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Activity Of Soil Enzymes In Constructed Wetlands Treated With Swine Wastewater

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Dedication

This thesis is dedicated to my parents, Linga Reddy Baddam and Bhudevi Baddam and my sister Niveditha Baddam for their love, support and encouragement.

Biographical Sketch

Ramgopal Baddam was born on June 15, 1984 in Nizamabad, Andhra Pradesh, India. He received his Bachelor's degree in Agricultural Sciences from Acharya. N.G. Ranga Agricultural University, India in 2009. He joined the Master's program in Plant, Soil and Environmental Science in North Carolina Agricultural and Technical State University in the Fall of 2009. He conducted research on the activity of soil enzymes in constructed wetlands treated with swine wastewater. He presented a poster on his thesis research at the American Society of Agronomy Annual Meeting (Oct. 16-19, 2011) in San Antonio, Texas.

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List of Nomenclature

SWE	Swine Waste Effluent
CW	Constructed Wetland
CM	Continuous Marsh Wetland
MPM	Marsh Pond Marsh Wetland
TC	Total Carbon
TN	Total Nitrogen
MBC	Microbial Biomass Carbon
Org N	Organic Nitrogen
TPF	Tri Phenyl Formazan
TTC	2, 3, 5 Triphenyltetrazolium Chloride
THAM	Tris Hydroxymethyl Amino Methane
MUB	Modified Universal Buffer
PNG	P-nitro Phenol β-D-glucoside
PNS	P-nitro Phenyl Sulfate
PNP	P-nitro phenol

Abstract

Increased swine production in North Carolina has resulted in higher waste production. Continuous application of swine wastewater from lagoons to agricultural land can pose surface and ground water pollution. Constructed wetland (CW) treatment is an alternate to the lagoon spray field system that reduces the nutrients concentration through physical, chemical and biological mechanisms. One of the biological processes in the CW is enzymatic activity which plays a major role in releasing nutrients from organic substances.

The objectives of this research were to investigate the activity of soil enzymes at different depths of CW treated with swine wastewater and to assess the relationship between the enzyme activity and nutrient concentration. One continuous marsh (CM) and one marsh-pond-marsh (MPM) wetland cells were studied, which were in operation for the last ten years treating swine wastewater.

The activities of dehydrogenase, urease, phosphatase, arylsulfatase, and β-glucosidase were significantly higher in the soil surface layer (0-3 cm) than lower depths (6-12 cm). Enzyme activities were higher in marsh soils of CM than pond soils of MPM. There was no significant difference in enzyme activity between inlet and outlet of CM and MPM. No significant relationship was found between the enzyme activity and nutrient concentration. Urease, phosphatase and arylsulfatase activity were correlated to soil C and N, whereas, β-glucosidase activity was correlated to soil C. The results suggest that lower enzyme activity is required for these wetlands to achieve high nutrient removal efficiency.

Keywords: Enzyme activity, Swine wastewater, Constructed wetlands.

CHAPTER 1

Introduction

1.1 Overview

United States of America is among the largest producers, consumers and exporters of pork products of the world. The number and size of concentrated animal operations in the USA makes the treatment of manure generated by these operations a challenge. This is particularly true in North Carolina, which ranks 2nd in the entire nation in swine industry, where the disposal of animal manure impacts water quality (Mallin, 2000). According to the 2002 National Agricultural Statistics, 16% of all USA hogs are produced by North Carolina. The number of hogs in North Carolina has increased from 2.8 million in 1990 to more than 9 million in 1996 (USDA-NASS, 2004). The growth in the swine industry has been leveled off since 1997 because of governmental regulations and laws. There are now more than 3,000 swine units in North Carolina and the majority are located in Duplin and Sampson counties. Although, decreasing a number of swine operations, the number of swine units has increased to ten million. The conversion from small independent swine farms to large swine industrial operations has create a need for innovative waste management systems. In general, the lack of effective treatments and disposal systems of swine waste effluent (SWE) is a current issue. Currently, main hog operations flush SWE into an anaerobic lagoon, and lagoon wastewater is broadcasted over the land surface. Currently approximately 4,000 active anaerobic lagoons and 1140 inactive lagoons exist. Although these methods meet the federal and state standards, wastewater application on land has lead to the accumulation of nitrogen (N) and phosphorus (P) in fields to levels of concern.

One of the methods to treat swine waste is using constructed wetlands (CWs) with

vegetation reduce the nutrient concentration prior to land application. CWs provide an efficient ecological system with low maintenance requirements and construction costs to remove nutrients from animal wastewater (Hill et al., 1999; Kadlec & Knight, 1996). CW systems are commonly used to treat domestic sewage, industrial or municipal wastewater and agricultural runoff (Kadlec et al., 2000; Robert H, 1999). Wetland technology removes excess nutrients from wastewater by the process of sedimentation, adsorption, organic matter accumulation, microbial assimilation, nitrification-denitrification, and ammonia volatilization (Brix, 1993; Johnstone, 1991). Wetland systems and their applications to remove nutrients from point and non-point pollution sources are gaining considerable international interest (Gopal, 1999; Sakadevan & Bavor, 1998, 1999). CWs have successfully treated large amounts of animal manure inflows (Knight et al., 2000). They have successfully treated animal wastewater prior to land application and reduced nutrient concentrations applied to crops and pastures (Knight et al., 2000; Reddy et al., 2001). Previous studies have shown that 70-75% of the nitrogen (N) and 40-45% of the phosphorus (P) are removed from swine wastewater when CWs are treated with 3 to 36 kg N per ha per day (Hunt et al., 2002; Poach et al., 2004; Reddy et al., 2001). CWs receive nutrients and organic matter from wastewater and in winter month's detritus from vegetation. Thus, the decomposition of organic matter is important in CWs, which depends on microbial enzymatic activities.

During the treatment of wastewater in CWs, high molecular weight organic compounds are degraded to low molecular weight organic compounds by the metabolism of microorganisms (Brix & Schierup, 1989) and the enzyme activity of the soil (Kang et al., 1998; Martens et al., 1992). Microbial enzymes play a major role in the process of decomposition of organic matter (Tabatabai, 1982). Soil enzyme activity includes enzymes excreted by microorganisms as part of

extracellular metabolism and enzymes immobilized on soil colloids and humic materials (Burns, 1982). Enzyme activity in constructed wetlands is affected by many factors, including biological factors (microbial populations, higher taxa, and fauna), soil factors (pH, texture, nutrient composition, depth profiles, organic matter content, etc.) and climatic factors (Duarte et al., 2008; Reboreda & Cacador, 2008; Zaman et al., 1999). Shackle et al., (2006) reported that addition of exogenous enzymes to CWs will increase the biodegradation processes. Microbial decomposition plays a major role in macronutrient cycles and energy flows of aquatic ecosystems (Wetzel, 1992). In wetlands, the continual availability of nutrient resources depends upon the microbial degradation of organic matter (Aerts & de Caluwe, 1997; Alvarez & Guerrero, 2000; Shaver & Melillo, 1984; Talling & Lemoalle, 1998). The rate of decomposition controlled by extracellular enzymes has long been identified (Schimel & Weintraub, 2003).

Most of the organic matter in wetlands is composed of high molecular weight compounds, of which a small portion is readily available to the microorganisms (Benner et al., 1984; Chrost, 1991). Complex organic compounds in wastewater such as proteins, carbohydrates, lipids and their derivatives are initially hydrolyzed by extracellular enzymes produced microbially. After extracellular enzyme hydrolysis, low molecular weight compounds are formed and utilized by microorganisms as an energy source (Chrost, 1991). Therefore, the role of extracellular enzymes in the decomposition process provides valuable information about the cycling of nutrients in ecosystems. Many researchers have recognized that the interaction between substrate, wetland plants and microorganisms in wetlands plays a major role in wastewater purification (Hammer, 1989; Reddy, 1983).

Enzymes in soil are present extracellularly or intracellularly. Location of soil enzymes and their interaction with living and non-living substrates are shown in Figure 1.

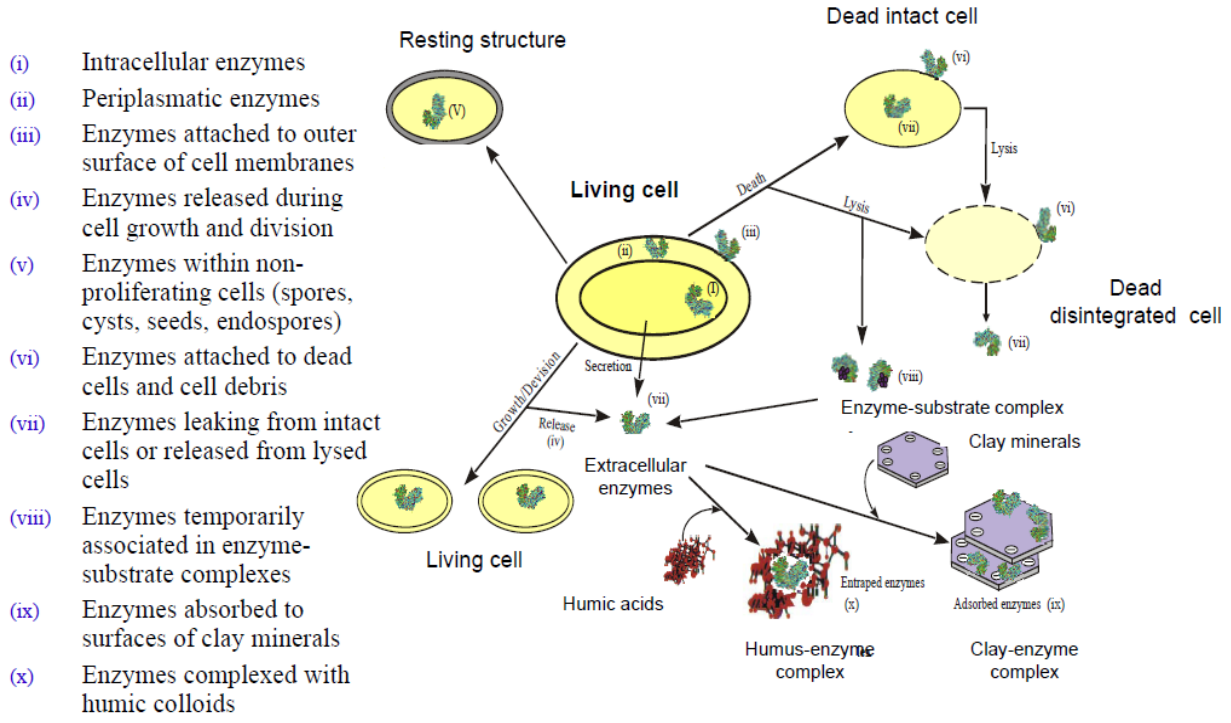
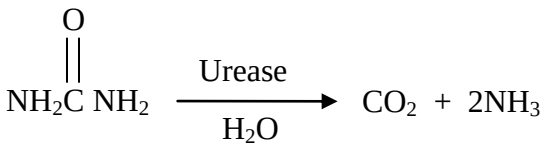


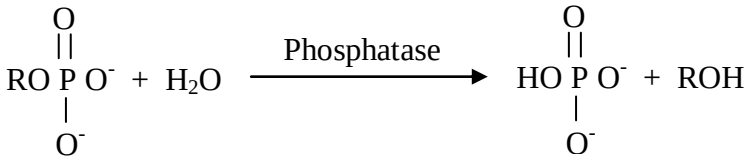
Figure 1. Location of Enzymes in Soils (Burns, 1982; Nannipieri et al., 1994).

In this research of CWs, we studied the activity of dehydrogenase, urease, arylsulfatase, phosphatase and β- glucosidase in soil samples collected at various depths. These enzymes are involved in metabolic processes such as C cycling (β- glucosidase, catalyzes the final step of cellulose degradation), N cycling (urease, that catalyzes the hydrolysis of urea in to carbon dioxide and ammonia), P cycling (acid Phosphatase, hydrolyzes organic-P compounds), S cycling (arylsulfatase, hydrolyzes the organic S compounds).

