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# Effect of Landscape Position and Dairy Manure Addition on Bioavailable Forms of Soil Phosphorus Using Enzyme Hydrolysis

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Themis Mahalia Stone
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# DEDICATION

I would like to dedicate this thesis to Katherine Sides… To an adored fellow country kid and top-notch nature scientist. You can take the kid out of the country, you can take the kid out of the trailer in Texas or Louisiana, but you can't take the love of the country out of the kid. Sides, I was so lucky to go to school with someone whose joy of exploration, clarity of thought, mad tech skills, and generosity with their time made something like graduate school possible, and even sometimes enjoyable.

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# ABSTRACT

Bioavailable phosphorus (P) has traditionally been measured as inorganic orthophosphate ($PO_4$) while organic P ($P_o$) has been considered of limited relevance to short-term biological consumption. However, enzymes secreted by bacteria and fungi which serve to mineralize $P_o$ are ubiquitous in the environment and may contribute significant bioavailable P over time. In order to assess environmentally-relevant, potentially bioavailable P in soils with added dairy wastes, microcosms of identical soil series but differing management histories and landscape positions were incubated at 24 C° for three weeks. Subsamples were taken weekly from microcosms and analyzed by: 1) Mehlich-3 extractant, 2) NaOH-EDTA subjected to sulfuric acid total digest, and 3) NaOH-EDTA subjected to enzymatic hydrolysis by one, two or three commercially-available phosphatase enzymes. A high throughput, microplate reader method (Johnson and Hill, 2010) was adapted to quantify the enzyme-hydrolysable P. There were significant microcosm changes ($P$ <0.05), with three week 24 C° incubation, in Mehlich-3 extractable P, with soils having low total P (TP) showing decreases and soils in both upland and depositional field positions increasing in Mehlich-3 extractable P. Also, over three weeks of study, low TP control soils subjected to three enzymes had a 58% increase in MRP over initial MRP in NaOH-EDTA soil extracts, whereas dairy farm upland soils had a 9% increase and depositional soils had an increase of 3%. Soils with higher levels of TP had smaller amounts of enzymatically hydrolysable P than soils containing low levels of TP. This points to limitations of this enzyme hydrolysis method for assessing potentially available $P_o$ in soils with very high Mehlich-3 and TP (Mehlich-3 P: >120 mg/kg; total P: >1,200 mg/kg). In conclusion, NaOH-EDTA extracts a range of $P_o$ species that can be transformed in the environment into P available to aquatic algae, but results from the Johnson-Hill method differ widely, even in identical series soils, possibly depending on field position and animal waste additions leading to high copper, zinc and TP.

# TABLE OF CONTENTS


Chapter Page

CHAPTER I LITERATURE REVIEW ..... 1
Phosphorus and water quality in Tennessee ..... 10
Biologically mediated phosphorus cycling ..... 17
Soil phosphorus testing ..... 25
Research Objectives ..... 29
CHAPTER II MATERIALS AND METHODS ..... 31
Site description ..... 31
Soil sampling, processing and pH testing ..... 36
Microcosm setup and sampling ..... 37
Enzymatic hydrolysis ..... 40
Mehlich-3 extractions ..... 42
Total P digests of soils and NaOH-EDTA extracts ..... 42
Model organic P compounds ..... 44
CHAPTER III RESULTS AND DISCUSSION ..... 47
Model compounds ..... 47
Soil characteristics ..... 53
Total P and molybdate-reactive P (MRP) in NaOH-EDTA soil extracts ..... 55
Effects of procedure physical conditions on transformations of $P_o$ to $P_i$ ..... 57
Microcosm Mehlich-3 extractable P ..... 58
Enzymatic hydrolysis in NaOH-EDTA microcosm extracts ..... 61
*Upland soils* ..... 61
*Depositional soils* ..... 64
*Control soils* ..... 67
*Time and treatments effects across all microcosm soils* ..... 72
CHAPTER IV CONCLUSIONS AND RECOMMENDATIONS ..... 76
Conclusions ..... 76
Recommendations ..... 77
REFERENCES ..... 80
APPENDIX A ..... 89
Vita ..... 122

# LIST OF TABLES

Table Page

Table 1. Examples of human intervention in the global biogeochemical cycles of carbon, nitrogen, phosphorus, sulfur, water, and sediments (Falkowski et al., 2000). ..... 2

Table 2. Phosphorus balance and efficiency of plant and animal uptake of P for the United States and several European countries (Carpenter et al., 1998 from Isermann, 1991). ..... 3

Table 3. Nitrogen and phosphorus discharges to surface waters (in $10^3$ mg/yr) from non-point and point sources in the United States (Carpenter et al., 1998). ..... 7

Table 4. U.S. stream and river reference and measured values for nitrogen (TN) and phosphorus (TP) (Dodds et al, 2009). ..... 11

Table 5. EPA water use categories (TDEC, 2008a) ..... 14

Table 6. Percent recovery of model Po compounds via enzymatic hydrolysis by three enzyme groups. ..... 49

Table 7. Percent recovery of two model $P_o$ compounds incubated with one, two or three enzymes and with or without EDTA……50

Table 8. Total soil P and total NaOH-EDTA extractable P by sulfuric acid digestion and Mehlich 3 extractable P, calcium (Ca), magnesium (Mg), potassium (K), copper (Cu) and zinc (Zn) of the three studied soils..... 54

Table 9. Multiple comparisons of microcosm sampling times across all soils and conditions (Tukey post-hoc tests: SPSS/PASW Statistics 18, 2010)..... 72

Table 10. Percent increase in bioavailable P in three microcosm soils subjected to one, two or three enzymes……74

Table 11. Tukey post hoc tests performed on 1-21 soil/treatment pairs. See legend above for description of “soils” 1-21 (SPSS/PASW Statistics 18, 2010). ..... 92

Table 12. Homogeneous subsets for treatments. Three soils of four sampling times each: N = 36 (PASW/SPSS Statistics 18, 2010)……119

## LIST OF FIGURES

Figure Page

Figure 1. Examples of human intervention in the global biogeochemical cycles of carbon, nitrogen, phosphorus, sulfur, water, and sediments. Data are for the mid-1900s (Falkowski et al., 2000). ..... 2

Figure 2. Median North Carolina Mehlich-3 soil test P index values for all crops by county for the year 2003. A soil test index value of >50 indicates a site on which crops are not expected to show a response to further P additions (Johnson et al., 2005). ..... 9

Figure 3. Relative impacts of pollution by source and percent of total Tennessee stream miles assessed (TDEC, 2008a). ..... 16

Figure 4. Model of phytase enzyme with an activator cation in right corner and phosphorus compounds as green, red and blue models (Wikipedia Commons, 2010). ..... 21

Figure 5. Location of Pond Creek sub-watershed within Watts Barr watershed of East Tennessee (Hagan and Walker, 2006). ..... 34

Figure 6. Pond Creek agricultural land uses (Walker and Carmichael, 2010). ..... 35

Figure 7. Enzyme-labile organic P quantification method (Johnson and Hill, 2010)……39

Figure 8. $H_2SO_4$ total P digests of microcosm NaOH-EDTA extracts versus NaOH-EDTA microcosm extract “Base P” (MRP) read at time of enzyme assays with Biotek plate reader. ..... 56

Figure 9. Comparisons of NaOH-EDTA soil extracts either processed in tandem (Chem P) or set aside before the enzymatic hydrolysis procedure (Base P). ..... 58

Figure 10. Comparisons of Mehlich-3 extractable P in three soils over 21 days. ..... 60

Figure 11. Upland soil $H_2SO_4$ total P digests of NaOH-EDTA extracts versus NaOH-EDTA microcosm extract “Base P” (MRP) and enzyme-labile Po hydrolyzed by 1 (PP), 2 (GP) or 3 (NP) enzymes. Mehlich-3 labile P is also shown. ..... 62

Figure 12. Depositional soil $H_2SO_4$ total P digests of NaOH-EDTA extracts versus NaOH-EDTA microcosm extract “Base P” (MRP) and enzyme-labile Po hydrolyzed by 1 (PP), 2 (GP) or 3 (NP) enzymes. Mehlich-3 labile P is also shown. ..... 65

Figure 13. Off-farm soil $H_2SO_4$ total P digests of NaOH-EDTA extracts versus NaOH-EDTA microcosm extract “Base P” (MRP) and enzyme-labile Po hydrolyzed by 1 (PP), 2 (GP) or 3 (NP) enzymes ..... 68

Figure 14. Percentages of $P_o$ in all microcosm experiment soils that were hydrolyzed by various enzyme combinations……70

Figure 15. Mehlich-3 phosphorus distribution in 56 hectare main dairy farm field (Graham, 2009). ..... 89

Figure 16. Mehlich 3 extractable potassium (A), calcium (B), magnesium (C) and pH (D) distribution in 56 hectare dairy farm main field (D. Graham, 2009). ..... 90

Figure 17. Aggregations of level III USEPA ecoregions for the national nutrient strategy (USEPA publication 822-B-01-012, 2001)……121

# CHAPTER I
# LITERATURE REVIEW

## The phosphorus problem in soils and aquatic systems

### *Introduction*

Since the Industrial Revolution, humans have altered between one third and one half of the Earth's surface for agricultural and other uses (Vitousek et al., 1997a). Accordingly, we have had profound effects on the global biogeochemical cycling of carbon (C), N and P (Table 1) (Falkowski et al., 2000; Smith et al., 1999). Industrial fixation of N has increased from less than 10 million metric tons in 1950 to over 80 million metric tons in 1990 and is predicted by some researchers to be around 135 million metric tons by 2030 (Vitousek et al., 1997b). While Falkowski et al. (2000) note that global N fluxes have changed by 108% due to human activities, P newly available to aquatic and terrestrial systems due to human activity has increased by 400% (Table 1). At present, mining is the primary source of P for use in the U.S. (Figure 1; Table 1). As is apparent from Figure 1, mining of P has increased dramatically in the decades since World War II (Falkowski et al., 2000). Like N applied to land for crop use, small but significant amounts of P are lost from agricultural systems to waterways (Brady and Weil, 1999). Phosphorus is often added to soils in excess of what plants are able to consume on a season-by-season basis (Table 2). This P either remains in the soil or is lost though erosion or leaching (Carpenter et al., 1998). While there is considerable variance worldwide, only about 30% of P applied to land in the U.S. is incorporated into plants (Table 2). Brady and Weil (1999) describe "the phosphorus problem in soil fertility" whereby: 1) native soils are initially low in P, 2) P compounds found in soil are usually unavailable for plants, and 3) soluble P additions from fertilizers are rapidly fixed when added to soils. Thus P has the potential to build up in soils.

Table 1. Estimates of human intervention in the global biogeochemical cycles of carbon, nitrogen, phosphorus, sulfur, water, and sediments. (from Falkowski et al., 2000).

| Element | Flux | Magnitude of flux (millions of metric tons per year) | | % change due to human activities |
|---|---|---|---|---|
| | | Natural | Anthropogenic | |
| C | Terrestrial respiration and decay $CO_2$ | 61,000 | | |
| | Fossil fuel and land use $CO_2$ | | 8,000 | +13 |
| N | Natural biological fixation | 130 | | |
| | Fixation owing to rice cultivation, combustion of fossil fuels, and production of fertilizer | | 140 | +108 |
| P | Chemical weathering | 3 | | |
| | Mining | | 12 | +400 |
| S | Natural emissions to atmosphere at Earth's surface | 80 | | |
| | Fossil fuel and biomass burning emissions | | 90 | +113 |
| O and H (as $H_2O$) | Precipitation over land | $111 \times 10^{12}$ | | |
| | Global water usage | | $18 \times 10^{12}$ | +16 |
| Sediments | Long-term preindustrial river suspended load | $1 \times 10^{10}$ | | |
| | Modern river suspended load | | $2 \times 10^{10}$ | +200 |

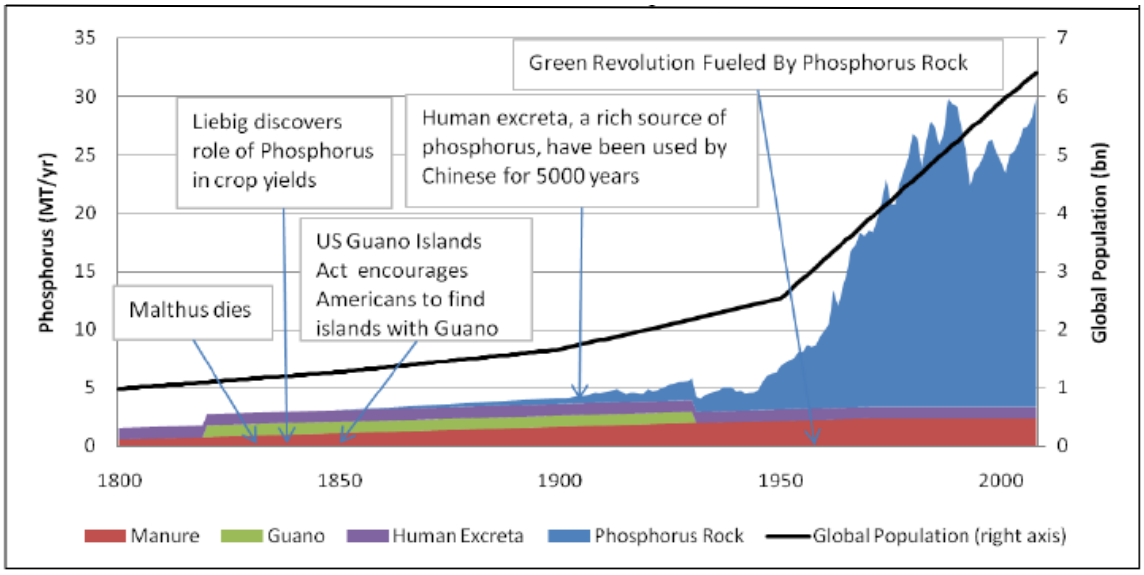



Figure 1. Historical sources and approximate U.S. use of P over the last two centuries. Primary y-axis is in millions of metric tons of marketable rock phosphate containing between 26% and 34% percent $P_2O_5$ (from Buckingham and Jasinski, 2009).

Table 2. Phosphorus balance and efficiency of plant and animal uptake of P for the United States and several European countries (from Carpenter et al., 1998).

| Nation | Area in agriculture ($10^6$ ha) | P input ($kg \cdot ha^{-1} \cdot yr^{-1}$) | | P output ($kg \cdot ha^{-1} \cdot yr^{-1}$) | | P surplus ($kg \cdot ha^{-1} \cdot yr^{-1}$) | Efficiency of uptake (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fertilizer | Feed | Animal | Plant | | Plant | Animal | Total |
| East Germany | 6.2 | 25 | 6 | 3 | 1 | 27 | 59 | 10 | 11 |
| West Germany | 12.0 | 27 | 10 | 10 | 3 | 24 | 76 | 34 | 35 |
| Ireland | 5.7 | 11 | 1 | 3 | 1 | 8 | 72 | 22 | 30 |
| Netherlands | 2.3 | 18 | 44 | 17 | 5 | 40 | 69 | 24 | 38 |
| Switzerland | 1.1 | 22 | 11 | 6 | 4 | 23 | 91 | 18 | 28 |
| United Kingdom | 18.5 | 9 | 3 | 2 | 1 | 9 | 55 | 18 | 25 |
| United States | 394.7 | 39 | 5 | 13 | 5 | 26 | 56 | 15 | 33 |

Of the essential elements for growth of primary producers, orthophosphate ($H_2PO_4^-$ and $HPO_4^{2-}$, hitherto referred to as $P_i$) is the least well represented in the Earth's crust, accounting for only about 0.1% of lithospheric materials (Quiquampoix and Mousain, 2005). Of the macronutrients needed for plant growth, P is present in the lowest quantities in the soil solution or in readily available forms in mineral soils (Brady and Weil, 1999). Productivity in terrestrial ecosystems is often determined by the chemical form (speciation) and stability of P, its adsorption and the average concentration of P in the soil solution (Quiquampoix and Mousain, 2005). Researchers have estimated average soil solution P concentrations to be around 1 μM P, or about 0.65 ppm (Quiquampoix and Mousain, 2005). These low available P concentrations often control primary productivity in both aquatic and terrestrial ecosystems. With P additions, this limiting factor is removed and primary producers grow until another factor is limiting. Many of the world's highly weathered soils, including Ultisols and Oxisols, are considered P deficient in relation to plant growth because of the aforementioned insolubility of P (Brady and Weil, 1999).

## Phosphorus and surface water quality

Phosphorus (P) is an essential nutrient for plants and animals but movement of excess P from soils to aquatic systems can have disastrous consequences if eutrophication results from the removal of P as the most-limiting factor for algal growth. Eutrophication can also be caused by additions of nitrogen (N) in previously N-limited environments. Excessive aquatic algal and aquatic plant growth as a result of human activities, also known as cultural eutrophication, is the most common problem facing surface waters today (Schindler, 2009; Smith et al., 1999; Carpenter et al., 1998). The extent of cultural eutrophication is so significant that it has been visualized by NASA satellites in various large water bodies such as the Baltic Sea (Schindler and Vallentyne, 2008). It follows that eutrophication has significant ecosystem and human costs. Ecosystem shifts include increased biomass of phytoplankton, macrophyte vegetation, benthic/epiphytic algae, and blooms of algal species which can result in substances produced that are toxic to other aquatic species and humans (Smith and Schindler, 2009). Ecosystem shifts can be defined as changes in biogeography which may be characterized by decreases in multiple species previously common to an ecosystem and replacement by a smaller range of species (Yamamuro et al., 2006; Beaugrand et al., 2002). Increases in aquatic primary production results in both decreased species diversity and excessive biomass availability for decomposers. Bacterial decomposition of algal biomass results in aquatic oxygen depletion which leads to fish kills and reductions in diversity of species sensitive to decreased oxygen (Smith and Schindler, 2009). Eutrophication costs immediately noticeable to humans include decreases in desirable shellfish and fish species and negative changes in water taste, odor, transparency and aesthetic value (Smith and Schindler, 2009). Phosphorus enrichment of freshwaters often substantially degrades aquatic ecosystems and prevents low cost water use for drinking, recreation and other purposes (Carpenter, 1998).

Economic costs associated with eutrophic waters are complex and, thus, difficult to precisely quantify. Pretty et al. (2003) note that consequences of eutrophication are largely known but the costs of these consequences have been poorly studied. These researchers addressed the economic costs of freshwater eutrophication in England and Wales and estimated costs to be $105-160 million U.S. dollars $yr^{-1}$ for this region alone (Pretty et al., 2003). These costs were subdivided into policy response costs, reduced recreational and waterfront property value, increased costs from chemical and biological water treatment, negative effects on biota, and economic losses from tourist industries (Pretty et al., 2003). Leffler (1997) estimates that tens of millions of dollars have been lost due to fish kills and closed beaches over the last two decades in coastal regions of the U.S. Pretty et al. (2005) reviewed negative externalities due to agricultural production in the United Kingdom (UK) and found a cost of $126.1 million U.S. dollars $yr^{-1}$ attributable to eutrophication. A recent study found that freshwater eutrophication in the U.S. costs about $2.2 billion $yr^{-1}$ (Dodds et al., 2009). Similar to the aforementioned UK study, these costs are due to loss in recreational water usage, waterfront real estate devaluation, spending on recovery of threatened and endangered species, and bottled drinking water costs (Dodds et al., 2009). This study was not able to quantify economic losses in commercial fishing industries and found gaps in reporting on frequency of algal blooms and fish kills. Thus the $2.2 billion $yr^{-1}$ estimated by Dodds et al. (2009) is weighted towards losses in recreational and property values and underestimates categories for which historical data is limited, such as economic costs of estuary destruction. Pretty et al. (2003) note that preventing the causative agents of eutrophication from entering biological systems is the most cost-effective way to prevent ecosystem damage and resulting costs. Likewise, preventing nitrogen (N) and P additions to surface waters is among the most cost-effective means of increasing water supplies for human use (Carpenter et al., 1998).

Because P is often the most limiting nutrient for algal growth in streams, rivers and lakes, additions of P can cause algae to rapidly grow until another nutrient becomes

limiting (Johnson and Hill, 2010; Smith et al., 1999). The idea of nutrient limitation is a cornerstone in the study of eutrophication and includes the concept that a single nutrient should be the primary limiting factor for plant growth in a given ecosystem (Smith et al., 1999). Therefore, growth of plants should be proportional to the influx rate of this nutrient and controlling eutrophication can be accomplished by restricting the influx of the controlling nutrient (Smith et al., 1999). Nitrogen is usually the most limiting nutrient in temperate estuaries and coastal waters, whereas P usually limits production in freshwater systems (Carpenter et al., 1998). Aquatic primary producers can respond to increases in dissolved reactive phosphorus (DRP) in the parts per billion range; however the exact quantities of DRP that will cause eutrophication in a given water body depend on a number of factors (McDowell et al., 2004). These facets include sorption capacity of stream sediments, climate, stream tree canopy cover and natural trophic status of a water body (Dodds et al., 2009). These biological, geochemical and physical facets are increasingly influenced by humans.

## Phosphorus loss from agricultural soils

### *Point versus non-point source pollution*

Phosphorus lost from agricultural catchments into waterways is considered a non-point source (NPS) of pollution. Carpenter et al. (1998) note that NPS N and P come from a range of rural landscapes (Table 3). Because nutrient inputs originate from extensive areas of land, it is difficult to trace their exact origin (Brady and Weil, 1999). In contrast, point source (PS) pollution has a single, identifiable location, such as a pipe from which pollutants are discharged. The EPA incorporates airborne pollution in its definition of NPS and describes NPS pollution as originating from “land runoff, precipitation, atmospheric deposition, drainage, seepage, or hydrologic modification”

Table 3. Estimated N and P discharges to surface waters (in Mg $yr^{-1}$) from non-point and point sources in the United States (from Carpenter et al., 1998).

| Source | Nitrogen | Phosphorus |
|---|---|---|
| Nonpoint sources | | |
| Croplands | 3204 | 615 |
| Pastures | 292 | 95 |
| Rangelands | 778 | 242 |
| Forests | 1035 | 495 |
| Other rural lands | 659 | 170 |
| Other nonpoint sources | 695 | 68 |
| Total | 6663 | 1658 |
| Total point sources | 1495 | 330 |
| Total discharge (nonpoint + point) | 8158 | 2015 |
| Nonpoint as percentage of total | 82% | 84% |

*Note:* Data are modified from Havens and Steinman (1995) and Gianessi et al. (1986).

(USEPA, 1999). Non-point pollution is difficult to measure and regulate because it is derived from activities dispersed over large areas (Carpenter et al., 1998). The effects of these activities are also variable due to spatially and temporally variable storm events (Carpenter et al., 1998). Point sources of N and P water pollution are important contributors to water quality degradation but can generally be eliminated through institutional and legislative action. Unlike PS pollutants, NPS discharges are unregulated by the Clean Water Act (CWA), and thus the CWA has reduced PS pollution but has not solved NPS problems (Houck, 1999).

According to the EPA's National Water Quality Inventory, NPSs contribute nearly two-thirds of water pollutants, and of this, around half is from agricultural sources (USEPA, 2008b). Agricultural activities which contribute to NPS pollution are grazing, plowing, planting, harvesting, fertilizing, pesticide applications and irrigation (USEPA, 2008c). Pollutants derived from these activities include pesticides, salts, sediments, pathogens and macronutrients such as N and P (USEPA, 2008c). Carpenter et al. (1998) note that NPS N and P pollution more than doubles that of PS inputs of these nutrients to waterways and that cropland is by far the single largest agricultural land use contributing to P pollution (Table 3).

While NPS pollution is diffuse in nature, there are certain areas within fields, watersheds and regions of the U.S. that contribute significantly higher amounts of pollution to surface waters than others. These areas are referred to as critical source areas and often have characteristics in common such as steep slopes, highly erodible soil, management which frequently leaves soil without vegetation, limited or no vegetative buffers between cropping fields and surface water, and livestock areas within close proximity to waterways (White et al., 2009). Conservation practices can reduce NPS nutrient pollution but funding, installation and placement of these practices is variable in both number and effectiveness (White et al., 2009).

***Animal feeding operations***

Regardless of how and why P is lost from agricultural systems, researchers have found that greater TP content in soils relates to increased dissolved and particulate P in runoff (Smith et al., 1999; Sharpley, 1995). The P problem in soil fertility has led to gradual soil TP accumulations in cropped soils in many areas of the developed world. Additionally, the concentration of animal feeding operations (AFOs) in certain parts of the U.S. has led to areas surrounding these operations having upper-profile soil test P (STP) and TP values two- to five-fold higher than surrounding unammended soils (Koopmans et al., 2007; Allen and Mallarino, 2006). Animal feeding operations are defined by the EPA as locations where animals are kept for 45 days or more in a 12 month period and where vegetation is not maintained during a typical growing season (USEPA, 2000). For example, North Carolina, a major poultry and hog-producing state, has a majority of counties with median STP values above levels necessary for crop production (Figure 2).

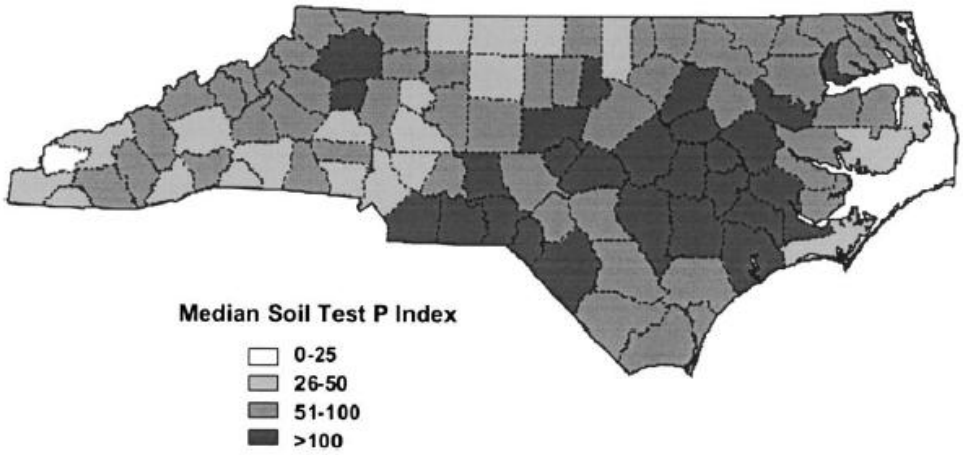



Figure 2. Median North Carolina Mehlich-3 soil test P index values for all crops by county for the year 2003. A soil test index value of >50 indicates a site on which crops are not expected to show a response to further P additions (from Johnson et al., 2005).

The importation of grains and other animal feedstuffs to AFO locations results in P-rich wastes being concentrated far from where the P is needed for growing crops. This animal waste must be disposed of locally without adding excessive transportation costs (Sharpley et al., 2004). Producers of swine AFOs typically own surrounding fields and pump liquid manure from lagoons onto these properties. Broiler and turkey AFOs typically produce dry manures and these wastes are applied on surrounding fields when possible and throughout local areas when necessary. The result of excessive P additions to soils can be that the sorption capacity of soils becomes saturated and the ability of a soil to "fix" or sorb further additions of P becomes limited (Allen and Mallarino, 2006). Soils in these areas become P-enriched over time and contribute to water quality problems in local and hydrologically-connected areas (Leytem et al., 2004; Sharpley et al., 2004). Many factors contribute to this P lost from agriculture landscapes including levels of STP and TP, soil texture, topography, climate, cropping operations, rate and timing of manure and inorganic fertilizer applications, soil biology and geochemistry, and soil vegetative cover (Hill and Cade-Menun 2009; Bunemann, 2008; McDowell et al., 2004).

Turner (2005) conceptualized loss of $P_o$ species from soils to waterways as a process driven by rainfall. Organic P species include phosphate monoesters, phosphate diesters and phosphonates which are soluble or sorbed to soil particles (Turner, 2005). These rainfall-driven processes include mobilization leading to dissolved forms of $P_o$, transport and transformations of this $P_o$ and ultimately P impact on waterbodies (Turner, 2005). The mobilization of soil $P_o$ species includes soil erosion (detachment) and solubilization via desorption, re-wetting and microbial turnover (Turner, 2005). Solution $P_o$ includes soluble, colloidal and particulate forms (Turner, 2005). Organic P transportation includes overland flow, tile drainage, interflow and leaching (Turner, 2005).

