Cattle manure was applied every four years at 269 kg N ha^{-1} for over a century and chemical fertilizers were applied every year at 67 kg N, 14.6 kg P, and 28 kg K ha^{-1} for 69 years. The highest Mehlich-3 extractable P was found in soils treated with P, followed by NP, NPK, NPKL, and cattle manure. The activities of alkaline phosphatase, inorganic pyrophosphatase, and phosphodiesterase were significantly higher in soils treated with cattle manure, but acid phosphatase activity was higher in soils treated with chemical fertilizers. Microbial biomass C and dehydrogenase activities were also the highest in manure-treated soils. Results suggested that long-term application of cattle manure at 269 kg N ha^{-1} (approximately 103 kg P

ha^{-1}) every four years promoted biological activities and P cycling, but did not result in accumulation of excessive Mehlich-3 extractable P in soil.

Introduction

Numerous reports demonstrated that repeated application of animal manure led to accumulation of phosphorus (P) in soil (Lemunyon and Gilber, 1993; Pesek et al, 1998; Reed et al., 1998). Soil test P may reach 247 kg ha^{-1} to 1121 kg ha^{-1} in Mehlich-3 extracts of soil samples taken from animal manure-treated soils (Reed et al., 1998). Soil test P levels of 73 kg ha^{-1} or less with Mehlich-3 extraction are generally associated with crop response to P fertilizer (Johnson et al., 1998). Animal manure application leads to soil test P levels exceeding the crop response range by several times. Thus, several states are calling for regulating manure land application based on P level in the soil and adjacent water bodies (McFarland et al., 1998).

Land application is the most economical procedure for waste disposal. Currently, animal waste is applied based on soil nitrogen (N) level. In the U.S., approximately 8.23×10^7 kg of P enters the environment in the form of animal manure per year (Wodzinski and Ullah, 1996).

Phosphorus accumulation in soils amended with animal waste is observed because of the lower N:P ratio (4:1) in manure (Gilbertson et al., 1979) than that taken up by major grain and hay crops (8:1, Fertilizer handbook, 1982). The

concern is that the released P from animal manure may be transported into rivers and lakes, and cause eutrophication in aquatic environments in which P is limiting (Carpenter et al., 1998).

Although excess P in soil systems may not benefit crop production, excess P may not be a threat to the environment, depending on many factors, including soil chemical, physical and biochemical characteristics, microbial population and activity, vegetative cover, and past P applications (Heathwaite et al., 1998; Gakstatter et al., 1978; Sharpley et al., 1994; Smith et al., 1998). Although some studies have been conducted to understand P runoff from agricultural land, little information is available on the impact of long-term application of animal waste on P cycling in soil.

Therefore, the objective of this work was to investigate the effects of long-term animal manure and chemical fertilizer treatments on P cycling in soil. Studies were conducted to evaluate effects of a century-long manure treatment and 69 years of fertilizer treatments on soil pH, organic C, total N, P contents, microbial biomass C, and activities of dehydrogenase and enzymes involved in P cycling, including acid phosphatase, alkaline phosphatase, inorganic pyrophosphatase, and phosphodiesterase.

Materials and Methods

Soil samples were obtained from a century-long continuous winter wheat (*Triticum aestivum* L.) experiment located at a research station at Oklahoma

State University, Stillwater, OK, U.S.A. The experiment was initiated on a Kirkland (fine, mixed, thermic Udertic Paleustolls) silt loam with a mean particle-size distribution of 37.5% sand, 40.0% silt, and 22.5% clay. The manure treatment plot was initiated in 1899. The chemical fertilizer treatment plots were initiated in 1929. There were six plots continued under investigation, including manure, P, NP, NPK, NPK plus lime and an untreated check. Cattle manure was applied every four years at 269 kg N ha^{-1} (approximately 103 kg P ha^{-1}). Chemical fertilizer plots received an annual application of 67 kg N, 14.6 kg P and 28 kg K ha^{-1}.

Composite soil samples included 18 cores per plot at 0-10, 10-20, and 20-30 cm, and were taken in October of 1998. The field-moist soil samples were sieved through a 2-mm screen and stored at 4°C. A portion of each sample was air-dried and a portion of air-dried samples was ground to pass an 80-mesh (180 μm) sieve. Soil pH values were determined using a combination glass electrode (soil:0.01 *M* $CaCl_2$ ratio = 1:2.5), and those of the organic C and total N by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Sulphur Analyzer (Schepers et al., 1989). Particle size distribution was determined by a pipette analysis (Kilmer and Alexander 1949). Water-soluble P levels were determined by the procedure described by Olsen and Sommers (1982). Phosphorus levels in Mehlich-3 extracts were determined by both Murphy and Riley method (MRP), and by Inductively Coupled Plasma Spectrometer (ICP-P; Mehlich, 1984; Murphy and Riley, 1962). The microbial biomass C was determined by the chloroform-fumigation-incubation method described by Jenkinson and Ladd (1981) using a

k_c factor of 0.45 with subtraction of the control. Dehydrogenase activity was assayed by the method described by Casida et al. (1964). Methods used for assaying phosphatases are summarized in Table 1.

Organic C and total N were determined using samples with particle size less than 180 μm. Soil pH and particle-size distribution were determined with air-dried samples that had less than 2-mm particle size. The microbial biomass C, dehydrogenase activity and phosphatase activity were determined using the < 2-mm field-moist samples. All results are expressed on a moisture-free basis. Moisture was determined after drying at 105°C for 48 h.

Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was done using the least significant difference (LSD) test. Percentage data was transformed using arc sine transformations before analysis (Gomez and Gomez, 1984). All results reported are averages of duplicated assays and analyses.

Results

Effects on Soil pH, Mehlich-3 extractable P, Organic C and Total N

The pH values in the tested 0-10 cm surface soils ranged from 4.2 to 5.7 with that of the untreated check soil at 4.8 (Fig. 1). Manure application increased soil pH while chemical fertilizer application resulted in lower soil pH (Fig. 1). The

Table 1. Methods used for assaying activities of enzymes involved in P cycling in soils.

Class/EC Number	Recommended name	Reaction	Substrate	Reference
3.1.3.1	Alkaline phosphatase	Orthophosphoric monoester + H_2O → alcohol + orthophosphate	*p*-Nitrophenyl phosphate	Eivazi and Tabatabai (1977)
3.1.3.2	Acid phosphatase	Orthophosphoric monoester + H_2O → alcohol + orthophosphate	*p*-Nitrophenyl phosphate	Tabatabai and Bremner (1969)
3.1.4.1	Phosphodiesterase	Orthophosphoric diester + H_2O → orthophosphoric monoester + alcohol or phenol or nucleoside	bis(*p*-Nitrophenyl) phosphate	Browman and Tabatabai (1978)
3.6.1.1	Inorganic Pyrophosphatase	Pyrophosphate + H_2O → 2 orthophosphate	Sodium pyrophosphate	Dick and Tabatabai (1978)

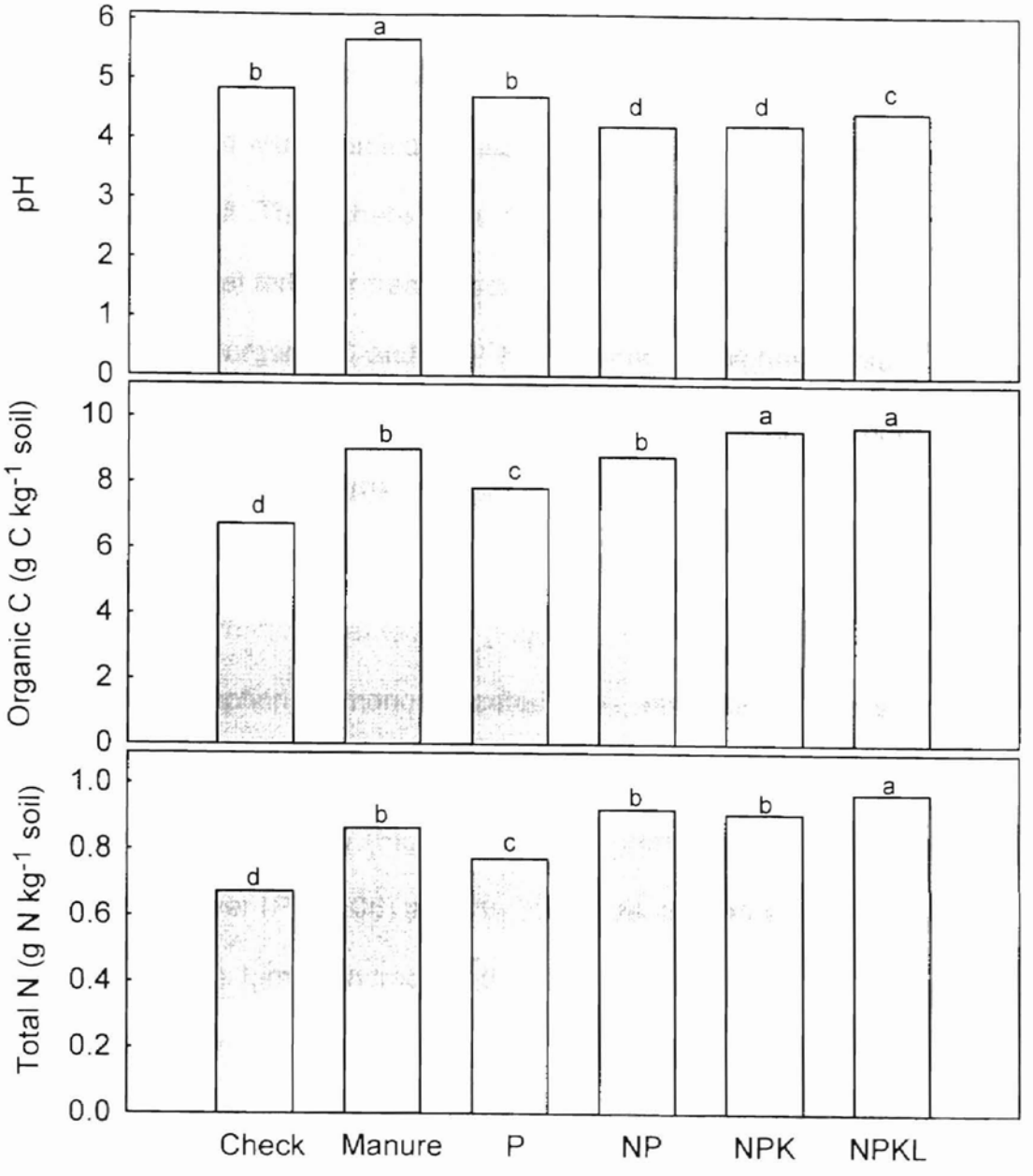

Figure 1. Effect of a long-term animal manure and chemical fertilizer application on pH values, organic C content, and total N content of surface soils (0-10 cm). Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test.

pH values from manure-treated soils were approximately 0.9 units higher than that of the untreated check soil. With the exception of the P-treated soil, the pH values in soils treated with chemical fertilizers were 0.3 to 0.6 pH units lower than that of the check soil. Thus, there were 1.2 to 1.5 pH unit differences between manure- and chemical fertilizer-treated soils.