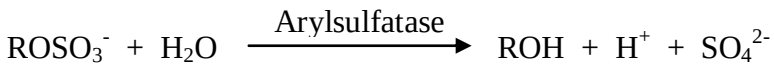
1.1.1 Urease. Urease is the enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia. The urea or uric acid in swine wastewater originates from swine urine. Soil urease originates from plants (Polacco, 1977) and microorganisms found intra and extracellularly (Blakeley & Zerner, 1984; Burns, 1986; Mobley & Hausinger, 1989; Mulvaney & Bremner, 1981)extracellularly (Blakeley & Zerner, 1984; Burns, 1986; Mobley & Hausinger, 1989; Mulvaney & Bremner, 1981). The urease enzymatic reaction is:



1.1.2 Phosphatase. Phosphatase is an extracellular hydrolytic enzyme that plays an important role in the formation of inorganic phosphorus from organic phosphorus esters (Chrost, 1991). Indigestible phosphorus is excreted by hogs and ends up in wastewater. The phosphorus in the excreta is in the organic and inorganic form. Phosphatases are enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid (Schmidt & Laskowski 1961). This enzyme plays a critical role in the P cycle (Speir & Ross, 1978). Bacteria, fungi and yeast produce phosphatases and their reaction differs depending on the substrate (Hollander, 1971). The phosphatase enzymatic reaction is:



1.1.3 Arylsulfatase. Arylsulfatase is responsible for the hydrolysis of organically bound sulfate esters (Tabatabai & Bremner, 1970). Arylsulfatase is an extracellular hydrolytic enzyme that plays a key role in the formation of inorganic sulfate ions from organic sulfate compounds. It is important to the S cycling processes in wetland soils. They are produced by soil bacteria and in response to sulphur limitations (McGill & Cole, 1981). The arylsulfatase enzymatic reaction is:



1.1.4 β-glucosidase. β-glucosidase catalyzes the hydrolysis of sugars resulting in the formation of β-linked monosaccharide (Eivazi & Tabatabai, 1988). This enzyme plays an important role because it is involved in the biodegradation of various β-glucosidase present in soil organic matter (Ajwa & Tabatabai, 1994; Martinez & Tabatabai, 1997). The final product of the enzymatic reaction is glucose which is an important carbon energy source for soil microorganisms (Esen, 1993).

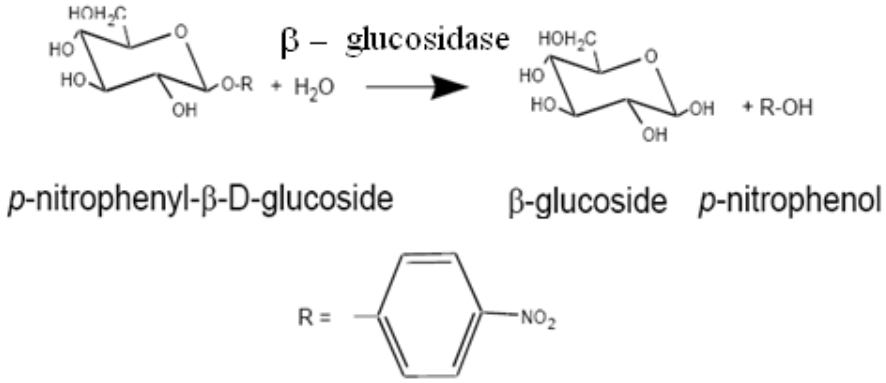


Figure 2. Reaction of β-glucosidase enzyme.

1.1.5 Dehydrogenase. Dehydrogenase activity is commonly used as an indicator of microbial activity in soils (Burns, 1978). This enzyme oxidizes soil organic matter by transferring electrons and protons from substrates to acceptors. This process is part of the respiration pathway of soil microorganisms and its activity is strongly related to soil type and soil air-water conditions (Doelman & Haanstra, 1979; Glinski & Stepniewski, 1985; Kandeler, 1996). Thus, dehydrogenase activity is very important to support microbial biochemical processes in soil.

These enzymes are involved in major nutrient cycles such as C cycling (β- glucosidase), N cycling (urease), P cycling (Phosphatase), and S cycling (arylsulfatase). So the above enzymes are important in our wetland study.

Table 1

Role of soil enzymes in decomposition of organic matter and nutrient cycling

Enzyme	Organic matter substances acted on	End product	Significance	Predictor of soil function
Urease	urea nitrogen	ammonia (NH_3) and carbon dioxide (CO_2)	plant available NH_4	nutrient cycling
Phosphatase	organic phosphorus	phosphate (PO_4)	plant available P	nutrient cycling
Arylsulfatase	organic sulfur	Sulfate (SO_4)	plant available S	nutrient cycling
β-glucosidase	carbon compounds	glucose	energy for microorganisms	organic matter decomposition

1.2 Hypothesis

1. Enzymes will act on complex organic compounds, decompose them into smaller molecules and release nutrients from organic matter.
2. Enzyme activity is occurs in the high concentration of nutrients found in CWs.
3. Enzyme activity has correlation with nutrients concentration, C and N content in soil.
4. Enzyme activity will differ in marsh area from the pond area of wetland cell.

1.3 Objectives

The objectives of this research were:

1. To study the activity of dehydrogenase, phosphatase, urease, β-glucosidase, arylsulfatase and measure the microbial biomass carbon (MBC) content in several soil depths of CWs treated with swine wastewater.
2. To correlate the specific enzymatic activities to total carbon (TC), total nitrogen (TN), NH_4, and PO_4 ions.

3. To investigate the differences in enzymatic activity in the pond area with marsh areas of marsh pond marsh (MPM) and continuous marsh (CM) constructed wetlands.

1.4 Justification

Waste effluent from concentrated swine operations is commonly stored and treated in anaerobic lagoons, and the liquid collected is sprayed on nearby fields. If nutrients in this liquid are in excess of crop uptake rates, they may seep through the surface into ground water or in high rainfall event nutrients may be subject to runoff and contaminate surface waters. To reduce the contamination of surface and ground water, alternative methods of treating wastewater should be implemented. One of the options under consideration is using CWs to reduce the nutrient concentration of swine wastewater before land application. During the treatment of wastewater in CWs, high molecular weight organic pollutants are degraded to low molecular weight compounds and inorganic products. This is mainly achieved by the metabolism of microorganisms and enzyme activity in soil. CWs have a living dynamic system containing many free enzymes such as immobilized extra-cellular enzymes and intra-cellular enzymes. The CWs treated with swine wastewater receive organic solids and nutrients in inorganic form. Also, in winter months vegetation will senesce and becomes part of the organic matter referred as detritus. Therefore, the nutrient availability in such wetlands is from wastewater content, decomposition of plant residue, and decomposition of organic solids. Previous studies (Poach et al., 2004; Reddy et al., 2001) have indicated that 70-75% of N and 40-45% of P is removed in these wetlands. However, the complete removal of these nutrients was not achieved because of the continuous decomposition that occurs in these wetlands. However, there is not much data available on enzymatic activity in CWs treated with swine wastewater. Also, no data exists in the literature to correlate the enzyme activity with nutrient concentration in highly nutrient loaded

CWs. The enzymatic approach represents a valuable method to assess decomposition processes in wetland sediments, and that characteristically low enzyme activities in the sediments may be important in the water quality amelioration function. Therefore, it is important to understand the enzymatic activities in different depths of CWs receiving high concentration of nutrients through swine wastewater application and how this work can be related to improve the efficiency of nutrient removal in such wetlands.

If these enzymes activity in wetland soils is higher, it is more likely that more nutrients are released in the system by microorganisms and therefore, less efficiency of wetlands can be observed. However, these CWs are very complicated systems having continuous flow of wastewater in to the CW carrying organic solids, nutrients, and microbial populations. In normal CW ecosystems treating wastewater will have low concentrations of nutrients and microbial populations and diversities. Whereas, in these CWs, not only high concentrations of nutrients input but also higher populations and diversity of microorganisms input into the system. Therefore, it is very essential to understand, if there is any relationship between enzymatic activities and nutrients concentration.

CHAPTER 2

Literature Review

2.1 Constructed wetlands (CWs)

CWs are constructed basins that contain stable water all over the year (at least during wet season) and differ from natural wetlands having shallow depth and great vegetation coverage. CWs are engineered systems designed and constructed to make use of natural processes such as vegetation, soils, and their related microbial activities to treat domestic wastewater, industrial wastewater and agricultural runoff. CWs work as alternative to conventional system for nutrient management to reduce nutrient concentration in agricultural wastewaters before application to the land (Hunt & Poach, 2001; Knight et al., 2000). CWs offer different mechanisms to improve water quality which include biochemical transformations, settling of suspended particulate matter, adsorption/desorption processes and absorption by plants and microbes (immobilization).

Two basic categories exist for the design of the CWs, surface flow (SF) and sub surface flow (SSF) wetlands. The surface flow wetlands have shallow depths over a rooting matrix supporting macrophytes. Because of the density of vegetation and configuration, surface flow wetlands contain shallow flow and low velocity. Surface flow CWs (SF) are parallel to the natural environments due to the permanent standing water and environment favorable for plant species (Scholz & Lee, 2005). In surface flow systems water depths are less than 0.4 m (Kadlec & Knight, 1996). Sub surface systems are classified by the direction of water flows either horizontally (horizontal sub surface flow constructed wetlands HSSFCW) or vertically (vertical) through the porous filter material. In sub surface systems water flows below the rooting matrix and they contain gravel of different diameter. Surface flow CW systems (SF) are less abundant than sub surface flow systems, even though free water surface wetlands are one of the oldest

designs in Europe (Vymazal, 2005). For efficient purification of wastewater hybrid systems have been introduced that consist of different types of constructed wetlands constructed in sequence (Vymazal et al., 2008). However, in USA for animal wastewater treatment surface flow CW systems have been preferred (Knight et al., 2000).

2.2 Vegetation role in treatment wetlands

In CWs, plants provide a substrate and a carbon source for microbes. Wetland plants increase the aerobic portion by oxygenating the substrate adjacent to their roots (Brix, 1993) and additionally during the growing season they remove nutrients from the incoming wastewater. A variety of plant species have been recommended for use in animal wastewater treatment wetlands. Desirable species are native so that they best suited to local conditions and they should have high productivity for rapid nutrient uptake, rhizome production and colonization. Desirable species should be able to tolerate high nutrient concentrations. Cattails and bulrushes are commonly used for treating wastewater in CWs.

Plants play an important role in purification of wastewater in CWs. Plants directly influence the soil enzyme activity by excreting organic compounds and exogenous enzymes and there by affect species composition and diversity of microbes. Also, plant roots release exudates and oxygen in to the rhizosphere for microbial proliferation. Wetland plants immobilize nutrients from the wastewater. The uptake of nutrients ranged from species to species of wetland plants. The uptake rates are from 2 to 10% of applied nutrients through wastewater (Reddy et al., 2001). Reboreda and Cacador (2008) reported positive correlation between root biomass and rhizosphere sediment enzyme activity of five enzymes. In CWs plant biomass residue undergoes decomposition by extracellular enzymes and mineralization of organic matter will occur. Purification in CWs is based on combined action between microbes and filter material which is

provided by plants. In wetlands, organic carbon mainly supplied by the vegetation and which is used as the carbon and energy source by microbes. Plant tissue also provides a large amount of surface area for microbial growth. When plants die litter decomposition leads to the mobilization of metals and their return to sediment and their rate depend upon the type of tissue and plant species (Windham et al., 2003). Wetland plants contribute removal of metals through important processes such as substrate stabilization, rhizosphere oxidation and supply of organic matter for microorganisms (Kosolapov et al., 2004). Wetland plants interact with microorganisms in the rhizosphere by supplying organic matter required for appropriate reactions and thus provide suitable environment for nutrients cycling (Jacob & Otte, 2003).

2.3 Nutrient removal in wetlands

Nutrients and solids removal in wetlands is mainly achieved by shallow water (which maximizes the sediment to water interface), accumulation of litter, high primary productivity, and the presence of aerobic and anaerobic sediments (Mitsch & Gosselink, 1993). Additionally slow water flow causes to settle suspended solids from the water column in wetlands. The most important constituents in animal wastewater are N, P, and total solids and these can be reduced by using CWs with vegetation (Poach et al., 2004; Reddy et al., 2001).