## Phosphorus and water quality in Tennessee

### *Reference streams*

In order to define costs of eutrophication, Dodds et al. (2009) defined the degree to which macronutrients (N and P) in water departed from reference values (Table 4). Surface water reference values represent natural waters with the least possible human impact and serve as points of comparison for the extent departure from unaltered states. Table 4 shows references values of total phosphorus (TP) from designated rivers and streams in all aggregated Level III Ecoregions of the continental U.S. and percentages of sampled waterways that had values above reference values. These data were gathered by the U.S. Environmental Protection Agency (EPA) from representative samples of the population of waterways in each ecoregion (Dodds et al., 2009). This study noted that all ecoregions had a majority of waterways with measured TP levels above reference values. Furthermore, Dodds et al. (2009) found that TP means were up to 3-fold higher than reference values and, in 9 out of 14 ecoregions, over 80% of sampled rivers and streams surpassed reference values. Ecoregion IX, Southeast

Table 4. US EPA reference and measured values for total phosphorus (TP) in U.S. streams and rivers by aggregated level III ecoregions (Figure 17, Appendix). Number of sampling stations is in parentheses (modified from Dodds et al, 2009).

| Ecoregion | Ecoregion name | Reference TP in mg $L^{-1}$ | Measured TP median in mg $L^{-1}$(n) | % of rivers > reference median |
|---|---|---|---|---|
| I | Willamette & Central Valleys | 0.016 | 0.088 (178) | 96 |
| II | Western Forested Mountains | 0.019 | 0.026 (1380) | 60 |
| II | Xeric West | 0.012 | 0.055 (808) | 75 |
| IV | Great Plains Grasslands | 0.046 | 0.087 (341) | 67 |
| V | Central Cultivated Plains | 0.049 | 0.184 (489) | 86 |
| VI | Corn Belt & N. Great Plains | 0.052 | 0.168 (815) | 90 |
| VII | Mostly Glaciated Dairy Region | 0.022 | 0.080 (910) | 87 |
| VIII | Nutrient-poor Upper Midwest & Northeast | 0.013 | 0.021 (608) | 65 |
| IX | Southeastern Temper. Forested Plains & Hills | 0.048 | 0.080 (2104) | 68 |
| X | TX/LA Coastal & MS Alluvial Plains | 0.048 | 0.176 (295) | 99 |
| XI | Central and East Forested Uplands | 0.020 | 0.022 (1591) | 53 |
| XII | Southern Coastal Plain | 0.025 | 0.103 (466) | 85 |
| XIII | Southern Florida Coastal Plain | 0.036 | 0.080 (n not available) | 87 |
| XIV | Eastern Coastal Plain | 0.015 | 0.077 (375) | 95 |

Temperate Forested Plains and Hills, has one of the highest reference TP values but was still found to have greater than 68% of measured waterways with TP concentrations over this threshold. Ecoregion IX includes much of the crop production acreage and lands on which animal operations are located in central and western Tennessee and central and eastern North Carolina.

Reference values are linked to climate and ecoregion-specific nutrient levels which have resulted in historically stable and biodiverse ecosystems over the last centuries. Reference values are set by ecoregion because region-specific climatic conditions, hydrological factors and species composition, among other factors, mediate nutrient availability. Reference values encapsulate TP values from waterways not significantly altered by anthropogenic inputs. According to Dodds et al. (2009), eutrophication may occur in streams in central Tennessee and central North Carolina (EPA Nutrient Ecoregion IX) above 0.048 ppm TP. Eastern Tennessee and western North Carolina (EPA Nutrient Ecoregion XI) have a lower stream reference value of 0.020 ppm TP (Dodds et al., 2009).

***Designated water uses***

The Tennessee Department of Environment and Conservation (TDEC) catalogs surface water impairment by monitoring and analyzing the degree to which a particular section of surface water departs from its designated uses as determined by the state of Tennessee. There are seven designated uses for waterways: 1) fish and aquatic life, 2) recreation, 3) irrigation, 4) livestock watering and wildlife, 5) drinking water supplies, 6) industrial water supplies, and 7) navigation (TDEC, 2008a). Due to a 2000 federal law, all streams, rivers, lakes and other water bodies in the state of Tennessee are designated as having at least two public uses, which are recreation and fish and aquatic species protection (TDEC, 2008a). In addition, Tennessee further classifies all water bodies as designated for irrigation, livestock and wildlife use (TDEC, 2008a). Drinking water

classifications apply to both waterbodies that are presently used for that function or are likely to be used in the future (TDEC, 2008a).

Waterbodies are assessed for impairment by comparing monitored water conditions to water quality standards for the waterbody's designated uses (TDEC, 2008a). Water quality criteria for drinking water use, for example, includes pH values in the range of 6-9, total dissolved solids that do not exceed 500 mg l-1, turbidity that can be reduced to acceptable concentrations by standard water treatment processes, *E. coli* concentrations that do not exceed a five sample mean of 630 per 100 ml and no toxic substances that materially affect the health and safety of humans or animals (TDEC, 2008c). Also, numeric limits for approximately 64 commonly occurring toxic substances are defined, and vary depending on the substances (TDEC, 2008c). For example, Toluene is allowed in concentration of 1,000 $\mu l\ l^{-1}$, whereas vinyl chloride, Alachlor and mercury (Hg) have allowable concentrations of 2 $\mu l\ l^{-1}$ (TDEC, 2008c).

After water quality, and thereby use support, is determined, waterbodies are assigned to one of five EPA-recommended impairment categories (Table 5) (TDEC, 2008a). As referenced in Table 5, when a waterbody is found to not meet one or more designated uses, it is placed in category 5 and is therefore added to the 303(d) list, a document compiling public waters in Tennessee that are "water quality limited" or are expected to be so in the following two years and need additional pollution control measures (TDEC, 2008b). These impaired waterbodies are prioritized for Total Maximum Daily Load (TMDL) studies. A TDML study, "(1) quantifies the amount of a pollutant in a stream, (2) identifies the sources of the pollutant, (3) and recommends regulatory or other actions that may need to be taken in order for the stream to no longer be polluted" (TDEC, 2008b).

Table 5. EPA water-use impairment categories (from TDEC, 2008a)

| | |
|---|---|
| **Category 1** | waters are **fully supporting** of all designated uses. These streams, rivers, and reservoirs have been monitored and meet the most stringent water quality criteria for all designated uses for which they are classified. The biological integrity of Category 1 waters is favorably comparable with reference streams in the same subecoregion and pathogen concentrations are at acceptable levels. |
| **Category 2** | waters are **fully supporting** of some designated uses, but have not been assessed for all uses. In many cases, these waterbodies have been monitored and are fully supporting of fish and aquatic life, but have not been assessed for recreational use. |
| **Category 3** | waters are **not assessed** due to insufficient or outdated data. However, streams previously identified as impaired are not moved to this category simply because data are old. |
| **Category 4** | waters are **impaired**, but a TMDL has been completed or is not required. Category 4 has been further subdivided into three subcategories. |
| | **Category 4a** impaired waters that have already had all necessary TMDLs approved by EPA. |
| | **Category 4b** impaired waters do not require TMDL development since "other pollution control requirements required by local, State or Federal authority are expected to address all water-quality pollutants" (EPA, 2003). An example of a 4b stream might be where a discharge point will be moved in the near future to another waterbody with more assimilative capacity. |
| | **Category 4c** impaired waters in which the impacts are not caused by a pollutant (e.g., flow alterations). |
| **Category 5** | waters have been monitored and found to not meet one or more water quality standards. These waters have been identified as **not supporting** their designated uses. Category 5 waterbodies are moderately to highly impaired by pollution and need to have TMDLs developed. These waters are included in the 303(d) List of impaired waters in Tennessee. |

### *United States EPA 303(d) list*

Once a stream is placed on the 303(d) list, it is considered a priority for water quality improvement measures (TDEC, 2008b). Water quality improvement efforts include regulatory actions such as halting permits to potential pollutant producers, not allowing dischargers to expand or locate on 303(d) listed waterways and not authorizing additions of any pollutant found to be the cause of use limitations (TDEC, 2008b). Further, efforts to limit pollution discharge may target land uses such as crop production and forestry that are usually exempted from pollution discharge permit requirements (TDEC, 2008b). These efforts may include generating and using county, state and federal funds for installation of Best Management Practices (BMPs) on privately owned lands (Hagan and Walker, 2006). Funding to support improvements in 303(d) listed watersheds in Tennessee can come from the Tennessee Valley Authority (TVA), the federal Natural Resources Conservation Service (NRCS), the Tennessee Department of Agriculture (TDA) and the University of Tennessee. Funding and technical expertise has assisted area landowners in implementing pollution control measures by providing free grass seed, free soil tests and installing infrastructure BMPs such as livestock fencing and heavy-use area gravel (Hagan and Walker, 2006). These measures address NPS pollutants and generally require voluntary action on the part of land owners.

### *The status of water quality in Tennessee*

The 2008 303(d) TDEC report, *The Status of Water Quality in Tennessee*, provides information on the relative impacts of various pollutants in Tennessee waterways (Figure 3)(TDEC 2008a). Nutrient, pathogen and habitat alterations constitute 59% of impairment in assessed Tennessee waterways (Figure 3). Of nutrient-impaired stream miles, excessive nitrate/nitrite was found in 1,371 miles and excessive phosphate/total P was found in 1,218 miles (TDEC, 2008a). A given stream can be impaired for both

excessive nitrate and phosphate. Water-borne pathogens and habitat alterations relate to uncontrolled livestock access to waterways (Walker and Carmichael, 2010). Nutrient impairment is tied to livestock operations on several tiers: uncontrolled animal access to waterways places N and P directly in waters, manures from grazing lands can be lost to surface water via runoff and erosion during storms, and land applications of wastes from animal feeding operations can lead to high soil nutrient contents resulting in NPS losses to waterways over time.

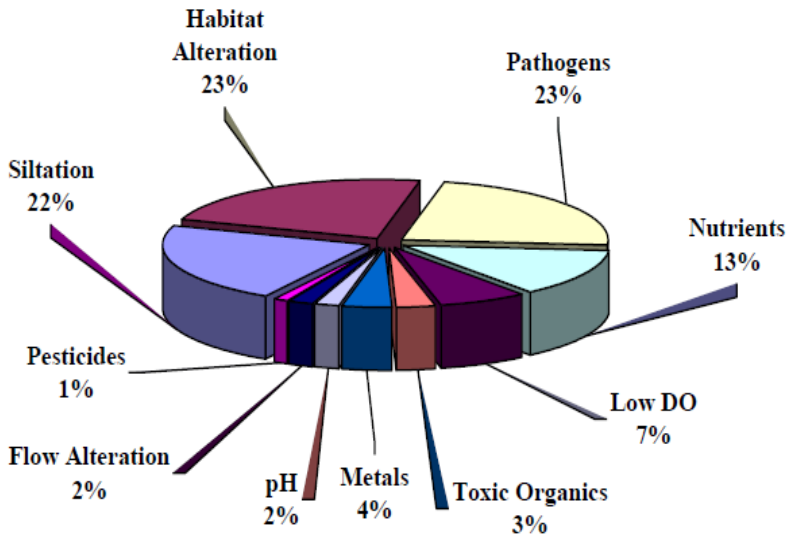



Figure 2. Relative impacts of pollution by source on Tennessee stream miles assessed (TDEC, 2008a).

Because P itself is not toxic to humans, recommended limits for P in domestic water supplies are not set. Instead, TDEC has set criteria in relation to P impacts on fish and wildlife such that P concentrations should not stimulate aquatic plant growth and substantially alter aquatic habitat and biological integrity (TDEC, 2008c). Fish and aquatic life and recreation are the only waterbody designated uses that have nutrient criteria (TDEC, 2008a). However, waters are not categorized as nutrient impaired unless

negative “biological or aesthetic impacts are also documented” (TDEC, 2008a). Because there is variability due to physical and hydrological characteristics amongst waterbodies at which excess N and P negatively impact biological integrity, chlorophyll *a* is sometimes measured as a nutrient response variable as a proxy for direct N and P assessments (TDEC, 2008a). Other nutrient response variables include observable characteristic such as turbidity and algal biomass (MDEC, 2011).

In addition to nutrients, pathogens and habitat alteration, siltation and low dissolved oxygen (DO) contribute to an additional 29% of impairment in assessed stream miles (Figure 3) (TDEC, 2008a). Low DO and significant diurnal DO fluctuations can be response variables for excessive nutrients (TDEC, 2008a). Added nutrients can lead to excessive algal growth which adds DO to waters during times of photosynthesis and depletes DO during respiration. Upon algal death and subsequent decay, DO levels can stay consistently low, causing both selection for low DO tolerant aquatic species and die-offs of species intolerant of low DO. Additionally, silt additions can have a number of negative effects, including smothering fish eggs and aquatic invertebrates, clogging fish gills, interfering with photosynthesis and changing water depth, flow and temperature. Also, water pollutants such as P can be bound to sediment and can contribute to eutrophication over time.

## Biologically mediated phosphorus cycling

A portion of the inorganic P added to soil is incorporated into soil food webs and becomes a component of soil organic matter (SOM). Soil organic matter consists of C, oxygen (O), hydrogen (H), N, S and P (Dalal, 1977). It has been estimated that the ratio of C:N:P in mineral soil organic matter is 110:9:1 (Dalal, 1977). Over time, some of soil organic matter becomes humic substances, a complex mixture of organic materials that have been significantly modified from their original form (Brady and Weil, 1999). About

60-80% of SOM is in the form of humic compounds and may be bound to clay particles and hydroxides and thus be protected from decomposition by microbial attack (Brady and Weil, 1999). A portion of a soil's $P_o$ is contained in humic substances and SOM, with high SOM content being associated with high P content in some soils (Dalal, 1977).

In some soils horizon with high biological activity, organic P ($P_o$) can represent between 20% and 80% of total P (He et al., 2004; Dalal, 1977). In soils with substantial amounts of insoluble $P_i$ but with relatively large quantities of accumulated $P_o$, overall P availability may be controlled by $P_o$ mineralization (Quiquampoix and Mousain, 2005). Mineralization is in turn controlled by both abiotic (Baldwin et al., 2005; Celi and Barberis, 2005) and biotic processes (Hill et al., 2007; Giri et al., 2006; Sinsabaugh, 1994). Biotic processes include release of organic acids by plants as well as minerization of $P_o$ compounds by reductase, oxidase and hydrolase enzymes secreted by microorganisms (Sinsabaugh, 1994). Enzymatically-mediated $P_o$ mineralization will be discussed shortly.

Abiotic mineralization of $P_o$ in the environment has two major pathways: hydrolytic and photolytic reactions (Baldwin et al., 2005). The relative importance of these pathways is tied to nature of the $P_o$ compound and the physical environment in which it is found (Baldwin et al., 2005). Photolytic reactions are most important in parts of the environment where light of specific wavelengths can penetrate, such surface waters (Baldwin et al., 2005). Hydrolytic $P_o$ mineralization occurs in the presence of water and degrades $P_o$ by cleaving P-O bonds (Baldwin et al., 2005). Additionally, divalent metal ions such as copper (Cu) and zinc (Zn) has been shown to increase the hydrolysis rate of $P_o$ compounds ADP and ATP by 60 and 12-fold, respectively. Abiotic processes can also inhibit $P_o$ mineralization. These abiotic processes include adsorption to minerals, incorporation into humic substances and precipitation reactions (Celi and Barberis, 2005). Organic P can be adsorbed to ferric oxides, as well as clays and sediments. Phosphate monoesters, specifically inositol phosphate, show high rates of

mineralization inhibition with around 20% of biotic $P_o$ as monoesters but over 50% of soil $P_o$ in this form (Celi and Barberis, 2005).

Of the elements essential to life, many are generally found in the environment in organic form (Bunemann, 2008). Organic P is variable in soils, with higher quantities typically present in the A horizon, the zone of organic matter accumulation (Brady and Weil, 1999). However, quantities of $P_o$ in A horizons vary across soil orders, management conditions, time, and many other factors (Brady and Weil, 1999). The upper 40 cm of an Ultisol or Mollisol can have approximately 60-80% of its TP as $P_o$ compounds, whereas an Aridisol may have closer to 20% of TP as $P_o$ (Brady and Weil, 1999; Quiquampoix and Mousain, 2005). Thien and Myers (1992) give an average percent of total soil P in organic forms as 30-50%. However, even in the upper profiles of soil orders with relatively high $P_o$, $P_o$ can comprise less than 0.1% of soil (Brady and Weil, 1999).

The enzymes that transform complex organic molecules into biologically-available nutrients are ubiquitous in natural environments (Sinsabaugh, 1994). Soil enzymes are pivotal in biogeochemical cycling of soil nutrients (Geisseler and Horwath, 2009). Chemical processes catalyzed by enzymes include depolymerization, oxidation and hydrolysis (Bunemann, 2008; Giri et al., 2006). These are all processes which reduce the complexity of $P_o$ molecules and allow them to be mineralized by microbes and, thus, used by plants and animals (Allison, 2006).

Enzymes are ubiquitous in soils because soil bacteria and fungi use a complex enzyme-driven metabolism to attain both needed energy from reduced molecules and nutrients. Enzymes facilitate numerous processes within cells and outside of cells via extracellular enzymes secreted by microbes (Quiquampoix and Mousain, 2005). Biogeochemical processes facilitated by microbes, and the enzymes they produce, include decomposition of organic matter, N fixation, N mineralization, nitrification, denitrification, P mineralization, sulfur oxidation and sulfur reduction. Soil microbes are essential to, or participate in, the Earth's C, N, sulfur and P cycles and profoundly affect

nutrient availability to both terrestrial and aquatic organisms and plants (Giri et al., 2006). More specifically, certain bacteria, fungi, plant roots and digestive tracks of invertebrates can produce extracellular enzymes which hydrolyze soil $P_o$ compounds (Quiquampoix and Mousain, 2005). While abiotic mechanisms leading to mobilization of P are constant in soils, these processes are considered to be less efficient than biologically-derived mechanisms by several orders of magnitude (Quiquampoix and Mousain, 2005). Enzymes, with their ability to reduce required activation energy for chemical reactions, facilitate the efficiency of biogeochemical nutrient cycling.

Of the many enzyme classes, phosphohydrolases, also known as phosphatases, play a major role in P cycling. Most organic matter is in polymerized form and is either adsorbed to soils or is too large to pass through plant/algal/fungal cell walls or bacterial cell membranes (Quiquampoix and Mousain, 2005). Phosphohydrolases are the broad class of enzymes responsible for reducing complex P-containing polymers to oligomers, monomers and phosphate ions such as $H_2PO_4^-$ and $HPO_4^{2-}$ (Quiquampoix and Mousain, 2005). Phosphatase enzymes remove phosphate groups from their substrates by hydrolyzing phosphoric acid monoesters into molecules with free hydroxyls and phosphate ions. A graphic representation of phytase, a phosphohydrolase and a high molecular weight protein, is provided by Figure 4.

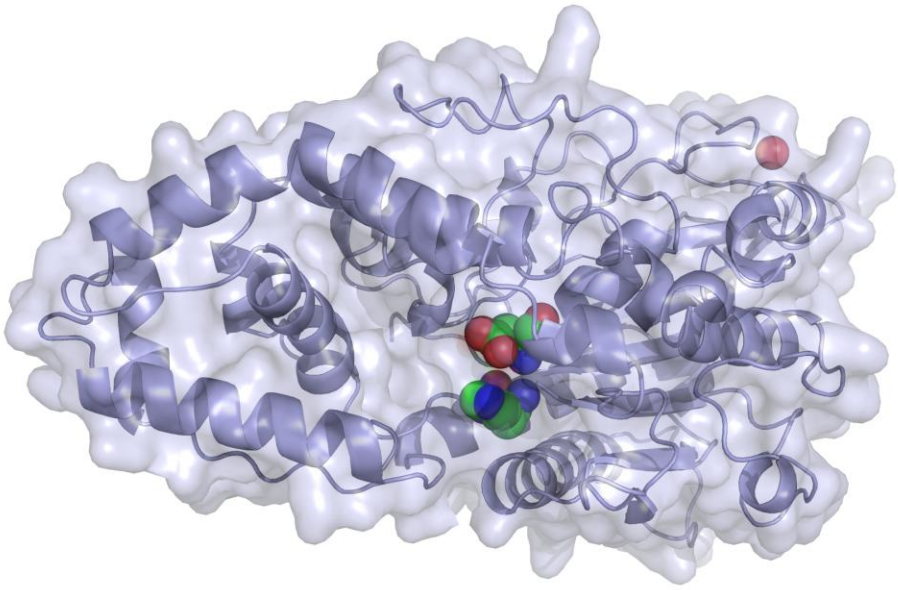


Figure 3. Model of phytase enzyme with an activator cation in right corner and phosphorus compounds as green, red and blue models (Wikipedia Commons, 2010: http://en.wikipedia.org/wiki/Phosphatase).

As was previously mentioned, phosphatase enzymes are found in both marine and terrestrial environments. Boavida and colleagues (1997) studied four Mediterranean lakes and found significant positive correlations ($P < 0.05$) between alkaline phosphatase activity and soluble reactive phosphorus (SRP) in lake waters. Additionally, this study found significant positive correlations between the presence of alkaline phosphatase, TP and chlorophyll *a* in lake waters. This finding was unexpected by the study's authors as it is generally assumed that high phosphatase activity in both soils and natural waters correlates with low P availability (Sinsabaugh, 1994). However, P shortages in soil rhizospheres and in areas of high microbial activity may cause induction of phosphatase producing genes, thereby resulting in high enzyme activities (Sinsabaugh, 1994). In contrast, it is possible that waterbodies accumulate enzyme activity due to past biological activity and without regards to current P needs of organisms.

Decomposition and P cycling in aquatic and terrestrial environments can be seen as the collective result of extracellular enzyme activities (Sinsabaugh, 1994). These activities are regulated by soil conditions such as moisture, temperature, and factors leading to adsorption, inhibition and stabilization (Sinsabaugh, 1994). Like minerals, humic compounds in soils play a role in both stabilizing and inhibiting enzyme activity (Alison, 2006; Quiquampoix and Mousain, 2005). Humic acids can bond to enzyme active sites and inhibit enzyme activity by blocking substrate access to these sites (Alison, 2006). Humic acid/enzyme bonding can cause both reduced enzyme activity in the short-term and long-term resistance to enzyme degradation in both terrestrial and aquatic ecosystems (Alison, 2006).

Phosphatase enzymes, including phytase, are activated and inhibited by a range of soil compounds, polyvalent anions and cations (Quiquampoix and Mousain, 2005). Many phosphatase and phytase enzymes are inhibited by fluoride, polyvalent anions such as phosphate, molybdate, arsenate and metal cations such as silver, zinc, mercury (II), copper (III), iron (II) and manganese (II) (Quiquampoix and Mousain, 2005). Further, chelating agents such as tartrate, oxalate and ethylenediaminetetraacetic acid (EDTA) have been found to inhibit phytase and phosphatase (Quiquampoix and Mousain, 2005). However, there may be variations among enzymes of the same class and function due to their diverse biological origins, but trends have been found in enzymes from specific organisms and plants (Quiquampoix and Mousain, 2005; http://www.brenda-enzymes.org/) The extensive on-line database BRENDA provides information on dozens of enzyme parameters for hundreds of scientifically characterized enzymes (http://www.brenda-enzymes.org/:Technical University of Braunschweig, Germany). The most general factors controlling enzyme activity, such as co-factors (activators), inhibitors, and pH/temperature optima will be discussed.

In addition to having a pH and temperature range at which protein conformation is correct for activity, many phosphatases and certain phytases are activated by divalent cations (Quiquampoix and Mousain, 2005). Specifically, phytase from *Bacillus subtilis*

has been found to require calcium cations for activation and acid phosphatase (Quiquampoix and Mousain, 2005). Also, in *Lysobacter enzymogenes* alkaline phosphatase, inhibition from the addition of EDTA was found to be reversible by the addition of a range of cations including magnesium, calcium, manganese and zinc (Quiquampoix and Mousain, 2005). As a general trend, phytase from wheat are activated by calcium and magnesium and phytase from Saccharomyces spp. are activated by iron (II) and copper (III) (Quiquampoix and Mousain, 2005). Optimal pH for phytase from various organisms and plants is broad, with examples ranging from 2.2 in *Pichia farinosa* to 7.5 in both *Bacillus subtilus* and *Phaseolus aureus* phytase. Alkaline and acid phosphatases, which are present in numerous living cell types, have a similarly wide pH range of 2.5 (*Aspergillus niger*) to 8.5 (*Lysobacter enzymogenes*) (Quiquampoix and Mousain, 2005).

Principle identified soil $P_o$ compounds that the above enzymes act upon are inositol phosphates (also known inprecisely as phytates or specifically as *myo-inositol hexa or pentakisphosphate*), simple labile phosphate monoesters (non-inositol phosphates), polyphosphates, phospholipids and nucleic acids (Quiquampoix and Mousain, 2005; He et al., 2004; Turner et al., 2002). The presence of $P_o$ compounds is variable across soils, time and farming practices, but multiple investigations put prevalence of $P_o$ forms in descending order similar to as listed above (Quiquampoix and Mousain, 2005; He et al., 2004; Turner et al., 2002). Phytate-like $P_o$ molecules may be up to 80% of $P_o$, whereas phospholipid concentrations may represent 0.5-7% of $P_o$ and nucleic acids are generally under 3% of soil $P_o$ (Quiquampoix and Mousain, 2005). In a study using nuclear magnetic resonance (NMR) spectroscopy of NaOH–EDTA-extractable P in Irish grassland soils, Bourke and colleagues (2008) found that *myo*-inositol hexakisphosphate was a dominant orthophosphate monoester. Similarly, He and colleagues (2004) found phytate-like $P_o$ to dominate the enzyme-labile NaOH-extracted $P_o$ fractions in non-farmed and cultivated Spodosols, with cultivated manured soils having almost double the phytate-like $P_o$ of uncultivated soils. Turner et al. (2002) found

proportions of phytate-labile, alkaline phosphomonoesterase and alkaline phosphomonoesterase/ phosphodiesterase-labile $P_o$ to vary across water-extracted pasture soils. In Turner et al.'s (2002) study, labile orthophosphate monoesters were from <0.1 % of molybdate-unreactive P (MUP) to 4.8% of MUP. Phosphomonoesterase/phosphodiesterase-labile $P_o$ represented 9-23% of MUP and phytase-labile $P_o$ was 12.3-33% of MUP (Turner et al., 2002). In their review of $P_o$ in the environment, Quiquampoix and Mousain (2005) concluded that inositol phosphates are more resistant to mineralization than other $P_o$ compounds and are present in soil in very stable salt forms which behave similarly to phosphate ions by bonding with iron, aluminum and calcium.

In summary, biological mechanisms to free complexed $P_o$ from soil include release of extracellular enzymes and mineral-dissolving molecules (Hill et al., 2007; Quiquampoix and Mousain, 2005). Quiquampoix and Mousain (2005) propose an approach to classifying soil $P_o$ on the basis of the biological mechanism by which it is moved into living cells, as opposed to historically used chemical soil extractants which may capture $P_o$ species with a range of biological accessibility. These categories include enzymatically hydrolysable phosphorus ($P_{enz}$), dissolvable phosphorus ($P_{diss}$) and membrane-permeant phosphorus ($P_{memb}$). Dissolvable phosphorus represents P that can be mobilized by the secretion of organic acid complexing molecules or by pH adjustment via proton excretion (Quiquampoix and Mousain, 2005). Lastly, $P_{memb}$ represents low molecular weight P that may be products of $P_{diss}$ and $P_{enz}$, as well as existing $P_i$ (orthophosphate), that can cross cell membranes through active and passive transport processes (Quiquampoix and Mousain, 2005). Orthophosphate is the majority of the $P_{memb}$ pool and represents the P pool that has been historically quantified using traditional analytic methods, namely molybdate reactive P (MRP). Dissolvable P ($P_{diss}$) may overlap with $P_i$ released by extractants such as Mehlich-3. Mehlich extractants use acetic acid and nitric acid, as well as EDTA and other compounds and may mimic acidified plant root zones.

## Soil phosphorus testing

Organic P has historically been considered of limited relevance as a source of P for plant growth and soil tests for P were developed using this paradigm. Turner and colleagues (2002) emphasized that $P_o$ contributes significantly to P transfer from land to waterways because it is a substantial portion of TP in overland flow, soil solution and leachate. As previously mentioned, mechanism by which $P_o$ contributes to algal growth is through the liberation of $P_i$ by phosphatase enzymes. These bacterial and fungal phosphatase enzymes are ubiquitous in soil and water environments (Hill and Cade-Menun, 2009; Lim et al., 2007; Allison, 2006; Turner et al., 2005).

Because the soils of many watersheds of the Southeast U.S. receive large nutrient inputs from animal wastes, it is relevant to compare various methods of quantifying soil P supplied by these manures. Mehlich-1 or -3 extractants are used to determine soil test P (STP) in many state soil testing labs in the Southeast U.S. While Mehlich extractants release soluble P, most P adsorbed to Fe- and Al-oxides, they only extract a small portion of $P_o$. This $P_o$ is released via hydrolyses by the acidic Mehlich reagents. In addition to flaws related to not capturing available P from the $P_o$ fraction, other artifacts exist in chemical extractant-based soil testing. For example, Sharpley and colleagues (1987) found that more variability in Bray 1, Mehlich 1, and Texas A&M P soil tests was accounted for by organic P fractions in highly weathered soils (17%), when compared to calcareous (4%) and sparingly weathered soils (1%). Thus it is possible that responses to additions of $P_o$ to soil would not be identified symmetrically across soil types by plant response-based extractants. Furthermore, Mehlich and other extractants used in agronomic soil testing labs typically capture a small proportion of both NaOH-EDTA-extractable P and TP (He et al., 2004).

Thien and Myers (1992) used a novel approach to elucidate both microbial influences on STP and total bioavailable P pools. These researchers added C and N to a range of soils with varying properties and incubated the soils for 7 days at 35 $^{o}$C. The addition of C and N was to place demand on the most bioavailable P forms and create sinks for this P (Thien and Myers, 1992). After incubation with C and N, termed the "bioactive treatment," $NaHCO_3$ extracts were tested for MRP. Results showed that the bioactive soils had less $NaHCO_3$-extactable $P_i$ than untreated soils. These researchers note that immobilization temporarily decreases $P_i$ available for plant uptake and transfers it to either the $P_o$ or microbial ($P_m$) pool. The amount of preincubation $P_i$ immobilized in bioactive soil treatment was from 28 to 91%, with an average of 66% (Thien and Myers, 1992). Conversely, Labile $P_o$ in bioactive soils had average values of 2.4 times that of untreated soils, whereas soils without added C and N remained nearly the same throughout incubation. Labile $P_o$ is assumed to be available to plants over time and was defined in this study as the sum of $P_o$ and $P_m$ minus immobilized P ($P_{immob}$) (Thien and Myers, 1992). In effect, bioavailable $P_o$ was found by reducing the hypothetically mineralizable pool ($P_o + P_m$) by the amount of immobilized $P_i$ (Thien and Myers, 1992). This study determined $P_o$ as the TP minus $P_i$, with total P being assessed by acid digestion and $P_i$ as $NaHCO_3$-labile P. Further, $P_{immob}$ was defined as the difference of $NaHCO_3$-extractable $P_i$ before and after incubation (Thien and Myers, 1992). Carbon and N additions to soils activated a chain of events which included microbial release of enzymes but do not necessarily translate to immediately higher STP. The results of this study illustrates the weakness of static P tests and gives insight into biochemical processes, such as microbial activity redistributing the most biologically available forms (Thien and Myers, 1992).This study's authors note that plants use P from multiple sources in a soil and that assessment of a soil's ability to supply P needs to take into account all of these sources (Thien and Myers, 1992). These researchers conclude that the idea of trying to identify the most readily labile pools of P is a commonality of many extraction approaches, however by using microbial assimilation as a bioextractant,

instead of chemical extraction, rhizospheres are more accurately mimicked and conditions are more reflective of those that will determine net P availability to plants over time (Thien and Myers, 1992).