The levels of organic C and total N were not the highest in surface soils treated with manure (Fig. 1). Organic C levels were significantly higher ($P<0.05$) in surface soils treated with NPK and NPKL than in the manure-treated soil. A significantly higher ($P<0.05$) level of total N was also measured in the NPKL-treated soil than the manure-treated soil (Fig. 1).

With the exception of manure-treated soils, pH values were significantly higher ($P<0.05$) in the lower profile (20-30 cm) than in the upper and middle profiles (0–10 and 10–20 cm) (Fig. 2). The pH values in manure treated soils were significantly lower ($P < 0.05$) at 20 to 30 cm depth than at 0 to 10 and 10 to 20 cm depths (Fig. 2). Liming increased the pH value of the middle and lower soil profiles in the NPK-treated soil significantly ($P < 0.05$).

Organic C contents were influenced by depth in all soils except the check (Fig. 2). Organic C in soils treated with NPK or NPKL decreased in the subsurface soil and fell significantly ($P < 0.05$) below that in the manure-treated soils (Fig. 2).

Water soluble P levels in these soils tested with Murphy and Riley method (1962) did not exceed 1 mg P kg^{-1} soil with the exception of P- treated surface soils (0-10 cm), which was about 1.5 mg P kg^{-1} soil (data not shown). The levels

Figure 2. Effect of a long-term animal manure and chemical fertilizer application on distribution of pH values, organic C and total N over soil profile 0-30 cm.

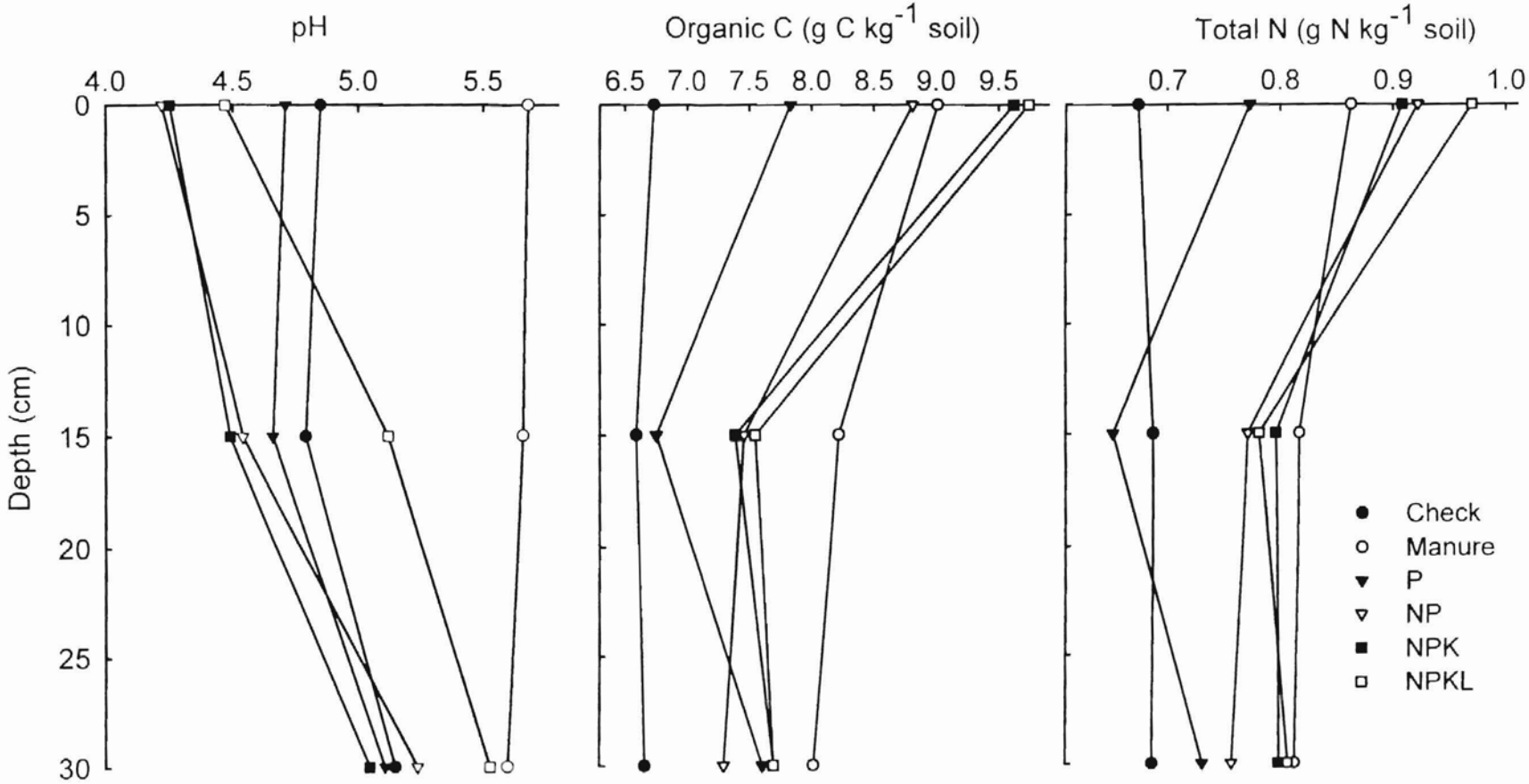

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Figure 3. Effect of a long-term animal manure and chemical fertilizer application on (A & C) MRP and ICP-P levels in surface 0-10 cm soils, and (B & D) distribution of MRP and ICP-P over soil profile 0-30 cm. Different letters in A indicate significantly different means at $P < 0.05$ according to least significant difference test.

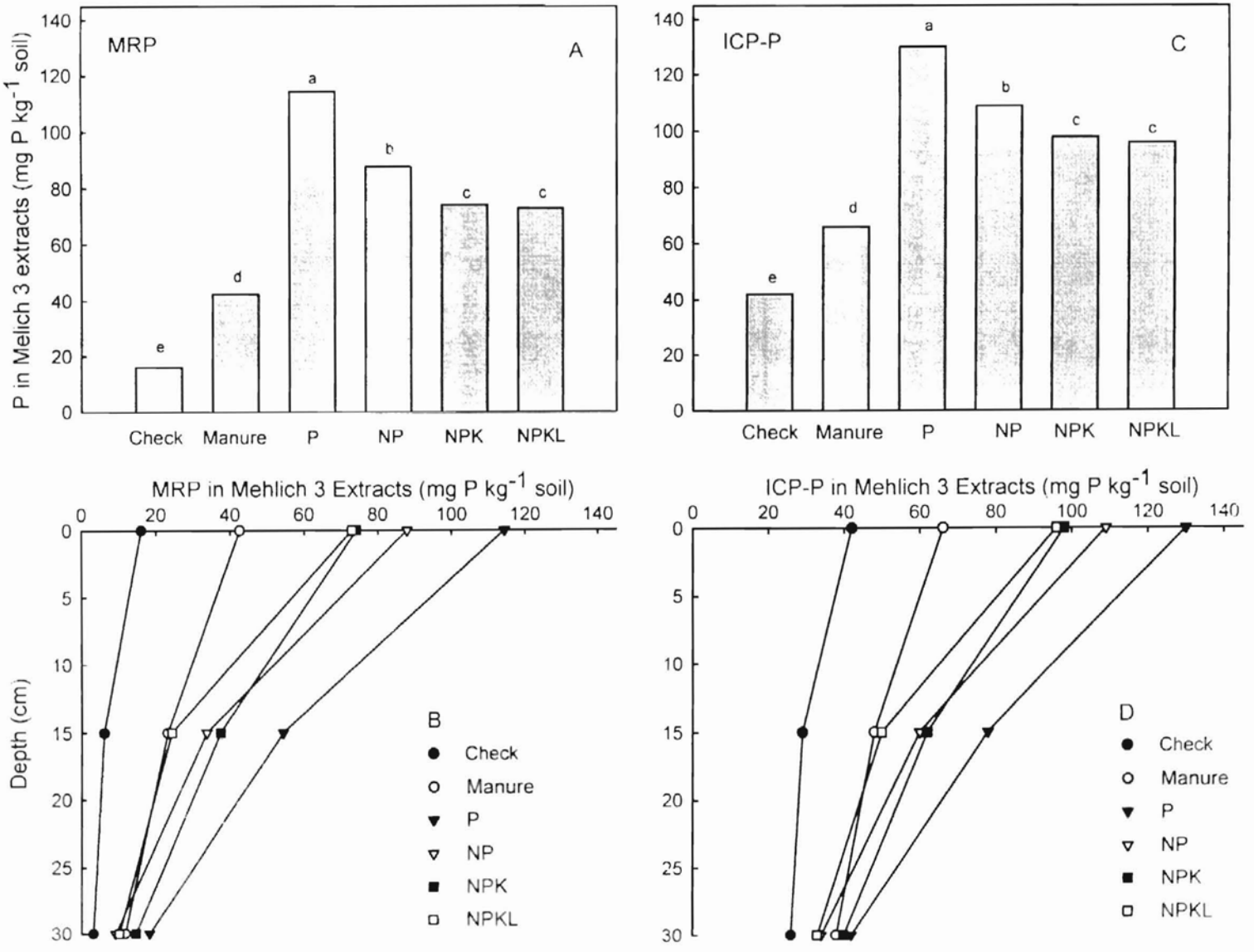
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Table 2. MRP expressed as percentages of ICP-P†.