2.3.1 Nitrogen removal. Nitrogen enters in animal wastewater treatment wetland as organic and inorganic forms. The inorganic forms are nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) and ammonium (NH_4^+).

Nitrogen may be lost from the wetland system through volatilization, plants or microbes immobilization, adsorption, nitrification and denitrification processes. Ammonia volatilization is a process of removal of N in wastewater, but it causes pollution to the environment (Asman, 1994). Research showed that ammonia volatilization accounted for less than 20% of the nitrogen

removal when Marsh Pond Marsh CWs were treated with swine wastewater (Poach et al., 2002). Nitrate and nitrite are removed from wastewater by plant uptake or denitrification (Gambrell & Patrick Jr, 1978). The major removal pathway of N in wetlands receiving wastewater is by denitrification, which convert wastewater N to gaseous form of N. Hammer (1994) reported that when wetland nitrate is limited, denitrification often times affected by nitrification. The removal of N from swine wastewater accounts less than 10% of the N load by plant accumulation (Hunt et al., 2002; Poach et al., 2004a; Reddy et al., 2001).

Several scientists reported that CWs were efficient in removing N, P, COD and TSS (Hunt et al., 1995; Payne et al., 1992; Reddy & De Busk, 1985). Nitrogen removal occurred at 51% and 36% when 16 and 32 kg N ha^{-1} day^{-1} was applied through swine wastewater respectively (Reddy et al., 2001). Hunt et al (2002) reported that CWs removed 75 to 50% nitrogen from the swine wastewater when they received nitrogen loads between 3 to 36 kg per ha per day. The average mass reduction of TKN was in the range of 46 to 72% (Hammer & Knight, 1994). Poach et al. (2004) reported that when wetlands were loaded at the range of 2-52 N kg/ha/day and the total N removal efficiency was in the range of 10 to 75%.

2.3.2 Phosphorus removal. Wetlands have a tendency to retain phosphorus through physical (Sedimentation and adsorption), chemical (Precipitation) and biological processes (Plant and microbial uptake) (Gale et al., 1994; Reddy et al., 1999). A decrease in contaminants is observed whenever a deposition of solids (e.g. phosphorus) takes place (Johnston et al., 1984). Bioavailable forms of phosphorus may be sorbed on to soil particles or taken up by plants. Phosphorus adsorption on soil matrix is the main process for phosphorus removal in wetlands (Richardson, 1985).

In CWs, inputs of phosphorus continue over a period of several years, Sorption sites in

the sediments become increasingly unavailable and ultimately lead to phosphorus release (Cyrus & Reddy, 2010; Kadlec, 1985). Plant uptake and decomposition of organic phosphorus increases with increase in the temperature. In growing season retention of phosphorus increase have been observed at domestic wastewater treatment wetlands (Gearheart et al., 1989). Addition of aluminum to the substrate also increases phosphorus removal (James et al., 1992; Reddy et al., 2011). Phosphorus adsorption is positively correlated to aluminum content in the substrate (Richardson, 1985). Periodic draining can allow oxidation and recharge sorption sites for greater phosphorus removal than under permanently reduced conditions (Faulkner & Richardson, 1989). CWs have used other techniques such as limestone, slag, wollastonite, calcite, shale, iron rich gravel and zeolite to improve the removal processes of precipitation and absorption (Brix et al., 2001). Shale based wetlands had 98 to 100% P removal capacity (Mann, 1997). Fisher (1998) reported 20% P removal by vertical-flow constructed gravel wetland treating urban wastewater.

2.4 Enzyme activity in wetlands

The accumulation of organic matter is a characteristic feature of both natural and CWs (Tanner et al., 1998). Accumulation of organic matter in soils provides long-term storage of carbon and nutrients (e.g. Nitrogen and Phosphorus). Nutrient retention function in wetlands closely related to the accumulation of organic matter and which is based on biological processes. Soil enzyme activity is important in CWs and is sensitive to changes in soil micro-environmental conditions such as soil pH, temperature, plant exudates and soil water chemistry (Shackle et al., 2000). Enzymes activities are useful indicators of microbial activity in a given ecosystem (Ravit et al., 2003; Sinsabaugh, 1994).

Enzymes present extracellularly in sediments by attached to clay minerals or complexed with humic colloids (Burns, 1982). Extracellular enzymes excreted by fungi and bacteria, to

metabolize the humic compounds by reducing their molecular weight and increase the solubility and mobility of potential carriers of heavy metals (Gramss et al., 1999). The maximum potential activity of enzymes occurring in sediment rather than the actual enzyme activity in water (Alef & Nannipieri, 1995). Microbial degradation of high molecular organic matter is initiated by extracellular hydrolysis (Marxsen & Witzel, 1991) and is completed intracellularly. Therefore extracellular enzymes play a vital role as initiators of organic pollutant removal in treatment wetlands (Kang et al., 1998). CWs supplemented with extracellular enzymes as a way to increase the rate and efficiency of soil decontamination (Cervelli & Perret, 1998) and waste treatment (Duran & Esposito, 2000; Michael D, 1993). Soil enzyme activity proposed as an important determinant of soil quality (Alef & Sparling, 1995) and water quality improvement in agricultural and wetland systems respectively (Freeman et al., 1997; Kang et al., 1998). Enzyme activity (activity on a per gram of soil basis) depends on both total microbial biomass and enzyme efficiency (enzyme activity on a per microbial biomass basis).

2.5 Soil enzymatic activities in CWs

Kong et al. (2009) studied the enzyme and root activities in wetlands constructed for domestic waste water purification by planting four different plant species and the correlations between contaminant removal and soil enzyme activity, root activity, and the plant growth were obtained. It was observed that the removal efficiency of NH_4^+ was significantly correlated with both urease and protease activity in all wetlands, and the removal of total phosphorus (TP) and soluble reactive phosphorus (SRP) was significantly correlated with phosphatase activity in most wetlands, while the removal of TN, NO_3^-, and chemical oxygen demand (COD) was significantly correlated with enzyme activity in a few instances. This strong correlation between root activity and enzyme activity indicated that plant root activity can affect enzyme activity, and that plant

roots have important effects on contaminant removal. Kang, et al. (1998) reported that soil enzymatic activities would be lower in wetland sediment than adjacent uplands.

Zhang and co-workers (2010) focused on the relationship between plant diversity (six species richness levels) and nutrient retention and enzyme activities associated with carbon, nitrogen and phosphorus cycling in a full-scale CW fed with post treatment domestic wastewater. They observed retention of NH_4 and NO_3 in the wetland substrate increased with increasing species richness, while phosphorus retention significantly decreased the richness level of 16 species per plot. It was also reported that activities of dehydrogenase, β-glucosidase, invertase, phenol oxidase, protease and nitrate reductase were affected by plant species richness, were strongly depended on the presence or absence of plants, while activities of urease and acid phosphatase were strongly affected by plant species richness. These findings suggest that by manipulating the quantity and quality of carbon supply in CWs, it may be possible to modify extracellular enzyme activities in order to maximize the efficiency of water treatment (Shackle et al., 2000). Microbial abundance and enzyme activities in the rhizospheres of nine plant species were investigated in an vertical-flow CW by Ge et al. (2011). They reported that the abundance of denitrifiers, as well as urease, acid, and alkaline phosphatase activities were positively correlated to plant root biomass and significant differences in rhizospheres enzyme activity among plant species were also observed.

2.6 Soil enzymatic activities in other agricultural eco-systems

Sustainable management practices in agriculture, including crop rotations and fertilization systems have significant effects on soil biochemical processes. Long-term cropping systems and fertilization can influence important soil properties such as soil structure and density, pH, the quantity, quality, and distribution of soil organic matter and nutrient cycles

within the soil profile. Moore et al. (2000) reported that highest enzyme activities, microbial biomass carbon and nitrogen contents are found in multicropping systems involving oats or meadow, and the lowest in continuous corn and soybean systems. Bergstrom et al. (1998) reported that the activity of dehydrogenase, phosphatase, and arylsulfatase was greater in no-tilled than in conventionally tilled fields. Soil urease, acid phosphatase and protease activities in the top 0-10 cm depth, and acid phosphatase, dehydrogenase, arylsulfatase, invertase, amidase and urease in the 0-7.5 cm depth were significantly higher in soil subjected to no-tillage than in ploughed ones (Dick, 1984; Doran, 1980; Klein & Koths, 1980). Verstraete and Votes (1977) showed that application of animal manure plus green manure increased phosphatase, urease, and saccharase and β- glucosidase activities over a 7-year period and that enzyme activity was related to soil organic matter. Decomposition of carbon substrates under anaerobic conditions of flooded soils is generally slower than in upland soil (Tate, 1979). Hu et al. (1999) and other short term studies have shown that organic amendments increase microbial biomass. Long-term cultivation in the absence of organic amendments causes decrease in organic C and total N content (Dick, 1992; Tate, 1987).

According to Acosta-Martinez and Harmel (2006) β- glucosidase activity 150 and170 µg g^{-1}, arylsulfatase activity 100 and 50 µg g^{-1} and phosphatase activities 600 and 400µg g^{-1} were observed in pasture and cultivated sites by different application rates of poultry litter amendments. Earlier studies in agricultural soils have reported that enzymatic activities for phosphatase 798 µg g^{-1}, dehydrogenase 93 µg g^{-1}, β- glucosidase 282 µg g^{-1}, and urease were 120 µg g^{-1} (Aon & Colaneri, 2001). Tiquia (2002) reported that relative abundance and activities of enzymes were higher in poultry manure composting than in pig manure. Municipal solid waste compost (MSW-C) application showed higher enzyme activity than vermicompost, ovine

manure and sewage sludge. The enzymatic activities MSW-C applied soils were observed for phosphatase 350 µg g^{-1}, arylsulfatase 35 µg g^{-1}, urease 65 µg g^{-1} and dehydrogenase 8 µg g^{-1} (Albiach et al., 2000).

CHAPTER 3

Materials and Methods

3.1 Field

3.1.1 Site and wetland cells design. The experiment was conducted in one continuous marsh (CM) and one marsh-pond-marsh (MPM) cell at the swine research facility of the North Carolina A&T State University farm in Greensboro. The 11 m wide × 40 m long wetland cells were constructed in 1995 (Reddy et al., 2001) to treat swine wastewater generated from university farm swine unit. MPM consisted of 11m width × 10 m length × 0.15 m water depth of the marsh sections at the both influent and effluent ends and an 11 m width × 20 m length × 0.75 m water depth pond section separating the marsh sections. The marsh sections of both wetland cell types were planted with *Typha latifolia* L. (broadleaf cattail) and *Schoenoplectus americanus* (pers) (bulrush). In the pond section of the MPM cell presence of duck weed was observed during the summer months. The CM cell is a continuous system planted with the cattail and bulrushes from the inflow end to the outflow end (Figure 3).

3.1.2 Soil sampling. Soil samples were collected in June 2010 from the constructed wetlands after being treated with swine wastewater. Twelve cores were hand augered along the length of each wetland cell and sectioned into depths 0-3 cm, 3-6 cm and 6-12 cm. The wet soil samples were bagged in polythene bags, transported in an ice chest to the laboratory, and stored in the refrigerator at 5 ºC for further analysis.

3.2 Soil analysis procedure

A soil subsample was air dried in the laboratory under room temperature for 2 days. The percent moisture of the original wet sample was determined. The air dried soil samples were crushed and sieved through 1.0 mm mesh to remove any plant material and sand particles, mixed

thoroughly and stored in the refrigerator in plastic bags.