As was just mentioned, a range of soil extractants and methods are used to assess plant-available P both in soil testing labs and for scientific study across the U.S. Among these are Bray-1, Morgan and Mehlich-1 and Mehlich-3 extractants. Mehlich-1 and Mehlich-3 extractants are most often used for the acidic, high clay soils common in the Southeast U.S. The Mehlich-3 extractant consists of 0.2 M $CH_3COOH$ + 0.25 M $NH_4NO3$ + 0.015 M $NH_4F$ + 0.13 M $HNO_3$ + 0.001 M EDTA (Chemical Methods of Soil Analysis, 2005). The Thien and Myers (1992) study mentioned above used 0.5 M $NaHCO_3$ (sodium bicarbonate) as a soil P extractant taken before biological activation of soils. Mehlich and $NaHCO_3$ extraction have been extensively validated and correlated to plant response over decades of use. Organic acids, such as citric acid and ammonium oxalate, have also been used in various studies (Shober and Sims, 2007; Hayes et al., 2000).

Numerous studies compare P extracted by the aforementioned extractants, as well as water, and use an acid/heat digest of soils as a TP reference point as a means to quantify total existing P in a soil ( Shober and Sims, 2007; He et al, 2004). More recent studies have subjected various soil extractant-labile P pools to enzymatic hydrolysis (He et al., 2004; Turner et al., 2002). Sodium hydroxide (NaOH) or NaOH-EDTA are often used in these studies to extract the pool of P for enzymes to act upon, i.e. enzyme-labile $P_o$ (Johnson and Hill, 2010; Bunemann, 2008; He et al., 2006, 2004; Hayes et al., 2000). In general, NaOH or NaOH-EDTA extracts the preponderance of TP when compared to water or $NaHCO_3$ extraction (Bourke et al., 2008; He et al., 2004). Other recent studies have used molecular and atomic spectrometry to assess the range of $P_o$ compounds found in NaOH-EDTA extracts of soil (Hill and Cade-Menun, 2009; Quiquampoix and Mousain, 2005; Worsfold et al., 2008). Bourke et al. (2008) used $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy on Irish grassland soils and found that NaOH-EDTA

extracted from 75 to 88% of soil TP. Hill and Cade-Menun (2009) found a similarly high proportion of soil and sediment TP on a chicken farm to be extractable by NaOH-EDTA. The $^{31}P$ NMR technique used by Bourke and colleagues (2008), and others, is further able to distinguish between classes of P compounds contained in NaOH-EDTA extracts. These four classes of P compounds were inorganic orthophosphate ($P_i$), orthophosphate monoesters, orthophosphate diesters and pyrophosphate (Bourke et al., 2008). The Bourke et al. (2008) study is typical in that it shows $P_i$ to be the single largest pool of NaOH-EDTA-extractable P, but other forms of P may represent significant quantities of bioavailable P over time.

Both the solution chemistry of NaOH-EDTA extracts and the relative rates of elements that this extractant releases may be variable across soil orders and levels of manure ($P_o$) addition. For example, it is probable that an NaOH-EDTA soil extract has variable aluminum, calcium, magnesium, zinc, copper, TP, $P_i$, clay and humic matter and that any combination of these factors can lead to variable rates of enzymatic hydrolysis and accuracy in colorimetric $P_i$ detection (Yaqoob et al., 2004). These factors are in turn controlled by native soil chemistry, human alterations and amendment additions, field position, climate and much else.

This study follows the method for enzymatic hydrolysis of NaOH-EDTA-extractable P developed by He et al. (2004) and further refined by Johnson and Hill (2010) and introduces parameters not considered in the aforementioned studies. Broadly, this study adds laboratory time-course, field position, fertilization regimen and soil order factors not previously considered. First, as previously discussed, soil P transforms in natural environments over time and assessing P species at different times has relevance. Comparing how these changes are reflected in P pools using various extractants may also provide useful information. This study compares Mehlich-3 extractable P with NaOH-EDTA extractable P over three weeks of soil/manure incubations at upper profile warm season conditions. Second, this study uses two highly manure amended Ultisol soils from upland and depositional field positions, as well as an

inorganic fertilizer amended Ultisol, and compares behavior over time amongst these soils. Lastly, Johnson and Hill (2010) used Berkshire and Marlow stony loam soil (coarse-loamy, isotic, frigid Typic and Oxyaquic Halplorthods) and He and colleagues (2004) used like reagents and 2 of the 3 same enzymes on other Northeastern Spodosols. Textures and chemical composition vary immensely between Spodosols and the Ultisol clay loams (fine, kaolinitic, thermic Rhodic Paleudults) used for this study. Paleudults, a well-developed deep Ultisol, are common in southeastern states which have a significant amount of the nation's poultry production. For example, Georgia, Arkansas and Alabama are the three top broiler producing states and North Carolina is second in both hog and turkey production (North Carolina Agricultural Statistics, 2010).

## Research Objectives

The previously mentioned Johnson and Hill (2010) enzyme-based $P_o$ assessment study sought to adapt a method developed by He et al. (2004) to a microplate reader format which could read 96 samples, all under 1 mL, simultaneously. Both the lower costs of reagents and enzymes and speed of analysis of the Johnson and Hill (2010) method could contribute to assessing the potentially hydrolysable $P_o$ in soils receiving high $P_o$ inputs that are susceptible to runoff P loss from agricultural areas. The current research will evaluate the applicability of the Johnson and Hill (2010) method to high clay soils found throughout the southeastern U.S. Further, this research aims to compare a traditional soil test extractant, namely Mechlich-3, for assessing available soil P with the enzyme hydrolysis method.

Specific research objectives are: 1) to adapt the high throughput enzyme hydrolysis method of Johnson and Hill (2010) to dairy manure-amended Ultisols; 2) to quantify environmentally-relevant $P_o$ species not captured in Mehlich-3 extractable P;

and 3) to assess enzymatic hydrolysis in soils with high organic P inputs from various landscape positions.

# CHAPTER II
# MATERIALS AND METHODS

## Site description

### *Pond Creek watershed*

Sample sites for this study were in the Pond Creek watershed, which is located in the Ridge and Valley physiographic region of southeastern Tennessee. Pond Creek watershed comprises part of the Upper Tennessee River Basin and is contained within the Watts Barr/Fort Loudon Watershed of Eastern Tennessee (Figure 5) (Hagan and Walker, 2006).This region is characterized by dolomite/limestone valleys surrounded by rolling hills (Hagan and Walker, 2006). Given the extremely diverse terrain in this area, field slopes range from 0% to over 16% across short distances with some areas being especially prone to erosion (Web Soil Survey, 2011). Pond Creek watershed is a sub-watershed of the Watts Bar watershed and contains row cropping and livestock operations which often occur on sloped land and adjacent to streams that have little or no riparian vegetation (Walker and Carmichael, 2010; Hagan and Walker, 2006). Hagan and Walker (2006) reported 12 large dairy operations in the Pond Creek watershed, accounting for over 2,500 cattle. Many of these operations are located adjacent to Pond Creek itself, as it is common for streams to bisect arable valley land (Hagan and Walker, 2006). Walker and Carmichael (2010) state that grazing in stream riparian zones accounts for 60% of agriculture's contribution to water impairment in this sub-watershed.

Hagan and Walker (2006) used RUSLE parameters to derive soil loss for the Pond Creek watershed and estimated a loss of 43,253 tons $yr^{-1}$ or 1.83 tons $ac^{-1}$ $yr^{-1}$. While disturbed mining areas had the highest soil losses, livestock loafing and feedlot areas had the second highest soil losses, contributing at a rate of 18.32 tons $ac^{-1}$ $yr^{-1}$ (Hagan and Walker, 2006). Also, low residue crop land and poor pasture had losses above

tolerance limits at 12.89 and 10.92 tons $ac^{-1}$ $yr^{-1}$ respectively (Hagan and Walker, 2006). Because overgrazed pasture is a significant land use in the Pond Creek watershed, this category contributed 43.9% of the total soil lost (Hagan and Walker, 2006). Medium and high crop residue on crop production lands mitigated losses, but the overall picture from overgrazed and poor pastures and crop lands in the Pond Creek watershed point to high nutrient inputs to waterways from soils.

About half of the 23,579 acres contained in Pond Creek Watershed are used for cattle grazing (Walker and Carmichael, 2010; Hagan and Walker, 2006). Other major land uses include forest and conventional cropping systems (Figure 6) (Walker and Carmichael, 2010). Pond Creek, which runs adjacent to the study site, is a tributary of Watts Bar Reservoir which is listed on the 2008 EPA 303(d) list of impaired water bodies (TDEC, 2008b). Pond Creek itself had 21.0 miles listed in 2006 by the EPA as impaired by nitrate, *E. coli* and habitat alterations (Walker and Carmichael, 2010).

The watershed and characteristic soils have been well characterized by previous investigations (Walker and Carmichael, 2010; Graham, 2009; Hagan and Walker, 2006). Mehlich-3 extractable P, calcium, magnesium, potassium and pH for the application field are available in Appendix A, Figures 13 and 14 (Graham, 2009). The aforementioned soil characteristics are relevant to this study in that they may affect soil extract solution chemistry and therefore enzyme assays performed in this liquid matrix. The mean air temperature in this region during 2005-2009 was 59 $^{o}$F (15 $^{o}$C), mean yearly precipitation was 43” (109 cm) and the yearly average temperature of the top 2” (5.1 cm) of the soil profile was 59.2 $^{o}$F (15.1 $^{o}$C) (USDA weather station #2077, Eastview Farm SE TN). June through August mean soil temperatures for the top two inches of the soil profile were 75 $^{o}$F (23.9 $^{o}$C) (USDA weather station #2077, Eastview Farm SE TN, 2005-2009).

***Study farm***

A 500-head dairy farm located within the Pond Creek watershed was used for this study. The owner of this dairy operation also had fields in the same valley farmed without the addition of dairy wastes. Thus soils with a range of TP concentrations and landscape positions, but in identical climates and soil series, were able to be located. Soil samples were taken from two separate fields, a waste application field and a field which received no dairy or other organic waste. The soils from the later field are referred control soils throughout this text. The amended field was planted to a warm season corn (*Zea mays*) and cool season winter wheat (*Triticum aestivum*) or rye (*Secale cereal)* rotation. Corn is cut for silage in the fall when winter wheat or rye is planted. Wheat is also cut and used for wheatlage production. Wheatlage is fed to on-site dairy cows in winter months when forage is not available (Graham, 2009). Between wheat harvest and corn planting, fields are disked to a depth of approximately 6". All seeds are planted using a seed drill (Graham, 2009). An inorganic 150-0-100 fertilizer is applied in spring (rate not available) and this field has been under this management scheme for approximately 10 years. In addition to being used for dairy feed production and winter grazing, this 56 hectare field is used as an application area for dilute dairy waste washed from milking parlor floors. This waste is stored in an anaerobic lagoon and is land-applied approximately twice a year after mixing with a prop agitator. Manure slurry is applied to fields by pumping from the lagoon through a 6" hose and a traveling big gun rig system (Graham, 2009). Field application rates are variable depending on the distance from lagoons and other factors, but one investigation found that dilute manure slurry was applied at a rate of 3 cm over field surfaces in the rig spray zones (Graham, 2009). Infiltration of these wastes may be variable and runoff to lower field depositional areas has been observed (D. Graham, personal communication).

A second field, from which control samples were obtained, had not received animal waste for at least the last ten years. This control site was approximately one half mile away and was planted to a soybean (*Glycine max*) and winter wheat rotation. This

site had been in a soy/corn/winter wheat or rye rotation for approximately 5 years. This site was fertilized with an inorganic 19-19-19 fertilizer once a year at a rate of approximately 350 lb $acre^{-1}$.

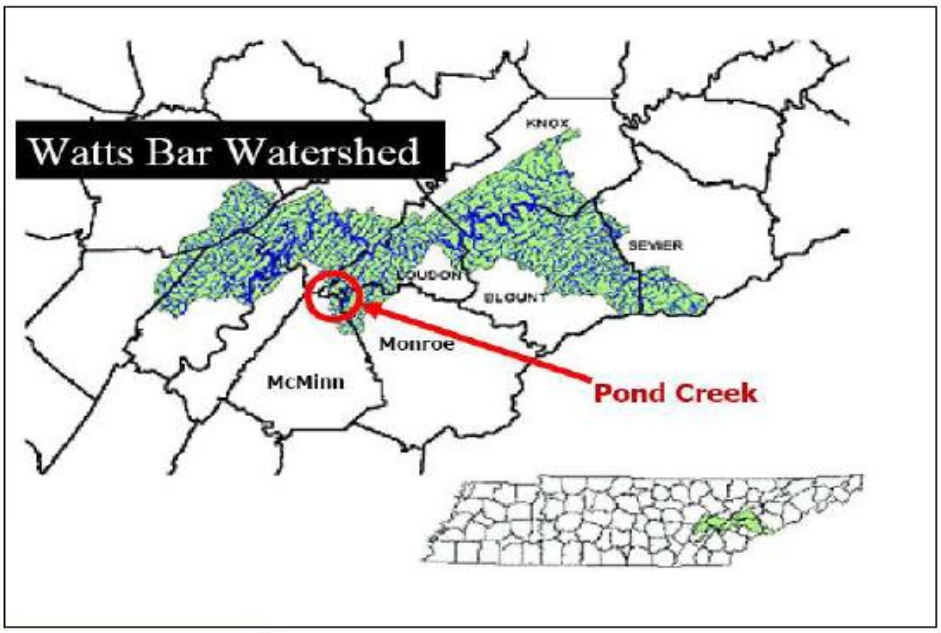



Figure 4. Location of Pond Creek sub-watershed within Watts Barr watershed of East Tennessee (Hagan and Walker, 2006).

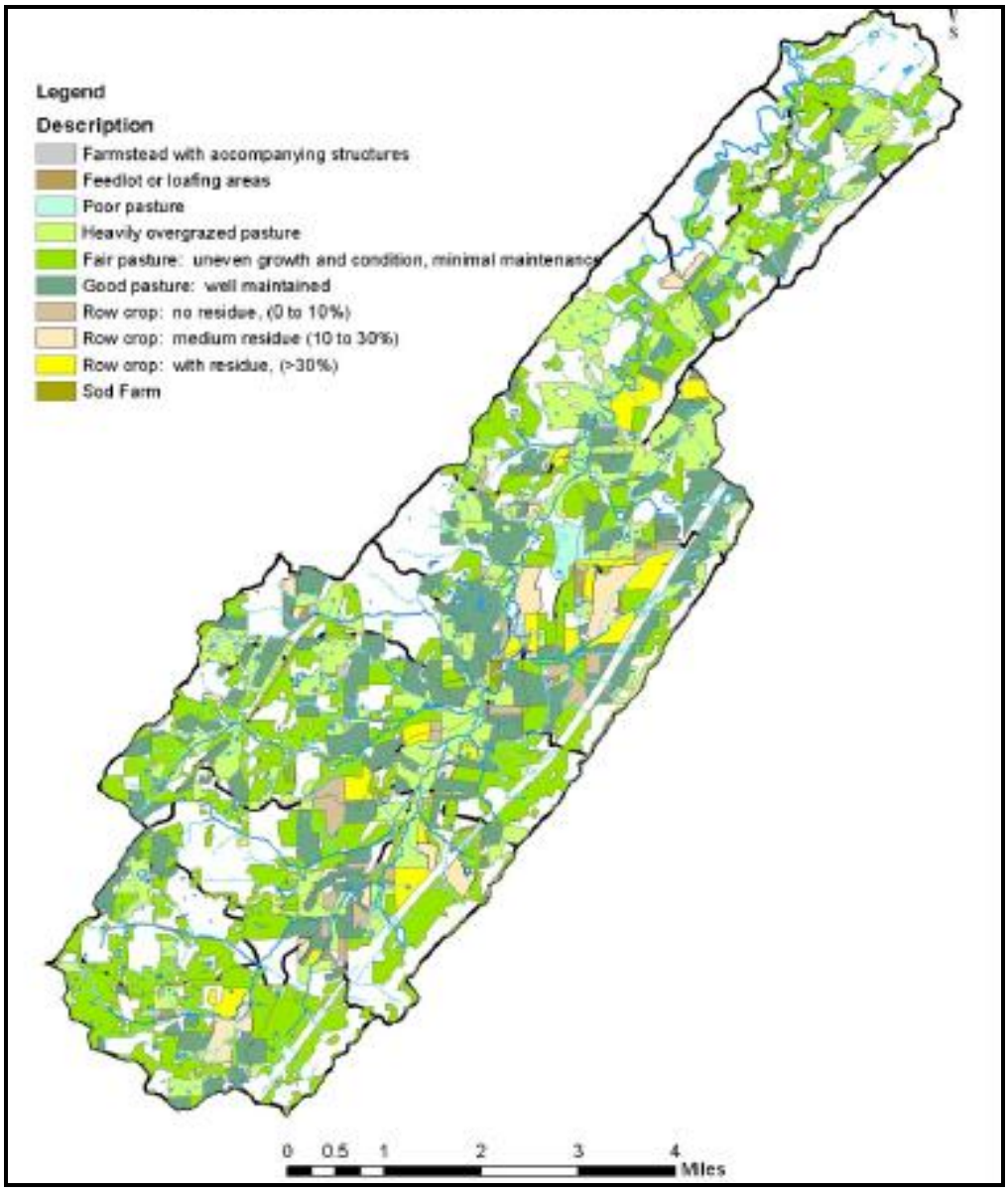



Figure 5. Pond Creek agricultural land uses (Walker and Carmichael, 2010).

Soils of both fields are classified as Decatur (Web Soil Survey, 2010). All studied soils can further be classified by the USDA system as fine, kaolinitic, thermic Rhodic Paleudults with completely exposed B horizons due to severe erosion (Web Soil Survey, 2010).

## Soil sampling, processing and pH testing

Soil samples were taken from two landscape positions from the waste-amended field, an upland area and a depositional area. The upland area was an eroded section of the field while the depositional area experiences accumulation of eroded soil from upland areas and was approximately 550 feet below the upland sampling area. The slope between upland and depositional field positions averaged approximately 7%, with a maximum of 10% and a minimum of about 4% slope (Graham, 2009). Soils from an un-amended field were sampled and served as a control. The slope of the control field was 4%. Upland and depositional soils from the waste-amended field were sampled on February 18$^{th}$ of 2010 and the control soil from the un-amended field was sampled on March 17$^{th}$ of 2010. Randomized soil cores in each area were taken with a standard 1 cm-width probe to a depth of 5 cm. All soils were refrigerated at 4 $^{o}$C until analysis.

Immediately after removal from fields, gravimetric soil moisture was determined from subsamples. Soils were homogenized and visible rocks (>2mm) and woody materials were removed prior to oven drying. Soil subsamples were air dried at room temperature until they were in the range of 12% moisture by weight. Soils were then sieved with a 2mm sieve, further homogenized and stored at 4 $^{o}$C until use in microcosms or other analyses. Total phosphorus (TP) and Mehlich-3 extractable P were measured in the aforementioned air dried soils, and then these analyses were run again on soil from microcosms. Microcosm creation, TP and Mehlich-3 procedures will be discussed shortly.

Soil pH was measured by using a calibrated pH meter. Two repetitions of 10 grams of soil were added to 10 mL of deionized water and mixed by hand-inverting for 1 minute and immediately measured.

Dilute cow waste was collected from the reel sprayer prior to field application on November 7th, 2009. Samples were collected in Nalgene bottles and kept frozen at -20 $^{o}$C until use.

## Microcosm setup and sampling

Three replicate microcosms of each of the three sample areas described above were created by adding 700 g of soil and 185 ml of dilute cow manures to wide mouth 32 oz mason jars. Care was taken to not destroy what was present of 2mm soil aggregates from sieving. Soils were gently tamped so each had the same height in the jar as other microcosms of the same soil**.** This soil line was marked on the side of jars to have a point of comparison if soils subsided when dilute manure solution was added. This was an attempt to quantify differences, if present, in bulk density between microcosm repetitions and soil types. Variable bulk density could lead to variable soil aeration and therefore differing redox conditions, microbial community selection and rates of P mineralization/ immobilization. In order to create the soil/manure microcosms described above, dilute cow manure was thawed overnight at 4 $^{o}$C and then in a warm water bath for about an hour and was added drop-wise to the microcosms. Based on the measurements of the microcosm jars, 185 mL of dilute waste was determined to approximate the observed field application rate of 3 cm determined previously by Graham (2009). Saturated pore space at the bottom of the microcosms was not visible. Each microcosm was left to equilibrate for an hour before time 0 sampling. Subsamples were taken from 0-9 cm depth with a ~1.25 cm diameter stainless

steel coring device. This depth was about 1 cm less than the entire depth of the microcosm. Each microcosm was weighed before and after sampling to gravimetrically maintain constant moisture. Each week, deionized water (DI) was added drop-wise to bring the microcosms to their weight immediately after their previous sampling. Cores contained approximately 10 grams of soil and were taken once a week for three weeks. Each micro-core was subdivided into 1.0 g portions for % moisture determination, 3.58 g (2.5 g dry wt) portions for Mehlich-3 extraction, 2.86 g (2 g dry weight) for NaOH-EDTA extraction and enzymatic hydrolysis (EH) and the remaining soil was air dried for approximately 24 hours and made available for TP analysis (Figure 7). Cores were homogenized thoroughly before these subdivisions. Both total P ($TP_{NaOH}$) analysis and EH was performed on 0.25M NaOH-0.05M EDTA extracts from microcosms at zero through 21 days (described below). Additionally, TP was measured via the sulfuric acid digestion method described below on microcosm soils at the 1-week sampling time.

After time 0 sampling, all microcosms were incubated in darkness at 24 $^{o}$C and only removed from the incubator for moisture maintenance, weekly sampling and sprout removal. For free gas exchange, microcosms had lids with 12 holes of about 2mm diameter punched in each.

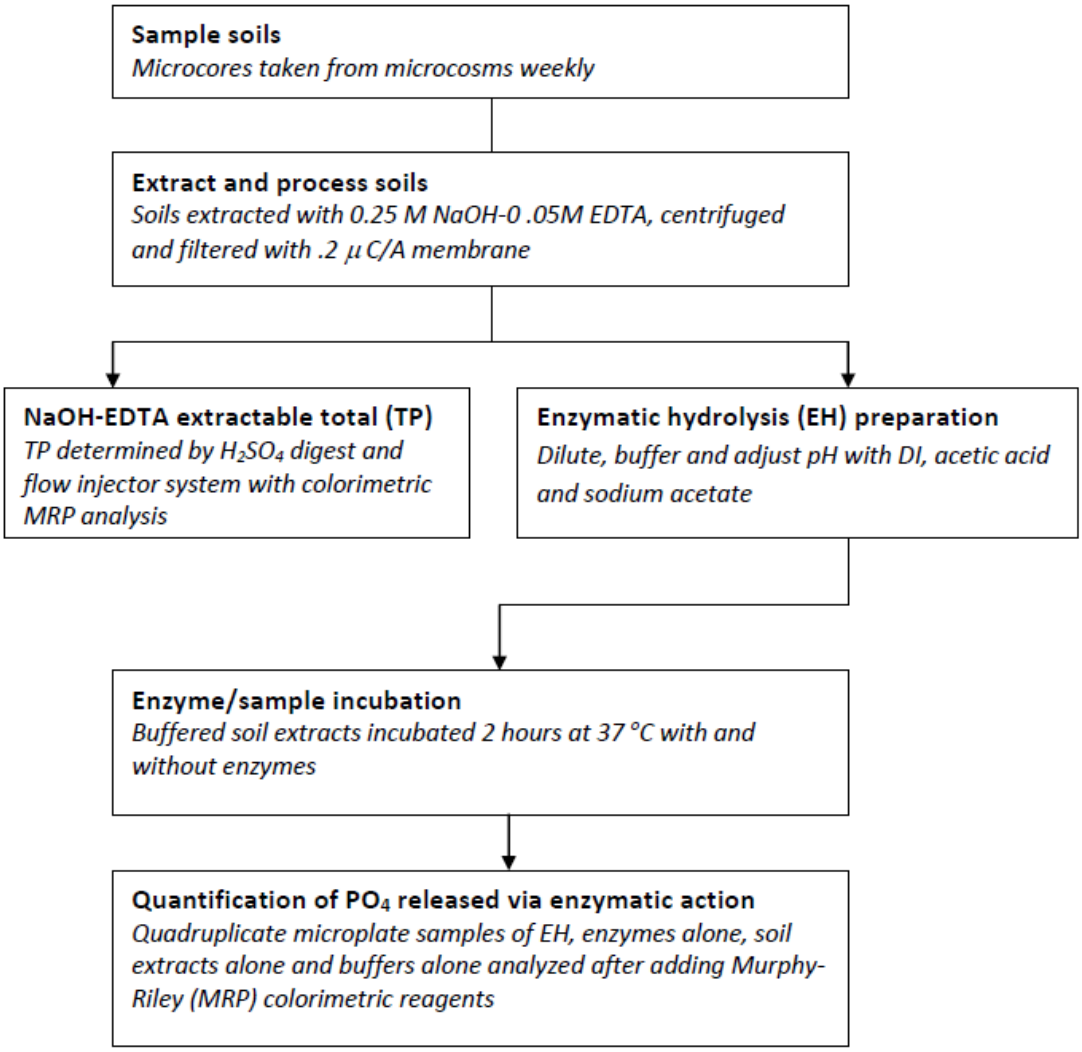



Figure 7. Enzyme-labile organic P quantification method (Johnson and Hill, 2010)

## Enzymatic hydrolysis

Soil subsamples taken from the microcosms were extracted with 0.25M NaOH-0.05M EDTA at a 1:15 soil to solution ratio by oven dry weight. Soils and extractants were added to sterile polypropylene 50 mL centrifuge tubes and shaken on a platform shaker at 200 rpms for 16 hours at room temperature. Soils/extractants were immediately centrifuged at 3,000 g for 30 minutes. The supernatant in each centrifuge tube was then filtered with syringe-mounted 0.20 μm Whatman cellulose acetate membrane filters.

Filtered soil extracts were then diluted with DI (5 mL filtered extractant brought up to 50 mL volume). Diluted extracts were then used for either $TP_{NaOH}$ analysis (described under total P digests of soil and NaOH-EDTA extracts) or EH. For EH, diluted NaOH-EDTA extracts were buffered in 0.4M sodium acetate-2.5M glacial acetic acid and deionized water. Specifically, 36.5 mL water, 7.5 mL 0.4M sodium acetate, 1 mL 2.5M glacial acetic acid and 5 mL diluted NaOH-EDTA soil microcosm extract were added to 50 mL centrifuge tubes in the aforementioned order. Adding acid directly to pure soil extracts may cause organic phosphorus hydrolysis, so this was avoided. Centrifuge tubes were then mixed for 30 seconds on a vortex shaker. The pH of the buffered NaOH-EDTA soil extracts from microcosms was then tested and adjusted, if needed, until pH values were in the range of 4.80-5.30. Average values for the buffered NaOH-EDTA soil extracts from microcosm weekly samples were pH 4.90 – 4.98.

The three enzymes used for EH were listed by Sigma Aldrich as active in the 4-7 pH range. For example, assayed values for acid phosphatase P1146 (EC 3.1.3.2) was one unit on enzyme for the hydrolysis of 1.0 μmole of p-nitrophenyl phosphate per minute at pH 4.8 and 37 $^{o}$C. The three enzymes used were: acid phosphatase from potato (cat. #P1146; EC 3.1.3.2) with an optima of pH 5.0-5.3, acid phosphatase from wheat germ (cat. #P3627; EC 3.1.3.2) with an optima of pH 5.7, and nuclease $P_1$ from *Penicillium citrinum* (cat. #N8630; EC 3.1.30.1) with an optima of pH 5.3. Prior to use for EH, these

three enzymes were buffered in 0.1M sodium acetate solution, pH adjusted to pH 5 +/- 0.1 and centrifuged for 10 minutes at 22,000 x g (Johnson and Hill, 2010). The buffered enzymes were pipetted into 1.5 mL centrifuge tubes and immediately stored at -20 $^{o}$C. The activity of buffered enzymes was 0.5 units (U) per mL for potato phosphatase (PP), 0.5 U/mL for wheat germ phosphatase (GP) and 5 U/mL for nuclease. In order to partition soil organic P into various compound classes, enzymes were blended in three separate groups: PP, PP/GP and PP/GP/NP (Johnson and Hill, 2010).