Treatment	Soil depth (cm)		
	0 - 10	10 - 20	20 - 30
	%		
Check	38[e]	21[d]	12[e]
Manure	65[d]	48[c]	31[c]
P	88[a]	70[a]	44[a]
NP	81[b]	56[bc]	27[d]
NPK	76[c]	60[b]	37[b]
NPKL	76[bc]	48[bc]	31[c]

† MRP and ICP-P are P concentrations (mg P kg^{-1} soil) in Melich-3 extracts determined by Murphy and Riley method (1962) and by Inductively Coupled Plasma Spectrometer (ICP), respectively. Different letters indicate significantly different means within sampling depths at $P < 0.05$ according to least significant difference test.

treated with animal manure, while the activities of acid phosphatase were significantly greater in soils treated with chemical fertilizers (Fig. 4). The highest acid phosphatase activity was detected in the soil treated with NP, giving an average of 373 mg ρ-nitrophenol released kg^{-1} soil h^{-1}, followed by soils treated with NPK, NPKL, and P (Fig. 4). Acid phosphatase activities were not significantly different in manure-treated and check soils. Activity of alkaline phosphatase in the manure-treated soil was more than 2-fold of those in the check or P-treated soil, and exceeded 4-fold of those in the soils treated with NP, NPK, or NPKL (Fig. 4). Activity of inorganic pyrophosphatase was the lowest in the NP-treated soil, which was less than half of that in the manure-treated soil (Fig. 4). Activities of phosphodiesterase averaged 29 mg ρ-nitrophenol released kg^{-1} soil h^{-1} for the soils tested with the exception of the manure-treated soil, which showed more than doubled activity (Fig. 4).

In the lower soil profiles (20 to 30 cm) tested, activities of all P cycling enzymes studied were the highest ($P < 0.05$) in the manure-treated soil (Fig. 5).

Effect on Microbial Biomass C and Dehydrogenase Activity

Microbial biomass C contents were the highest in the 0-10 cm surface soil treated with manure and the lowest in those of check and P-treated soils (Fig. 6). A similar trend was found in the lower soil profiles (20 to 30 cm, Fig. 6B). Microbial biomass C contents decreased significantly ($P < 0.05$) with increasing soil depth in all the soils tested.

Figure 4. Effect of a long-term animal manure and chemical fertilizer application on the activities of acid phosphatase (mg ρ-nitrophenol kg^{-1} soil h^{-1}), alkaline phosphatase (mg ρ-nitrophenol kg^{-1} soil h^{-1}), inorganic pyrophosphatase (mg PO_4^{-3} kg^{-1} soil h^{-1}), and phosphodiesterase (mg ρ-nitrophenol kg^{-1} soil h^{-1}) in surface soils (0-10 cm). Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test.

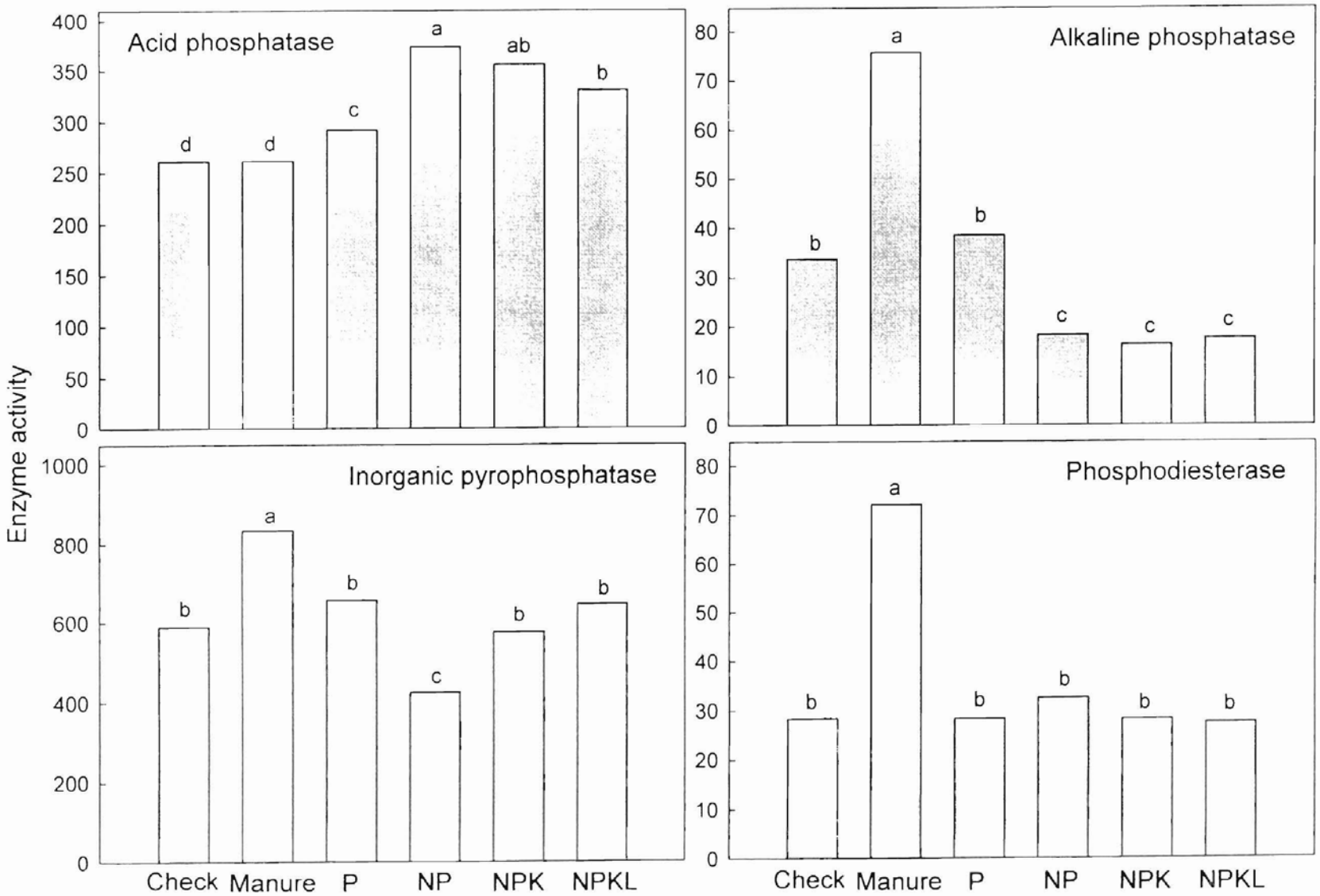
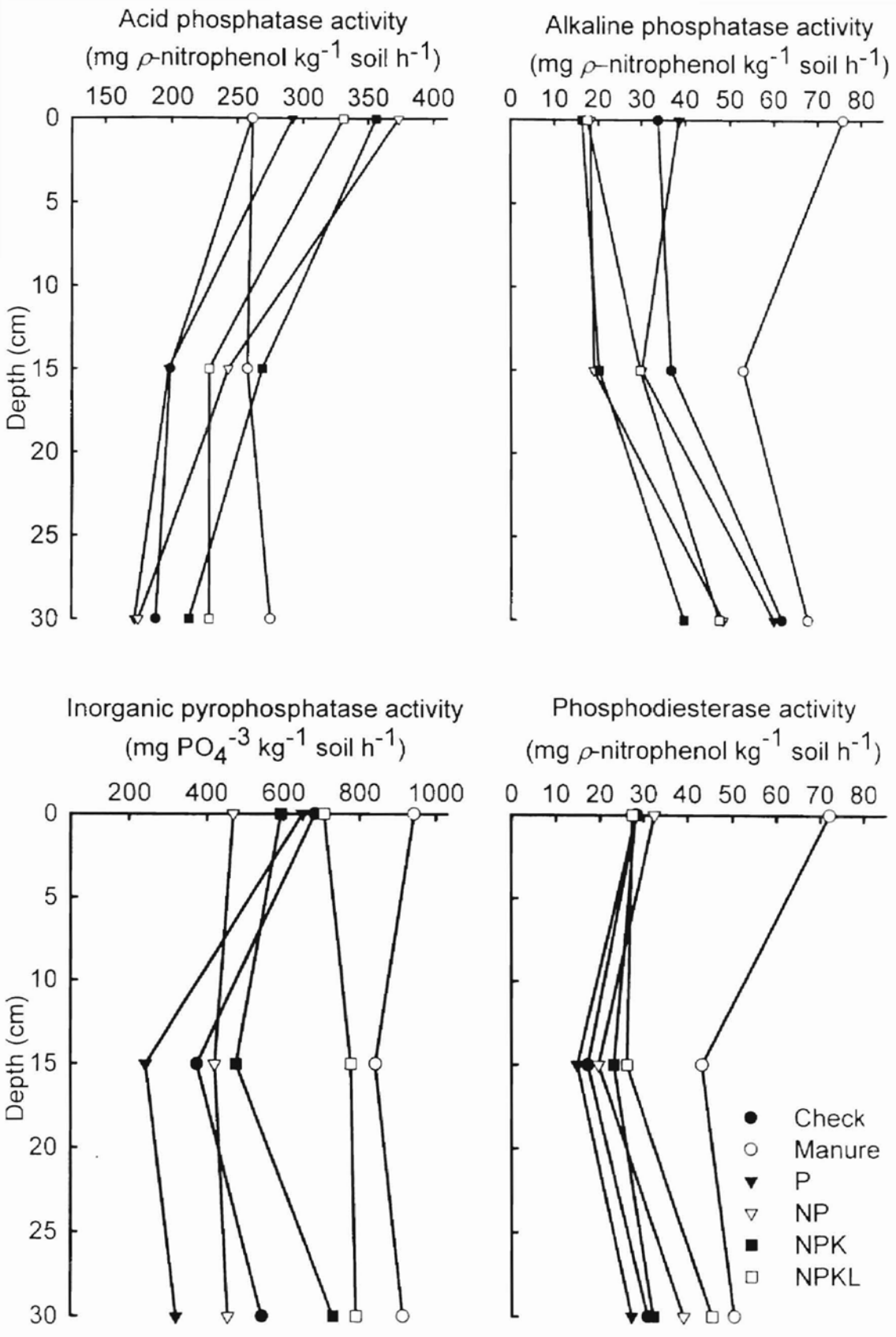