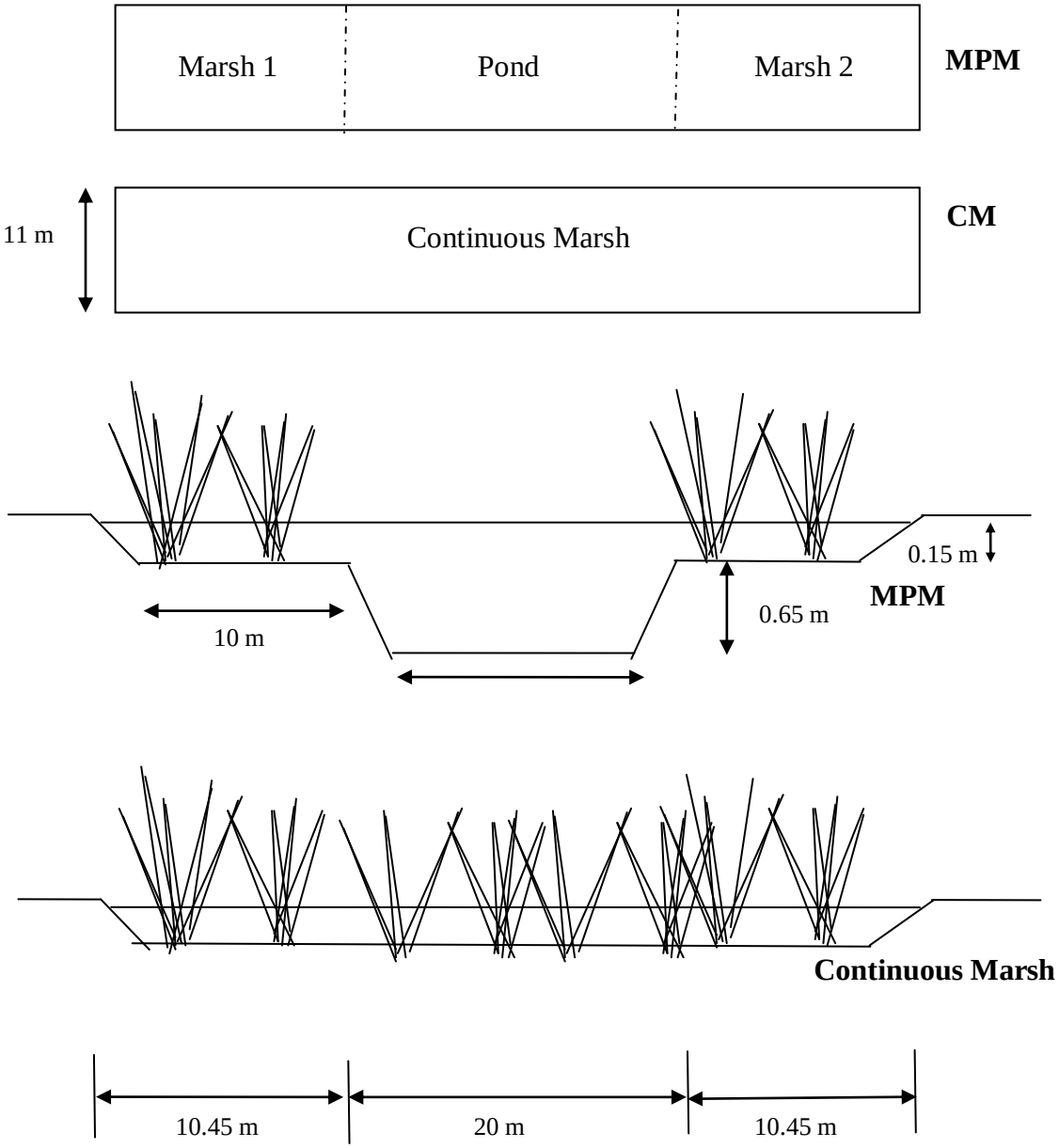


Figure 3. Schematic diagram showing continuous marsh (CM), and pond section separates marsh areas in marsh pond marsh (MPM) constructed wetland cells.

3.3 Soil Samples Analysis

The pH of each soil sample was determined using pH meter (Orion 3 star pH bench top), Total carbon and nitrogen was determined using a CHN analyzer (Perkin Elmer series 2 model:

2400), Microbial biomass carbon (MBC) by using a Schimadzu Total organic carbon analyzer (Model TOC: VSCN), ammonium (NH_4), nitrate (NO_3) and orthophosphate (PO_4) were analyzed using flow injection analyzer (FIA) (Lachat instruments Quick chem. 8500 series 2).

3.4 Soil Enzyme activity assay

The activity of dehydrogenase, urease, phosphatase, β- glucosidase and arysulfatase was determined in each soil sample.

3.4.1 Urease. Urease activity was determined according to the procedure of Klose & Tabatabai (2000). A solution containing 10 mL phosphate buffer (pH 6.7), 0.5 mL toluene and 10 mL 10% aqueous urea was added to 5 g air-dry soil (< 2 mm), and the mixture was incubated for 48 h at 37^0 C. At the end of incubation period, 20 mL of 1M KCL was added and the culture solution was shaken thoroughly for 30 min and filtered. The released ammonium (NH_4^+) in the filtrate was measured by using FIA (Flow Injection Analyzer) and the urease activity was expressed as µg $NH_4^+ g^{-1}$ of soil.

3.4.2 Phosphatase. Phosphatase activity was determined by using the procedure described by Tabatabai and Bremner, (1969) and Eivazi and Tabatabai (1977). One gram of air-dry soil (< 2mm) was placed in a 50 ml Erlenmeyer flask and 4 mL of modified universal buffer (MUB) (pH 6.5), 0.25 mL of toluene and 1 mL of P-nitro phenol (PNP) solution were added as working substrate for the enzyme and the contents were mixed for a few seconds, covered in a flask and placed in an incubator at 37 ºC for 1 hr. After 1hr, it was removed and 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added and swirled thoroughly for a few seconds and then filtered. The yellow color intensity of the filtrate was measured by using a spectrophotometer at 420 nm.

The P-nitrophenol content of the filtrate was calculated by using a calibration graph

plotted from the results obtained with standards containing 0, 10, 20, 30, 40, and 50 µg of p-nitrophenol. For the standard graph, 1 mL of standard p-nitrophenol solution diluted to 100 mL was taken in a volumetric flask and mixed the solution thoroughly and then piped out 0, 1, 2, 3, 4, and 5 mL aliquots of this diluted standard solution into 50 mL Erlenmeyer flasks and adjusted the volume to 5 ml by addition of water. After that 1 ml of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added and mixed the solution thoroughly and filtered the resultant suspension through a whatman No. 4 filter paper and measured the yellow color intensity of filtrate by using spectrophotometer at 420 nm.

Using these results standard p-nitrophenol graph was plotted. Controls also were performed for each soil which allowed no color development from p-nitrophenol released by Phosphatase activity. For this controls the same procedure was followed as described for samples.

3.4.3 Arylsulfatase. Arylsulfatase activity was determined by using the procedure of Tabatabai and Bremner (1970). One gram of soil was placed in a 50 ml Erlenmeyer flask and 4 mL 0.5 M acetate buffer (pH 5.8), 0.25 mL of toluene and 1 mL of P-nitro phenyl sulfate (PNS) solution (as substrate) were added and mixed for few seconds, covered and placed in an incubator at 37 ºC for 1 hr. After 1 hr incubation, the parafilm cover was removed and 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added and swirled thoroughly for few seconds and filtered the soil suspension through a whatman No. 4 filter paper. The yellow color intensity of the filtrate was measured by using a spectrophotometer at 420 nm.

For the calibration graph and the controls used the same procedure was followed as for the phosphatase assay. Controls were performed for each soil in which no color development occurred from p-nitrophenol released by arylsulfatase activity.

3.4.4 β-glucosidase. β-glucosidase activity was measured according to the procedure described by Eivazi & Tabatabai (1988). One gram of soil was placed in a 50 ml Erlenmeyer flask and 4 mL Modified Universal Buffer (MUB) (pH 6.0), 0.25 mL of toluene and 1 mL of P-nitro phenol β-D-glucoside (PNG) solution (as substrate) were added and swirled the contents for few seconds and covered the flask with parafilm and placed in incubator at 37 ºC for I hr. After 1hr incubation, parafilm was removed and 1mL of 0.5 M $CaCl_2$ and 4 mL of 0.1 M Tris Hydroxymethyl Amino Methane (THAM) buffer at pH 12 were added and swirled thoroughly for few seconds and filtered the soil suspension through a whatman No.4 filter paper. The yellow color intensity of the filtrate was measured by using a spectrophotometer at 420 nm.

For calibration graph and controls same procedure was followed as for phosphatase assay. Controls performed for each soil which allowed no color development from P-nitrophenol (PNP) released by β- glucosidase activity. It is important to treat the incubated soil sample with THAM buffer pH 12 instead of the 0.5 M of NaOH used for extraction of p-nitrophenol in assay of phosphatase and arysulfatase because the substrate of β- glucosidase, PNG is hydrolyzed with time in the presence of excess NaOH. The $CaCl_2$ and THAM treatment served extraction of p- nitrophenol released in the assay of β- glucosidase activity and the activity of β- glucosidase was expressed as µg p-nitrophenol g^{-1} of soil.

3.4.5 Dehydrogenase. Dehydrogenase activity was determined according to the procedure described by Casida, et al. (1964). Six grams of air dried soil was mixed with 0.07 g of $CaCO_3$, 1 mL (3% aqueous solution) 2, 3, 5 Triphenyltetrazolium Chloride (TTC) and 2.5 mL distilled water. Mixed the contents of each tube, covered with parafilm and placed it in incubator at 37 ºC for 24 h. After 24 h incubation, parafilm was removed and 10 mL of methanol was added and shaked for 1 min and solution was filtered through a glass funnel plugged with

absorbent cotton in to a 100 mL volumetric flask. Washed the tube with methanol, quantitatively transferred the soil to the funnel and then added additional methanol to the funnel until the reddish color was disappeared from the cotton plug, Diluted the filtrate to 100 mL and measured the intensity of reddish color by using spectrophotometer at a wavelength of 485 nm against blank.

The amount of Tri Phenyl Formazan (TPF) produced was calculated by reference to a calibration graph prepared from TPF standards. For this calibration graph diluted 10 mL of TPF standard solution to 100 mL with methanol (100 µg of TPF mL^{-1}) and then piped out 0.5, 1, 2.5, 5, 7.5, 10 and 15 mL of this solution in to 100 mL volumetric flasks and diluted to 100 mL with methanol and mixed thoroughly and measured the intensity of reddish color of TPF by using spectrophotometer at a wavelength of 485 nm against blank. The absorbance readings were plotted on a graph against TPF concentration.

3.5 Statistical Analysis

Relationship between enzymatic activities to TC, TN, NH_4 and PO_4 ions were evaluated using linear correlation analysis. Correlation coefficients (r) were presented for all possible pairs of correlations. For each enzyme mean values and standard errors were used to test differences with depth at each site. Differences between means were tested using paired-sample *T*-tests. The level of significance for all analyses was tested at $P<0.05$.

CHAPTER 4

Results and Discussion

Table 2 shows the measured parameters decreased with increasing depth in CM and MPM. The highest values were observed in the upper 0-3 cm depth. TC and TN were found to decrease from 1.35 to 0.75% and 0.13 to 0.07 % in CM and 0.7 to 0.43% and 0.09 to 0.06 % in pond area of MPM, respectively. NH_4 and NO_3 nutrient concentrations were decreased from surface depth to deeper soil depths. Contrary to earlier studies (Poach et al., 2004) that showed a lower concentration of NH_4 in the pond area due to higher ammonia volatilization compared to the marsh area, we observed higher NH_4 concentration in the pond area compared to the marsh area. This is attributed to the higher dissolved O_2 found in the pond area relative to marsh areas resulting in exceeded mineralization than nitrification rate.