For EH, 1 mL of each single or enzyme blend was added to 1 mL of the buffered soil extract in a 50 mL centrifuge tube and incubated in a 37 $^{o}$C water bath for 2 hours with 100 rpm shaking. Simultaneously, buffered soil extracts with no enzymes, buffers without soil extract or enzymes, and enzymes alone were incubated under the same conditions (Johnson and Hill, 2010). Enzymatic hydrolysis and heat-induced chemical change was terminated after 2 hours by bringing samples from 37$^{o}$C to 4 $^{o}$C. Buffered soil extracts were saved as baselines for comparisons of chemical hydrolysis of organic P due to the 2-hour incubation time alone. Orthophosphate content of all aforementioned samples was determined within 24 hours of extracting and buffering microcosm soil extracts. A modified Murphy-Riley method and a photospectrometric microplate read at 850 nm was used to determine orthophosphate content of samples (Johnson and Hill, 2010; He and Honeycutt, 2005). In brief, 25 μL of sodium dodecyl sulfate (SDS), 100 μL of 0.1M ascorbic acid-0.5M trichloroacetic acid, 25μ L 0.01M ammonium molybdate and 50 μL of 1M sodium citrate-0.2M sodium arsenite-5% glacial acetic acid were added in the aforementioned order to all microplate wells. Each sample type had four replicates. The reagent addition procedure above was followed but SDS was excluded in $PO_4$ standard curve wells and all other wells without added enzymes. Four wells of enzyme buffering solution were used as blank well subtractants.

A Biotek Synergy HT spectrophotometer with $KC^4$ software was used for $PO_4$ determination. If $R^2$ values of the standard curve of 0.98 to 1.00 were not obtained, the microplate was discarded from the data set. Orthophosphate was determined in

quadruplicate samples of NaOH-EDTA soil extracts which had not been incubated along with samples which had gone through the enzymatic hydrolysis procedure. Thus original $PO_4$ content was known in buffered soil extracts without enzymes which had and had not been incubated for two hours or subjected to enzymes. Using this Johnson and Hill (2010) method, $PO_4$ contamination from enzymes and buffers, as well as non-enzyme hydrolysable P in soils before EH, could be subtracted from hydrolyzed enzyme/substrate combinations.

## Mehlich-3 extractions

The Mehlich-3 extractant consists of glacial acetic acid, nitric acid, ammonium nitrate, ammonium fluoride and ethylenediaminetetraacetic acid (EDTA)(Kuo, 2005). For Mehlich-3 extraction, 2.5 g of soil by dry weight (3.58 g wet weight at time of removal from microcosm) was added to 25 mL of Mehlich-3 soil extractant in 50 mL centrifuge tubes and shaken horizontally on a platform shaker at 200 rpm for 5 minutes. Supernatant was filtered with 0.45 μm Whatman cellulose acetate membrane filters and stored at 4 $^{o}$C until analysis. A molybdate blue colorimetric method based Skalar San$^{++}$ flow injection apparatus (Catnr 503-365-1 w/r; Norcross, GA) was used for $PO_4$ determination of Mehlich-3 extracts. The Skalar system was housed at University of Tennessee's water quality testing lab and was operated following USEPA procedures.

## Total P digests of soils and NaOH-EDTA extracts

***Total P of soils***

Total P in soil samples collected from the field sites was determined by sulfuric acid digestion using the Lachat QuikChem method 13-155-01-1-B (Diamond, 1998). This method involves the digestion of soil samples on an aluminum block digester followed by analysis via the molybdate blue colorimetric method on a Skalar San$^{++}$ system. Briefly, 0.400 g of 0.84 mm sieved soil, 1.50 g of potassium sulfate, 0.125 g copper sulfate

pentahydrate and 3.5 mL of concentrated sulfuric acid was added to digestion tubes and allowed to sit overnight. The next morning, 4 Hengar boiling stones were added to each tube and tubes were placed on a Lachat block digester that had been preheated to 160 $^{o}$C. The digest continued with glass tube stoppers in place for 4 hours once the digester reached approximately 380 $^{o}$C. After digestion, 46.5 mL of deionized water was added to each digestion tube to bring the total volume to 50 mL. The contents of each tube were analyzed using the Skalar San$^{++}$ flow injection spectrophotometer system previously mentioned.

***Total P of NaOH-EDTA extracts***

Additionally, TP in 0.25M NaOH-0.05M EDTA liquid extracts (referred to as $TP_{NaOH}$) from all microcosm sampling times was determined using the US EPA method, which uses the block digester and molybdate blue colorimetric method mentioned above (Eaton et al., 2005). The procedure consisted of mixing 134 g of potassium sulfate, 200 mL of concentrated sulfuric acid and 25 mL of mercuric sulfate solution and 650 mL of DI water. The mercuric sulfate solution was prepared by dissolving 8 g red mercuric oxide (HgO) in 50 mL of 1:4 sulfuric acid:DI water and diluting to 100 mL with DI water. The final digestion solution was then diluted to a volume of 1 L. Twenty mL of the diluted NaOH-EDTA extract solution was mixed with 5 mL of the digestion solution and 4 Hengar white alumina boiling stones in glass digestion tubes. Digestion tubes were mixed for about 10 seconds at high on a vortex mixer. Digestion tubes were then placed on a Latchat block digester for one hour at 160 $^{o}$C and then the heat was raised to 380 $^{o}$C. Once the digester reached 380 $^{o}$C, the digestion process was continued for 2 ½ hours. After approximately 15 minutes of cooling, 49 mL of DI water was added to each flask, bringing the total solution volume to 50 mL. Total P in this solution was determined colorimetrically within 24 hours via the molybdate blue colorimetric method on the Skalar San$^{++}$ autoanalyzer previously mentioned.

## Model organic P compounds

Model compounds equivalent to 50 ppm $P_o$ were mixed in DI water and stored at 4 $^oC$ in opaque Nalgene HDPE bottles. Model compounds and DI water were mixed for about a half hour on magnetic stir plates and were used within 24 hours for enzymatic hydrolysis and ten days for total P digests. All compounds were readily soluble in water except DNA which had to be mixed with ~ 2 mL of water and pulverized into water-soluble gel. Model compound sources and details are as follows:

- Deoxyribonucleic acid (DNA). Acros Organics product #406, lot #A0243992 with an assayed value of 8.5% P (0.294 g dissolved in 500 mL DI water).
- Phytic acid ($C_6H_{18}O_{24}P_6$*Na * $yH_2O$). M.W. 660.3, Sigma product # P8810-10g, lot # 068K0133 (0.089 g dissolved in 500 mL DI water).
- Glycerophosphate disodium salt pentahydrate ($C_3H_9O_6PNa$*$H_2O$). M.W. 306, MP product # 102914, lot # 2494J. (0.247 g dissolved in 500 mL DI water).
- Sodium pyrophosphate decahydrate ($Na_4P_2O_7$*$10H_2O$). M.W. 446.06, Fisher product #S390-500, lot # 076735 (0.1800 g dissolved in 500 mL DI water).
- Adenosine 5' triphosphate (ATP). $C_{10}H_{14}N_5O_{13}P_3$*2Na. M.W. 505.2, Alexis product #480-021-G001, lot #L13696 (0.136 g dissolved in 500 mL DI water).
- p-Nitrophenyl phosphate disodium salt hexahydrate ($C_6H_4NO_6PNa_2$*$6H_2O$) M.W. 371.14, Fisher product BP25341, lot #093275 (0.300 g dissolved in 500 mL DI water).
- D-glucose-6-phosphate barium salt hydrate ($C_6H_{11}O_9PBa$) M.W. 395.5, MP product #101786, lot #6497E (0.320 g dissolved in 500 mL DI water).

Model compounds were subjected to enzymatic hydrolysis using the same procedure as was used for hydrolysis of soil extracts from microcosms. Accordingly, 50

ppm P of model $P_o$ compounds were diluted in DI water-0.4M sodium acetate-2.5M glacial acetic acid solutions similarly to NaOH-EDTA extracted soils from microcosms. Because NaOH-EDTA soil extract solutions were alkaline due to the presence of NaOH, buffers with a lower pH than that of used for model compounds had to be created. The pH of the model $P_o$ compound buffer solutions were 5.0, and 4.7 for NaOH-EDTA soil extract solutions. Specifically, model $P_o$ compound buffer solutions consisted of 75 mL 0.4M sodium acetate, 6 mL 2.5M glacial acetic acid and 419 mL DI water. Five mL of 50 ppm P model $P_o$ compounds were pipetted into 45 mL of the above buffer and vortexed for 30 seconds in 50 mL polypropylene centrifuge tubes. This 5 ppm P solution was stored at 4 $^{o}$C and was subjected to enzymatic hydrolysis within 24 hours.

Testing the efficacy of enzymes on the model compounds above in a pH 5.0 buffer and DI water matrix represents an idealized condition in which there are no known sources of interference with enzyme activity. However this matrix does not contain the EDTA that diluted soil extracts did. EDTA is known to interfere with enzyme activity by chelation of activator cations. Accordingly, a correction factor was included to account for the level of interference EDTA had on enzyme activity. The correction factor was determined by following the procedure described in the last paragraph with 50 ppm P adenosine 5' triphosphate (ATP) and p-nitrophenyl phosphate disodium salt hexahydrate (nitrophenyl P) mixed in both 0.25 M NaOH-0.05 M EDTA and DI water and DI water alone. The model compounds in their separate matrices were then buffered and pH-adjusted to 5.0 with sodium acetate and acetic acid and diluted to 5 ppm P and subjected to enzymatic hydrolysis using the same method as was used on all other model compounds and soil extracts (Figure 7, boxes 4-5). Results of the EDTA and non-EDTA matrices were then compared and the percent difference of each enzyme group in the two matrices was applied to all conditions containing enzymes and EDTA. Specifically, correction factors created from the average differences of enzymatic hydrolysis of ATP and nitrophenyl P in DI and EDTA matrices were applied to the negative controls of all experimental data containing enzymes.

Additionally, 25 ppm model $P_o$ compounds were subjected to the total P digestion procedure previously mentioned (Eaton et al., 2005). This was to verify the total P digestion procedure on organic P compounds. All digests were performed in triplicate with 20 mL sample volume. Samples were digested to approximately 1 mL and then 49 mL of DI water was used for dilution. Samples were mixed for 30 seconds on the vortex apparatus and analyzed for P content using the Skalar flow injector system previously mentioned. Deionized water blanks and Fisher P standards were used for quality control in this and other mentioned analyses.

## Statistics

IBM SPSS PASW Statistics 18 software was used to perform univariate post-hoc multiple comparisons for observed means on all soil microcosm results. Specifically, Tukey HSD post-hoc tests of significance were performed on all microcosm soil treatments at all times. Outputs of these analyses are available in the appendix. The SPSS default significance level of $P<0.05$ was accepted for all analyses. Additionally, Microsoft Excel Office 2007 software was used to perform individual t-tests across single treatments and microcosm extraction times. Two-tailed, type-3 (samples with unequal variance) tests were used as it was not known if samples had equal variance. T-tests were performed at the $P<0.05$ significance level.

# CHAPTER III
# RESULTS AND DISCUSSION

## Model compounds

Model organic P ($P_o$) compounds used in this study for enzyme activity verification were deoxyribonucleic acid (DNA), phytic acid ($C_6H_{18}O_{24}P_6$*Na * y$H_2O$), glycerophosphate disodium salt pentahydrate ($C_3H_9O_6PNa$*$H_2O$), sodium pyrophosphate decahydrate ($Na_4P_2O_7$*10$H_2O$), adenosine 5' triphosphate (ATP: $C_{10}H_{14}N_5O_{13}P_3$*2Na), *p*-nitrophenyl phosphate disodium salt hexahydrate ($C_6H_4NO_6PNa_2$*6$H_2O$) and D-glucose-6-phosphate barium salt hydrate ($C_6H_{11}O_9PBa$). These model compounds were selected to represent major P-containing organic molecule classes. These common biologically-derived molecules include sugar and glycerophosphates (D-glucose-6-phosphate and glycerophosphate, respectively), nucleic acids (DNA), energy-transfer compounds (adenosine 5' triphosphate: ATP), 6-carbon ring phosphates (*p*-nitrophenyl phosphate) and phytic acid, the primary plant $P_o$ storage molecule. *Myo*-inositol hexakisphosphate, more generally known as phytate or phytic acid, is the only compound that is found in soils, plants (with the highest concentrations often in grains), animals and microbes (Quiquampoix and Mousain, 2005). Sodium pyrophosphate, a compound classified as a condensed phosphate and technically not a $P_o$ compound because it does not contain carbon, was also subjected to enzymatic hydrolysis because it is commonly quantified by analytic tools which capture $P_o$ (Ahlgren et al, 2007; He et al., 2004). For example, $P_o$ is often determined to be the difference between TP by sulfuric acid ($H_2SO_4$) digestion and the inorganic phosphate ($P_i$) extractable by any number of soil extractants before $H_2SO_4$ digestion (He et al., 2004). Condensed phosphates, such as pyrophosphate, would not be read as $P_i$ because they do not contain orthophosphate ions and therefore will

not react with Murphy-Riley colorimetric reagents (Matula, 2010). However, upon $H_2SO_4$ digestion, pyrophosphate is disassembled into individual phosphate moieties and would thus be de facto quantified as $P_o$ (He et al., 2004).

In order to hydrolyze the aforementioned model $P_o$ compounds, various enzymes were used. The rationale for using these single enzymes and enzyme blends follows that of Johnson and Hill (2010). A summary of this rationale is as follows. Simple labile monoesters are hydrolyzed by PP, phytate-like P should be hydrolyzed by the combination of PP/GP and the previous $P_o$ groups plus DNA-like P should be captured by PP/GP/NP (Johnson and Hill, 2010). Recall that PP is potato phosphatase, GP is wheat germ phosphatase and NP is nuclease. Thus PP/GP/NP should be capable of hydrolyzing the majority of environmental $P_o$ compounds, including nucleic acids. However, as a model compound example, PP/GP/NP should release no more molydate-reactive P (MRP) from glycerophosphate than PP would because glycerophosphate is a simple monoester. On the other hand, phytic acid is a more recalcitrant compound and requires either phytase or the combination of PP/GP to release some or all the phosphate groups from its six P-O-cyclohexane ring (Quiquampoix and Mousain, 2005). Similarly, NP does not directly cleave P-O bonds, but cleaves polynucleotides thereby producing mononucleotides which can be disassembled by monoesterases such as PP and GP (He et al., 2004).

Results of hydrolysis of seven model $P_o$ compounds in DI water and enzyme buffer solution and subjected to one, two or three enzymes are presented in Table 6. As can be seen in Table 6, no enzyme combinations resulted in 100% hydrolysis of $P_o$ to $P_i$. Glycerophosphate and pyrophosphate had the highest conversions, with over 90% of the $P_o$ in these model compounds being hydrolyzed to $P_i$ in the presence of potato phosphatase (PP, Table 6). *P*-nitrophenyl phosphate and ATP had $P_o$ to $P_i$ conversion rates in the 70% range in the presence of PP and PP + GP (potato phosphatase and wheat germ phosphatase, respectively). In the presence of PP+GP, phytic acid and D-

glucose-6 phosphate had the lowest hydrolysis rates of around 40% and 50% respectively.

Table 6. Mean percent recovery of model $P_o$ compounds by three enzyme groups. Standard deviations are in parentheses.

| | | % Recovery† | |
|---|---|---|---|
| **Model Compound** | **Class** | **PP§** | **PP+GP¶** | **PP+GP+NP#** |
| **Glycerophosphate** | phosphate monoester | 94.5(7.7) | 78.5(15.4) | 88.0(13.0) |
| **Phytic acid** | phosphate monoester | 11.3(8.4) | 40.2(1.9) | 28.4(13.1) |
| **Sodium pyrophosphate** | polyphosphate | 94.1(6.9) | 82.1(13.4) | 84.2(6.8) |
| **Adenosine triphosphate (ATP)** | condensed phosphate | 67.0(8.1) | 76.3(7.2) | 55.6(8.5) |
| ***p*-Nitrophenyl phosphate** | phosphate monoester | 71.1(5.5) | 69.7(11.4) | 59.8(9.6) |
| **D-glucose-6-phosphate** | phosphate monoester | 50.6(8.1) | 50.8(13.4) | 62.9(3.4) |
| **Deoxyribonucleic acid (DNA)** | nucleic acid | ‡ | ‡ | 71.7(9.6) |

† Percent recovery calculated by comparing known 5 ppm $P_o$ in model compounds and $P_i$ after enzyme assays; n=4

‡ Not determined

§ Acid phosphatase from potato; ¶ Acid phosphatase from wheat germ; # Nuclease P1 from *Penicillium citrinum*

Of model compounds that were used in this study and by others, He et al. (2004) and Johnson and Hill (2010) had average $P_o$ to $P_i$ conversion rates of 95-100% in all but phytic acid and glycerophosphate. Johnson and Hill (2010) were only able to recover 75.6% of phytic acid and 76.3% of glycerophosphate. Both He and colleagues (2004) and Johnson and Hill (2010) found phytic acid to be the most resistant to enzymatic hydrolysis.

Table 7 has data comparing two model $P_o$ compounds in the DI water and sodium acetate matrix previously mentioned with a matrix containing the same amount of EDTA as was present in soil extract and enzyme incubations. This data set was added after the completion of the microcosm experiments and was meant to approximate some of the interferences created by EDTA and accommodate for the experimental design flaw of not having EDTA in model compound incubations.

Model $P_o$ compounds incubated in the presence of NaOH-EDTA did have less hydrolysis than model $P_o$ compounds incubated in identical conditions without NaOH-EDTA. To illustrate, PP incubated in a NaOH-EDTA matrix hydrolyzed only 85.6% of the p-nitrophenyl phosphate and ATP model compounds hydrolyzed in a water matrix (Table 7). The difference between recovery of model compounds in EDTA and non-EDTA matrices was most significant with PP+GP enzymes and ATP as the model compound, where just over half of the $P_o$ recovered by enzymatic hydrolysis in a water matrix was recovered in the presence of EDTA ($P$<0.05).

Table 7. Mean percent recovery of two model $P_o$ compounds by enzyme groups dissolved in either DI water or EDTA. Standard deviations are in parentheses.

| | | % Recovery† | |
|---|---|---|---|
| **Model Compound** | **Class** | **PP‡** | **PP+GP§** | **PP+GP+NP¶** |
| ***Model compounds in DI/buffer matrix:*** | | | | |
| ***p*-Nitrophenyl phosphate** | phosphate monoester | 76.1(2.4) | 78.9(8.9) | 76.7(8.2) |
| **Adenosine triphosphate (ATP)** | condensed phosphate | 69.5(19.2) | 77.6(4.7) | 66.4(13.0) |
| ***Model compounds in DI/buffer/NaOH-EDTA matrix:*** | | | | |
| ***p*-Nitrophenyl phosphate** | phosphate monoester | 63.2(9.8) | 62.6(2.7) | 71.3(6.5) |
| **Adenosine triphosphate (ATP)** | condensed phosphate | 61.2(3.0) | 42.6(6.3) | 51.0(7.1) |
| ***Mean recovery of model compounds in EDTA as a percent of that recovered with no EDTA present*** | | 85.6 | 67.1 | 84.9 |

† Percent recovery calculated by comparing known 5 ppm $P_o$ in model compounds and $P_i$ after enzyme assays; n=4

‡ Acid phosphatase from potato; § Acid phosphatase from wheat germ; ¶ Nuclease P1 from *Penicillium citrinum*

Kowalenko and Babuin (2007) found that the addition of 0.006M EDTA to a 3 mg P $L^{-1}$ solution substantially reduced blue color formation of Murphy-Riley reagents. Kowalenko and Babuin (2007) also found the reduction in color intensity in the presence of EDTA was partially negated by Al, Ca, Mg, K and Fe, but that these elements negated color development in the presence of EDTA differently. This has implications for this present study, beyond analyses of $P_o$ compounds, in that different soils would have variable Al, Ca, Mg, K and Fe and EDTA is present in all soil extract solutions. Also, mean standard deviations of model compound hydrolysis in an EDTA matrix were lower: 6.4%,

4.5% and 6.8% for the three mentioned enzyme groups respectively (Table 7). Lower standard deviations could be related to the aforementioned reduction of color development in the presence of EDTA (Kowalenko and Babuin (2007).

While quantifying $P_o$ hydrolysis of model compounds by subjecting them to enzyme assays in a pH-adjusted DI water and sodium acetate matrix represents a means of verifying the activity of the purchased enzymes, this matrix was not equivalent to that of the soil extracts which were subjected to enzymatic hydrolysis. Because 0.25M NaOH-0.05M EDTA was required to extract soil $P_o$ and $P_i$, the extracted solution contained NaOH-EDTA. Possible enzyme activity interferences could have been introduced by EDTA's ability to chelate enzyme activator cations such as Ca and Mg (Hill and Cade-Menun, 2007). In addition to enzyme activity interferences caused by EDTA, compounds extracted from soils such as $PO_4$, Zn, and Cu in the matrix in which enzymes were suspended could have caused reductions in enzyme activity. For example, excess enzyme products such as $PO_4$ are known to inhibit enzyme activity. Thus verification that the enzymes used in this study were capable of hydrolyzing common environmental $P_o$ compounds, to varying degrees, was achieved but the extent that identical amounts of $P_o$ hydrolysis occurred in the soil extract matrix from microcosms was unclear. In future research, buffered model $P_o$ compounds and enzymes should be incubated in a 0.25M NaOH-0.05M EDTA soil extract matrix. This would indicate the extent to which the likes of EDTA, Cu, Zn and high $P_i$ interfered with enzyme activity.

It is also unclear why hydrolysis rates and standard deviations from this study differed from those obtained by Johnson and Hill (2010). He and colleagues (2004) obtained even lower standard deviations which could possibly be explained by these researchers using 1 mL total assay volumes, compared to microplates wells in the current study which contained 0.30 mL of enzymes, substrates and Murphy-Riley reagents. While sample volumes can explain variations in standard deviations, it does not address the overall lower rates of model compound hydrolysis found in the current investigation. It is possible that there were enzyme batch and/or model compound

differences between this and Johnson and Hill's 2010 study. However, all purchased enzymes are required to be tested for activity rates at specified temperatures and pH values. The enzymes used in this study were no exception and were sold by measured units of activity. For example, Sigma acid phosphatase (potato) product P1146 has a unit activity definition of the ability to hydrolyze 1.0 μmole of *p*-nitrophenyl phosphate per minute at pH 4.8 and 37 $^{o}$C, with a pH optima of 5.0-5.3 (Sigma-Aldrich product data sheet). However, this product is partially purified and has a stated activity of 3-10 units/mg solid. It is possible that this study and that of Johnson and Hill (2010) had differing ratios of enzyme activity to substrate concentration. The current study, used assays of 0.5 units (U)/mL of PP and GP activity and 5 U/mL NP activity with 50 μL of enzymes added to 50 μL 5 ppm P model compounds. Johnson and Hill (2010) had the same enzyme activity in their soil extract/enzyme assays but model $P_o$ compound and enzyme concentrations are unclear.

Throughout the experiment, microplate wells without added enzymes had even color upon the addition of Murphy-Riley reagents, whereas enzyme contamination tests, enzyme/model compound and enzyme/soil extract wells periodically had deeper blue colors as specs or rafts on the surface of wells (to be discussed shortly). Despite being stirred within seconds of the spectrographic read, uneven color distribution within the microplate wells was observed. A similar phenomenon was noticed throughout this experiment in soil/enzyme and enzyme-alone conditions and was addressed, as best possible, by mixing uneven wells with new pipette tips as close to absorbance determination as possible. The error that uneven enzyme suspension represents in this study is unclear. Throughout the study, PP/GP and PP/GP/NP enzyme blends had more uneven surface color than PP alone by about four times. As an example, glycerophosphate and pyrophosphate subjected to hydrolysis by PP perhaps represents both the least recalcitrant $P_o$ compounds and the least introduction of variable levels of P contamination through the addition of enzymes. This is due to PP being the only condition under which there is not the addition of an unpurified enzyme (GP). As was

previous noted, glycerophosphate and pyrophosphate had the highest rates of enzymatically-mediated conversion of $P_o$ to $P_i$.

## Soil characteristics

Soils from the dairy waste application field, in both landscape positions, had consistently higher pH values when compared with control soils. Further, microcosm soils (soils and dairy wastes) had pH values that were higher than control soils. Average pH values for upland soil microcosms were 6.5, 6.4 for depositional soil and 5.9 for control soil, whereas the corresponding soils before use in microcosm had pH values of 5.7, 5.7 and 5.2, respectively (Table 8). This is due to the high pH of dairy wastes applied to fields surrounding milking parlors (Graham, 2009). In the soils used for this study, Mehlich-3 extractable P, Ca, Mg, K, Cu and Zn were found to be higher in soils receiving dairy waste compared to control soils. High concentrations of these elements can be attributed to additions of dairy wastes to soils at levels beyond crop uptake (Carpenter et al., 1998). Mehlich-3 extractable P, Ca, Cu and Zn were higher in the lower field position compared to upland. Mehlich-3 extractable P and Zn had the most extreme enrichment in depositional soils, being about 5 times that of control soils (Table 8).

Table 8. Characteristics of air dried soil used in study and NaOH-EDTA TP ($TP_{NaOH}$) of microcosm extracts at week 1. Values in parentheses denote standard deviations.

| Soil type and | *Total P* | *CEC* | *§ pH* | *Ca* | *Mg* | *P* | *K* | *Cu* | *Zn* |
|---|---|---|---|---|---|---|---|---|---|
| **sample location** | *mg/kg soil* | *meq/cm $^3$* | *§* | *Mehlich-3 extractable elements in mg/kg soil* | | | | | |
| †Upland Microcosm Soil TP | 903(33.5) | 10.6(0.5) | 5.7(0) | 609(7.5) | 148(1.9) | 236(28) | 420(19.4) | 2.5(0.3) | 11(1) |
| ‡Upland Microcosm $TP_{NaOH}$ | 686(47.4) | | | | | | | | |
| | | | | | | | | | |
| Depositional Microcosm Soil TP | 1,262(23.8) | 11.8(0.3) | 5.7(0) | 726(8.3) | 130(2.0) | 285(0.8) | 285(19.4) | 3.8(0.2) | 17.7(1.1) |
| Depositional Microcosm $TP_{NaOH}$ | 1,210(148.9) | | | | | | | | |
| | | | | | | | | | |
| Control Microcosm Soil TP | 570(17.6) | 9.3(0.1) | 5.2(0) | 544(6.6) | 116(6.5) | 45(7.6) | 157.4(4.1) | 1.3(0.2) | 3.3 (0.7) |
| Control Microcosm $TP_{NaOH}$ | 219(13.1) | | | | | | | | |
| | | | | | | | | | |
| Dilute manure total P (as applied) | 46 ppm(6.5) | | | | | | | | |

†Total P in microcosm soil with manure by sulfuric acid digest; sampled at one week of microcosm incubation; n=3

‡ Microcosm $TP_{NaOH}$ is NaOH-EDTA-extractable total P after .2 um filtration and sulfuric acid digest at one week of microcosm incubation; n=3

§ pH and Mehlich-3 extractable elements were tested in air-dried field soils; n=2

The same pattern seen for Mehlich-3 extractable P was also seen for microcosm soil total P (TP) in that control soils had the lowest TP (570 mg/kg soil), upland field positions had the second lowest TP values (903 mg/kg soil) and depositional soils had the highest TP (1,262 mg/kg soil). In control soils, Mehlich-3 extracted a much a lower percentage of the total soil P than it did in soils from the application field (Table 8). Total P of NaOH-EDTA microcosms extracts ($TP_{NaOH}$) did not reflect TP in microcosm soils (measured after 1 week). Levels of $TP_{NaOH}$ were only about a third that of TP in control soils whereas $TP_{NaOH}$ was two thirds that of TP in upland soils, and depositional soils had similar quantities of $TP_{NaOH}$ and TP. This suggests that in heavily waste-amended soils NaOH-EDTA is capable of capturing the majority of $P_i$ and $P_o$ but the percentage of soil TP extracted by NaOH-EDTA can vary considerably by management and landscape position even within the same soil type. As one example, Hill and Cade-Menun (2007) found that in two poultry waste-amended soils, TP in NaOH-EDTA extracts determined by ICP-OES analysis was 65 to 79% that of soil TP determined by digestion.

## Total P and molybdate-reactive P (MRP) in NaOH-EDTA soil extracts

Depositional soils had significantly higher ($P$<0.05) amounts of $TP_{NaOH}$ than NaOH-EDTA extractable P ($P_{NaOH}$) (Figure 8). The mean difference between $TP_{NaOH}$ and $P_{NaOH}$ in depositional soils was 384.5 mg P kg soil$^{-1}$, whereas the mean difference in upland soils was 87.2 mg P kg soil$^{-1}$. The difference between $TP_{NaOH}$ and $P_{NaOH}$ at the time of incubation with enzymes is assumed to approximate potentially enzymatically hydrolysable $P_o$. The combination of NaOH and EDTA extracts an indeterminate pool of $P_o$ and $P_i$ as it is known that NaOH can extract $P_i$ associated with amorphous Fe, Al and organic matter (Turner and Leytem, 2004). Numerous studies have shown NaOH-EDTA soil extracts to contain both $P_i$ and $P_o$ (Turner et al., 2003; Hill and Cade-Menun, 2007). Hill and Cade-Menun (2007) extracted crop soils amended with poultry litter with NaOH-EDTA and characterized the $P_o$ and $P_i$ species composition by $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy. In this study, Hill and Cade-Menun (2007) found orthophosphate ($P_i$) to be the majority of extracted P but $P_o$ compounds such as orthophosphate monoesters, orthophosphate diesters, polyphosphate and phytate combined in one soil represented 29% of total P as measured by NMR. However the aforementioned $P_o$ components of $TP_{NaOH}$ are largely not detectable using the Murphy-Riley colorimetry applied throughout this study. Enzymes can potentially transform the $P_o$ contained in $TP_{NaOH}$ to $P_i$ which is then measurable using Murphy-Riley colorimetric methods (Johnson and Hill, 2010).