Figure 5. Effect of a long-term animal manure and chemical fertilizer application on the activities of acid phosphatase, alkaline phosphatase, inorganic pyrophosphatase, and phosphodiesterase over soil profiles 0-30 cm.


Acid phosphatase activity
(mg ρ-nitrophenol kg-1 soil h-1)
150 200 250 300 350 400
Alkaline phosphatase activity
(mg ρ-nitrophenol kg-1 soil h-1)
0 10 20 30 40 50 60 70 80
Inorganic pyrophosphatase activity
(mg PO4-3 kg-1 soil h-1)
200 400 600 800 1000
Phosphodiesterase activity
(mg ρ-nitrophenol kg-1 soil h-1)
0 10 20 30 40 50 60 70 80
Depth (cm)
0 5 10 15 20 25 30
Check
Manure
P
NP
NPK
NPKL

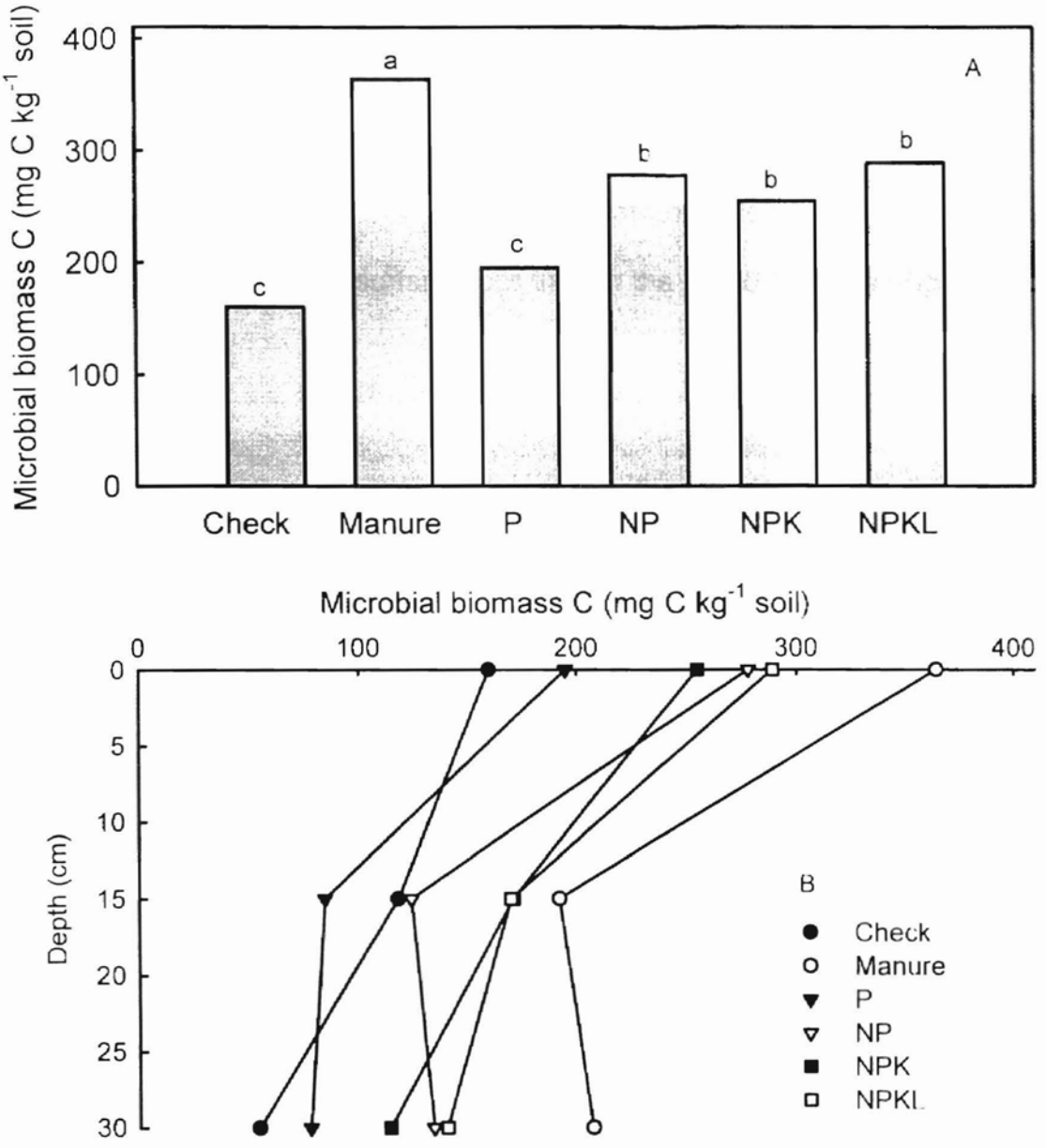


Figure 6. Effect of a long-term animal manure and chemical fertilizer application on (A) microbial biomass C in surface soils (0-10 cm), and (B) distribution of microbial biomass C over soil profile 0-30 cm. Different letters in A indicate significantly different means at $P < 0.05$ according to least significant difference test.

The trends for dehydrogenase activity in the soils tested were similar to those for microbial biomass C contents (Fig. 7). The highest dehydrogenase activity among surface soils was in soil treated with animal manure while the lowest was in the check soil. For each soil treatment, dehydrogenase activity was significantly higher in the surface soils than in the 20-30 cm soils (Fig. 7B).

Discussion

One would expect that animal manure applications enrich soil organic matter and increase total N content. Results from this study showed that soil organic matter and total N content were actually lower in the 0-10 cm surface soil treated with animal manure when compared with some of the soils treated with chemical fertilizers. One explanation is that manure promoted biological and microbial activities, which accelerated break down of organic substances and resulted in reduced soil organic matter and total N content. The enhanced biological activities in manure-treated soils are evidenced by relatively high phosphatase activities, microbial biomass C and dehydrogenase activity. As suggested by Jenkinson and Ladd (1981), microbial biomass not only contains a labile pool of nutrients but also drives the cycling of organic matter and nutrients in soil.

These results do not agree with those reported by Suri and Puri (1997). Their study indicated significant build up of organic C in the plots treated with P and farmyard manure. Their results, however, were obtained from a one-year