Table 2

Parameters measured (Mean±SD) of CM and pond area of MPM at three different depths

Soil	**CM**			**M-P-M (Pond Area)**		
Property	**0-3 cm**	**3-6 cm**	**6-12 cm**	**0-3 cm**	**3-6 cm**	**6-12 cm**
pH	6.64±0.12	6.73±0.13	6.91±0.24	7.88±0.12	7.59±0.20	7.57±0.18
	……. µg g^{-1}…….					
TC	13558±6175	8800±2326	7500±2055	7025±4945	5925±4684	4325±2117
TN	1375±685	875±205	733±187	950±102	666±152	650±70
NH_4^+	24.84±42	12.45±21	3.62±3	45.17±10	40.40±19	56.45±37
NO_3^-	7.90±13.70	2.20±2.60	2.80±4.00	0.40±0.50	0.10±0.07	0.08±0.09
PO_4^{-2}	408±143	237±128	81±80	183±83	92±102	34±31
Org N	1342±646	860±197	726±183	904±237	625±246	593±262
MBC	622±56	591±68	552±57	505±31	446±24	415±7

4.1 Enzyme activity in wetlands

4.1.1 Urease activity. In CM and MPM, urease activity decreased with increasing soil depth (Figure 4). In CM, at inlet and outlet points significant decrease was observed between 0-3 and 6-12 cm depths. In both wetlands from inlet to outlet, the soil samples at 0-3 cm (300 to 450 μg g^{-1} soil) showed highest urease activity than in the lower depths. The least enzymatic activity (<200 μg g^{-1} soil) was observed in the 6-12 cm depth. In CM, the activity of urease increased from the inlet to the outlet from 363 to 436 μg/g at the 0-3 cm depth and 280 to 340 μg/g at the 3-6 cm depth. In MPM, the activity of urease decreased from inlet to outlet 407 to 301 μg/g at 0-3 cm depth and 340 to 227 μg/g at 3-6 cm depth. We could not find a significant difference in enzyme activity between inlet and outlet in both CM and MPM (Table 3). Urease activity depends on the availability of NH_4^+ in the inlet and the outlet of the CM and MPM. In MPM, highest urease activities were observed in the marsh area than in the pond area. In comparison to MPM, urease activity was higher in CM but the difference was not statistically significant (P<0.05).

In both wetlands, highest urease activities were observed in comparison to other four enzymes. It may be attributed to the low availability of NH_4^+ in our wetlands due to the nitrification and denitrification processes as evident by the data shown in Table 2. Previous studies at this site showed that 70-95% nitrogen (N) removal from the swine wastewater when the wetland cells were loaded with 3 to 36 kg N per ha per day (Poach et al., 2004a; Reddy et al., 2001). The desired removal mechanism of wastewater N from wetland is denitrification, which converts N to gaseous forms of nitrogen and NH_3 volatilization. However, the volatilization of ammonia as it relates to acid rain is of concern. Past research conducted on constructed wetland systems treating swine wastewater found that ammonia volatilization accounted for less than

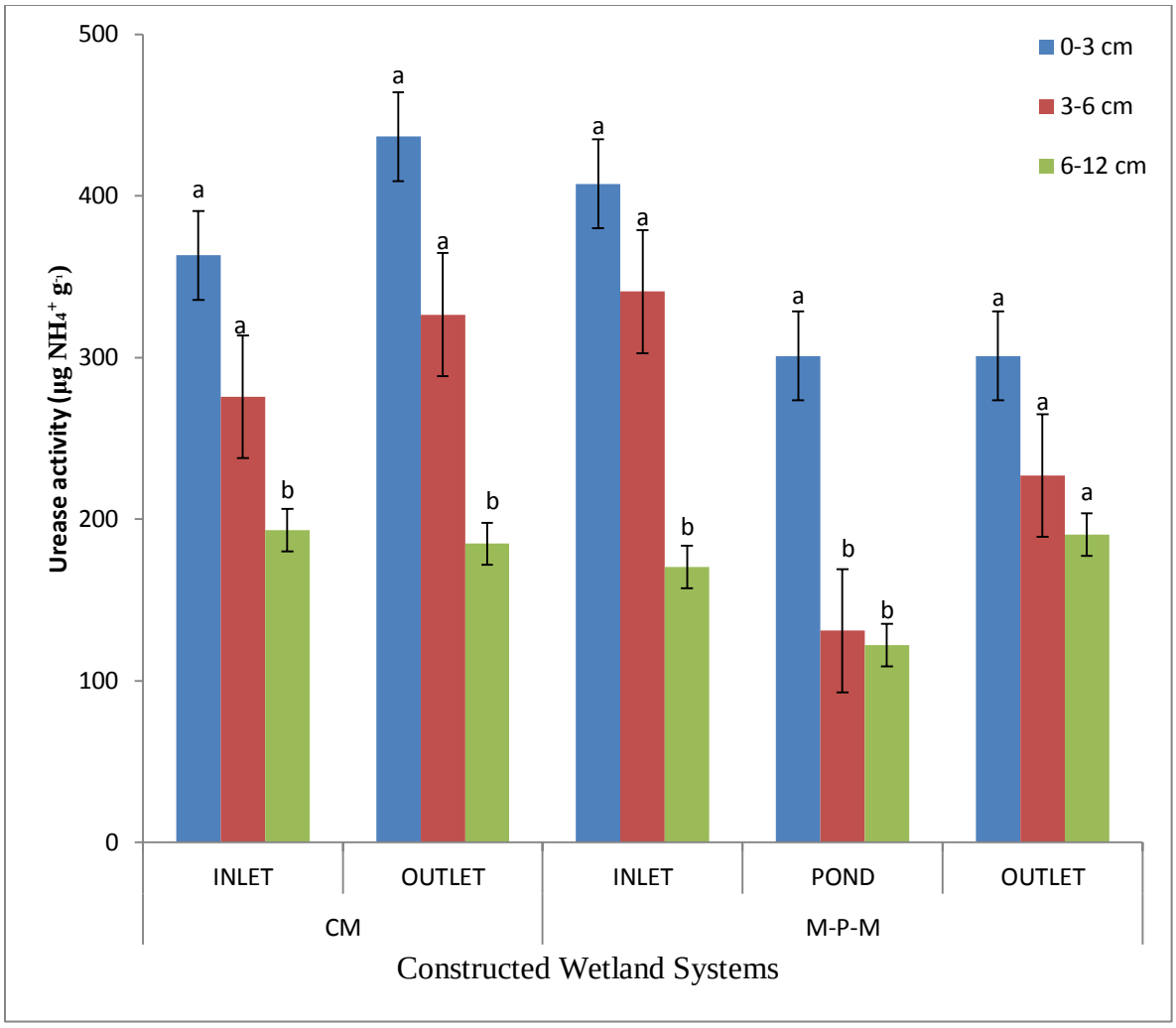


Figure 4. Urease activity in each site at different soil depths in the Continuous marsh (CM) and Marsh pond marsh (MPM) constructed wetlands (mean± SE, n = 4).

Note: Means having the same letters in common are not significantly different at the 5% level of probability for each site at different soil depths of CM and MPM as determined from t-test pair comparison.

20% of the N removed by the wetlands (Poach et al., 2002). Denitrification is the desired mechanism for N removal because N accumulation by plant accounts for less than 10% of the N load (Hunt et al., 2002; Poach et al., 2004; Reddy et al., 2001).

Urease activity in the species-specific surface flow constructed wetlands was observed to be in the range of 240 to 260 µg g^{-1}, 160 to 180 µg g^{-1}, and 140 to 160 µg g^{-1} for 0-5 cm,

15-20 cm, and 35-40 cm, respectively (Kong et al., 2009). However, comparatively higher enzyme activities were observed in case of our constructed wetland systems treated with swine waste water which was 367 μg g^{-1} at 0-3 cm depth, 282 μg g^{-1} at 3-6 cm depth and 186 μg g^{-1} at 6-12 cm depth in CM and 336 μg g^{-1} at 0-3 cm depth, 232 μg g^{-1} at 3-6 cm depth and 161 μg g^{-1} at 6-12 cm depth in MPM. On the other hand, our results were consistent with the urease activities reported by Zhang et al. (2010), wherein they found that, depending upon the species richness in the full scale constructed wetlands obtained on the treatment of domestic wastewater activities ranged from 120 to 400 μg g^{-1}. This may be due to removal of nitrogen is more or availability of ammonium is less in our CWs when compared to other systems. This is a continuous input of nutrients, uric acid and microbes into the wetland system. Therefore, higher urease enzyme activity is evident with high TN in sediment (soil) samples. Since, higher microbial populations (Dong & Reddy, 2010) require higher amounts of NH_4 as a N source and due to the lack of required NH_4 concentration, microbes produce urease enzyme to hydrolyze uric acid which is a part of total N.

4.1.2 Phosphatase activity. In CM and MPM phosphatase activity showed highest in top 0-3 cm depth than other two depths (Figure 5). Inlet portion of CM samples showed significantly decreased phosphatase activity between 0-3 and 3-6 cm whereas, in outlet the difference was not significant among the depths. The lowest enzymatic activity (12 μg g^{-1} soil) was observed in 6-12 cm depth of CM inlet and MPM pond area. In CM, phosphatase activity was observed similarly at 0-3 cm soil samples in inlet and outlet, whereas higher activity was observed at 3-6 cm and 6-12 cm depth samples in outlet portion of wetland. In MPM, the activity of phosphatase increased from inlet to outlet at all three depths. Phosphatase activity was highest in marsh (outlet) area than the pond area and the difference was significant (P<0.05). Although,

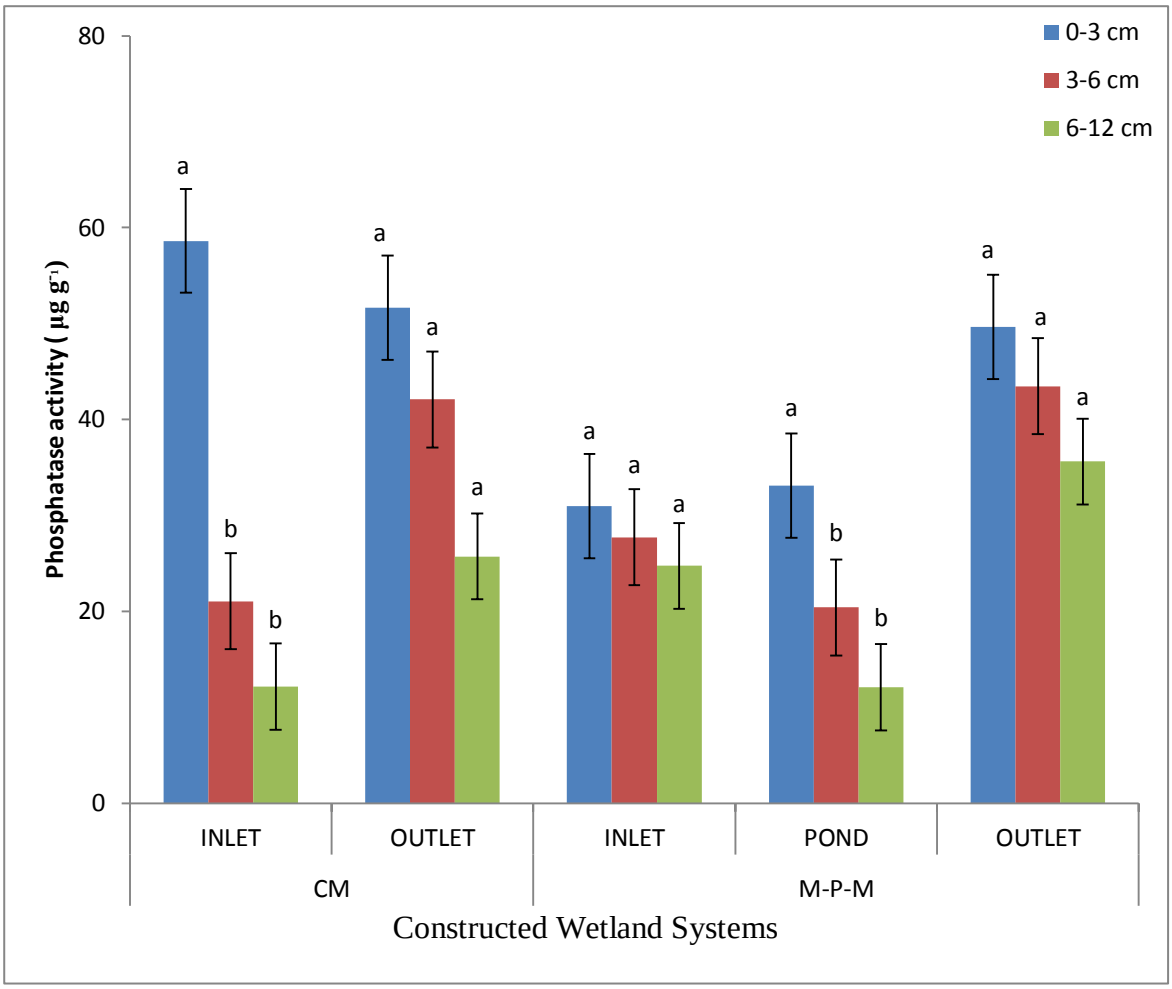


Figure 5. Phosphatase activity in each site at different soil depths in the Continuous marsh (CM) Marsh pond marsh (MPM) constructed wetlands (mean± SE, n = 4).