Figure 8 shows control and depositional soils have the highest amounts of $P_o$ compounds available for hydrolysis, assuming that $TP_{NaOH}$ minus $P_{NaOH}$ equals $P_o$ compounds available for enzyme hydrolysis. Whether this is a valid assumption would have to be tested by $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy, for example, of NaOH-EDTA soil extracts (Hill and Cade-Menun, 2007). Upland dairy farm soils had the smallest amount of potentially enzyme-labile $P_o$. This could be due to the loss of $P_o$ from this landscape position after manure applications due to erosion and/or runoff.

Depositional field positions tend to accumulate P via erosion and runoff from upland areas and this P can be incorporated into both plant and microbial biomass as $P_o$. Conversely, the P in NaOH-EDTA extracts of depositional soils could have not been in organic forms, and instead could be controlled by any number of factors, including P-bonding to fine soil textures related to being in a depositional field position. Control soils had slightly higher mean differences between $TP_{NaOH}$ and $P_{NaOH}$, than upland soils (Figure 8) but about 30% less soil TP (Table 8).

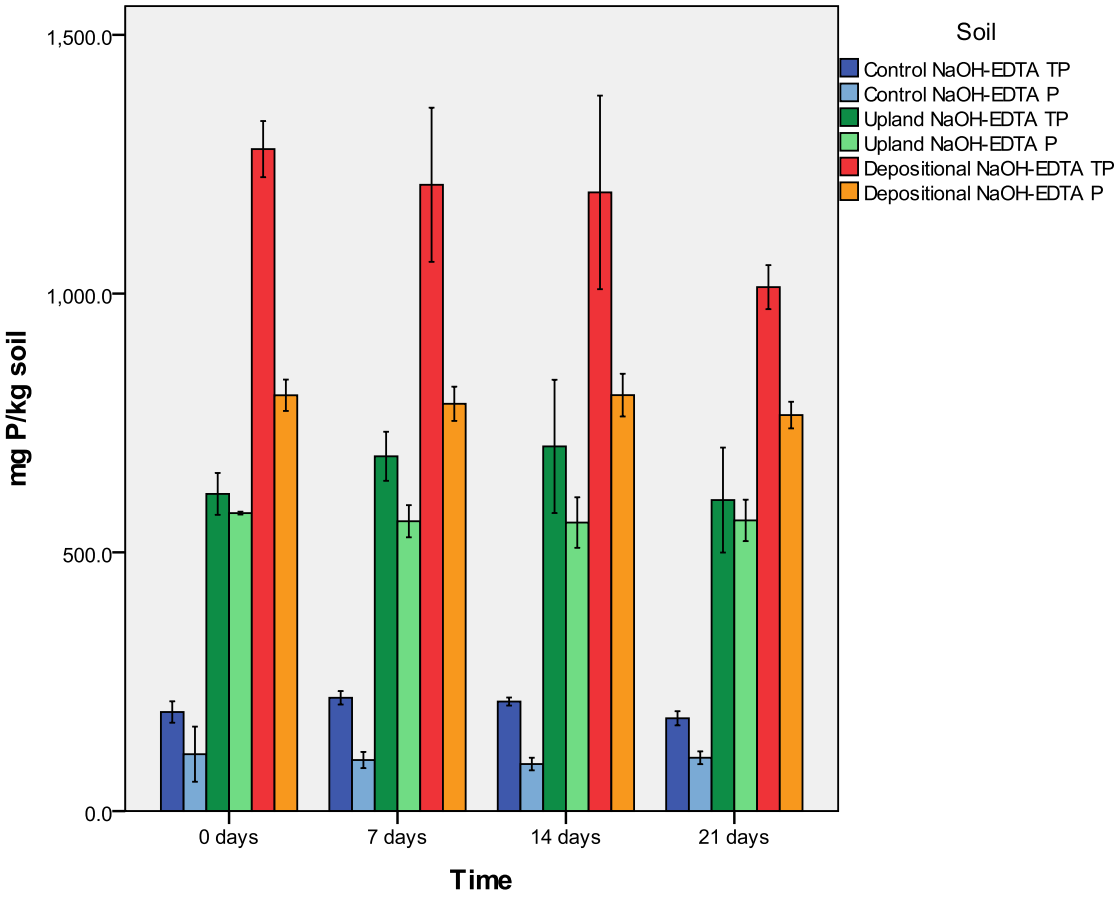



Figure 8. Total P of NaOH-EDTA extracts ($TP_{NaOH}$) and NaOH-EDTA extractable P ($P_{NaOH}$) from microcosm subsamples taken weekly.

## Effects of procedure physical conditions on transformations of $P_o$ to $P_i$

In order to assess the action of enzymatic hydrolysis process itself upon the conversion of molybdate unreactive P (MUP) to molybdate reactive (MRP or $P_i$) all microplates included quadruplicate wells which had and had not been incubated at 37 $^oC$ for two hours with buffered enzymes (Johnson and Hill, 2011). Thus microplates contained samples run with and without use of the enzyme hydrolysis protocol to assess the degree to which incubating soil extracts to 37 $^oC$ for 2 hours with shaking would promote hydrolysis of $P_o$ through chemical and physical mechanisms. This procedure allowed for creation of pre enzyme hydrolysis MRP measurements which were subtracted from final MRP readings, thereby allowing quantification of $P_i$ released due to enzymatic hydrolysis alone.

Figure 9 shows the three soils used in this study, with one set of samples processed in conditions which could cause chemical hydrolysis of MUP (NaOH-EDTA incubated P in Figure 9) and another set of samples kept under refrigeration until the time of plate reading(NaOH-EDTA P in Figure 9, $P_{NaOH}$ in text ). Neither NaOH-EDTA incubated P nor $P_{NaOH}$ had significantly higher MRP at the time of microplate reading ($P<0.05$). The difference between NaOH-EDTA incubated P and $P_{NaOH}$ in all soils was smaller than the differences between these any other treatments, such as Mehlich-3 or enzyme additions, applied during this study. In summary the enzymatic hydrolysis procedure, conducted without enzymes, did not cause significant adsorption and release of $P_i$ at various times. No single soil showed that the incubation procedure itself caused an increase in conversion of $P_o$ to $P_i$. Thus future applications of the Johnson and Hill (2010) method using similar soils may only require one set soil extract samples to be removed from the enzymatic hydrolysis procedure for initial soil $P_i$ subtraction.

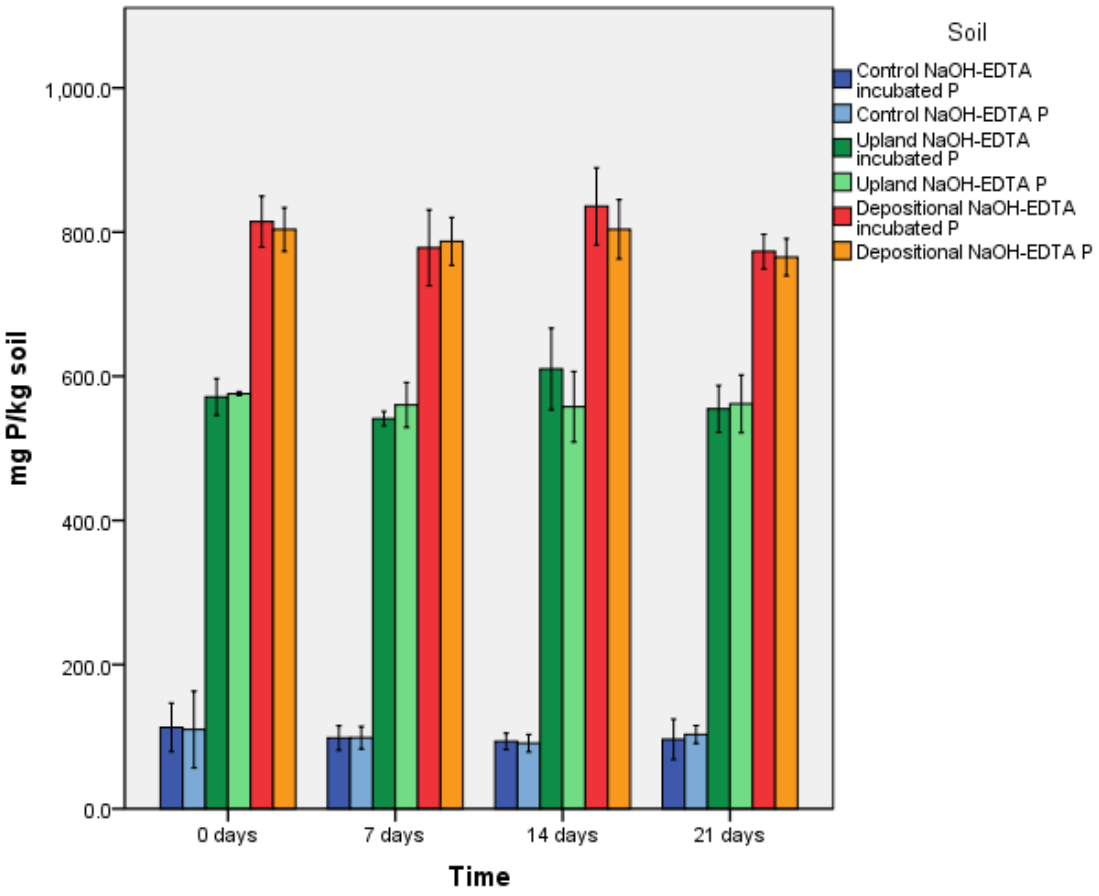



Figure 9. Comparisons of microcosm MRP before and after 2-hour incubation at 37 C. NaOH-EDTA soil extracts were either incubated in tandem (NaOH-EDTA incubated P) or set aside before the enzymatic hydrolysis procedure (NaOH-EDTA P).

## Microcosm Mehlich-3 extractable P

Mehlich-3 extractable P in soil/manure microcosms over time did not increase in the same proportions as microcosm soil TP (Table 8; Figure 10). Mean microcosm soil TP by $H_2SO_4$ digest was about 570 mg P/kg soil in control soils, 903 mg P/kg soil in waste-application field upland soils and 1,262 mg P/kg soil in waste-application field

depositional soils (Table 8). Mean Mehlich-3 P for combined sampling times was 4.7 mg P $kg^{-1}$ soil in control soil microcosms, 34.7 mg P $kg^{-1}$ soil in upland microcosms and 101.5 mg P $kg^{-1}$ in depositional soil microcosms ( Figure 10). Depositional microcosm soils had approximately twice the TP of control soils but around 20-fold the Mehlich-3 extractable P. As soils receive heavy long-term P application, data such as the aforementioned may reflect increasingly environmentally labile P and therefore potential loss to waterways. Additionally, Figure 10 shows that depositional soils had approximately 3 times the Mehlich-3 extractable P of upland soils. In a situation where application of P-containing wastes are presumed to be uniform, this illustrates that slope grade is a strong factor controlling P movement. Were this field typical in that the depositional areas were located at field edge rather than in sink hole features, loss of soil and P-containing runoff to surface waters would be likely if field-edge buffers were not installed.

Mehlich-3 extractable P sampled at 0 and 3 weeks from soil microcosms significantly increased in depositional and upland dairy farm soil ($P< 0.05$), whereas values significantly decreased in control soils ($P< 0.05$). These changes were small relative to both soil TP and $T_{PNaOH}$, but were significant nonetheless.

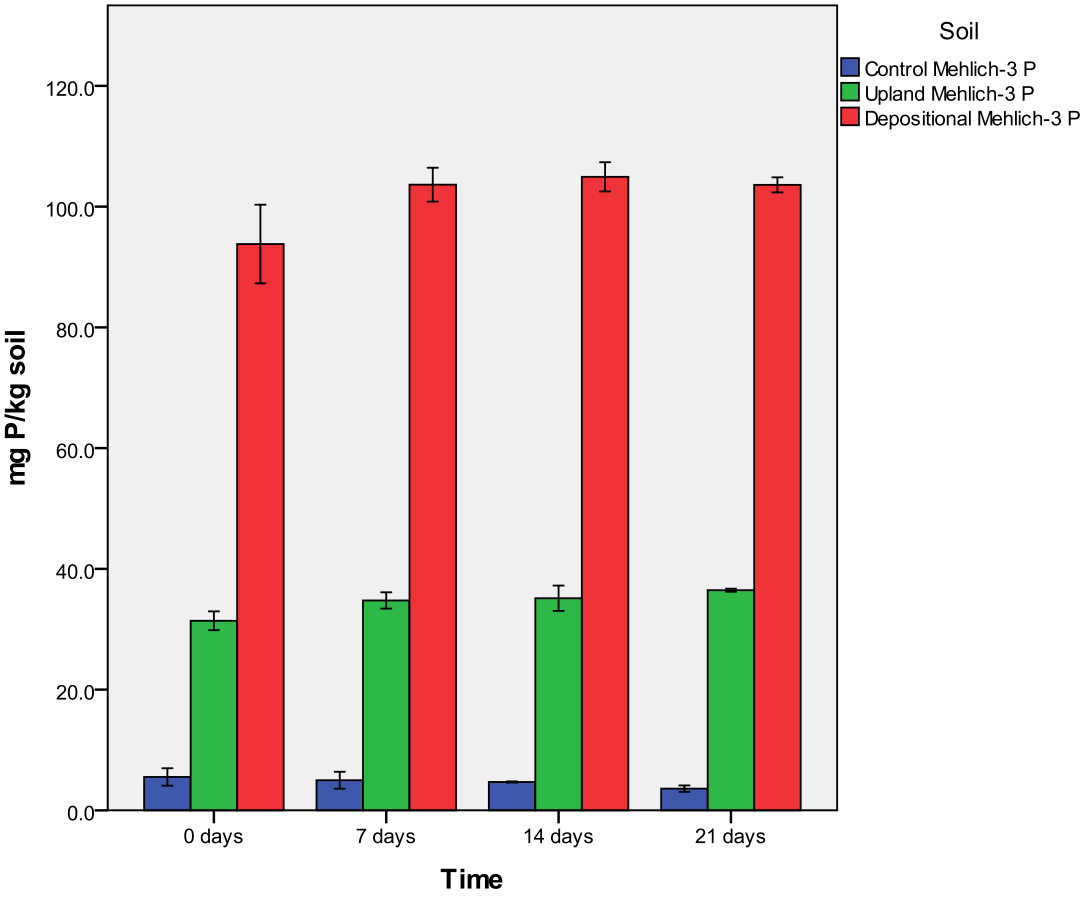



Figure 10. Comparisons of Mehlich-3 extractable P in three microcosm soils over 21 days.

Control soils had a mean Mehlich-3 extractable P of 5.5 mg P kg soil$^{-1}$ at the initial sampling time and 3.6 mg kg-1 by 21 days of microcosm incubation which represented a significant decrease ($P$< 0.05). Upland soils had mean Mehlich-3 extractable P of 31.4 mg P kg soil$^{-1}$ at the initial sampling time and 36.5 mg kg$^{-1}$ at 21 days, a significant increase ($P$< 0.05). Depositional soils had mean Mehlich-3 extractable P of 93.8 mg P kg soil$^{-1}$ at the initial sampling time and 103.6 mg kg$^{-1}$ at 21 days showing a significant increase over the time period ($P$< 0.05). Increased Mehlich-3 extractable P in high TP waste-amended upland and depositional soils may be due to increased microbial population growth under warm microcosm incubation conditions. It is possible that microbes were utilizing $P_o$-containing reduced carbon compounds and could not consume all the $P_i$ bonded to these molecules. This is in keeping with Loria and Sawyer (2005) who found that Mehlich-3 extractable P significantly increased ($P$<0.05) from day 0 to 14 in a silty clay loam Mollisol incubated at 22 $^{o}$C with raw swine manure. Specifically, swine manure application rates of 37.5 and 50 mg P kg$^{-1}$ in a Mollisol beginning with a Mehlich-3 value of 50 mg P kg$^{-1}$ showed the greatest increases in Mehlich-3 P over the first 14 days of the study (Loria and Sawyer, 2005).Conversely, in soils where P is limited, like control soils, it is likely that microbial activation would result in the consumption of labile P and therefore lowered Mehlich-3 extractable P.

## Enzymatic hydrolysis in NaOH-EDTA microcosm extracts

### *Upland soils*

Sodium hydroxide and EDTA extracts ($P_{NaOH}$) of upland dairy farm soils incubated in microcosms sampled over all sampling times combined had more MRP ($P_i$) after the addition of potato phosphatase (PPase) and PPase combined with wheat germ phosphatase (GPase) (Figure 11).

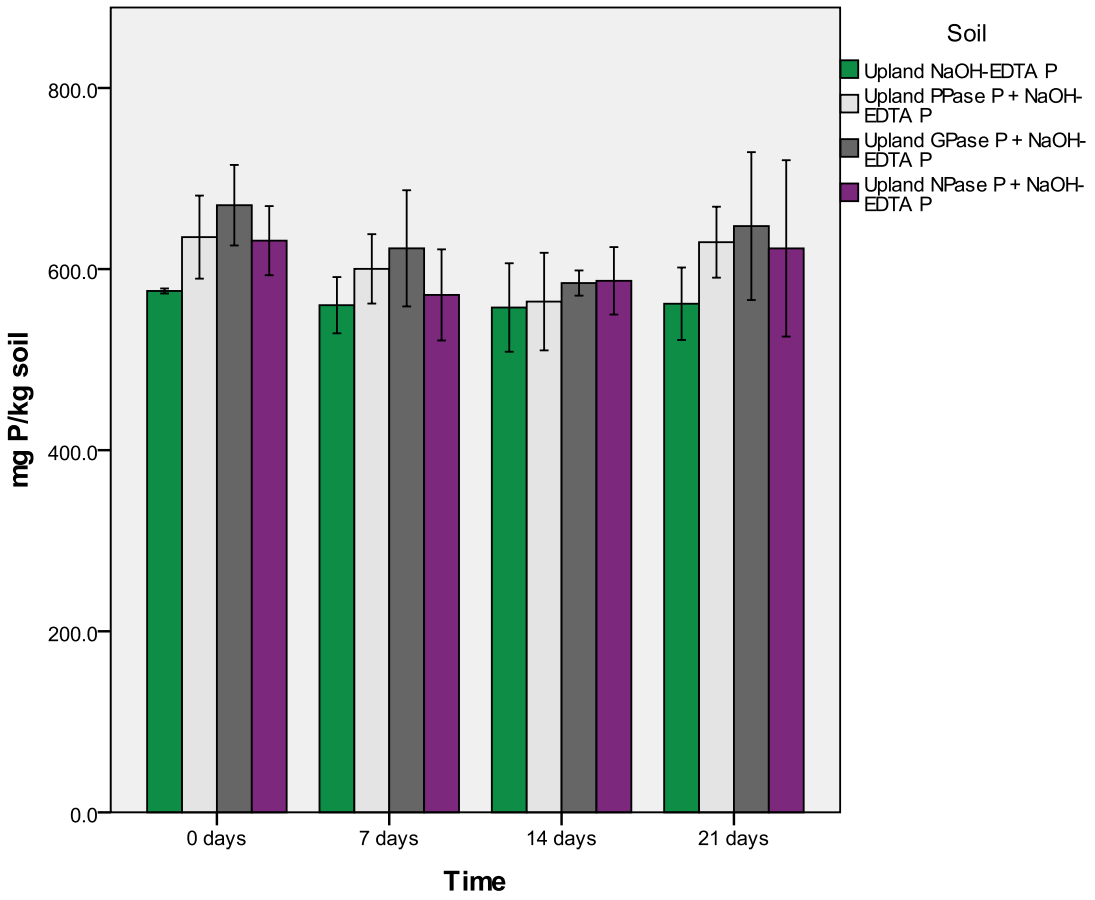



Figure 11. Differences between upland soil NaOH-EDTA microcosm extract ($P_{NaOH}$ or MRP) before and after the addition of 1 (PP), 2 (GP) or 3 (NP) enzymes. Error bars +/- 1 standard deviation.

Mean release of MRP from all four sampling times in NaOH-EDTA extracts taken from upland soil microcosms was 44.8 (27.2) mg P $kg^{-1}$ from PPase, 68.9 (30.0) mg P $kg^{-1}$ from PPase/GPase, 40.7 mg $kg^{-1}$ (21.1) from PPase/GPase/NPase. Samples taken at 14 days and PPase/GPase/NPase (NPase in Figure 11) had comparatively low MRP after initial NaOH-EDTA-extracted $P_i$ and $P_i$ from enzyme contamination was subtracted. Variability in enzyme-hydrolysable P was large throughout this experiment, as shown by the error bars in Figure 11. Accordingly mean release of MRP due to enzyme hydrolysis

of any enzyme cocktail was not significant during any of the four weekly sampling times (Excel 2-tailed type 3 t-test, $P<0.05$). Additionally, Tukey HSD post-hoc tests ($P<0.05$) showed no significant enzymatically-mediated release of MRP for each enzyme cocktail at combined sampling times (Table 11 in Appendix).

A possible reason for the high variability in the amount of MRP released from microcosm soils after enzyme additions is variability among the soil microcosms themselves. This can be seen by examining the amount of MRP released from enzyme hydrolysis in 3 replicate microcosms of the same treatment: 80.2, 0 and 69.2 mg P kg soil$^{-1}$ for upland soil microcosms at time 0 sampling. A better test of the Johnson and Hill (2010) enzyme hydrolysis microplate method may have been to collect 3 replicate samples from each microcosm at each sampling event as well as utilizing replicate microcosms for each treatment. This would give some idea of the degree of the variability between microcosms of the same treatment, which would allow a separation of the variability due to individual microcosms from other sources of variability inherent in the procedure, such as enzyme efficiency and analytical method. These results highlight that even with sieving and thoroughly homogenizing soil samples before weighing out into individual microcosms, soil microscale chemical/ biological differences may exert considerable influence on enzymatic action.

Additional sources of error in measurements of enzyme-hydrolysable P were introduced from enzyme $P_i$ contamination and non-uniform mixing of enzymes in microplate wells. Regarding contamination, each microplate run included 4 repetitions of enzyme contamination tests (negative controls) and contamination levels had a range for all four upland microcosm sampling times of 0.2-0.9 ppm P for potato phosphatase (PPase), 0.9-1.7 ppm for PPase and wheatgerm phosphatase (GPase) combined and 1.3-2.4 ppm for nuclease phosphatase (NPase) and PPase/GPase . In addition to enzyme contamination, a non-uniform distribution of Murphy-Riley colorimetric reagents was seen in some individual wells containing only enzymes (contamination test). Wells containing enzymes, particularly multiple enzymes experienced more intense blue color

concentrating on the surface of the well while deeper in the well the color intensity was much less. This phenomenon rarely occurred in wells containing soil extracts along with enzymes.

***Depositional soils***

Depositional dairy farm soils incubated in microcosms and sampled over three weeks had a cumulative mean release of 19.1 mg P kg soil$^{-1}$ after the addition of PPase, PPase/GPase and PPase/GPase/NPase enzymes (Figure 12). This result was much lower than the amount of enzyme hydrolysis occurring in uplands soils. This despite larger differences between $TP_{NaOH}$ and $P_{NaOH}$ than in upland soils (Figure 8). Recall that the difference between $TP_{NaOH}$ and $P_{NaOH}$ is the P pool upon which enzymatic conversion of organic and polyphosphates to orthophosphate ($P_i$) is possible. Presumably, the larger this difference, the more enzymatic hydrolysis is possible if adequate enzyme activity is present. Additionally this potentially enzymatically hydrolysable P was variable over the course of the microcosms incubation. For example, depositional soil sampled at zero weeks had an approximate mean difference between $TP_{NaOH}$ and $P_{NaOH}$ of 475 mg P kg soil$^{-1}$. By 21 days, the mean difference between $TP_{NaOH}$ and $P_{NaOH}$ was 247 mg P kg soil$^{-1}$. Mean enzyme hydrolysis over the entire study from additions of PPase, PPase/GPase and PPase/GPase/NPase were 3.8 (7.5), 38.0 (15.7) and 15.5 (8.8) mg P kg soil$^{-1}$, respectively. The amount of MRP released via enzyme hydrolysis was not significant for all enzyme combinations at $P<0.05$ (Appendix, Table 11). Possible reasons for insignificant enzymatic hydrolysis will be discussed shortly.

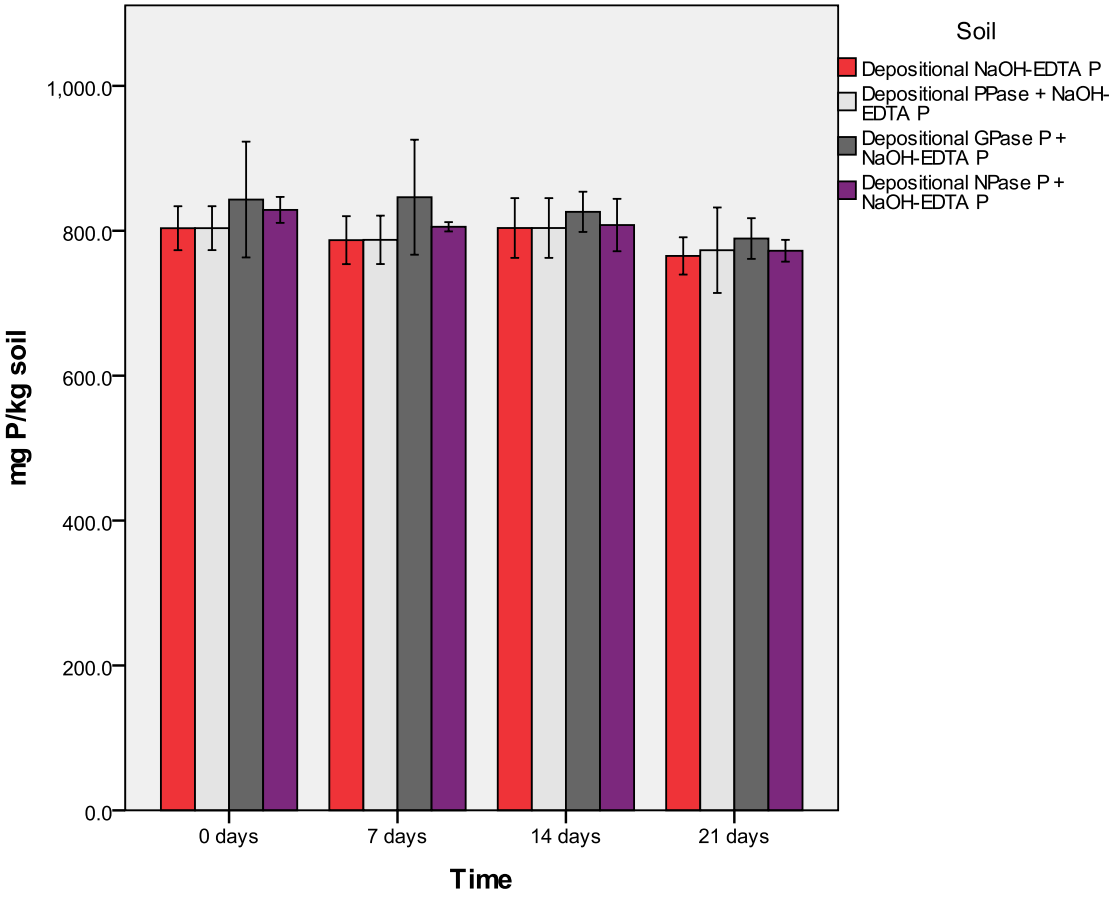



Figure 12. Differences between depositional soil NaOH-EDTA microcosm extract ($P_{NaOH}$ or MRP) before and after the addition of 1 (PP), 2 (GP) or 3 (NP) enzymes. Error bars +/- 1 standard deviation.

Because PPase hydrolyzes polyphosphates and $P_o$ monoesters, with the exception of phytate, the PPase-labile fraction is expected to be lower than PPase /GPase and PPase/GPase/NPase. Accordingly, PPase /GPase should capture the aforementioned $P_o$ compounds plus phytate and PPase/GPase/NPase should these compounds and P-containing nucleic acids. Therefore PPase/GPase/NPase-labile $P_o$ (15.5(8.8) mg P kg soil$^{-1}$), should not be lower than PPase/GPase-labile $P_o$ (38.0(15.7) mg P kg soil$^{-1}$), as was seen in depositional soils. Possible reasons for the enzyme cocktail with the most enzymes not resulting in the highest $P_o$ hydrolysis include increased analytic interference with Murphy-Riley colorimetric reagents with more enzymes. However, the last two mentioned hydrolysis numbers were within each other's standard deviations and furthermore, the NPase-labile $P_o$ fractions in soils can be lower than 1% of TP in soils as determined by NMR (Hill and Cade-Menun, 2009).

Possible reasons for the comparatively low MRP released by enzyme hydrolysis for depositional soil extracts as opposed to upland and control soils include high orthophosphate in the soil extract solution subjected to enzymatic hydrolysis (Table 8, Figure 8 ). Also, high zinc ($Zn^{2+}$) and copper ($Cu^{2+}$) were found in dairy farm upland and depositional soils (Table 8). High concentrations of $Zn^{2+}$ are known to inhibit acid phosphatase (EC 3.1.3.2) from both wheat germ and potato tubers and high $Cu^{2+}$ may inhibit wheat germ phosphatase. Mean concentrations of Mehlich-3 extractable $Cu^{2+}$ and $Zn^{2+}$ in depositional soils were 3.8 and 17.7 mg kg soil $^{-1}$, respectively. As a point of reference, off-farm control soils had mean concentrations of Mehlich-3 extractable $Cu^{2+}$ and $Zn^{2+}$ of 1.3 and 3.3 mg kg soil $^{-1}$, respectively (Table 8). Concentrations of $Cu^{2+}$ and $Zn^{2+}$ in $P_{NaOH}$ extracts of dairy farm soils were not available but would make an interesting point of study.