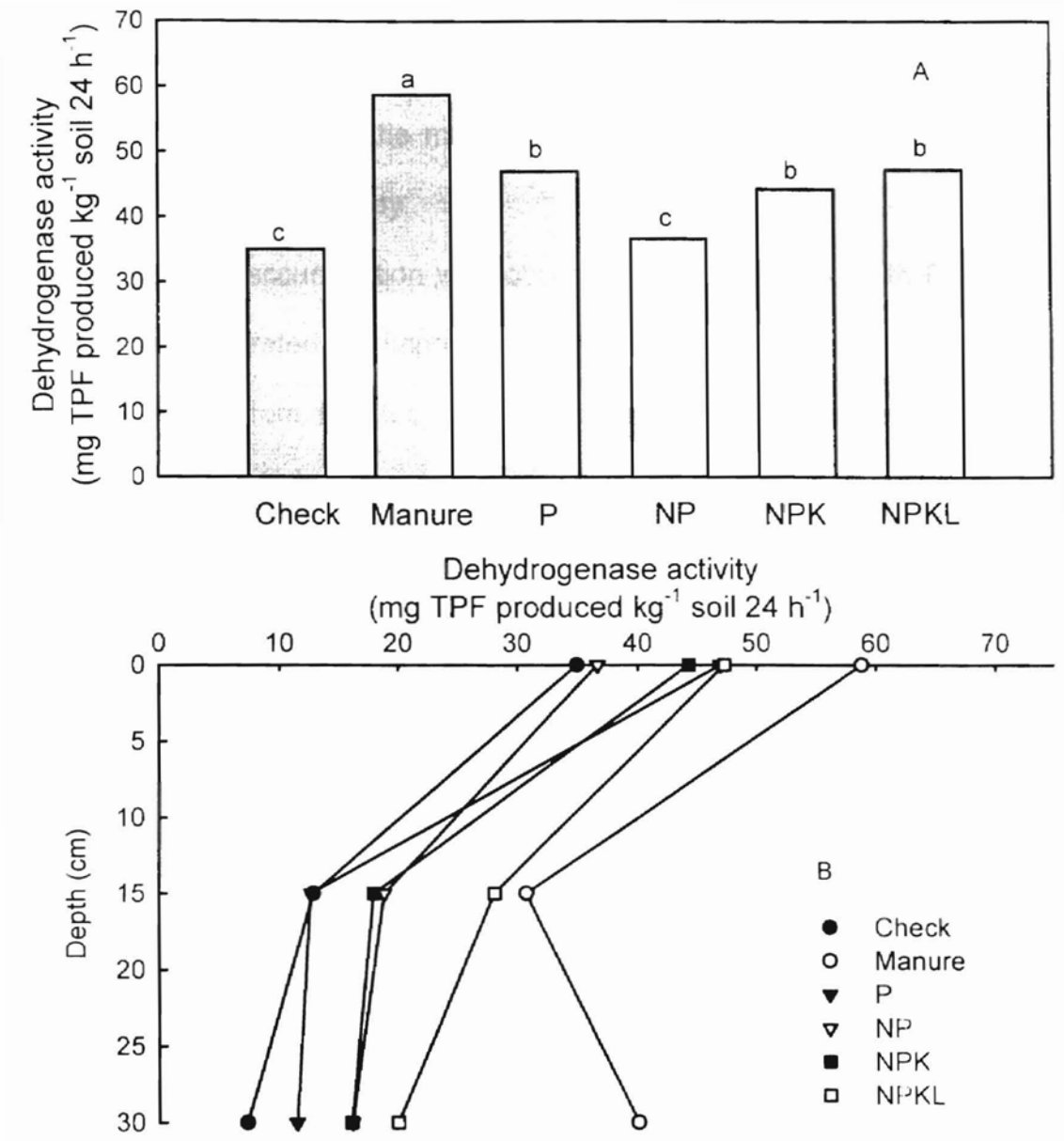


Figure 7. Effect of a long-term animal manure and chemical fertilizer application on (A) dehydrogenase activity in surface soils (0-10 cm), and (B) distribution of dehydrogenase activity over soil profile 0-30 cm. Different letters in A indicate significantly different means at $P < 0.05$ according to least significant difference test.

field experiment. Enrichment of soil organic matter by animal manure was reported in soils under manure treatment for 45 years (Agbenin and Goladi, 1997). However, the total cattle manure application rate in their study was 20 times of those used in this study.

Phosphorus accumulation was observed in soils treated with P fertilizer alone. This demonstrated the importance of balancing nutritional needs for crop production. Results from this study are consistent with those reported by Agbenin and Goladi (1998), who found that addition of P fertilizer increased the concentration of labile P.

Mehlich-3 P levels in soil have been found to be highly correlated with dissolved P, a potential indicator of P enrichment due to runoff (Sharpley, 1997). Results from this study indicated that MRP in the manure-treated surface soil was only 43 kg P ha^{-1} after applied manure at 269 kg N ha^{-1} (approximately 103 kg P ha^{-1}) every four years for over a century. The P rate in manure application is almost double of that in chemical fertilizer treatments. However, the soil test P in the manure-treated soil is only 60% or less of those in chemical fertilizer-treated soils, and is far below 247 kg ha^{-1} to 1121 kg ha^{-1} reported by Reed et al. (1998). Our result is consistent with those reported by Schlegel and Alam (1999; personal communication). They found that P accumulation was observed in soils amended with animal manure for 3 years, but not for 10 or 30 years. One possible explanation is that the microbial community adapted to the conditions with time and mobilized P compounds in the animal waste. The released P can

either be taken up by plants, transported to the subsoil, or transported in water for considerable distances.

When MRP was expressed as percentages of ICP-P in Mehlich-3 extracts, application of chemical P increased this ratio considerably. Chardon et al. (1997) attributed the difference between MRP and ICP-P in solutions to dissolved organic P.

Manure application showed a greater impact over the 0-30 cm soil profile while the influence of chemical fertilizer applications were mostly on the soil surface 0 to 10 cm. This is strongly demonstrated by phosphatase activities. Distributions of soil organic C and total N contents over the soil profiles indicated movement of soluble manure compounds from the surface to lower soil profiles. Deeper impact may result in a relatively longer-term effect. Poultry manure applied to a rice field at 120 or 180 kg N ha^{-1} showed a residual effect on wheat, which followed rice, and this residual effect was equivalent to 40 kg N ha^{-1} (Singh et al., 1997).

The different trend for acid phosphatase activity in these soils may be accounted for by changes in soil pH. Although it has been reported that the optimal pH for the activities of soil acid phosphatases is around pH 6.5 (Tabatabai and Bremner, 1969), this optimum pH may vary depending upon soil type and microbial community structure. In this study, higher acid phosphatase activity was detected in soils with lower soil pH despite the fact that the highest pH value in soils tested did not exceed 5.25. It is possible that the main source

and type of acid phosphatases in these soils differ from those Iowa soils studied by Tabatabai and Bremner (1969).

Dehydrogenase is active only inside intact living cells. Thus, it is generally accepted that dehydrogenase activity may be used to assess microbial activity (Tabatabai, 1994). The higher microbial biomass C content and dehydrogenase activity in manure-treated soils at 20-30 cm depth than those at 10-20 cm suggest possibly higher microbial population and activities in the lower soil profiles. Similar trends were also observed in phosphatase activities. However, this trend was not observed in the chemical parameters tested. It seems that manure application affected microbial populations and activities in a peculiar way. Since the surface 15 cm is usually considered the plow layer, samples taken at 10-20 cm were located in the transition zone of plow and subsoil layers. Constantly changing and disturbing the environment due to tillage, development of plant root system, and soil moisture fluctuation may not favor establishment of any particular microbial community. As a result, biological activities were relatively lower than that in the soil profiles above and below this layer. This trend has not been reported in other research studies. One of the possible reasons is that most other studies on biological activities over soil profiles were conducted with samples taken at 15-cm increments (Tabatabai and Bremner, 1970) or 5-cm increments up to 15 cm only (Deng and Tabatabai, 1997).

Manure application not only altered biological activities and P cycling in soil, but also provided adequate nutrients for wheat production. After a century long continuous wheat experiment, wheat yield in the past 64 years (1930-1994)

was not significantly different between the manure-treated plot and plots treated with NP, NPK, or NPKL (Boman et al., 1996). These results also suggested that application of animal manure at 269 kg N ha^{-1} every four years for over a century did not result in accumulation of excessive amount of Mehlich-3 extractable P, but maintained wheat yield.

In conclusion, animal manure application may promote biological activities and P cycling in soil. The results obtained from this study may be useful to guide understanding the impact of long-term application of animal manure on P cycling and P accumulation in soil.

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Chapter V

IMPACT OF ANIMAL MANURE ON SOIL MICROBIAL COMMUNITY STRUCTURE

Abstract

Microorganisms play intricate roles in nutrient cycling, soil fertility, and soil organic matter turnover. Microbial community structures in soils under different management systems in a long-term continuous wheat experiment were characterized by plate count methods. These soils were treated with cattle manure, P, NP, NPK, or NPK plus lime. Soils amended with cattle manure had the highest numbers of total culturable bacteria, as well as the highest percentage of bacteria that could be characterized as r-strategists. The bacterial communities in the non-treated check soil and soils treated with P and NP were more distributed toward K-strategists. Soil treated with manure showed the lowest counts of culturable fungi, but the highest counts of culturable bacteria. DNA recovered was the most abundant in the manure-treated soil.

Introduction

Agricultural practices play an intricate role in manipulating soil microbial activities and community structure (Paul and Clark, 1989). Soils amended with organic matter generally have higher active microbial populations than soils amended with inorganic fertilizers (Bolton et al., 1985). This is demonstrated in

studies conducted by Bolton et al. (1985), Fauci and Dick (1994), and Ancheng and Xi (1994). Bolton et al. (1985) found that green manure increased soil microbial biomass and enzyme activities more than inorganic fertilizers. Results from Fauci and Dick (1994) indicated that organic inputs supported 80 to 400% greater microbial biomass C than the control. Results from Ancheng and Xi (1994) showed that organic manure increased the total number of fungi, actinomycetes, bacteria, and P-solubilizing bacteria. In addition, crop rotation may also play a role in altering microbial activity in soils. Fraser et al. (1988) reported that manure applications in combination with crop rotations resulted in increased microbial biomass, bacterial and fungal populations, dehydrogenase activity, and CO_2 evolution in surface soils when compared to a monocrop system with organic fertilizers.

Although it is well established that agricultural practices, such as addition of organic amendments, alter microbial activity in soil, little is known about their impact on microbial community structure. Microbial communities remain among the most challenging communities to characterize and enumerate. To fully describe microbial community structure in an environment, species diversity, species evenness, and the physiological role of species in the environment needs to be described (Tiedje et al., 1999). Since there may be as many as a trillion bacterial species globally (Dukheiqen, 1998), it is both difficult and time consuming to identify each species present. It is, therefore, easier to observe microbial populations with techniques that analyze broad groups within a community rather than each individual member.

Methods for analyzing broad groups within a community include culturing and enumerating microorganisms, and analyzing the total extractable soil DNA, such as determining the guanine plus cytosine (G+C) content, amplified ribosomal DNA restriction analysis (ARDRA), denaturing and thermal gradient gel electrophoresis (DGGE/TGGE), single Strand Conformation Polymorphism (SSCP), and more recently, Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Tiedje et al., 1999; Zuberer, 1994).

Recent reviews have revealed disadvantages associated with determining community structure using the G+C content of soil. For instance, this method requires the extraction of DNA from soil, which can cause bias towards certain phylotypes (Spring et al. 2000). It has also been argued that genomic DNA in dead cells can survive for long periods of time in the environment, thereby, making changes in microbial community structure over time difficult to determine using G+C content (Spring et al., 2000). Furthermore, G+C content is related to taxonomy and does not give conclusive indications as to the interactions among soil microorganisms, which is a requirement for a full description of community structure (Tiedje et al., 1999).

Plate count methods have been commonly used. These methods produce quantitative yields of microorganisms, allow for physiological classification of microorganisms, and provide specific information as to the biodiversity of the soil microorganisms (DeLeij et al., 1993; Øvreas and Torsvik, 1998). Therefore, the objectives of this study were to determine the effects of long-term animal manure and chemical fertilizer treatments on bacterial and fungal communities by

enumerating microbial population and determining ecophysiological indices (EP-indices), growth strategy, as well as total extractable soil DNA.