Note: Means having the same letters in common are not significantly different at the 5% level of probability for each site at different soil depths of CM and MPM as determined from t-test pair comparison.

CM showed highest phosphatase activity, significant difference was not found between CM and MPM wetland systems. Phosphatase activity showed significant difference between inlet and outlet in MPM, whereas in CM the difference was not significant (Table 3).

In the earlier studies (Kong et al., 2009) phosphatase activity in surface flow constructed wetlands was reported as 120-140 µg g^{-1} for 0-5 cm depth, 90-110 µg g^{-1} for 15-20 cm depth, 85-100 µg g^{-1} for 35-40 cm depth depending upon the species used in the study. On the other

hand, in natural wetlands at fen site very high phosphatase activity (4675-9680 μg g^{-1}) was observed (Kang & Freeman, 1999). Our observations on constructed wetland systems treated with swine wastewater which was 66 μg g^{-1} in CM and 37 μg g^{-1} in MPM were comparable to Kong et al. (2009) but were very low compared to Kang and Freeman (1999). This may be due to the fact that treated swine wastewater contains lot of inorganic phosphate or available phosphorus in the sediments (Table 2) that might have decreased the phosphatase activity in our constructed wetlands. This is in agreement with Reddy et al. (2001), Poach et al. (2004a), Hunt et al. (2002) work, indicated that 40-45% of phosphorus (P) was removed from the swine wastewater or 55-60% of P available in wastewater when constructed wetlands were treated with 3 to 36 kg N per ha^{-1} day^{-1}.

4.1.3 Arylsulfatase activity. Arylsulfatase activity was decreased with the increasing depths in CM and MPM wetlands (Figure 6). In CM inlet portion showed significant decrease in enzyme activity between 0-3 and 6-12 cm depths, whereas in outlet no significant difference was observed between the depths. In MPM, inlet and outlet points showed significant difference in enzyme activity between 0-3 and 6-12 cm depths and in pond area no significant decrease was observed among three depths. In CM, the activity of arylsulfatase decreased from inlet to outlet 65 to 57 μg/g at 0-3 cm depth and no difference was found at 3-6 cm depth.

In MPM, arylsulfatase activity was higher in marsh area compared to pond area and the difference was statistically significant ($P<0.05$) among them. No significant difference in enzyme activity was observed between inlet and outlet in both CM and MPM (Table 3). CM wetland showed significantly ($P<0.05$) higher arylsulfatase activity than in MPM wetland. The microbial demand for sulfur is higher in CM as compared to MPM. Finally, arylsulfatase activity depends on the availability of sulphate ion in both CM and MPM wetlands.

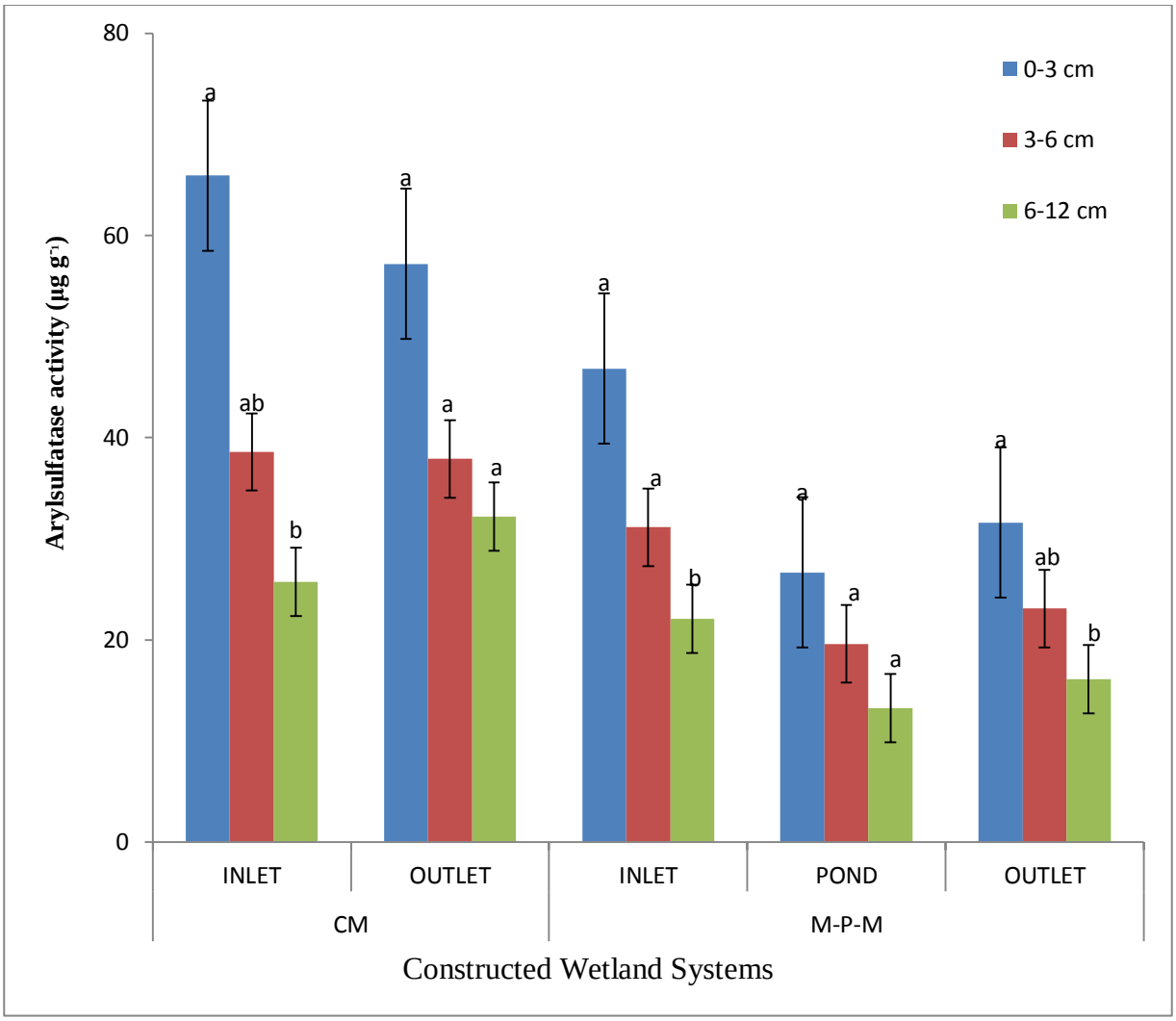


Figure 6. Arylsulfatase activity in each site at different soil depths in the Continuous marsh (CM) and Marsh pond marsh (MPM) constructed wetlands (mean± SE, n = 4).

Note: Means having the same letters in common are not significantly different at the 5% level of probability for each site at different soil depths of CM and MPM as determined from t-test pair comparison.

Microbial arylsulfatase enzyme activity in the Laurentian Great Lakes wetlands was reported to be 24-239 nmol g C^{-1} h^{-1}(Hill et al., 2006). Whereas in natural wetlands at fen site it was observed in the range of 200-700 µg g^{-1} (Kang & Freeman, 1999). The enzyme activities in our constructed wetland systems were 58 µg g^{-1} and 35 µg g^{-1} in CM and MPM wetlands, respectively. Lower arylsulfatase enzyme activity could be due to lower uptake of sulphur by

plants and microbes which is a more important process than the lower mineralization by which SO_4 is released into the wetland or treated swine wastewater contains higher amounts of inorganic sulfate or available sulphur in wetlands that might decrease the enzyme activity compared to other systems in our study.

4.1.4 β-glucosidase activity. β-glucosidase activity showed highest at 0-3 cm depth in CM and MPM (Figure 7). A decreased enzymatic activity was observed with increasing depths in both wetland systems, and the difference was not significant (P<0.05). In CM system β- glucosidase activity decreased from inlet to outlet 66 to 60 µg/g at 0-3 cm depth. In MPM, the activity of enzyme decreased from inlet to outlet 48 to 35 µg g^{-1} at 0-3 cm depth and 35 to 29 µg g^{-1} at 3-6 cm depth. The pond section showed less enzyme activity than marsh area in MPM system and the difference among them was highly significant (P<0.05). The enzyme activity was significantly (P<0.05) higher in CM compared to MPM wetland. In CM and MPM, the activity of β-glucosidase was observed no significant between inlet and outlet (Table 3).

Hill and co-workers (2006) in their study on Laurentian Great Lakes wetlands found the β-glucosidase activity to be in the range of 581 to 1262 nmol g C^{-1} h^{-1}. In naturally decomposing litter at medium salinity rate, 2200 to 3670 µg g^{-1} range of β-glucosidase activity was observed (Rejmankova & Sirova, 2007). Lower enzyme activities compared to these activities were observed in our constructed wetland systems which were 54 µg g^{-1} and 32 µg g^{-1} in CM and MPM wetlands, respectively. Lower enzyme activity may be due to an excess of low molecular weight carbon in our wetland sediments compared to other wetlands. When such readily metabolized soluble carbon is freely available, it has been suggested that there is no need for microorganisms to acquire it enzymatically (Chrost & Rai, 1993). We observed lower β-glucosidase activity when compared to other eco-systems.

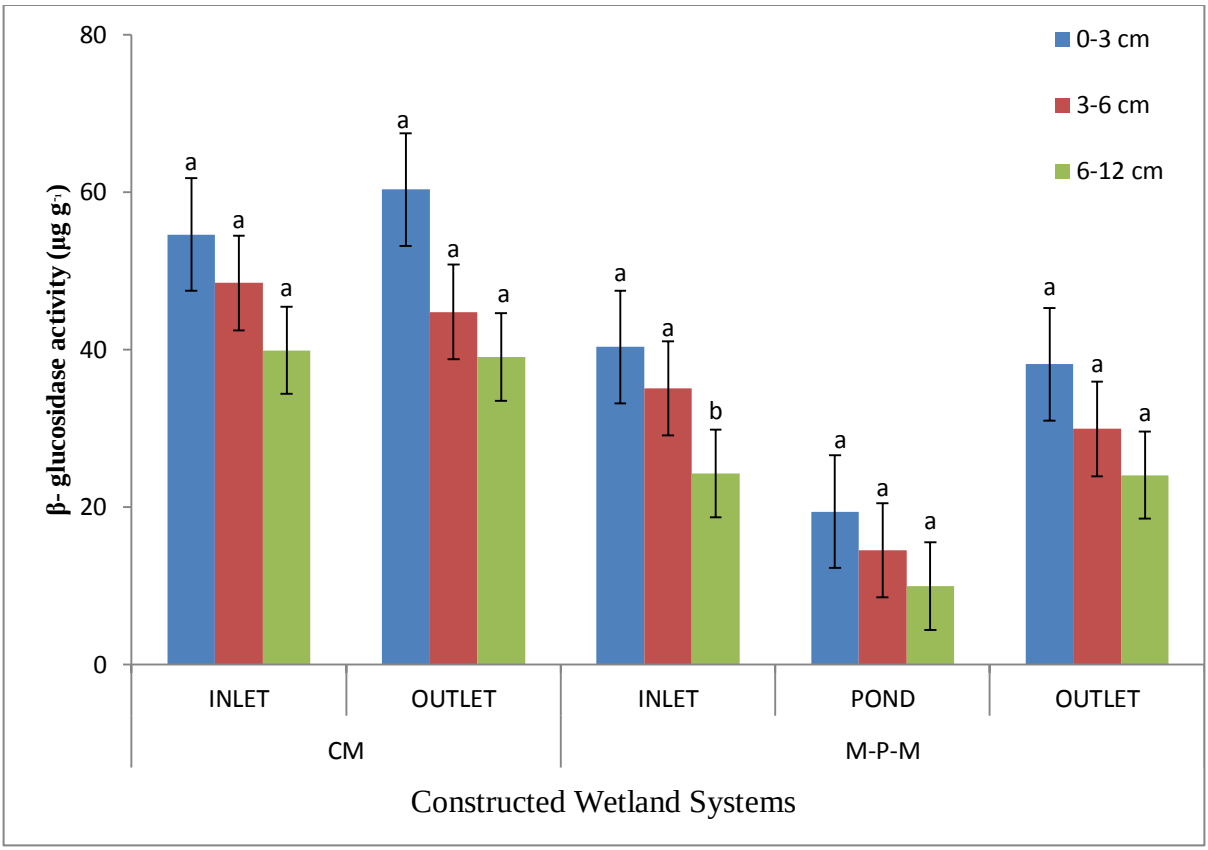


Figure 7. β-glucosidase activity in each site at different soil depths in the Continuous marsh (CM) and Marsh pond marsh (MPM) constructed wetlands (mean± SE, n = 4).