High background P was illustrated by NaOH-EDTA extractable P at all microcosm sampling times, as well as total soil by analysis by $H_2SO_4$ digestion at day seven of microcosm incubations (Figure 8, Figure 9, Table 8). Enzymes are typically inhibited by an excess of their product. A mechanism for this includes enzyme active sites being

physically blocked by $PO_4$ molecules which disallow $P_o$ compounds the access necessary for hydrolysis (Figure 4). It is quite possible that the high concentration of inorganic P in the depositional soil inhibited $P_o$ hydrolysis. It is also possible that some $P_o$ was hydrolyzed but the orthophosphate anion bonded with cations or organic matter in solution and thereby became colorimetrically undetectable by Murphy-Riley reagents. Variability caused by enzyme contamination of inorganic P is illustrated by the highest standard deviations most often being paired with the presence of wheat germ phosphatase (GPase), the least purified of the enzymes used. In eleven of twelve depositional soil extract/enzyme microplate analyses, conditions with GPase had the highest standard deviations.

***Control soils***

Control soils incubated in microcosms and sampled over three weeks had a net release of MRP after the addition of PPase, PPase/GPase and PPase/GPase/NPase (Figure 13). Mean release of MRP after incubation with PPase, GPase/NPase and PPase/GPase/NPase additions at the initial sampling time were 46.4, 39.6 and 31.6 mg P kg $soil^{-1}$, respectively (Figure 13). By the final 21 day sampling time, standard deviations decreased and more enzyme-labile P was found in the PPase/GPase/NPase fraction than at the initial sampling time with MRP concentrations being 53.0, 46.0 and 96.6 mg P kg $soil^{-1}$ for the aforementioned enzyme groups (Figure 13). Proportionally, control soils had the largest $P_o$ to $P_i$ transformations after enzyme incubations of the three studied soils. One possible reason for this is the lower TP, Cu and Zn when compared to soils receiving dairy waste applications. Also, control soils, in contrast to depositional soils, had very small portions of their TP that was captured by the Mehlich-3 extractant. A reason for this could be that less of the control soil's TP was in Al-P and Fe-P compounds that the acidifying Mehlich-3 extractant acts upon and more existed as $P_o$ compounds.

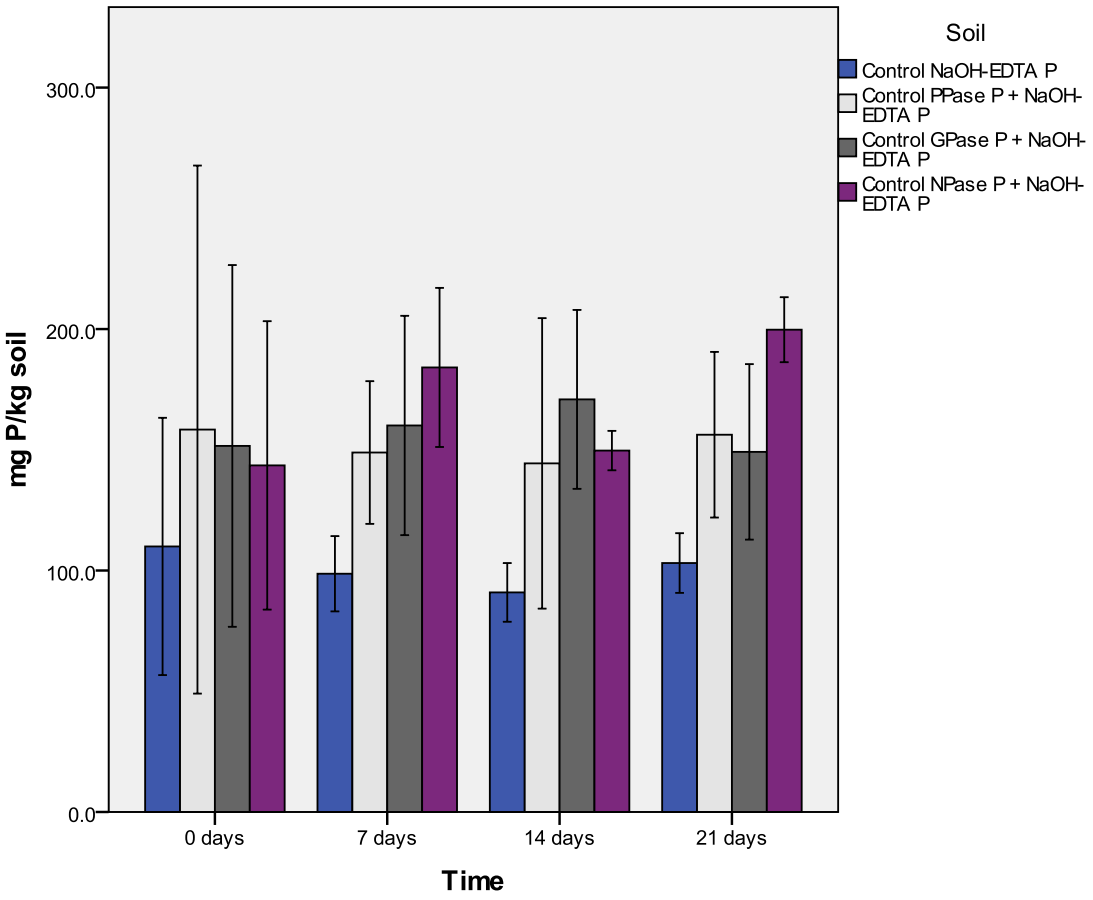



Figure 13. Differences between control soil NaOH-EDTA microcosm extract (NaOH-EDTA P or MRP) before and after the addition of 1 (PP), 2 (GP) or 3 (NP) enzymes. Error bars +/- 1 standard deviation.

Control soils had both lower soil TP and NaOH-EDTA-extractable TP than upland and depositional soils receiving dairy waste. Potentially enzyme-hydrolysable $P_o$ was higher than depositional soils but lower than upland soils. Also, as previously mentioned, mean Mehlich-3 extractable $Cu^{2+}$ and $Zn^{2+}$ were 1.3 and 3.3 mg kg soil $^{-1}$, respectively values about one forth that of depositional soils (Table 8). The persistence of a high degree of variability in enzymatic hydrolysis results for control soils having low Cu and Zn and comparatively low MRP in solution points to the possibility that other interferences may be reducing the accuracy of this method. For example, matrix EDTA could be chelating activator cations and thus be causing decreases in enzyme activity levels.(Omburo et al., 1992) Enzymes such as PPase require activator cations such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$, but are also inhibited by high $Zn^{2+}$, as well as $Al^{3+}$ (Sigma-Aldrich datasheet, product P1146). Additionally, potential problems of uneven levels of enzyme $PO_4$ contamination and non-uniform distribution of color reagents in microplate wells remain.

Perhaps a more useful way of looking at the efficacy of the Johnson and Hill (2010) method across soils is to compare the rate of enzymatic hydrolysis of the P contained in the difference between $H_2SO_4$ total P digests of NaOH-EDTA microcosm soil extracts ($TP_{NaOH}$) and MRP before enzyme incubation ($P_{NaOH}$). The difference between $TP_{NaOH}$ and $P_{NaOH}$ is hypothesized $P_o$, as was discussed previously. In other words, given a quantity of $P_o$ an enzyme could act upon, how much does soil management and field position affect the quantification of $P_o$ by the Johnson and Hill (2010) method? Figure 14 illustrates the strong effect of both field position and soil management on results of the aforementioned method. For example, dairy farm depositional soil, when combining all sampling times, had around 2% of its total $P_o$ that was hydrolysable by potato phosphatase (PPase) alone (Figure 14).

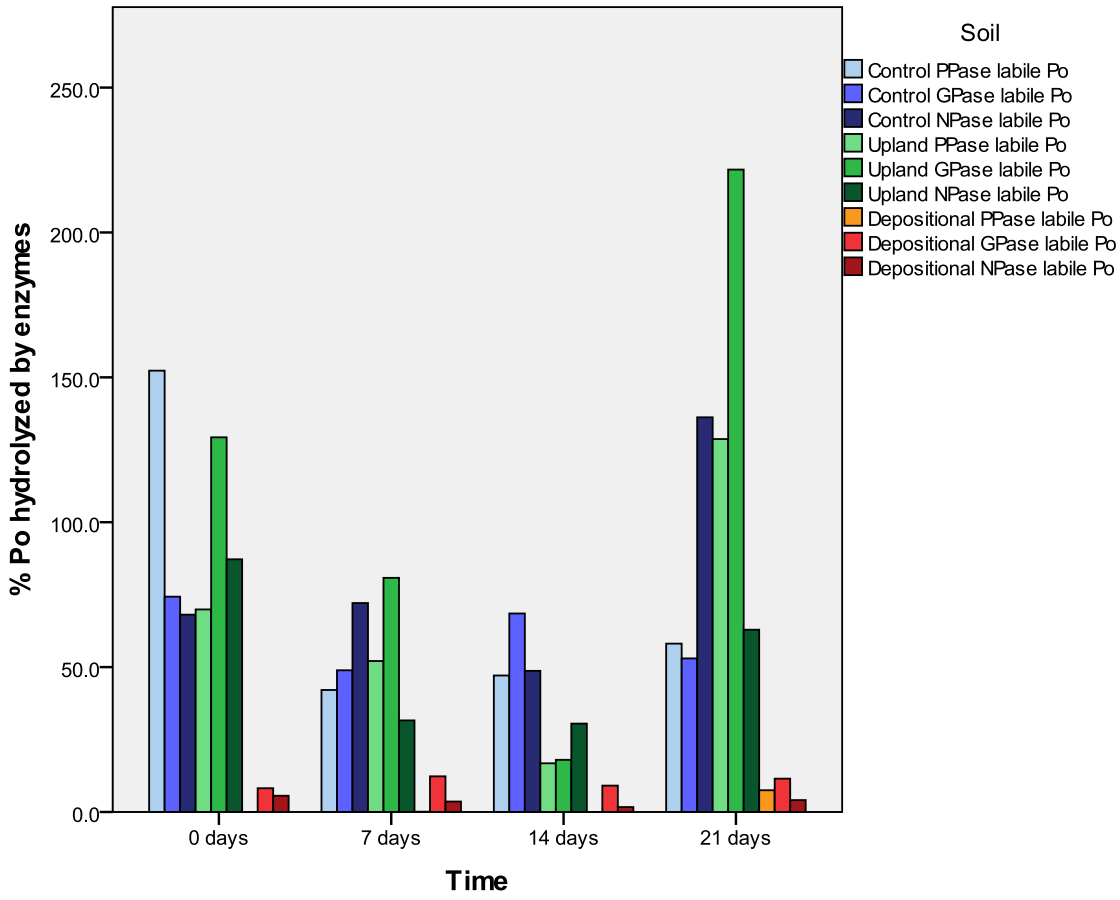



Figure 14. Percentages of $P_o$ in all microcosm experiment soils that were hydrolyzed by various enzyme combinations. The difference between $H_2SO_4$ total P digests of NaOH-EDTA microcosm soil extracts,$TP_{NaOH}$, and $P_{NaOH}$ before enzyme incubations is hypothesized $P_o$.

This number increased to around 10% of hypothetically available $P_o$ when the combination of potato and wheat germ phosphatase (GPase) was used. However, PPase produced no measurable hydrolysis in three of the four depositional soil microcosm sampling times (Figure 14). Why the soil the highest $TP_{NaOH}$ (Figure 8) had the lowest percentages of its hypothetically available $P_o$ hydrolyzed by enzymes was discussed previously and will be touch on again shortly.

Conversely, both upland and control soils had PPase-labile $P_o$ averages for the entire microcosm experiment of generally near over 50%, with the exception of the 14 day sampling time in upland soils (Figure 14). The combination of PPase and GPase enzymes used in upland soil extracts diverged with control soils in that GPase-labile $P_o$ was over 75% of hypothetical $P_o$ in all but the 14 day sampling time (Figure 14). That any soil had over 100% of its $P_o$ fraction being hydrolyzed by any single enzyme or enzyme cocktail illustrates probable quantification errors.

The NPase enzyme condition (nuclease phosphatase, GPase and PPase) should theoretically contain the highest percentages, as it is the combination of three enzymes. This was only the case in control soils sampled at 7 and 21 days and upland soils sampled at 14 days (Figure 14). Because nucleic acids can be less than 1% of the TP in NaOH-EDTA soil extracts (Hill and Cade-Menun, 2009), the PPase/GPase/NPase combination should at least approximate PPase/GPase. However, PPase/GPase was as much as 150% greater than PPase/GPase/NPase applied to upland soils sample at 21 days. Additionally, GPase/NPase hydrolyzed around 75% more of hypothetically available $P_o$ than PPase/GPase/NPase incubated with control soils and sampled at day 0 of the microcosm experiments (Figure 14). The GPase/NPase the PPase/GPase/NPase cocktails should serve as a sort of technical replication in this study. That these fractions were very different on several occasion shows that further study is needed using this method and similar soils.

***Time and treatments effects across all microcosm soils***

No significant differences between enzyme hydrolysis of microcosm samples at different sampling times were found (Table 9). However, while sampling times were not significant overall, there were certain treatments and sampling times that did have significant variance**.** For example, upland soils had mean PPase-labile P of 59.5, 45.4, 6.5 and 67.9 mg P $kg^{-1}$ soil at sample weeks zero through three, respectively (Figure 11). The difference between sampling week two (14 days) and three (21 days) were significant at the *P* <0 .05 level. It is unclear whether these results reflect analytical errors or biological processes within upland soil microcosms.

Table 9. Multiple comparisons of microcosm sampling times across all soils and conditions (Tukey post-hoc tests: SPSS/PASW Statistics 18, 2010).

mg P/kg soil

Tukey HSD

| (I) Time | (J) Time | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | 3.266 | 8.1134 | .978 | -17.788 | 24.319 |
| | 3 | 3.078 | 8.1134 | .981 | -17.976 | 24.131 |
| | 4 | 15.843 | 8.1134 | .210 | -5.211 | 36.897 |
| 2 | 1 | -3.266 | 8.1134 | .978 | -24.319 | 17.788 |
| | 3 | -.188 | 8.1134 | 1.000 | -21.241 | 20.866 |
| | 4 | 12.577 | 8.1134 | .410 | -8.476 | 33.631 |
| 3 | 1 | -3.078 | 8.1134 | .981 | -24.131 | 17.976 |
| | 2 | .188 | 8.1134 | 1.000 | -20.866 | 21.241 |
| | 4 | 12.765 | 8.1134 | .397 | -8.289 | 33.819 |
| 4 | 1 | -15.843 | 8.1134 | .210 | -36.897 | 5.211 |
| | 2 | -12.577 | 8.1134 | .410 | -33.631 | 8.476 |
| | 3 | -12.765 | 8.1134 | .397 | -33.819 | 8.289 |

In addition to assessing time as an individual variable, multiple comparison tests were performed on individual treatments compared across all soils and sampling times. Results showed that MRP before and after subjecting samples to the enzymatic hydrolysis procedure, without the addition of enzymes, was not significantly different (Appendix, Table 11). Moreover, there were not statistically significant differences between enzyme fractions, in any soil, when PPase, PPase/GPase or PPase/GPase/NPase enzyme cocktails were incubated with $P_{NaOH}$ soil extracts ($P < 0.05$). For example, when all upland microcosm soil sampling times were combined, any given enzyme combination did not yield significantly more $PO_4$ than any other enzyme combination (Appendix, Table 11). Also notably, there was enough variation across soils and sampling times that the mean enzymatic hydrolysis of any given soil, when sampling times were combined, was not significantly different ($P<0.05$) (Appendix Table 11). In their 2010 study, Johnson and Hill did not compare soils from different landscape positions, so it is not known whether or not they would have found similar enzymatic hydrolysis levels in soils with diverging quantities of $TP_{NaOH}$. As a point of comparison, Johnson and Hill (2010) found that during the six weeks of their study and combining sampling depths, a 25 to 35% increase in MRP over initial MRP in NaOH-EDTA in the extracts of a single soil subjected to enzymatic hydrolysis was found. Our study found that over three weeks of study, control soils subjected to three enzymes had a 58% increase in MRP over initial MRP in NaOH-EDTA soil extracts, whereas dairy farm upland soils had a 9% increase and depositional soils only had an increase of 3% (Table 10). The aforementioned percentages are means of all sampling times for NaOH-EDTA soil extract MRP and all three enzymes used throughout this study.

The different findings of this study and that of Johnson and Hill (2010) illustrate that an enzyme-limited reaction may be present in our study and this may be controlled by any number of factors including high soil TP, Zn, soil texture and mineralogy.

Table 10. Mean percent increase in bioavailable P in three microcosm soils subjected to one, two or three enzymes over 3 weeks. Mehlich-3 P as a percentage of NaOH-EDTA extractable P is also provided for comparison.

| | | *------Net percent change in bioavailable P†--------* | | | |
|---|---|---|---|---|---|
| **Soil** | | **PP‡** | **PP+GP§** | **PP+GP+NP¶** | **Mehlich P/PNaoH (in %)** |
| | | | | | |
| ***Control*** | | 50 | 56 | 68 | 5 |
| | | | | | |
| ***Upland*** | | 8 | 12 | 7 | 6 |
| | | | | | |
| ***Depositional*** | | 0 | 5 | 2 | 13 |
| | | | | | |

† Increase in bioavailable MRP due to enzyme labile P (enzyme hydrolysis/P from NaOH-EDTA microcosm soil extracts)

‡ Acid phosphatase from potato; § Acid phosphatase from wheat germ; ¶ Nuclease P1 from *Penicillium citrinum*

While Johnson and Hill (2010) used soils with high TP, soil texture and mineralogy diverged from our study and Zn/Cu content was not mentioned by Johnson and Hill. Enzyme-limited reactions do not allow for all possible substrates ($P_o$) to be transformed and occur when where is an excess of substrate in relation to the amount of enzyme activity (Sinsabaugh, 1994). This can occur when there is a lack of enzymes or the activity of enzymes is hampered by any number of factors, including binding with humic acids (Allison, 2006). Some of these possible factors were discussed previously and include interactions of EDTA and the cations necessary for enzyme activation. Also, soil texture, humic acid content and mineralogy can shape the composition of NaOH-EDTA extracts and this in turn could result in enzyme inhibition not found in Johnson and Hill's study. For example, Johnson and Hill (2010) note that NaOH-EDTA had a low TP extraction efficiency in their study (11-64% of TP) and that this was due to high molar ratios of Al and Fe to NaOH-EDTA. It is possible that the EDTA in the enzyme/substrate solution of Johnson and Hill's study was bound to Al and Fe and this did not allow the EDTA to chelate enzyme activator cations and/or kept elements that could bind with free $PO_4$ bonded to EDTA. Possible reasons for differing results between Johnson and Hill's 2010 study and this investigation are numerous. Characterization of the solution

chemistries of the NaOH-EDTA soil extracts used in both studies would be a useful place to begin.

In conclusion, enzymatic additions to the soil-dairy waste matrices studied did not allow for $P_o$ species fractionation and provided variable results on quantities of $P_o$ that could be hydrolyzed in soil. Soils from depositional field positions and receiving dairy waste applications had low MRP release after addition of enzymes in proportion to the concentration of total P compared to the other soil treatments. Both high soil TP concentrations and addition of metals to soils from intensive dairy and confinement poultry operations is typical. Use of the Johnson and Hill (2010) method in these situations appears to be problematic when clay loam Ultisols are used, as opposed to the stony loam Spodosols (11% clay) of the region from which Johnson and Hill gathered soils (Johnson and Hill, 2010). It is unfortunate that this method appears to have low efficacy on Ultisols, as they are common soils in US states with numerous confinement animal operations and are situations with potentially high $P_o$ accumulation and potential P loss to waterways (North Carolina Agricultural Statistics, 2010). Further investigation of the applicability of the Johnson and Hill (2010) method is needed in both diverse soil orders, as well as soils with a range of TP and metal concentrations.

# CHAPTER IV
# CONCLUSIONS AND RECOMMENDATIONS

## Conclusions

In addition to non-uniform distribution of Murphy-Riley reagents in microplate wells, possibly resulting from variable enzyme mixing and $PO_4$ contamination of enzymes, this study had two major design flaws. Both of these flaws were alluded to in the last section. First, the efficacy of enzymes on hydrolyzing model $P_o$ compounds was tested in a pH 5.0 sodium acetate and DI water matrix. While this validated that enzymes had the ability to liberate some $P_i$ from a range of $P_o$ compounds, it did not reflect the actual soil extract matrices the enzymes were to operate in, namely a solution with EDTA and unknown quantities of humic acids, metals, base cations and other elements and compounds. Several weeks into this study, it was realized that the EDTA matrix that enzymes were being subjected to could introduce the interferences with enzyme activity previously discussed. Model compounds subjected to enzymes in matrices with EDTA showed that about four-fifths the enzymatic hydrolysis as those in buffered DI matrices (Table 7). It is worth noting that aforementioned DI/buffer/EDTA/enzyme solution only approximates lowered enzyme activity in the presence of EDTA and not the interaction of EDTA and the soil extract solutions used throughout this study with their suite of other chemical characteristics.

The second major experimental design flaw, previously discussed in some detail, was that replicate microcosms of the same treatment could result in soil extracts with greatly differing chemical composition from one another. A large degree of variability between microcosms of the same treatment was seen in this study. Without the aforementioned design flaws, it may have been possible to isolate, or move closer to isolating, causes of variability in assessed enzymatic hydrolysis.

Despite the experimental design flaws, there is useful information in this study. For example, depositional soils, which may have the most sediment-like texture, seem the most problematic for this high-throughput microplate reader method. This result highlights that more research is needed before applying this method to stream and lake sediments. This work also illustrates the importance of landscape position in the efficacy of this method and that lower landscape positions are accumulating P, Cu and Zn additions from animal waste disproportionately. These additions are some of many factors which may interfere with enzyme activity. While it is unclear which factor in the lower field positions of the dairy farm studied impacted measured enzyme-mediated $P_o$ to $P_i$ transformations, it is clear that soils like these require further investigation.

Additionally, the standard deviations found in results from the colorimetric analysis of P using the enzyme-base microplate-reader method contrast sharply with those found from colorimetric analysis of Mehlich-3 extracts analyzed by a flow-injection auto-analyzer system. The greater precision in the flow-injection auto-analyzer method may be partially explained by the fact that this method used a much greater sample volume than the microplate reader (10 mL vs. 1 mL).

## Recommendations

Interferences with enzyme activity can be established by adding known quantities of model $P_o$ compounds to the exact matrix in which enzyme assays are to be performed. Spiking soil extracts with model $P_o$ compounds is necessary because it can illustrate whether or not enzymatic hydrolysis is occurring to the extent possible given known quantities of added $P_o$. Our study has shown that enzyme assays may perform differently, even when using identical soil series, thus it cannot be taken for granted that the Johnson and Hill (2010) enzymatic hydrolysis procedure behaves similarly in all

soils. Our study has shown that animal waste additions and field position alone exert considerable influence and should be considered.

Testing this microplate-based method on stream and lake waters directly with no prior extraction would be a way to bypass potential interferences imposed by both EDTA and high $P_i$ concentrations. Further, subjecting natural waters to enzymatic hydrolysis makes sense because it is ultimately the $P_o$ to $P_i$ transformations in this matrix that are directly linked to eutrophication. Enzyme-based analyses of natural waters would require either pH adjustment and/or use of alkaline phosphatase enzymes. Purified alkaline phosphatase enzymes are more readily available commercially than acid phosphatase enzymes. Ideally, purified broad-substrate phosphatase should be used with purified phytase with the same pH optima.

Given problems with microcosm replications and multiple unknown sources of error in the enzymatic hydrolysis process, further recommendations include multiple tests of the exact same water or soil extract sample (technical replicates). Also, using large sample sizes, higher numbers of repetitions and an MRP analysis system such as an auto-analyzer system could help establish sources of variability. Further, the auto-analyzer system uses a read time of a couple minutes, as opposed to the 30 minute color development time used in microplate analyses. Uneven blue color development in microplate-Murphy-Riley incubations usually occurred in the last 10 minutes of incubation and thus may not occur using a flow injector system.

Lastly, and in lieu of an accurate enzyme-based environmentally-labile P test, the 0.25M NaOH-0.05M EDTA used in this study showed that it is possible to extract the majority of soil TP via this extractant in situations of high organic P inputs to soils. Inorganic P extracted by NaOH-EDTA can be analyzed using either microplate or flow-injector MRP systems, with the former costing less and the latter having higher precision. It has been shown that NaOH-EDTA is capable of extracting both organic and inorganic P compounds (Hill and Cade-Menun, 2009; He et al., 2004). However, a portion of NaOH-EDTA extract containing pyrophosphate, polyphosphates and organic P

compounds would be inaccessible unless subjected to acid digest (He et al., 2004). Because NaOH-EDTA captures both substantial portions of both organic and inorganic soil P, it may be more environmentally relevant than Mehlich-3 alone (Ahlgren et al., 2007; Johnson and Hill, 2010). However, whether NaOH-EDTA captures P in proportion to what can be lost to waterways through leaching or runoff is not known.

# REFERENCES


Ahlgren, J., H. De Brabandere, K. Reitzel, E. Rydin, A. Gogoll, M. Waldeback. 2007. Sediment phosphorus extractants for phosphorus-31 nuclear magnetic resonance analysis: a quantitative evaluation. Journal of Environmental Quality 36: 892-898.

Allen, B.L., A. P. Mallarino. 2006. Relationships between extractable soil phosphorus and Phosphorus saturation after long-term fertilizer or manure application. Soil Science Society of America Journal 70: 454-463.

Allison, S.D. 2006. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. Biogeochemistry 81: 361–373.

Baldwin, D.S., J.A. Howitt, J.K. Beattie. 2005. Abiotic degradation of organic phosphorus compounds in the environment. In B.L. Turner, E. Froussard, D.S. Baldwin (eds.) Organic Phosphorus in the Environment. CABI Publishing, UK; 75-88.

Beaugrand, G., P.C. Reid, F. Ibanez, J. A. Lindley, M. Edwards. 2002. Reorganization of North Atlantic marine copepod biodiversity and climate. Science 296:1692-1694.

Boavida, M.J., W. Hamza, D. Ruggiu, R.T. Marques. 1997. Eutrophication: alkaline phosphatase revisited. Mem. Ist. Ital. Idrobiol. 56: 15-21.

Bosak, A. B. Barlovic-Tusek, E. Reiner. 2008. Differentiation of EDTA-sensitive from EDTA-insensitive human serum esterases hydrolysing phenylacetate. Journal of Enzyme Inhibition and Medicinal Chemistry 23(4): 521–525.

Bourke, D., P. Dowding, H.Tunney, J. E. O'Brien, D. W. Jeffrey. 2008. The organic phosphorus composition of an Irish grassland soil. Biology & Environment 108 (1): 17-28.

Brady, N.C., R.R. Weil. 1999. The Nature and Properties of Soil (12$^{th}$ edition). Prentice Hall, New Jersey; 540-569.

Buckingham, D.A., S.M. Jasinski. 2009. Phosphate rock statistics, United States Geological Survey. [Online.] Available online at http://seekingalpha.com/article/182522-taking-stock-of-phosphorus-and-biofuels (verified 4/6/11).

Bunemann, E.K. 2008. Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. Soil Biology and Biochemistry 40: 2116-2129.

Carpenter, S.R., N. F. Caraco, D. L. Correll, R. W. Howarth, A. N. Sharpley, and V. H. Smith. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecological Applications 8(3): 559-568.

Celi, L., E. Barberis. 2005. Abiotic stabilization of organic phosphorus in the environment. In B.L. Turner, E. Froussard, D.S. Baldwin (eds.) Organic Phosphorus in the Environment. CABI Publishing, UK; 75-88.

Dodds, W.K., W.W. Bouska, J.L. Eitzmann, T.J. Pilger, K.L. Pitts, A.J. Riley, J.T. Schloesser, and D.J. Thornbrugh. 2009. Eutrophication of US freshwaters: analysis of potential economic damages. Environmental Science & Technology 43: 12-19.

Dalal, R.C. 1977. Soil Organic Phosphorus. Advances in Agronomy 29: 83-117.

Diamond, D. 1998. Determination of phosphorus in soils and plants by flow injector analysis (block digestor method). Lachat QuikChem method 13-155-01-1-B. Zellweger Analytics, Inc. Milwaukee, WI.

Falkowski, P., R. J. Scholes, E. Boyle, J. Canadell, D. Canfield, J. Elser, N. Gruber, K. Hibbard, P. Hogberg, S. Linder, F. T. Mackenzie, B. Moore III, T. Pedersen, Y. Rosenthal, S. Seitzinger, V. Smetacek, W. Steffen. 2000. The global carbon cycle: a test of our knowledge of Earth as a system. Science 290: 291-296.

Eaton, A.D., L.S. Clesceri, E.W. Rice, A.E. Greenberg, M.A.H Franson, eds. 2005. Standard Methods for Examination of Water and Wastewater: Centennial Edition. American Public Health Association.

Finzi, A. C., and S.T. Berthrong. 2005. The uptake of amino acids by microbes and trees in three cold-temperate forests. Ecology 86:3345–3353.

Geisseler, D., W.R. Horwath. 2009. Short-term dynamics of soil carbon, microbial biomass, and soil enzyme activities as compared to longer-term effects of tillage in irrigated row crops. Biological Fertility of Soils 46:65–72.