Materials and Methods

Soil samples

Soil samples were obtained from a century-long continuous winter wheat (*Triticum aestivum* L.) experiment located at a research station at Oklahoma State University, Stillwater, OK, U.S.A. The experiment was initiated on a Kirkland (fine, mixed, thermic Udertic Paleustolls) silt loam with a mean particle-size distribution of 37.5% sand, 40.0% silt, and 22.5% clay. The properties of the soils are reported in Table 1. The manure treatment plot was initiated in 1899. The chemical fertilizer treatment plots were initiated in 1929. There were six plots under investigation, including manure, P, NP, NPK, NPK plus lime and an untreated check. Cattle manure was applied every four years at 269 kg N ha^{-1} (approximately 103 kg P ha^{-1}). Chemical fertilizer plots received an annual application of 67 kg N, 14.6 kg P and 28 kg K ha^{-1}.

Soil samples (0-10 cm) were taken in January of 2000. The field-moist soil samples were sieved through a 2-mm screen and stored at 4°C. Soil pH values were determined using a combination glass electrode (soil:0.01 *M* $CaCl_2$

Table 1. Properties of the soils used[†]

Soil	pH[‡]	Total N (g kg^{-1})*	Organic C (g kg^{-1}) *
Check	5.1^{b}	0.68^{c}	7.29^{f}
Manure	5.5^{a}	0.91^{a}	10.17^{b}
P	4.5^{c}	0.74^{c}	7.98^{e}
NP	4.3^{d}	0.83^{b}	8.43^{d}
NPK	4.3^{d}	0.90^{ab}	9.40^{c}
NPKL	4.5^{c}	0.95^{a}	10.81^{a}

[†] Different letters indicate significantly different means at $P<0.05$ according to least significant difference test.

[‡] Soil : 0.1 *M* $CaCl_2$ ratio = 1:2.5

*The organic C and total N contents were determined by dry combustion using a Carlo-Erba NA 1500 N/C/S Analyzer

ratio = 1:2.5), and those of the organic C and total N by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Sulphur Analyzer (Schepers et al., 1989). Particle size distribution was determined by a pipette analysis (Kilmer and Alexander 1949).

Plate Counts

Bacteria were cultured on 0.1-strength Tryptone soya agar plates (TSA) at 25°C (Katoh and Itoh, 1983; De Leij et al., 1993). All bacterial colony forming units (CFU) appearing within 24 h were designated as r-strategists, and the remaining as K-strategists (De Leij et al., 1993). EP-Indices (H') were calculated using the equation $H' = \Sigma(p_i * \log_{10} p_i)$ with p_i representing CFU on each day as a proportion of the total CFU in that sample in 10 days incubation. TSA plates with 10 to 200 colonies were selected for enumeration.

Fungi were cultured on 0.1-strength malt extract agar plates (MEA) at 23°C (Parkinson, 1994). Fungal colonies were observed after 72 hours of incubation with countable plates of the highest dilution being enumerated.

DNA Extraction

DNA in soil was extracted from 5 g of sample using the method described by Zhou et al. (1996). Briefly, soil samples were mixed with a DNA extraction buffer containing Tris-HCl, EDTA, sodium phosphate, sodium chloride, CTAB (hexadecyltrimethylammonium bromide), and proteinase K. Bacterial cells were lysed by SDS at 65°C. Extracted DNA was pelleted by centrifugation and

resuspended in TE buffer. Two μl of crude DNA was loaded to a 2% agarose gel for quantification.

Data analysis

Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was done using the least significant difference (LSD) test. Linear regression was used to determine relationships. Percentage data was transformed before analysis using arc sine transformations (Gomez and Gomez, 1984). Results reported are averages of 5 replicated plate counts.

Results

Effect of soil treatments on bacterial communities

Numbers of total culturable bacteria were significantly higher in the manure-treated soil than those of the other soils tested (Fig. 1A). The amount of extractable crude soil DNA, which was approximately 20 kb in length, was also the highest in the manure-treated soil as shown in Figure 1B. The evenness of bacterial growth strategies, as indicated by EP-indices (Fig. 2B), indicated significantly less evenness in the NPK-treated soil than detected in the remaining soils. Bacteria numbers after 24 h incubation (r-strategist bacteria) were shown

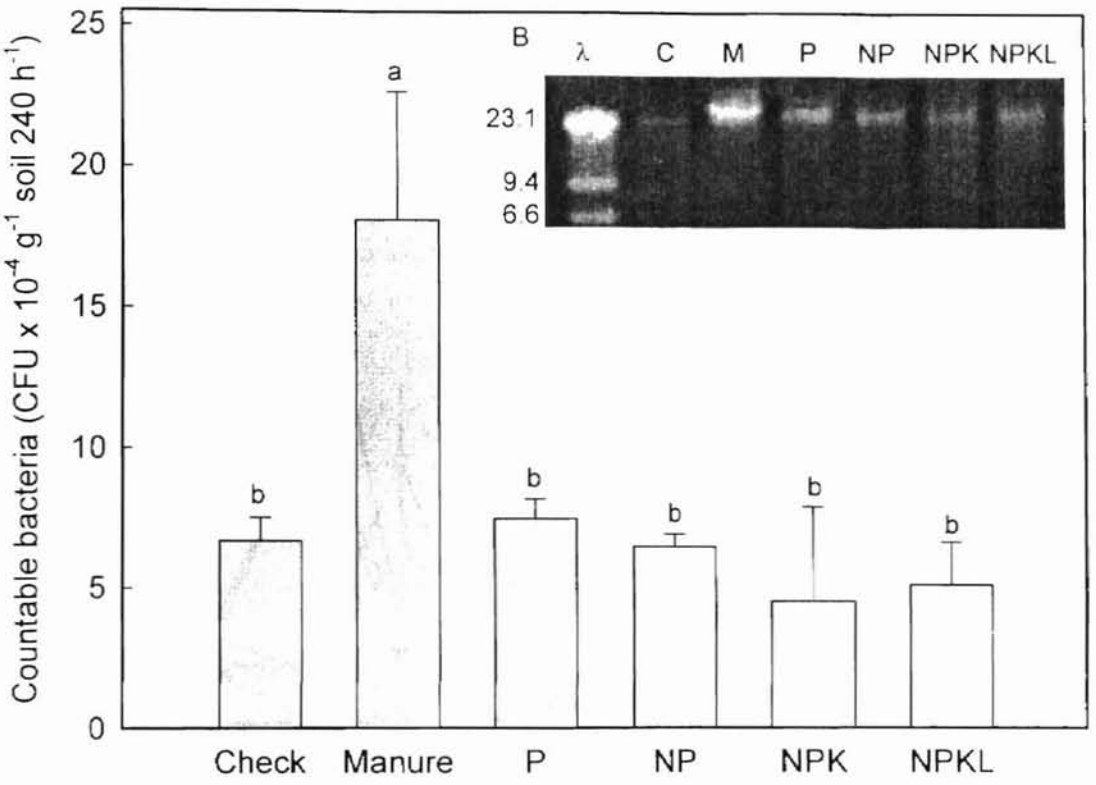

Figure 1. Total bacteria colony forming units after 10 days incubation. Different letters indicate significantly different means at $P<0.05$ according to least significant difference test. B. Crude DNA extract from soil.

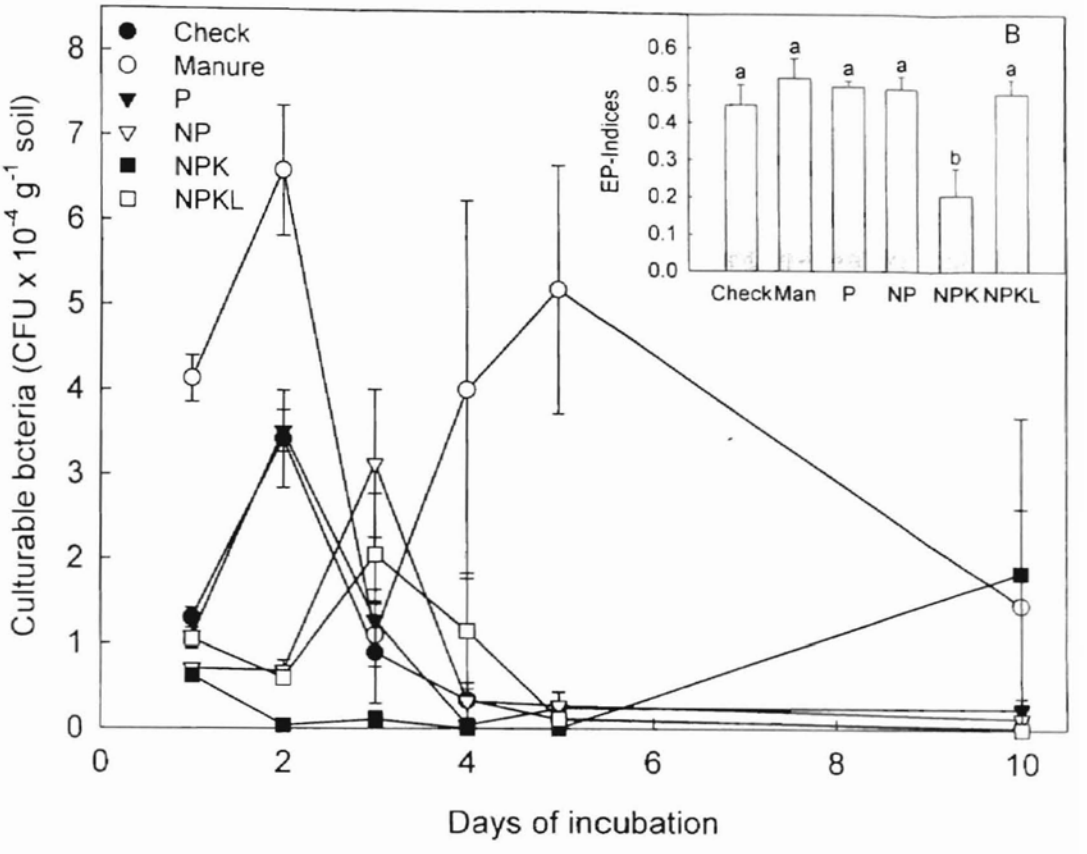

Figure 2. Bacterial CFU enumerated up to 10 days in soils under different management systems. B. EP-indices in soils under different management systems. Different letters indicate significantly different means at $P<0.05$ according to least significant difference test.

to be most numerous in the manure-treated soil and least numerous in the NPK-treated soil (Fig. 3). R-strategist bacteria also appear to have a positive relationship with soil pH (Fig. 3B). The percent of the total bacterial community comprised of r-strategist bacteria ranged from 1% in the check-treated soil to 24% in the manure-treated soil (Fig. 4).