Note: Means having the same letters in common are not significantly different at the 5% level of probability for each site at different soil depths of CM and MPM as determined from t-test pair comparison.

4.1.5 Dehydrogenase activity. In CM and MPM dehydrogenase activity decreased with the depth of wetland (Figure 8). In CM, enzyme activity decreased from inlet to outlet showing no significant difference among distance in wetland.

The distance from inflow to outflow did not show any significant difference in enzyme activity in MPM. In pond area the activity was less compared to marsh area in MPM system. Dehydrogenase activity was significantly (P<0.05) higher in CM Compared to MPM. The activity of dehydrogenase was observed no significant between inlet and outlet in both CM and

MPM (Table 3). Enzyme activity was not significantly different among 3-6 and 6-12 cm depth in all samples taken in CM and MPM.

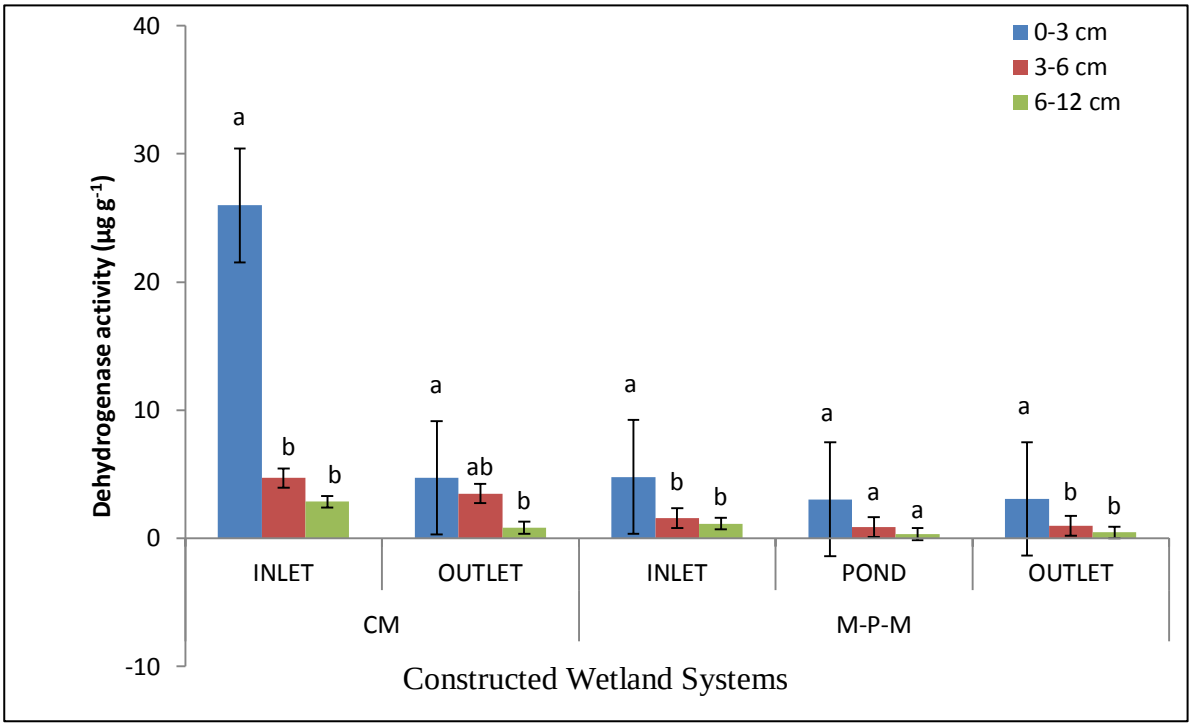


Figure 8. Dehydrogenase activity in each site at different soil depths in the Continuous marsh (CM) and Marsh pond marsh (MPM) constructed wetlands (mean± SE, n = 4).

Note: Means having the same letters in common are not significantly different at the 5% level of probability for each site at different soil depths of CM and MPM as determined from t-test pair comparison.

The dehydrogenase activities in our study were observed to be 15 µg g^{-1} in CM and 3 µg g^{-1} in MPM. On the contrary, Zhang et al. (2010) reported 4-6 times higher activities for domestic wastewater treated full scale constructed wetland.

Enzyme activities were higher in upper 0-3 cm depth for all five enzymes understudy in CM and MPM systems. Earlier studies have also reported that activities of enzymes were higher in upper sediment depths (Aon & Colaneri, 2001; Niemi et al., 2005). In our study, enzyme activities were decreased with the depth of wetland. Factors that could influence the soil enzyme

activities in these wetlands are oxygen availability, organic carbon availability, pH and soil nutrients. In these CW, swine wastewater first flowed from inlet area to outlet area and surface soil is exposed to nutrient and organic carbon rich wastewater and therefore, could influence the rate of microbial growth and enzyme activity in upper 0-3 cm sediment depth. The decrease in the enzyme activities could be due to changes in microbial population, decrease in novo synthesis of enzymes or increase of inhibitors such as metal ions under the reduced condition (Freeman et al., 1996; Pulford & Tabatabai, 1988).

Table 3

Enzyme activity (µg/g) in the inlet and outlet points of Continuous marsh and Marsh pond marsh wetland cells.

Enzymes	**CM**		**MPM**	
	Inlet†	**Outlet†**	**Inlet†**	**Outlet†**
Urease	832 [a]	948 [a]	918 [a]	718 [a]
Phosphatase	91 [a]	119 [a]	83 [a]	128 [b]
Arysulfatase	130 [a]	127 [a]	100 [a]	70 [a]
β-glucosidase	143 [a]	144 [a]	99 [a]	92 [a]
Dehydrogenase	33 [a]	9 [a]	7 [a]	4 [a]

Note: † Means having the same letters in common are not significantly different at the 5% level of probability for inlet and outlet points of CM and MPM as determined from t-test pair comparison.

For all five enzymes in our study, we could not find any significant difference between inlet and outlet values in both CM and MPM (Table 3). This may be due to 10 year long utilization of our CWs for swine wastewater treatment which would have affected the settling of organic matter, nutrient and microbial influx in inlet and outlet areas of both the wetlands.

In MPM, our results clearly indicated that enzyme activities were higher in marsh area in comparison to pond area (Table 4). Because the presence of plants could influence the changes of enzyme activities in marsh area by supplying organic carbon and modifying hydrochemistry in rhizosphere. Choi et al. (2009) reported that the organic carbon supplemented by root exudates, root debris and plant residue played an important role in increasing enzyme activities in the sediments with plants. He also reported sediments with wetland plants exhibited significantly higher enzyme activities of β-glucosidase, arysulfatase, phosphatase and N-acetylglucosaminidase. He observed significant differences in sediment organic matter between vegetated and nonvegetated sediments and found sediment organic matter was higher in the vegetated sediments.

Table 4

Enzyme activity difference in the marsh and pond areas of Marsh pond marsh wetland cell

Enzymes	**Marsh†** ….µg/g….	**Pond†**
Urease	918[a]	554[a]
Phosphatase	128[a]	65[b]
Arysulfatase	100[a]	59[b]
β-glucosidase	99[a]	43[b]
dehydrogenase	7[a]	4[a]

Note: † Means having the same letters in common are not significantly different at the 5% level of probability for MPM as determined from t-test pair comparison.

The lowest enzymes activities were detected in the pond, which might explain the slow decomposition rate in the pond area. Activities of phosphatase, β- glucosidase and arysulfatase were found significantly (P<0.05) higher in marsh area than pond area (Table 4). Urease and

dehydrogenase activities were higher in marsh area but not significant. In this study, all enzyme activities were higher in CM than in MPM. This suggested that the characteristics of CM wetland were different from MPM wetland.

4.2 Relationship between enzyme activities to nutrient concentration

In CM and MPM at each site at different depths, the relationship between urease activity to ammonium concentration and phosphatase activity to phosphate concentration were determined by linear correlation analysis. No significant ($P<0.05$) correlation was found between urease activity and ammonium concentration and phosphatase activity and phosphate concentration in CM and MPM at all three depths.

From an ecological point of view, it is believed that an inverse relationship would exist between nutrient availability and enzyme activity (Sinsabaugh et al., 1993). The fundamental relationships between them are repression-depression and end product inhibition of the enzyme (Chrost, 1991). High enzyme activity indicates nutrient limitation (Sinsabaugh et al., 1993), and sometimes a pattern of increasing enzyme activity with decreasing nutrient availability found in soil. For example phosphatase activity increases as P declines (Allison & Vitousek, 2005; McGill & Cole, 1981) in soil. Activity of the N-releasing enzyme, chitinase, increases as N declines (Olander & Vitousek, 2005) in soil. However, the negative relationship between enzymatic activity and nutrient availability has not been established. There are contradictory reports concerning the relationship between enzymes and inorganic nutrient content in the soil (Speir & Ross, 1978). In CM and MPM systems, we could not find a relationship between urease activity to ammonium concentration and phosphatase activity to phosphate concentration and this observation was due to several factors. First, either enough nutrients are not available in our CWs to microbes that are heterotrophic and autotrophic in nature. Secondly, treated swine

wastewater contains very high autotrophic microbial populations (Dong & Reddy, 2010) that demand more ammonium and become competitive to heterotrophs. Thirdly, continuous process where input or source and output or removal of nutrients occurring simultaneously in our wetland systems. Fourthly, the soil enzymes are largely stabilized by humus and clay, and are thus independent of microbial regulation (Burns, 1982). These base line enzyme activities do not respond to low nutrient availability (Clarholm & Rosengren-Brinck, 1995). Finally, treated swine wastewater contains excess amounts of microorganisms, inorganic nitrogen and phosphorus that might have affected the relationship between them.

4.3 Microbial Biomass Carbon (MBC) at different depths

MBC was determined at three depths (0-3 cm, 3-6 cm and 6-12 cm) in CM and pond area of MPM wetlands (Table 5). Highest biomass carbon was observed in 0-3 cm depth of soil samples. In both wetlands MBC decreased with the depth and no significant ($P<0.05$) difference was found between the depths and this is due to the sampling in close soil depths.

Biomass carbon was higher in marsh area compared to pond area and the difference was not significant ($P<0.05$) among them. These results indicated that high amount of microbial activity carried out in marsh area than pond area. This is true in our study, where higher enzymatic activities carried out in marsh area compared to pond area for all five enzymes in CM and MPM systems.

4.4 Relationship between enzyme activity to TC and TN

Relationship between enzyme activity to TC and TN was determined by correlation analysis (Table 6 and 7). It was found that urease, phosphatase and arylsulfatase activities were strongly correlated to TC and TN at 0-3 cm depth in CM. In MPM, urease and phosphatase activities showed significant correlation with TC and TN. The results indicate that urease,

arylsulfatase and phosphatase activities are strongly dependent on the availability of total carbon and total nitrogen in the soil organic matter. β-glucosidase activity was significantly correlated with total carbon in CM.