Giri, B., P.H. Giang, R. Kumari, R. Prasad, and A. Varma. 2006. Microbial diversity in soils. In F. Buscot, and A. Varma (eds.) Microorganisms in Soils: Roles in Genesis and Functions. Springer, New York; 19-55.

Graham, D.C. 2009. Phosphorus Transport from a Field Receiving Long-Term Liquid Dairy Manure Application in a Karst Landscape. Master's Thesis University of Tennessee, Knoxville. Available online at http://trace.tennessee.edu/utk_gradthes/528/ (verified 4/6/11)

Leytem, A.B., J. T. Sims, and F. J. Coale. 2004. Determination of phosphorus source coefficients for organic phosphorus sources: laboratory studies. Journal of Environmental Quality 33: 380–388.

Loria, E.R., J.E. Sawyer. 2005. Extractable soil phosphorus and inorganic nitrogen following application of raw and anaerobically digested swine manure. Agronomy Journal 97:879–885.

Hagan, J., and F. R. Walker. 2006. Pond Creek watershed restoration plan. [Online]. Available at http://pondcreek.ag.utk.edu/Publications/Chapters_1-3.pdf (verified 4/6/11)

Hansen, N.C., T.C. Daniel, A.N. Sharpley, and J.L. Lemunyon. 2002. The fate and transport of phosphorus in agricultural systems. Journal of Soil and Water Conservation 57: 408-417.

Hayes, J.E., A.E. Richardson, R.J. Simpson. 2000. Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase. Biological Fertility of Soils 32:279-286.

He, Z., T.S. Griffin, C.W. Honeycutt. 2004. Enzymatic hydrolysis of organic phosphorus in swine manure and soil. Journal of Environmental Quality 33: 367-372.

He, Z., G.S. Toor, C.W. Honeycutt, and J.T. Sims. 2006. An enzymatic hydrolysis approach for characterizing labile phosphorus forms in dairy manure under mild assay conditions. Bioresource Technology 97: 1660-1668.

Hill, J.E. D. Kysela, M. Elimelech. 2007. Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVa Peninsula. Environmental Microbiology 9: 3100-3107.

Hill, J.E. B.J. Cade-Menum. 2009. Phosphorus-31 nuclear magnetic resonance spectroscopy transect study of poultry operations on the Delmarva Peninisula. Journal of Environmental Quality 38: 130-138.

Houck, O. 1999. The Clean Water Act TMDL Program: Law, Policy, and Implementation. Environmental Law Institute, Washington, D.C.

Johnson, A.M., D. L. Osmond, S. C. Hodges. 2005. Predicted impact and evaluation of North Carolina's Phosphorus Indexing Tool. Journal of Environmental Quality 34:1801–1810.

Johnson, N.R., J.E. Hill. 2010. Phosphorus species composition of poultry manure-amended soil using high-throughput enzyme hydrolysis. Soil Science Society of America Journal 74: 1786-1791Kowalenko, C.G. and D. Babuin. 2007.

Kowalenko, C.G., D. Babuin. Interference problems with phosphoantimonylmolybdenum colorimetric measurement of phosphorus in soil and plant materials. Communications in Soil Science and Plant Analysis 38: 1299-1316.

Koopmans, G. F., W. J. Chardon, R. W. McDowell. 2007. Phosphorus movement and speciation in a sandy soil profile after long-term animal manure applications. Journal of Environmental Quality 36: 305–315.

Kuo, S., 2005. In, D.L. Sparks, ed. Chemical Methods of Soil Analysis. Soil Science Society of America. Madison, Wisconsin. pg. 758.

Hays, J.E., A.E. Richardson, R.J. Simpson. 2000. Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase. Biological Fertility of Soils 32: 279-286.

Hedley, M.J., J.W.B. Stewart, B.S. Chauhan. 1982. Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. Soil Science Society of America Journal 46: 970-976.

Hill, J.E. D. Kysela, M. Elimelech. 2007. Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVa Peninsula. Environmental Microbiology 9: 3100-3107.

Hill, J.E., M.J. Cade-Menun. 2009. Phosphorus-31 nuclear magnetic resonance spectroscopy transect study of poultry operations on the Delmarva Peninsula. Journal of Environmental Quality 38: 130-138.

Leffler, M. 1997. Harmful algal blooms on the move. Maryland Marine Notes July-August: 4.

Lim, B.L., P. Yeung, C. Cheng, J. E. Hill. 2007. Distribution and diversity of phytate-mineralizing bacteria. The ISME Journal 1: 321–330

Massachusetts Department of Environmental Protection (MDEC). 2011. Total Maximum Daily Load for Nutrients in the Upper/Middle Charles River, Massachusetts. [Online] Available at: http://www.mass.gov/dep/water/resources/ucharles.pdf (verified 7/1/11).

Matula, J. 2010. Differences in available phosphorus evaluated by soil tests in relation to detection by colorimetric and ICP-AES techniques. Plant Soil Environment 56(6): 297-304.

McDowell, R.W., B.J.F. Biggs, A.N. Sharpley, L. Nguyen. 2004. Connecting phosphorus loss from agricultural landscapes to surface water quality. Chemistry and Ecology 20: 1-40

Nixon, S.W. 1995. Coastal marine eutrophication: a definition, social causes and future concerns. Ophelia 41: 199-219.

North Carolina Agricultural Statistics. 2010. North Carolina Department of Agriculture and Consumer Services 212: 9.

Omburo, G.A., J.M. Kuo, L. S. Mullins and F.M. Raushel. 1992. Characterization of the zinc binding site of bacterial phosphotriesterase. The Journal of Biological Chemistry 267:13278-13283.

Pretty, J.N., C.F. Mason, D.B. Nedwell, R.E. Hine, S. Leaf, and R. Dils. 2003. Environmental costs of freshwater eutrophication in England and Wales. Environmental Science & Technology 37:201-208.

Pretty, J.N. A.S. Ball, T. Lang, J.I.L. Morison. 2005. Farm costs and food miles: an assessment of the full cost of the UK weekly food basket. Food Policy 30: 1–19.

Quiquampoix,H. and D. Mousain. 2005. Enzymatic hydrolysis of organic phosphorus. In Turner, B.L., E. Frossard, D.S. Baldwin, eds. Organic Phosphorus in the Environment. CAB Publishing International.

Schindler, D.W., J.R. Vallentyne. 2008. The Algal Bowl: Overfertilization of the World's Freshwaters and Estuaries. University of Alberta Press.

Sharpley, A.N. H.Tiessen, C.V. Cole. 1987. Soil phosphorus forms extracted by soil tests as a function of pedogenesis. Soil Science Society of America Journal 51: 362-365.

Sharpley, A.N. 1995. Dependence of runoff phosphorus on extractable soil phosphorus. Journal of Environmental Quality 24: 920–926.

Sharpley, A.N., R. W. McDowell, P. J. A. Kleinman. 2004. Amounts, forms, and solubility of phosphorus in soils receiving manure. Soil Science Society of America Journal. 68: 2048–2057.

Shober, A.L, J.T. Sims. 2007. Integrating phosphorus source and soil properties into risk assessments for phosphorus loss. Soil Science Society of America Journal 71: 551–560.

Sims, J.T., R.O. Maguire, A.B. Leytem, K.L. Gartley, M.C. Pautler. 2002. Evaluation of Mehlich 3 as an agri-environmental soil phosphorus test for the mid-Atlantic United States. Soil Science Society of America Journal 66:2016–2032.

Sinsabaugh, R.L. 1994. Enzymic analysis of microbial patterns and processes. Biological Fertility of Soils 17: 69-74.

Smith, V.H., G.D. Tilman, J.C. Nekola. 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental Pollution 100: 179-196.

Smith, V.H., D.W. Schindler. 2009. Eutrophication science: where do we go from here? Trends in Ecology and Evolution 24: 201-207.

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. Available at http://websoilsurvey.nrcs.usda.gov/ (accessed 5/1/2011).

Tennessee Department of Environment and Conservation (TDEC). 2007. Use Classification for Surface Waters. [Online.] Available at http://tn.gov/sos/rules/1200/1200-04/1200-04-04.pdf (verified 4/6/11)

Tennessee Department of Environment and Conservation (TDEC). 2008a. 2008 305(b) Report: The Status of Water Quality in Tennessee. [Online.] Available at http://www.state.tn.us/environment/wpc/publications/pdf/2008_305b.pdf (verified 4/6/11).

Tennessee Department of Environment and Conservation (TDEC). 2008b. Final Year 2008 303 (d) List. [Online.] Available at http://tn.gov/environment/wpc/publications/#305b10 (verified 4/6/11)

Tennessee Department of Environment and Conservation (TDEC). 2008c. Rules of Tennessee Department of Environment and Conservation Water Quality Control Board Division Water Pollution Control Chapter 1200-4-3 General Water Quality Criteria. [Online.] Available at http://www.state.tn.us/sos/rules/1200/1200-04/1200-04-03.pdf (verified 7/2/11)

Thien, S.J., R. Myers. 1992. Determination of bioavailable phosphorus in soil. Soil Science Society of America Journal 56:814-818.

Turner, B.L. I.D. McKelvie, P.M. Haygarth. 2002. Characterization of water-extractable soil organic phosphorus by phosphatase hydrolysis. Soil Biology & Biochemistry 34: 27-35.

Turner, B. L., B.J. Cade-Menun. 2003. Organic phosphorus composition and potential bioavailability in semi-arid arable soils of the western United States. Soil Science Society of America Journal 67:1168-1179.

Turner, B. L., A.B. Leytem. 2004. Phosphorus compounds in sequential extracts of animal manures: chemical speciation and a novel fractionation procedure. Environmental Science Technology 38: 6101-6108.

Turner, B.L. B.J. Cade-Menun, L.M. Condron, S. Newman. 2005. Extraction of soil organic phosphorus. Talanta 66:294-306.

Turner, B.L., E. Frossard, D.S. Baldwin, eds. 2005. Organic Phosphorus in the Environment. CAB Publishing International.

U.S. Environmental Protection Agency. 1999. Guidance Specifying Management Measures for Sources of Nonpoint Pollution in Coastal Waters. Office of Water. [Online.] Available at http://www.p2pays.org/ref/04/03686/index-1.html (verified 4/6/11)

U.S. Environmental Protection Agency. 2000. Guidance Manual and Sample NPDES Permit for Concentrated Animal Feeding Operations, Final Internal Review Draft. [Online.] Available at http://www.epa.gov/npdes/pubs/dman_afo-2000.pdf (verified 4/6/11).

U.S. Environmental Protection Agency. 2008b. Nonpoint Source Pollution: The Nation's Largest Water Quality Problem. EPA841-F-96-004A [Online.] Available at http://www.epa.gov/owow/NPS/facts/point1.htm (verified 4/6/11).

U.S. Environmental Protection Agency. 2008c. Managing Nonpoint Source Pollution from Agriculture. EPA841-F-96-004F [Online.] Available at http://www.epa.gov/owow/nps/facts/point6.htm (verified 4/6/11).

Vitousek, P.M., H.A. Mooney, J. Lubchenko, J.M. Melillo. 1997a. Human domination of Earth's ecosystems. Science 277: 494-499.

Vitousek, P.M., J. Aber, R.W. Howarth, G.E. Likens, P.A. Matson, D.W. Schindler, W.H. Schlesinger, G.D. Tilman. 1997b. Human alteration of the global nitrogen cycle: causes and consequences. Ecological Applications 7: 737-750.

Walker, F., L.B. Carmichael. 2010. Watershed management in the Ridge and Valley Region: lessons learned from the Pond Creek Watershed,Tennessee. University of Tennessee Institute of Agriculture. [Online.] http://www.usawaterquality.org/conferences/2010/WednesdayPDF's/Concurrent_Sessions/SessionQ_120pm-5pm_AnimalAgriculture/Walker.pdf (verified 4/6/11)

Web Soil Survey. 2010. Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. Available online at http://websoilsurvey.nrcs.usda.gov/ (verified 5/1/2011).

White, M.J., D. E. Storm, P. R. Busteed, S. H. Stoodley, Shannon J. Phillips. 2009. Evaluating nonpoint source critical source area contributions at the watershed scale. Journal Environmental Quality 38:1654–1663.

Worsfold, P.J., P. Monbet, A.D. Tappin, M.F. Fitzsimons, D.A. Stiles, I.D. McKelvie. 2008. Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: a review. Analyica Chimica Acta 624:37-58.

Yamamuro, M., J. Hiratsuka, Y. Ishitobi, S. Hosokawa, Y. Nakamura. 2006. Ecosystem shift resulting from loss of eelgrass and other submerged aquatic vegetation in two estuarine lagoons, Lake Nakaumi and Lake Shinji, Japan. Journal of Oceanography 62: 551-558

Yaqoob, M., A. Nabi, P.J. Worsfold. 2004. Determination of nanomolar concentrations of phosphate in freshwaters using flow injection with luminol chemiluminescence detection. Analytica Chimica Acta 510(2):213-218.

# APPENDIX A

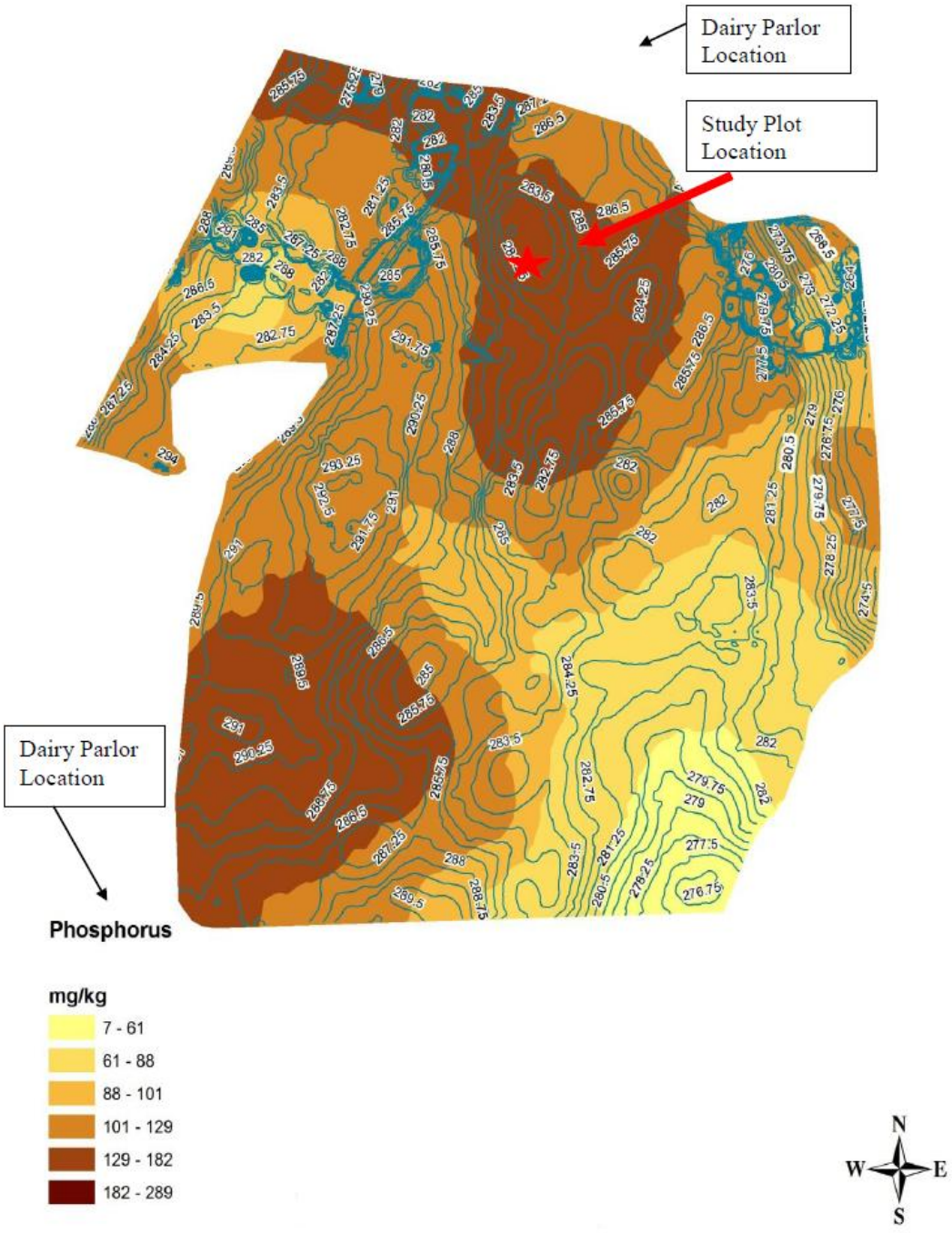



Figure 15. Mehlich-3 phosphorus distribution in 56 hectare main dairy farm field (Graham, 2009). Depositional soils for the microcosm study were from a trough ~ 100 m S of "Study Plot Location" and upland soils were from upslope (W) of depositional soils.

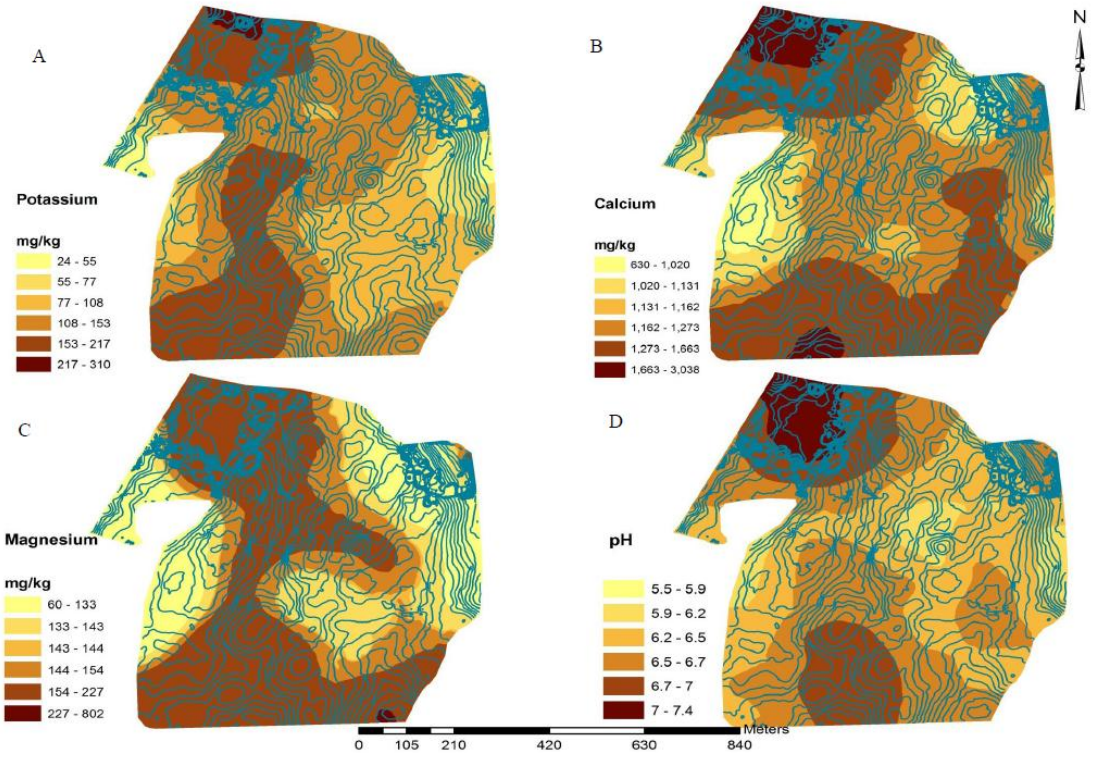



Figure 16. Mehlich 3 extractable potassium (A), calcium (B), magnesium (C) and pH (D) distribution in 56 hectare dairy farm main field (D. Graham, 2009).

**Tukey post hoc tests: soils and treatments combined in table below.**
"Soil" is any of the three microcosm soils subjected to treatments 1-39:
(Soils 1 to 9 are $P_{NaOH}$ incubated with enzymes minus beginning $P_{NaOH}$)

1 = Upland PPase P
2 = Upland PPase/GPase P
3 = Upland PPase/GPase/NPase P
4 = Depositional PPase P
5 = Depositional PPase/GPase P
6 = Depositional PPase/GPase/NPase P
7 = Control PPase P
8 = Control PPase/GPase P
9 = Control PPase/GPase/NPase P
10 = Upland Mehlich-3 P
11 = Depositional Mehlich-3 P
12 = Control Mehlich-3 P
13 = Upland NaOH-EDTA TP ($TP_{NaOH}$)
14 = Depositional NaOH-EDTA TP($TP_{NaOH}$)
15 = Control NaOH-EDTA TP ($TP_{NaOH}$)
16 = Upland chemically hydrolyzed P (incubated $P_{NaOH}$)
17 = Upland base P ($P_{NaOH}$)
18 = Depositional chemically hydrolyzed P (incubated $P_{NaOH}$)
19 = Depositional base P ($P_{NaOH}$)
20 = Control chemically hydrolyzed P (incubated $P_{NaOH}$)
21 = Control base P ($P_{NaOH}$)
22 = Control PPase-labile Po
23 = Control PPase/GPase-labile Po
24 = Control PPase/GPase/NPase-labile Po
25 = Upland PPase-labile Po
26 = Upland PPase/GPase-labile Po
27 = Upland PPase/GPase/NPase-labile Po
28 = Depositional PPase-labile Po
29 = Depositional PPase/GPase-labile Po
30 = Depositional PPase/GPase/NPase-labile Po
31 = Control PPase P + MRP ($P_{NaOH}$)
32 = Control PPase/GPase P + MRP ($P_{NaOH}$)
33 = Control PPase/GPase/NPase P + MRP ($P_{NaOH}$)
34 = Upland PPase P + MRP ($P_{NaOH}$)
35 = Upland PPase/GPase P + MRP ($P_{NaOH}$)
36 = Upland PPase/GPase/NPase P + MRP ($P_{NaOH}$)
37 = Depositional PPase P + MRP ($P_{NaOH}$)
38 = Depositional PPase/GPase P + MRP ($P_{NaOH}$)
39 = Depositional PPase/GPase/NPase P + MRP ($P_{NaOH}$)

**ANOVA**

mg P/ kg soil

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4.458E7 | 38 | 1173240.309 | 508.143 | .000 |
| Within Groups | 824268.735 | 357 | 2308.876 | | |
| Total | 4.541E7 | 395 | | | |

Table 11. Tukey post hoc tests performed on 1-39 soil/treatment pairs. See legend above for description of "soils" 1-39 (SPSS/PASW Statistics 18, 2010).

*The mean difference is significant at the 0.05 level. See * in Mean Difference (I-J) column

Tukey HSD

Mg P per kg soil

| | | | | | | |
|---|---|---|---|---|---|---|
| Upland PPase P | Upland GPase P | -24.0417 | 19.6166 | 1.000 | -100.741 | 52.658 |
| | Upland NPase P | 4.1250 | 19.6166 | 1.000 | -72.575 | 80.825 |
| | Depositional PPase P | 41.0167 | 19.6166 | .988 | -35.683 | 117.716 |
| | Depositional GPase P | 6.8167 | 19.6166 | 1.000 | -69.883 | 83.516 |
| | Depositional NPase P | 29.3500 | 19.6166 | 1.000 | -47.350 | 106.050 |
| | Control PPase P | -5.8250 | 19.6166 | 1.000 | -82.525 | 70.875 |
| | Control GPase P | -11.8167 | 19.6166 | 1.000 | -88.516 | 64.883 |
| | Control NPase P | -23.1583 | 19.6166 | 1.000 | -99.858 | 53.541 |
| Upland GPase P | Upland PPase P | 24.0417 | 19.6166 | 1.000 | -52.658 | 100.741 |
| | Upland NPase P | 28.1667 | 19.6166 | 1.000 | -48.533 | 104.866 |
| | Depositional PPase P | 65.0583 | 19.6166 | .273 | -11.641 | 141.758 |
| | Depositional GPase P | 30.8583 | 19.6166 | 1.000 | -45.841 | 107.558 |
| | Depositional NPase P | 53.3917 | 19.6166 | .736 | -23.308 | 130.091 |
| | Control PPase P | 18.2167 | 19.6166 | 1.000 | -58.483 | 94.916 |
| | Control GPase P | 12.2250 | 19.6166 | 1.000 | -64.475 | 88.925 |
| | Control NPase P | .8833 | 19.6166 | 1.000 | -75.816 | 77.583 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Upland NPase P | Upland PPase P | -4.1250 | 19.6166 | 1.000 | -80.825 | 72.575 |
| | Upland GPase P | -28.1667 | 19.6166 | 1.000 | -104.866 | 48.533 |
| | Depositional PPase P | 36.8917 | 19.6166 | .998 | -39.808 | 113.591 |
| | Depositional GPase P | 2.6917 | 19.6166 | 1.000 | -74.008 | 79.391 |
| | Depositional NPase P | 25.2250 | 19.6166 | 1.000 | -51.475 | 101.925 |
| | Control PPase P | -9.9500 | 19.6166 | 1.000 | -86.650 | 66.750 |
| | Control GPase P | -15.9417 | 19.6166 | 1.000 | -92.641 | 60.758 |
| | Control NPase P | -27.2833 | 19.6166 | 1.000 | -103.983 | 49.416 |
| Depositional PPase P | Upland PPase P | -41.0167 | 19.6166 | .988 | -117.716 | 35.683 |
| | Upland GPase P | -65.0583 | 19.6166 | .273 | -141.758 | 11.641 |
| | Upland NPase P | -36.8917 | 19.6166 | .998 | -113.591 | 39.808 |
| | Depositional GPase P | -34.2000 | 19.6166 | 1.000 | -110.900 | 42.500 |
| | Depositional NPase P | -11.6667 | 19.6166 | 1.000 | -88.366 | 65.033 |
| | Control PPase P | -46.8417 | 19.6166 | .925 | -123.541 | 29.858 |
| | Control GPase P | -52.8333 | 19.6166 | .757 | -129.533 | 23.866 |
| | Control NPase P | -64.1750 | 19.6166 | .302 | -140.875 | 12.525 |
| Depositional GPase P | Upland PPase P | -6.8167 | 19.6166 | 1.000 | -83.516 | 69.883 |
| | Upland GPase P | -30.8583 | 19.6166 | 1.000 | -107.558 | 45.841 |
| | Upland NPase P | -2.6917 | 19.6166 | 1.000 | -79.391 | 74.008 |
| | Depositional PPase P | 34.2000 | 19.6166 | 1.000 | -42.500 | 110.900 |
| | Depositional NPase P | 22.5333 | 19.6166 | 1.000 | -54.166 | 99.233 |
| | Control PPase P | -12.6417 | 19.6166 | 1.000 | -89.341 | 64.058 |
| | Control GPase P | -18.6333 | 19.6166 | 1.000 | -95.333 | 58.066 |
| | Control NPase P | -29.9750 | 19.6166 | 1.000 | -106.675 | 46.725 |
| Depositional NPase P | Upland PPase P | -29.3500 | 19.6166 | 1.000 | -106.050 | 47.350 |
| | Upland GPase P | -53.3917 | 19.6166 | .736 | -130.091 | 23.308 |
| | Upland NPase P | -25.2250 | 19.6166 | 1.000 | -101.925 | 51.475 |
| | Depositional PPase P | 11.6667 | 19.6166 | 1.000 | -65.033 | 88.366 |
| | Depositional GPase P | -22.5333 | 19.6166 | 1.000 | -99.233 | 54.166 |
| | Control PPase P | -35.1750 | 19.6166 | .999 | -111.875 | 41.525 |
| | Control GPase P | -41.1667 | 19.6166 | .987 | -117.866 | 35.533 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Control PPase P | Upland PPase P | 5.8250 | 19.6166 | 1.000 | -70.875 | 82.525 |
| | Upland GPase P | -18.2167 | 19.6166 | 1.000 | -94.916 | 58.483 |
| | Upland NPase P | 9.9500 | 19.6166 | 1.000 | -66.750 | 86.650 |
| | Depositional PPase P | 46.8417 | 19.6166 | .925 | -29.858 | 123.541 |
| | Depositional GPase P | 12.6417 | 19.6166 | 1.000 | -64.058 | 89.341 |
| | Depositional NPase P | 35.1750 | 19.6166 | .999 | -41.525 | 111.875 |
| | Control GPase P | -5.9917 | 19.6166 | 1.000 | -82.691 | 70.708 |
| | Control NPase P | -17.3333 | 19.6166 | 1.000 | -94.033 | 59.366 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Control GPase P | Upland PPase P | 11.8167 | 19.6166 | 1.000 | -64.883 | 88.516 |
| | Upland GPase P | -12.2250 | 19.6166 | 1.000 | -88.925 | 64.475 |
| | Upland NPase P | 15.9417 | 19.6166 | 1.000 | -60.758 | 92.641 |
| | Depositional PPase P | 52.8333 | 19.6166 | .757 | -23.866 | 129.533 |
| | Depositional GPase P | 18.6333 | 19.6166 | 1.000 | -58.066 | 95.333 |
| | Depositional NPase P | 41.1667 | 19.6166 | .987 | -35.533 | 117.866 |
| | Control PPase P | 5.9917 | 19.6166 | 1.000 | -70.708 | 82.691 |
| | Control NPase P | -11.3417 | 19.6166 | 1.000 | -88.041 | 65.358 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Control NPase P | Upland PPase P | 23.1583 | 19.6166 | 1.000 | -53.541 | 99.858 |
| | Upland GPase P | -.8833 | 19.6166 | 1.000 | -77.583 | 75.816 |
| | Upland NPase P | 27.2833 | 19.6166 | 1.000 | -49.416 | 103.983 |
| | Depositional PPase P | 64.1750 | 19.6166 | .302 | -12.525 | 140.875 |
| | Depositional GPase P | 29.9750 | 19.6166 | 1.000 | -46.725 | 106.675 |
| | Depositional NPase P | 52.5083 | 19.6166 | .769 | -24.191 | 129.208 |
| | Control PPase P | 17.3333 | 19.6166 | 1.000 | -59.366 | 94.033 |
| | Control GPase P | 11.3417 | 19.6166 | 1.000 | -65.358 | 88.041 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Upland NaOH-EDTA P | Upland PPase P | 518.9583* | 19.6166 | .000 | 442.259 | 595.658 |
| | Upland GPase P | 494.9167* | 19.6166 | .000 | 418.217 | 571.616 |
| | Upland NPase P | 523.0833* | 19.6166 | .000 | 446.384 | 599.783 |
| | Depositional PPase P | 559.9750* | 19.6166 | .000 | 483.275 | 636.675 |
| | Depositional GPase P | 525.7750* | 19.6166 | .000 | 449.075 | 602.475 |
| | Depositional NPase P | 548.3083* | 19.6166 | .000 | 471.609 | 625.008 |
| | Control PPase P | 513.1333* | 19.6166 | .000 | 436.434 | 589.833 |
| | Control GPase P | 507.1417* | 19.6166 | .000 | 430.442 | 583.841 |
| | Control NPase P | 495.8000* | 19.6166 | .000 | 419.100 | 572.500 |
| | Upland Mehlich-3 P | 529.3750* | 19.6166 | .000 | 452.675 | 606.075 |
| | Depositional Mehlich-3 P | 462.3250* | 19.6166 | .000 | 385.625 | 539.025 |
| | Control Mehlich-3 P | 559.1108* | 19.6166 | .000 | 482.411 | 635.810 |
| | Upland NaOH-EDTA TP | -87.2500* | 19.6166 | .007 | -163.950 | -10.550 |
| | Depositional NaOH-EDTA TP | -610.5750* | 19.6166 | .000 | -687.275 | -533.875 |
| | Control NaOH-EDTA TP | 363.4417* | 19.6166 | .000 | 286.742 | 440.141 |
| | Upland NaOH-EDTA incubated P | -5.5583 | 19.6166 | 1.000 | -82.258 | 71.141 |
| | Depositional NaOH-EDTA incubated P | -236.6083* | 19.6166 | .000 | -313.308 | -159.909 |
| | Depositional NaOH-EDTA P | -226.0917* | 19.6166 | .000 | -302.791 | -149.392 |
| | Control NaOH-EDTA incubated P | 463.6167* | 19.6166 | .000 | 386.917 | 540.316 |
| | Control NaOH-EDTA P | 463.1500* | 19.6166 | .000 | 386.450 | 539.850 |
| | Control PPase labile Po | 488.9167* | 27.7421 | .000 | 380.447 | 597.386 |
| | Control GPase labile Po | 502.6417* | 27.7421 | .000 | 394.172 | 611.111 |
| | Control NPase labile Po | 482.5417* | 27.7421 | .000 | 374.072 | 591.011 |
| | Upland PPase labile Po | 496.9417* | 27.7421 | .000 | 388.472 | 605.411 |
| | Upland GPase labile Po | 451.3667* | 27.7421 | .000 | 342.897 | 559.836 |
| | Upland NPase labile Po | 510.7667* | 27.7421 | .000 | 402.297 | 619.236 |
| | Depositional PPase labile Po | 561.9167* | 27.7421 | .000 | 453.447 | 670.386 |