Effect of soil treatments on fungi

Fungal colony forming units were lowest in the manure-treated soil with no differences detected between the check soil and chemically treated soils (Fig. 5). There was very little diversity among fungi from the manure-treated soil. Virtually all fungi counted from manure-treated soil were white in color with similar sizes in appearance. Fungi counted from the remaining treatments were mixtures of many sizes and colors including white, yellow, red, and green. It was also noticed that increasing soil pH appeared to have a negative effect on fungal counts (Fig. 5).

Discussion

Plate counting techniques for the enumeration of microorganisms has been widely used since the pioneering microbiologists in the late 1800's (Zuber, 1994). There are well known limitations of using plate counts to determine microbial community structure that have come to be generally accepted (Zuber, 1994). One of such limitations is that only a small fraction (approximately 1-10%)

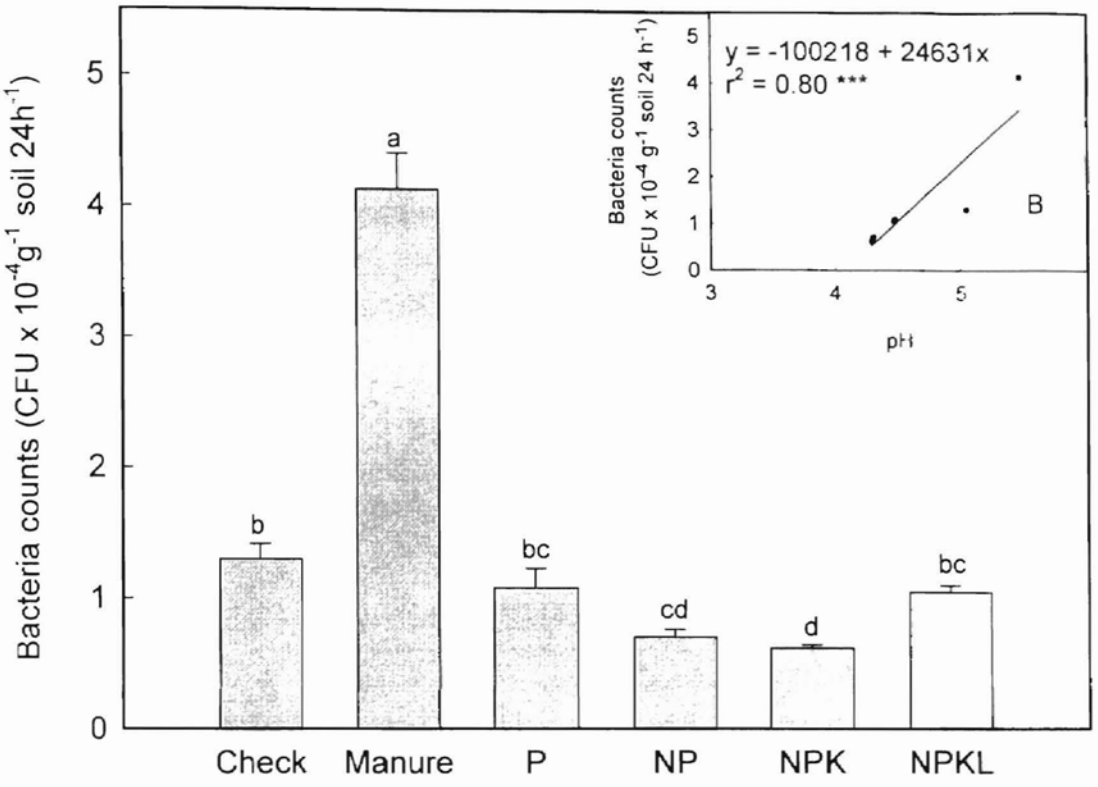

Figure 3. Bacteria CFU enumerated after 24 h incubation. Different letters indicate significantly different means at $P<0.05$ according to least significant difference test. B. Effect of soil pH on bacteria CFU g^{-1} soil 24 h^{-1}. *** Indicates significance at $P<0.01$.

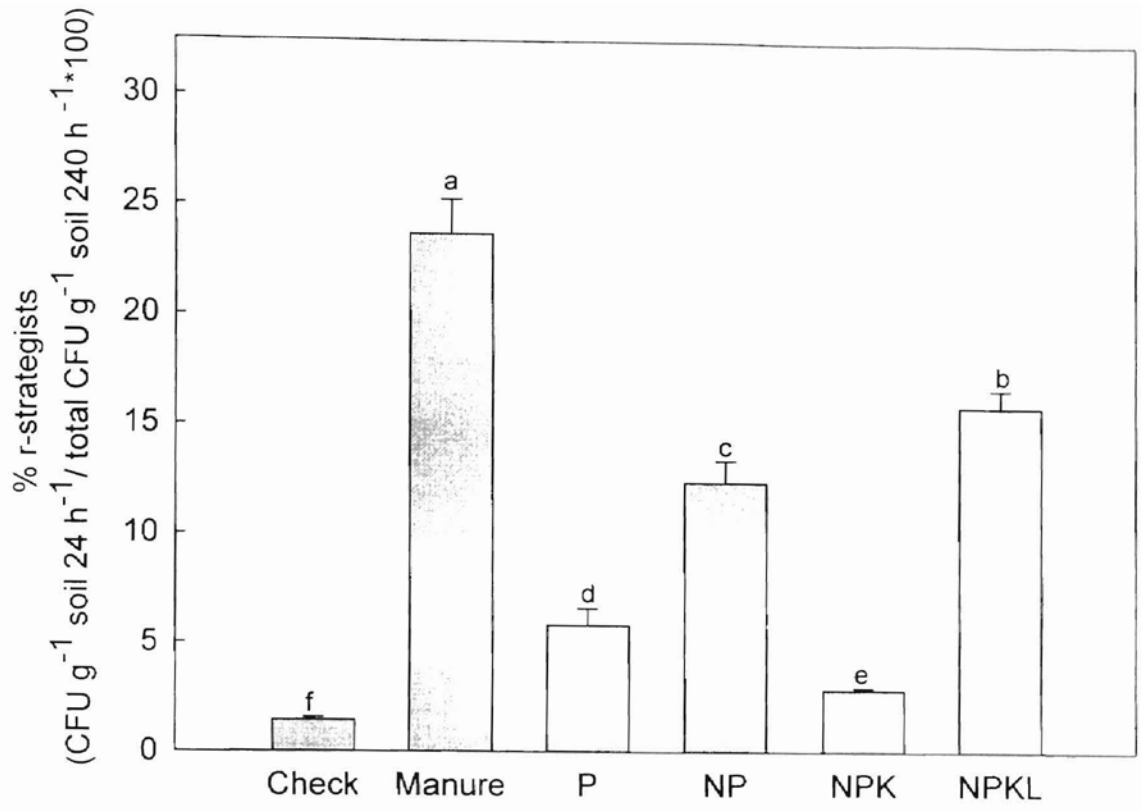

Figure 4. Bacterial counts in 24 h of incubation expressed as percentage of total bacteria CFU in 10 days (% r-strategists). Different letters indicate significantly different means at $P<0.05$ according to least significant difference test.

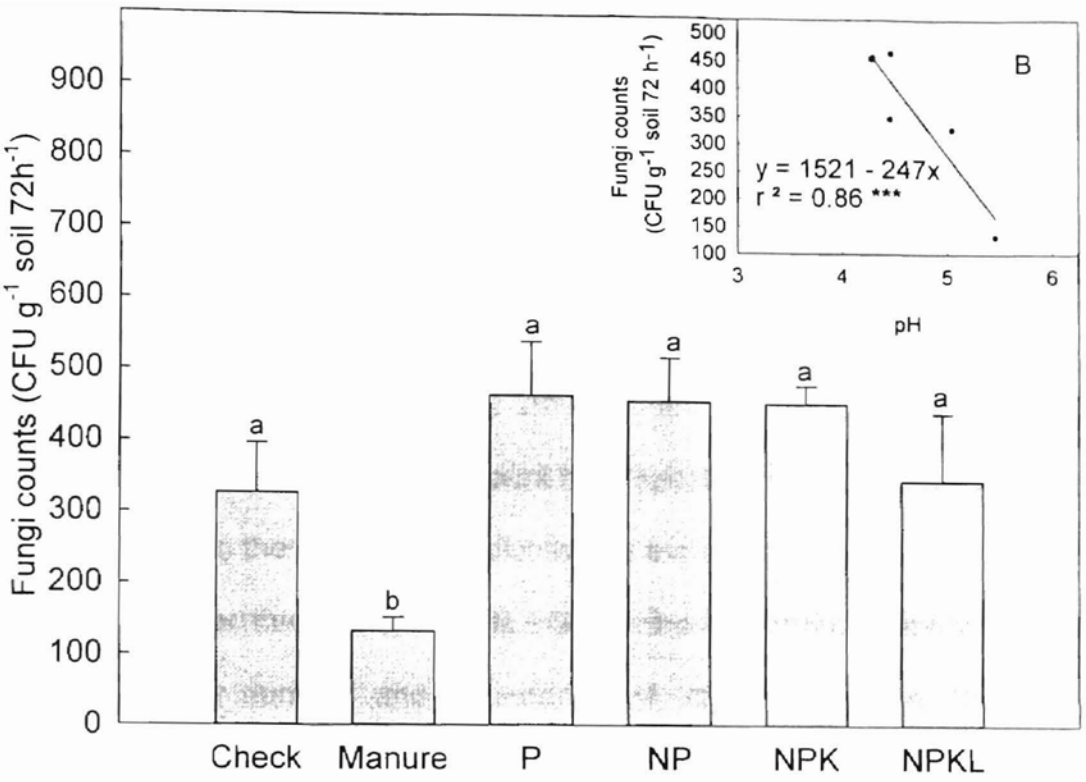

Figure 5. Total fungal population enumerated after 72 h of incubation. Different letters indicate significantly different means at $P<0.05$ according to least significant difference test. B. Regression between soil pH and total fungal colonies formed. *** Indicates significance at $P<0.01$.