Table 5

Microbial Biomass Carbon (MBC) values at three different depths in CM and pond area of MPM

	CM			**(Pond) MPM**		
	…….mg kg^{-1}……..					
Sub Sample	**Depth (cm)**					
	0-3	**3-6**	**6-12**	**0-3**	**3-6**	**6-12**
S1	574.7	659.9	490.6	533.7	465.7	424.1
S2	609.5	590.1	602.6	472.3	455.6	411.1
S3	684.4	522.9	565.3	511.2	418.5	410.5
Mean	622.9	591.0	552.8	505.7	446.6	415.2

This suggests that organic matter which contain different carbohydrates have been hydrolyzed into two sugar units in order for glucosidase was actively engaged to form glucose units for heterotrophs to consume as their carbon source. These results suggested that TC and TN in CWs influencing enzyme activity and decomposition ability. Our results shown that, still some enzymatic activities carried out in CWs even they received high concentration of nutrients through swine wastewater application.

Table 6

Correlation coefficients between soil enzyme activity and TC and TN at 0-3 cm depth in Continuous marsh wetland

Enzymes	Total C†	Total N†
Urease	0.92**	0.95**
Phosphatase	0.75**	0.71**
Arylsulfatase	0.89**	0.76**
β-glucosidase	0.71**	0.59*
dehydrogenase	0.63*	0.80**

Note: † * Represent significant correlations at $p < 0.05$.
** Represent significant correlations at $p < 0.01$.
^{NS} Represent no significant correlations at $p < 0.05$.

Table 7

Correlation coefficients between soil enzyme activity and TC and TN at 0-3 cm depth in Marsh pond marsh wetland

Enzymes	Total C†	Total N†
Urease	0.97**	0.97**
Phosphatase	0.95**	0.96**
Arylsulfatase	0.78**	0.69*
β-glucosidase	0.67*	0.63*
dehydrogenase	0.42^{NS}	0.51^{NS}

Note: † * Represent significant correlations at $p < 0.05$.
** Represent significant correlations at $p < 0.01$.
^{NS} Represent no significant correlations at $p < 0.05$.

CHAPTER 5

Conclusion

The constructed wetlands (1 CM and 1 MPM) studied at North Carolina A&T State University's swine unit have clearly shown that all five enzyme activities were decreased from 0-3 to 6-12 cm soil depths. Our results show that the presence of wetland plants can increase the enzyme activities in both CM and MPM wetlands. Phosphatase, arysulfatase and β-glucosidase activities were observed significantly higher in the marsh area compared to pond area of MPM wetland, whereas, urease and dehydrogenase activities were found to be not significant. For all five enzymes, highest enzymatic activity was carried out in CM wetland in comparison to MPM wetland. The above results indicated that MPM wetlands are effective in purifying the swine wastewater by retaining the excess nutrients in the wetland sediments. We observed some enzymatic activity in our CWs, even they received high concentration of nutrients through swine wastewater application. In the present study, various exudates from plants, high inorganic nutrients, microorganisms and total solids from swine wastewater, detritus of plants and wetland microorganisms seems to contribute to the biogeochemistry of the wetlands. These excess inorganic nutrients may not be sufficient for our wetland microorganisms so they released enzymes, these are acted on organic matter and decomposed into inorganic nutrients for their growth and development. Urease, arylsulfatase and phosphatase enzymes in this study were strongly correlated to TC and TN at 0-3 cm depth in CM and MPM. No significant correlations were found between urease activity to inorganic ammonium and phosphatase activity to inorganic phosphate concentration in both CM and MPM.

In general higher enzymatic activities are observed in agricultural soils and wetland sediments where lower nutrients concentration exists. In order for CWs to be efficient in

nutrients removal the enzyme activity must be lower. Previous studies indicated that higher the nutrient loading into these wetlands, lower the nutrients removal efficiency was observed. Even at 15 kg N loading, N and P removal was only 60% and 35%, respectively. It suggests that enzyme activity is continuously contributing to the release of inorganic nutrients which will reduce the wetlands efficiency.

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Appendix A

Nutrients data in CM and MPM Wetlands

A.1 TC data in CM and MPM systems

Wetland	**CM**			**MPM**		
	0-3 cm	**3-6 cm**	**6-12 cm**	**0-3 cm**	**3-6 cm**	**6-12 cm**
	…….(µg g^{-1} soil)…….					
Inlet	21800	9300	9200	52600	18100	9500
	10500	6900	8500	19400	7900	5300
	9700	9300	3900	8500	8500	6600
	11200	8000	7200	12000	20400	3900
Mid	11400	7700	8000	11100	4100	4000
	21400	6200	4400	11300	12900	7300
	7600	6000	9700	4200	3900	3700
	26500	9400	7800	1500	2800	2300
Outlet	14000	11400	5400	7900	5000	6200
	8000	8900	7800	9000	6400	7600
	10700	8100	10700	9900	9700	7100
	9900	14400	7400	17800	8800	5700

A.2 TN data in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	…….(µg g^{-1} soil)…….					
Inlet	2700	1100	1100	5100	2000	1100
	1100	800	800	1700	900	600
	1000	1000	400	1000	1000	700
	1200	800	700	1200	1100	500
Mid	1200	800	700	900	800	800
	2200	700	600	1800	2100	1300
	700	500	800	700	700	600
	2500	900	900	400	500	700
Outlet	1200	1000	500	900	700	800
	800	800	700	900	700	600
	1000	800	900	1000	1000	900
	900	1300	700	1700	1000	600

A.3 Ammonium (NH_4) data in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	155	80.8	15	37.2	22.2	21.4
	35.8	13.9	2.57	9.32	4.44	1.61
	16.9	4.99	1.45	15.2	18.5	30
	19.7	9.36	3.41	8.5	7.56	26.8
Mid	6.64	5.3	1.86	49.8	132	220
	4.42	3.41	2.3	186	228	191
	2.9	3.53	1.39	33.7	30.7	50.3
	35.1	15.4	6.95	51.8	50.1	62.6
Outlet	10.3	3.83	1.73	3.25	2.99	2.58
	2.74	2.48	1.57	2.05	1.96	1.38
	3.53	2.59	2.56	2.01	3.3	1.49
	5.08	3.84	2.7	3.92	3.51	1.25

A.4 Nitrate (NO_3) data in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	48.1	9.42	13.3	142	54.6	18.2
	6.61	4.28	3.59	20.1	6.7	2.17
	4.66	3.41	7.48	7.72	4.57	2.29
	7.8	3.62	5.42	17.5	1.74	0.381
Mid	0.982	0.816	0.633	1.29	0.136	0
	1.98	0	0.159	0.177	0	0
	0.2	0	0.641	0.251	0.112	0.18
	19.5	1.33	0.906	0.147	0.163	0.123
Outlet	0.339	1.73	0.377	4.51	1.14	1.39
	0	0.225	0.187	3.26	1.1	0.634
	3.99	1.09	0.234	2.76	1.38	0.69
	1.63	1.58	0.945	8.72	1.7	0

A.5 Ortho Phosphate (PO_4) data in CM and MPM systems

Wetland	**CM**			**MPM**		
	0-3 cm	**3-6 cm**	**6-12 cm**	**0-3 cm**	**3-6 cm**	**6-12 cm**
	…….(µg g^{-1} soil)…….					
Inlet	819	545.3	246.4	616.7	488.6	350.7
	467.6	298.9	53.9	480.2	270.2	79.1
	331.1	118.3	3.073	375.9	163.1	32.13
	419.3	350.7	25.9	563.5	303.1	53.76
Mid	277.2	129.5	31.36	284.9	34.16	7.91
	336.7	108.5	88.2	81.2	16.03	10.78
	404.6	124.6	10.5	178.5	79.1	43.68
	478.8	266.7	214.9	189	241.5	75.6
Outlet	387.8	318.5	34.93	325.5	200.2	196.7
	333.9	158.9	103.6	341.6	120.4	119.7
	296.8	213.5	133	333.2	291.9	185.5
	348.6	216.3	28.84	403.9	287	109.9

Appendix B

Enzymes Data in CM and MPM Wetlands

B.1 Urease activity in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	1477.4	387	324.6	2597.4	1093.4	755
	498.2	311.8	196.6	555.8	317.4	143.8
	219	176.6	86.2	330.2	513.4	245.4
	372.6	227.8	165.4	336.6	191.8	122.2
Mid	363	211.8	151	316.6	196.6	163.8
	1053.4	282.2	153.4	285.4	507.4	377.4
	239	226.2	210.2	83.8	105.4	91.8
	1157.4	264.6	215.8	84.6	91	152.6
Outlet	671	456.6	187.8	206.2	127	191.8
	251.8	247	155	231	202.2	147
	585.4	353.4	252.6	359	356.6	264.6
	239	249.4	143.8	407.8	222.2	158.2

B.2 Phosphatase activity in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	116.58	25.32	20.1	391.69	118.5	35.56
	26.52	14.56	42.34	35.94	31.6	32.14
	87.06	17.74	0.75	13.5	21.24	6.52
	62.3	26.56	15.57	26.04	30.28	45.23
Mid	81.54	65.25	33.6	44.85	21.6	71.88
	283.2	39.5	81.74	33.52	15.52	36.38
	32.82	35.16	112.81	20.89	4.84	10.76
	149.4	93.85	50.12	9.04	24.12	13.43
Outlet	109.14	82.6	19.2	26.76	46.06	41.64
	40.68	43	34.63	27.12	37.05	25.05
	51.66	41.2	23.35	42.08	14.12	54.18
	62.64	64.04	69.39	79.72	47.3	40.16

B.3 Arysulfatase activity in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	…….(µg g^{-1} soil)…….					
Inlet	130.16	33.44	31.07	62.70	51.64	43.05
	54.37	48.73	53.17	50.80	26.38	14.34
	35.43	46.19	15.87	27.74	33.05	23.05
	43.72	26.01	30.19	46.10	13.47	7.95
Mid	56.20	30.72	34.4	45.76	20.03	7.11
	195.36	40.8	38.17	24.14	35.8	23.52
	50.52	18.37	40.83	10.08	16.41	10.67
	306.42	49.84	38.73	4.39	6.15	11.7
Outlet	148.84	36.92	30.41	27.52	20.22	17.23
	69.30	34.11	33.07	27.23	20.31	12.64
	118.20	42.68	45.68	30.98	45.97	22.77
	45.10	29.97	33.07	40.70	28.77	11.89

B.4 β- glucosidase activity in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	59.45	48.1	36	55.00	62.95	32.46
	49.80	49.7	42.42	16.20	39.15	22.41
	15.80	47.62	16.45	40.56	36.58	26.5
	30.50	55.54	41.3	25.40	29.48	15.71
Mid	36.95	102.28	38.04	33.17	21.87	5.29
	93.96	41.31	32.27	15.46	29.38	21.11
	17.40	31.22	48.28	9.62	13.88	9.48
	106.62	39.28	28.71	3.28	7.76	3.98
Outlet	95.36	44.2	35.25	12.28	23.16	22.01
	23.68	54.41	25.85	7.80	29.38	20.07
	95.70	55.54	56.38	39.42	55.44	31.84
	26.60	24.96	38.88	36.85	37.27	22.32

B.5 Dehydrogenase activity in CM and MPM systems

Wetland	CM			MPM		
	0-3 cm	3-6 cm	6-12 cm	0-3 cm	3-6 cm	6-12 cm
	……(µg g^{-1} soil)……					
Inlet	63.026	12.419	12.245	6.855	8.69	2.668
	16.438	6.771	6.432	4.162	2.549	0.279
	11.313	4.965	1.413	3.755	1.523	1.158
	13.118	2.406	0.723	4.395	0.679	0.501
Mid	4.405	2.205	0.523	5.511	1.216	0.123
	27.617	5.993	3.255	11.564	7.604	5.343
	1.668	1.344	0.612	0.591	0.487	0.279
	14.493	3.726	1.278	0.134	0.928	0.59
Outlet	7	3.812	0.345	2.768	0.813	3.322
	4.251	3.094	1.389	2.329	0.353	0.59
	3.279	3.582	1.034	3.463	1.293	1.323
	2.263	5.161	0.545	3.81	0.852	0.345