of microbial cells which can be observed with a microscope can be recovered from soil using plate count procedures (Zuber, 1994). Therefore, microbial counts using the plate count method are not of total bacteria and fungi, they are of total recoverable CFU (Zuber, 1994). As long as samples are treated uniformly, plate counts are useful tools for comparing the effects of various soil treatments on recoverable bacteria and fungi, as well as the diversity of bacteria and fungi, under the conditions of the experiment (De Leij et al., 1993; Zuber, 1994).

One way to describe community diversity is by using the Shannon diversity index. The Shannon diversity index (Shannon, 1948) is a measure of community diversity that takes into account both species richness and evenness. The higher the index, the more evenly distributed the species within a community are and the healthier that community is. Since the Shannon diversity index is calculated from the number and abundance of different species, the name diversity index is not appropriate for quantifying classes of bacteria with similar developmental characteristics on agar plates. Therefore, the term eco-physiological index (EP-index) is used (De Leij et al., 1993). In this study, the EP-indices indicate the evenness of the amount of culturable bacteria with similar developmental characteristics appearing on each of the six enumeration days for each soil treatment. The higher the indices, up to a maximum of 0.816, the more even the distribution of groups.

The NPK-treated soil exhibited a lower EP-index and a lower percentage of r-strategists than expected. This can be explained as a result of amensalism.

Exudates were noticed on NPK plates during counting that appeared to prevent the formation of additional colonies on those plates. As is characteristic of K-strategists, the colonies that were formed after 10 days incubation may have been resistant to these toxins (De Leij et al., 1993).

Although a significant difference in EP-indices was only detected in the NPK-treated soil, differences in growth patterns during the 10 days of incubation were noticed. The manure-treated soil had two distinct growth peaks, at day 2 and day 5. The other soils only experienced one major growth peak. This is possibly due to less uniformity in manure versus chemical fertilizers. Since bacterial communities can change over short distances and increasingly diverse environments result in more ecological niches that can be filled, the number of groups of microorganisms that can be expected to be present in a diverse environment is greater than in a more uniform environment (De Leij et al., 1993).

The growth strategy of bacteria can also be use to determine microbial community structure. Growth strategists can be split into two classes; r-and K-strategists. R-strategist organisms proliferate in uncrowded, nutrient-rich environments, but they are often inefficient at metabolizing recalcitrant substrates (Luckinbill, 1978; De Leij et al., 1993). K-strategists, on the other hand, do well in environments that have reached their carrying capacity, are more efficient at breaking down recalcitrant substrates, and are less sensitive to toxins (Luckinbill, 19778; De Leij et al., 1993). Results from this study indicated that with the exception of the NPK-treated soil, each additional substance added to the chemical treatments increased the percent of r-strategists. The manure-treated

soil had increased total culturable numbers and percent of r-strategists when compared to the chemical treatments. Based on the definition of r-strategists, these results indicate that manure is either utilized by bacteria more easily than chemical fertilizers, there is less competition in the manure-treated soil, there are fewer substances that are toxic to bacteria in the manure-treated soil, or a combination of the three.

Differences in total bacteria and fungi CFUs enumerated after 10 days may have been a result of a pH effect. The positive relationship between pH and bacteria and the negative relationship between pH and fungi may have contributed to the increased total bacteria counts and extractable DNA and the decreased total fungi counts in the manure-treated soil. These results are consistent with Pennaned (2001), Staddon et al. (1998), Lei and Vandergheynst (2000), and Kelly et al. (1999) that reported changes in pH were at least partially responsible for changes in microbial community structure.

Decreased fungal counts in manure-treated soil may be due to additional effects besides pH. It was noticed that one group of morphologically distinct fungi was dominant on most manure plates and absent on most plates from the other soils. Further studies are needed to determine if these were the only fungi that could survive in the soil environment created by manure additions, or if manure additions increase the growth of these fungi allowing them to out compete fungi present in the other soils tested.

In conclusion, results from this study indicate that cattle manure application promotes bacterial growth, but may suppress fungal populations in

soil. These results may be useful for further understanding the effects of long-term cattle manure and chemical fertilizer applications on soil biology and nutrient cycling.

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Chapter VI

SUMMARY AND CONCLUSION

To meet the world's increasing demand for food, agricultural production must continue to increase while cautions are exercised to preserve the environment and sustain agricultural production. By being raised in an agricultural setting, I am aware that many farmers deeply care about the environment while manage to remain financially competitive. Agricultural scientists are, therefore, obligated to assist agricultural producers to develop management systems that preserve the environment while allow producers to profit.

If the environmental impact resulting from agricultural practices are to be minimized, techniques for reducing chemical applications, such as biological control, need to be further developed and the most ecologically sound methods for handling animal waste from concentrated animal feeding operations needs to be determined. This is a large task requiring cooperative efforts from scientists in many fields. This study is just a small corner of the research needed to integrate agriculture into an environmentally friendly process. Based on the objective proposed in this study, results indicated that

1. the activity of β-glucosaminidase in soils can be detected and quantified with the simple colorimetric method developed;

2. it appeared that β–glucosaminidase in the two soils tested originated from similar sources and demonstrated similar values of pH optimum and energy of activation;
3. animal manure application may promote biological activities and activities of enzymes involved in P transformation in soil;
4. cattle manure application increased r-strategist bacteria as well as total bacteria, but decreased the total number of fungi present in the soil; and
5. the quantity of DNA recovered appeared to correlate well with the recoverable microbial populations in the soils tested.

Hopefully, these findings will aid in the advancement of biological control and provide government regulators beneficial information for creating animal waste disposal guidelines. With cooperative efforts from farmers, researchers, and lawmakers, the impact of agriculture on the environment can be minimized.

APPENDIX

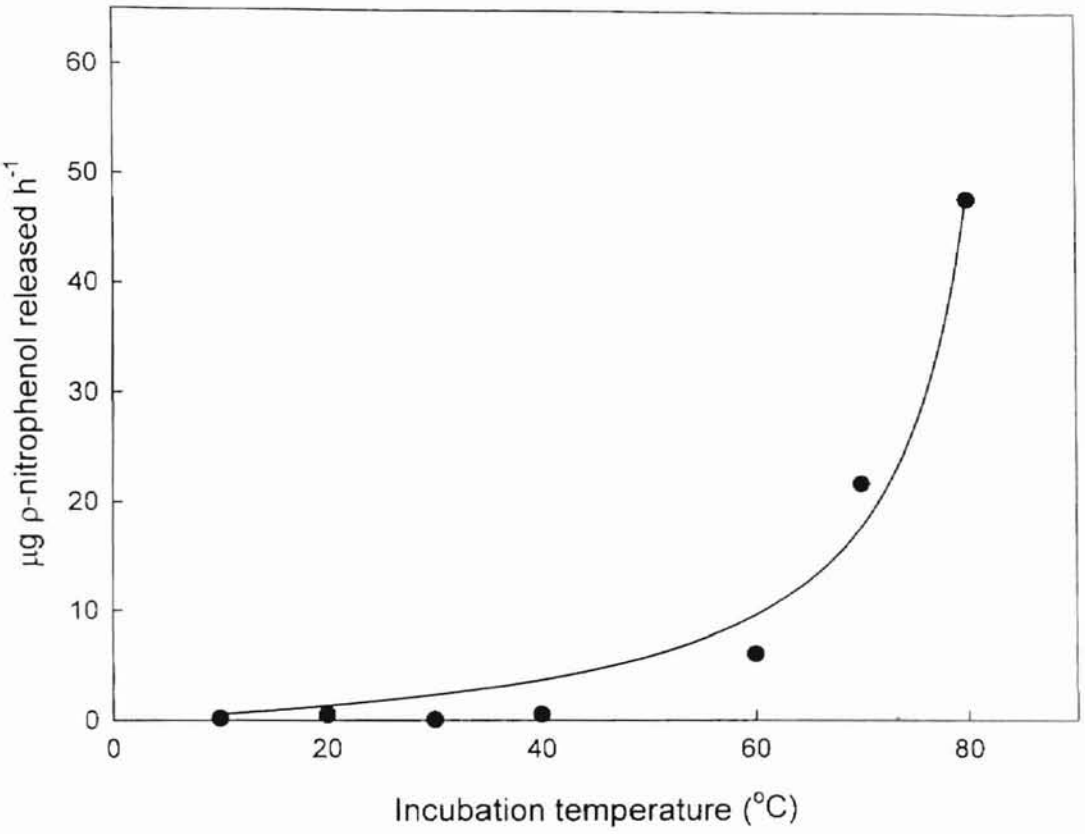

Figure 1. Effect of incubation temperature on autohydrolysis of ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

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