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|---|---|---|---|---|---|---|
| Depositional NaOH-EDTA P | Upland PPase P | 745.0500* | 19.6166 | .000 | 668.350 | 821.750 |
| | Upland GPase P | 721.0083* | 19.6166 | .000 | 644.309 | 797.708 |
| | Upland NPase P | 749.1750* | 19.6166 | .000 | 672.475 | 825.875 |
| | Depositional PPase P | 786.0667* | 19.6166 | .000 | 709.367 | 862.766 |
| | Depositional GPase P | 751.8667* | 19.6166 | .000 | 675.167 | 828.566 |
| | Depositional NPase P | 774.4000* | 19.6166 | .000 | 697.700 | 851.100 |
| | Control PPase P | 739.2250* | 19.6166 | .000 | 662.525 | 815.925 |
| | Control GPase P | 733.2333* | 19.6166 | .000 | 656.534 | 809.933 |
| | Control NPase P | 721.8917* | 19.6166 | .000 | 645.192 | 798.591 |
| | Upland Mehlich-3 P | 755.4667* | 19.6166 | .000 | 678.767 | 832.166 |
| | Depositional Mehlich-3 P | 688.4167* | 19.6166 | .000 | 611.717 | 765.116 |
| | Control Mehlich-3 P | 785.2025* | 19.6166 | .000 | 708.503 | 861.902 |
| | Upland NaOH-EDTA TP | 138.8417* | 19.6166 | .000 | 62.142 | 215.541 |
| | Depositional NaOH-EDTA TP | -384.4833* | 19.6166 | .000 | -461.183 | -307.784 |
| | Control NaOH-EDTA TP | 589.5333* | 19.6166 | .000 | 512.834 | 666.233 |
| | Upland NaOH-EDTA incubated P | 220.5333* | 19.6166 | .000 | 143.834 | 297.233 |
| | Upland NaOH-EDTA P | 226.0917* | 19.6166 | .000 | 149.392 | 302.791 |
| | Depositional NaOH-EDTA incubated P | -10.5167 | 19.6166 | 1.000 | -87.216 | 66.183 |
| | Control NaOH-EDTA incubated P | 689.7083* | 19.6166 | .000 | 613.009 | 766.408 |
| | Control NaOH-EDTA P | 689.2417* | 19.6166 | .000 | 612.542 | 765.941 |
| | Control PPase labile Po | 715.0083* | 27.7421 | .000 | 606.539 | 823.478 |
| | Control GPase labile Po | 728.7333* | 27.7421 | .000 | 620.264 | 837.203 |
| | Control NPase labile Po | 708.6333* | 27.7421 | .000 | 600.164 | 817.103 |
| | Upland PPase labile Po | 723.0333* | 27.7421 | .000 | 614.564 | 831.503 |
| | Upland GPase labile Po | 677.4583* | 27.7421 | .000 | 568.989 | 785.928 |
| | Upland NPase labile Po | 736.8583* | 27.7421 | .000 | 628.389 | 845.328 |
| | Depositional PPase labile Po | 788.0083* | 27.7421 | .000 | 679.539 | 896.478 |

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|---|---|---|---|---|---|---|
| Control NaOH-EDTA P | Upland PPase P | 55.8083 | 19.6166 | .639 | -20.891 | 132.508 |
| | Upland GPase P | 31.7667 | 19.6166 | 1.000 | -44.933 | 108.466 |
| | Upland NPase P | 59.9333 | 19.6166 | .464 | -16.766 | 136.633 |
| | Depositional PPase P | 96.8250* | 19.6166 | .001 | 20.125 | 173.525 |
| | Depositional GPase P | 62.6250 | 19.6166 | .357 | -14.075 | 139.325 |
| | Depositional NPase P | 85.1583* | 19.6166 | .010 | 8.459 | 161.858 |
| | Control PPase P | 49.9833 | 19.6166 | .851 | -26.716 | 126.683 |
| | Control GPase P | 43.9917 | 19.6166 | .966 | -32.708 | 120.691 |
| | Control NPase P | 32.6500 | 19.6166 | 1.000 | -44.050 | 109.350 |
| | Upland Mehlich-3 P | 66.2250 | 19.6166 | .237 | -10.475 | 142.925 |
| | Depositional Mehlich-3 P | -.8250 | 19.6166 | 1.000 | -77.525 | 75.875 |
| | Control Mehlich-3 P | 95.9608* | 19.6166 | .001 | 19.261 | 172.660 |
| | Upland NaOH-EDTA TP | -550.4000* | 19.6166 | .000 | -627.100 | -473.700 |
| | Depositional NaOH-EDTA TP | -1073.7250* | 19.6166 | .000 | -1150.425 | -997.025 |
| | Control NaOH-EDTA TP | -99.7083* | 19.6166 | .000 | -176.408 | -23.009 |
| | Upland NaOH-EDTA incubated P | -468.7083* | 19.6166 | .000 | -545.408 | -392.009 |
| | Upland NaOH-EDTA P | -463.1500* | 19.6166 | .000 | -539.850 | -386.450 |
| | Depositional NaOH-EDTA incubated P | -699.7583* | 19.6166 | .000 | -776.458 | -623.059 |
| | Depositional NaOH-EDTA P | -689.2417* | 19.6166 | .000 | -765.941 | -612.542 |
| | Control NaOH-EDTA incubated P | .4667 | 19.6166 | 1.000 | -76.233 | 77.166 |
| | Control PPase labile Po | 25.7667 | 27.7421 | 1.000 | -82.703 | 134.236 |
| | Control GPase labile Po | 39.4917 | 27.7421 | 1.000 | -68.978 | 147.961 |
| | Control NPase labile Po | 19.3917 | 27.7421 | 1.000 | -89.078 | 127.861 |
| | Upland PPase labile Po | 33.7917 | 27.7421 | 1.000 | -74.678 | 142.261 |
| | Upland GPase labile Po | -11.7833 | 27.7421 | 1.000 | -120.253 | 96.686 |
| | Upland NPase labile Po | 47.6167 | 27.7421 | 1.000 | -60.853 | 156.086 |
| | Depositional PPase labile Po | 98.7667 | 27.7421 | .147 | -9.703 | 207.236 |

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|---|---|---|---|---|---|---|
| Control PPase P + MRP | Upland PPase P | 107.1000* | 19.6166 | .000 | 30.400 | 183.800 |
| | Upland GPase P | 83.0583* | 19.6166 | .016 | 6.359 | 159.758 |
| | Upland NPase P | 111.2250* | 19.6166 | .000 | 34.525 | 187.925 |
| | Depositional PPase P | 148.1167* | 19.6166 | .000 | 71.417 | 224.816 |
| | Depositional GPase P | 113.9167* | 19.6166 | .000 | 37.217 | 190.616 |
| | Depositional NPase P | 136.4500* | 19.6166 | .000 | 59.750 | 213.150 |
| | Control PPase P | 101.2750* | 19.6166 | .000 | 24.575 | 177.975 |
| | Control GPase P | 95.2833* | 19.6166 | .001 | 18.584 | 171.983 |
| | Control NPase P | 83.9417* | 19.6166 | .013 | 7.242 | 160.641 |
| | Upland Mehlich-3 P | 117.5167* | 19.6166 | .000 | 40.817 | 194.216 |
| | Depositional Mehlich-3 P | 50.4667 | 19.6166 | .837 | -26.233 | 127.166 |
| | Control Mehlich-3 P | 147.2525* | 19.6166 | .000 | 70.553 | 223.952 |
| | Upland NaOH-EDTA TP | -499.1083* | 19.6166 | .000 | -575.808 | -422.409 |
| | Depositional NaOH-EDTA TP | -1022.4333* | 19.6166 | .000 | -1099.133 | -945.734 |
| | Control NaOH-EDTA TP | -48.4167 | 19.6166 | .892 | -125.116 | 28.283 |
| | Upland NaOH-EDTA incubated P | -417.4167* | 19.6166 | .000 | -494.116 | -340.717 |
| | Upland NaOH-EDTA P | -411.8583* | 19.6166 | .000 | -488.558 | -335.159 |
| | Depositional NaOH-EDTA incubated P | -648.4667* | 19.6166 | .000 | -725.166 | -571.767 |
| | Depositional NaOH-EDTA P | -637.9500* | 19.6166 | .000 | -714.650 | -561.250 |
| | Control NaOH-EDTA incubated P | 51.7583 | 19.6166 | .795 | -24.941 | 128.458 |
| | Control NaOH-EDTA P | 51.2917 | 19.6166 | .811 | -25.408 | 127.991 |
| | Control PPase labile Po | 77.0583 | 27.7421 | .693 | -31.411 | 185.528 |
| | Control GPase labile Po | 90.7833 | 27.7421 | .301 | -17.686 | 199.253 |

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|---|---|---|---|---|---|---|
| | Control NPase labile Po | 70.6833 | 27.7421 | .851 | -37.786 | 179.153 |
| | Upland PPase labile Po | 85.0833 | 27.7421 | .455 | -23.386 | 193.553 |
| | Upland GPase labile Po | 39.5083 | 27.7421 | 1.000 | -68.961 | 147.978 |
| | Upland NPase labile Po | 98.9083 | 27.7421 | .145 | -9.561 | 207.378 |
| | Depositional PPase labile Po | 150.0583* | 27.7421 | .000 | 41.589 | 258.528 |
| | Depositional GPase labile Po | 141.6833* | 27.7421 | .000 | 33.214 | 250.153 |
| | Depositional NPase labile Po | 148.2083* | 27.7421 | .000 | 39.739 | 256.678 |
| | Control GPase P + MRP | -5.9583 | 19.6166 | 1.000 | -82.658 | 70.741 |
| | Control NPase P + MRP | -17.3000 | 19.6166 | 1.000 | -94.000 | 59.400 |
| | Upland PPase P + MRP | -455.3717* | 19.6166 | .000 | -532.071 | -378.672 |
| | Upland GPase P + MRP | -479.4108* | 19.6166 | .000 | -556.110 | -402.711 |
| | Upland NPase P + MRP | -451.2308* | 19.6166 | .000 | -527.930 | -374.531 |
| | Depositional PPase + MRP | -640.0417* | 19.6166 | .000 | -716.741 | -563.342 |
| | Depositional GPase P + MRP | -674.2250* | 19.6166 | .000 | -750.925 | -597.525 |
| | Depositional NPase P + MRP | -651.6917* | 19.6166 | .000 | -728.391 | -574.992 |
| Control GPase P + MRP | Upland PPase P | 113.0583* | 19.6166 | .000 | 36.359 | 189.758 |
| | Upland GPase P | 89.0167* | 19.6166 | .005 | 12.317 | 165.716 |
| | Upland NPase P | 117.1833* | 19.6166 | .000 | 40.484 | 193.883 |
| | Depositional PPase P | 154.0750* | 19.6166 | .000 | 77.375 | 230.775 |
| | Depositional GPase P | 119.8750* | 19.6166 | .000 | 43.175 | 196.575 |

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|---|---|---|---|---|---|---|
| | Depositional NPase P | 142.4083* | 19.6166 | .000 | 65.709 | 219.108 |
| | Control PPase P | 107.2333* | 19.6166 | .000 | 30.534 | 183.933 |
| | Control GPase P | 101.2417* | 19.6166 | .000 | 24.542 | 177.941 |
| | Control NPase P | 89.9000* | 19.6166 | .004 | 13.200 | 166.600 |
| | Upland Mehlich-3 P | 123.4750* | 19.6166 | .000 | 46.775 | 200.175 |
| | Depositional Mehlich-3 P | 56.4250 | 19.6166 | .613 | -20.275 | 133.125 |
| | Control Mehlich-3 P | 153.2108* | 19.6166 | .000 | 76.511 | 229.910 |
| | Upland NaOH-EDTA TP | -493.1500* | 19.6166 | .000 | -569.850 | -416.450 |
| | Depositional NaOH-EDTA TP | -1016.4750* | 19.6166 | .000 | -1093.175 | -939.775 |
| | Control NaOH-EDTA TP | -42.4583 | 19.6166 | .979 | -119.158 | 34.241 |
| | Upland NaOH-EDTA incubated P | -411.4583* | 19.6166 | .000 | -488.158 | -334.759 |
| | Upland NaOH-EDTA P | -405.9000* | 19.6166 | .000 | -482.600 | -329.200 |
| | Depositional NaOH-EDTA incubated P | -642.5083* | 19.6166 | .000 | -719.208 | -565.809 |
| | Depositional NaOH-EDTA P | -631.9917* | 19.6166 | .000 | -708.691 | -555.292 |
| | Control NaOH-EDTA incubated P | 57.7167 | 19.6166 | .558 | -18.983 | 134.416 |
| | Control NaOH-EDTA P | 57.2500 | 19.6166 | .578 | -19.450 | 133.950 |
| | Control PPase labile Po | 83.0167 | 27.7421 | .516 | -25.453 | 191.486 |
| | Control GPase labile Po | 96.7417 | 27.7421 | .179 | -11.728 | 205.211 |
| | Control NPase labile Po | 76.6417 | 27.7421 | .705 | -31.828 | 185.111 |
| | Upland PPase labile Po | 91.0417 | 27.7421 | .295 | -17.428 | 199.511 |
| | Upland GPase labile Po | 45.4667 | 27.7421 | 1.000 | -63.003 | 153.936 |

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|---|---|---|---|---|---|---|
| | Upland NPase labile Po | 104.8667 | 27.7421 | .076 | -3.603 | 213.336 |
| | Depositional PPase labile Po | 156.0167* | 27.7421 | .000 | 47.547 | 264.486 |
| | Depositional GPase labile Po | 147.6417* | 27.7421 | .000 | 39.172 | 256.111 |
| | Depositional NPase labile Po | 154.1667* | 27.7421 | .000 | 45.697 | 262.636 |
| | Control PPase P + MRP | 5.9583 | 19.6166 | 1.000 | -70.741 | 82.658 |
| | Control NPase P + MRP | -11.3417 | 19.6166 | 1.000 | -88.041 | 65.358 |
| | Upland PPase P + MRP | -449.4133* | 19.6166 | .000 | -526.113 | -372.714 |
| | Upland GPase P + MRP | -473.4525* | 19.6166 | .000 | -550.152 | -396.753 |
| | Upland NPase P + MRP | -445.2725* | 19.6166 | .000 | -521.972 | -368.573 |
| | Depositional PPase + MRP | -634.0833* | 19.6166 | .000 | -710.783 | -557.384 |
| | Depositional GPase P + MRP | -668.2667* | 19.6166 | .000 | -744.966 | -591.567 |
| | Depositional NPase P + MRP | -645.7333* | 19.6166 | .000 | -722.433 | -569.034 |

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| Upland PPase P + MRP | Upland PPase P | 562.4717* | 19.6166 | .000 | 485.772 | 639.171 |
| | Upland GPase P | 538.4300* | 19.6166 | .000 | 461.730 | 615.130 |
| | Upland NPase P | 566.5967* | 19.6166 | .000 | 489.897 | 643.296 |
| | Depositional PPase P | 603.4883* | 19.6166 | .000 | 526.789 | 680.188 |
| | Depositional GPase P | 569.2883* | 19.6166 | .000 | 492.589 | 645.988 |
| | Depositional NPase P | 591.8217* | 19.6166 | .000 | 515.122 | 668.521 |
| | Control PPase P | 556.6467* | 19.6166 | .000 | 479.947 | 633.346 |
| | Control GPase P | 550.6550* | 19.6166 | .000 | 473.955 | 627.355 |
| | Control NPase P | 539.3133* | 19.6166 | .000 | 462.614 | 616.013 |
| | Upland Mehlich-3 P | 572.8883* | 19.6166 | .000 | 496.189 | 649.588 |
| | Depositional Mehlich-3 P | 505.8383* | 19.6166 | .000 | 429.139 | 582.538 |
| | Control Mehlich-3 P | 602.6242* | 19.6166 | .000 | 525.925 | 679.324 |

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| | Upland NaOH-EDTA TP | -43.7367 | 19.6166 | .969 | -120.436 | 32.963 |
| | Depositional NaOH-EDTA TP | -567.0617* | 19.6166 | .000 | -643.761 | -490.362 |
| | Control NaOH-EDTA TP | 406.9550* | 19.6166 | .000 | 330.255 | 483.655 |
| | Upland NaOH-EDTA incubated P | 37.9550 | 19.6166 | .997 | -38.745 | 114.655 |
| | Upland NaOH-EDTA P | 43.5133 | 19.6166 | .971 | -33.186 | 120.213 |
| | Depositional NaOH-EDTA incubated P | -193.0950* | 19.6166 | .000 | -269.795 | -116.395 |
| | Depositional NaOH-EDTA P | -182.5783* | 19.6166 | .000 | -259.278 | -105.879 |
| | Control NaOH-EDTA incubated P | 507.1300* | 19.6166 | .000 | 430.430 | 583.830 |
| | Control NaOH-EDTA P | 506.6633* | 19.6166 | .000 | 429.964 | 583.363 |
| | Control PPase labile Po | 532.4300* | 27.7421 | .000 | 423.960 | 640.900 |
| | Control GPase labile Po | 546.1550* | 27.7421 | .000 | 437.685 | 654.625 |
| | Control NPase labile Po | 526.0550* | 27.7421 | .000 | 417.585 | 634.525 |
| | Upland PPase labile Po | 540.4550* | 27.7421 | .000 | 431.985 | 648.925 |
| | Upland GPase labile Po | 494.8800* | 27.7421 | .000 | 386.410 | 603.350 |
| | Upland NPase labile Po | 554.2800* | 27.7421 | .000 | 445.810 | 662.750 |
| | Depositional PPase labile Po | 605.4300* | 27.7421 | .000 | 496.960 | 713.900 |
| | Depositional GPase labile Po | 597.0550* | 27.7421 | .000 | 488.585 | 705.525 |
| | Depositional NPase labile Po | 603.5800* | 27.7421 | .000 | 495.110 | 712.050 |

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| | Control PPase P + MRP | 455.3717* | 19.6166 | .000 | 378.672 | 532.071 |
| | Control GPase P + MRP | 449.4133* | 19.6166 | .000 | 372.714 | 526.113 |
| | Control NPase P + MRP | 438.0717* | 19.6166 | .000 | 361.372 | 514.771 |
| | Upland GPase P + MRP | -24.0392 | 19.6166 | 1.000 | -100.739 | 52.660 |
| | Upland NPase P + MRP | 4.1408 | 19.6166 | 1.000 | -72.559 | 80.840 |
| | Depositional PPase + MRP | -184.6700* | 19.6166 | .000 | -261.370 | -107.970 |
| | Depositional GPase P + MRP | -218.8533* | 19.6166 | .000 | -295.553 | -142.154 |
| | Depositional NPase P + MRP | -196.3200* | 19.6166 | .000 | -273.020 | -119.620 |
| Upland GPase P + MRP | Upland PPase P | 586.5108* | 19.6166 | .000 | 509.811 | 663.210 |
| | Upland GPase P | 562.4692* | 19.6166 | .000 | 485.770 | 639.169 |
| | Upland NPase P | 590.6358* | 19.6166 | .000 | 513.936 | 667.335 |
| | Depositional PPase P | 627.5275* | 19.6166 | .000 | 550.828 | 704.227 |
| | Depositional GPase P | 593.3275* | 19.6166 | .000 | 516.628 | 670.027 |
| | Depositional NPase P | 615.8608* | 19.6166 | .000 | 539.161 | 692.560 |
| | Control PPase P | 580.6858* | 19.6166 | .000 | 503.986 | 657.385 |
| | Control GPase P | 574.6942* | 19.6166 | .000 | 497.995 | 651.394 |
| | Control NPase P | 563.3525* | 19.6166 | .000 | 486.653 | 640.052 |
| | Upland Mehlich-3 P | 596.9275* | 19.6166 | .000 | 520.228 | 673.627 |
| | Depositional Mehlich-3 P | 529.8775* | 19.6166 | .000 | 453.178 | 606.577 |
| | Control Mehlich-3 P | 626.6633* | 19.6166 | .000 | 549.964 | 703.363 |
| | Upland NaOH-EDTA TP | -19.6975 | 19.6166 | 1.000 | -96.397 | 57.002 |
| | Depositional NaOH-EDTA TP | -543.0225* | 19.6166 | .000 | -619.722 | -466.323 |
| | Control NaOH-EDTA TP | 430.9942* | 19.6166 | .000 | 354.295 | 507.694 |

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| | Upland NaOH-EDTA incubated P | 61.9942 | 19.6166 | .381 | -14.705 | 138.694 |
| | Upland NaOH-EDTA P | 67.5525 | 19.6166 | .200 | -9.147 | 144.252 |
| | Depositional NaOH-EDTA incubated P | -169.0558* | 19.6166 | .000 | -245.755 | -92.356 |
| | Depositional NaOH-EDTA P | -158.5392* | 19.6166 | .000 | -235.239 | -81.840 |
| | Control NaOH-EDTA incubated P | 531.1692* | 19.6166 | .000 | 454.470 | 607.869 |
| | Control NaOH-EDTA P | 530.7025* | 19.6166 | .000 | 454.003 | 607.402 |
| | Control PPase labile Po | 556.4692* | 27.7421 | .000 | 448.000 | 664.939 |
| | Control GPase labile Po | 570.1942* | 27.7421 | .000 | 461.725 | 678.664 |
| | Control NPase labile Po | 550.0942* | 27.7421 | .000 | 441.625 | 658.564 |
| | Upland PPase labile Po | 564.4942* | 27.7421 | .000 | 456.025 | 672.964 |
| | Upland GPase labile Po | 518.9192* | 27.7421 | .000 | 410.450 | 627.389 |
| | Upland NPase labile Po | 578.3192* | 27.7421 | .000 | 469.850 | 686.789 |
| | Depositional PPase labile Po | 629.4692* | 27.7421 | .000 | 521.000 | 737.939 |
| | Depositional GPase labile Po | 621.0942* | 27.7421 | .000 | 512.625 | 729.564 |
| | Depositional NPase labile Po | 627.6192* | 27.7421 | .000 | 519.150 | 736.089 |
| | Control PPase P + MRP | 479.4108* | 19.6166 | .000 | 402.711 | 556.110 |
| | Control GPase P + MRP | 473.4525* | 19.6166 | .000 | 396.753 | 550.152 |
| | Control NPase P + MRP | 462.1108* | 19.6166 | .000 | 385.411 | 538.810 |

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|---|---|---|---|---|---|---|
| | Upland PPase P + MRP | 24.0392 | 19.6166 | 1.000 | -52.660 | 100.739 |
| | Upland NPase P + MRP | 28.1800 | 19.6166 | 1.000 | -48.520 | 104.880 |
| | Depositional PPase + MRP | -160.6308* | 19.6166 | .000 | -237.330 | -83.931 |
| | Depositional GPase P + MRP | -194.8142* | 19.6166 | .000 | -271.514 | -118.115 |
| | Depositional NPase P + MRP | -172.2808* | 19.6166 | .000 | -248.980 | -95.581 |
| Upland NPase P + MRP | Upland PPase P | 558.3308* | 19.6166 | .000 | 481.631 | 635.030 |
| | Upland GPase P | 534.2892* | 19.6166 | .000 | 457.590 | 610.989 |
| | Upland NPase P | 562.4558* | 19.6166 | .000 | 485.756 | 639.155 |
| | Depositional PPase P | 599.3475* | 19.6166 | .000 | 522.648 | 676.047 |
| | Depositional GPase P | 565.1475* | 19.6166 | .000 | 488.448 | 641.847 |
| | Depositional NPase P | 587.6808* | 19.6166 | .000 | 510.981 | 664.380 |
| | Control PPase P | 552.5058* | 19.6166 | .000 | 475.806 | 629.205 |
| | Control GPase P | 546.5142* | 19.6166 | .000 | 469.815 | 623.214 |
| | Control NPase P | 535.1725* | 19.6166 | .000 | 458.473 | 611.872 |
| | Upland Mehlich-3 P | 568.7475* | 19.6166 | .000 | 492.048 | 645.447 |
| | Depositional Mehlich-3 P | 501.6975* | 19.6166 | .000 | 424.998 | 578.397 |
| | Control Mehlich-3 P | 598.4833* | 19.6166 | .000 | 521.784 | 675.183 |
| | Upland NaOH-EDTA TP | -47.8775 | 19.6166 | .904 | -124.577 | 28.822 |
| | Depositional NaOH-EDTA TP | -571.2025* | 19.6166 | .000 | -647.902 | -494.503 |
| | Control NaOH-EDTA TP | 402.8142* | 19.6166 | .000 | 326.115 | 479.514 |
| | Upland NaOH-EDTA incubated P | 33.8142 | 19.6166 | 1.000 | -42.885 | 110.514 |
| | Upland NaOH-EDTA P | 39.3725 | 19.6166 | .994 | -37.327 | 116.072 |
| | Depositional NaOH-EDTA incubated P | -197.2358* | 19.6166 | .000 | -273.935 | -120.536 |

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|---|---|---|---|---|---|---|
| | Depositional NaOH-EDTA P | -186.7192* | 19.6166 | .000 | -263.419 | -110.020 |
| | Control NaOH-EDTA incubated P | 502.9892* | 19.6166 | .000 | 426.290 | 579.689 |
| | Control NaOH-EDTA P | 502.5225* | 19.6166 | .000 | 425.823 | 579.222 |
| | Control PPase labile Po | 528.2892* | 27.7421 | .000 | 419.820 | 636.759 |
| | Control GPase labile Po | 542.0142* | 27.7421 | .000 | 433.545 | 650.484 |
| | Control NPase labile Po | 521.9142* | 27.7421 | .000 | 413.445 | 630.384 |
| | Upland PPase labile Po | 536.3142* | 27.7421 | .000 | 427.845 | 644.784 |
| | Upland GPase labile Po | 490.7392* | 27.7421 | .000 | 382.270 | 599.209 |
| | Upland NPase labile Po | 550.1392* | 27.7421 | .000 | 441.670 | 658.609 |
| | Depositional PPase labile Po | 601.2892* | 27.7421 | .000 | 492.820 | 709.759 |
| | Depositional GPase labile Po | 592.9142* | 27.7421 | .000 | 484.445 | 701.384 |
| | Depositional NPase labile Po | 599.4392* | 27.7421 | .000 | 490.970 | 707.909 |
| | Control PPase P + MRP | 451.2308* | 19.6166 | .000 | 374.531 | 527.930 |
| | Control GPase P + MRP | 445.2725* | 19.6166 | .000 | 368.573 | 521.972 |
| | Control NPase P + MRP | 433.9308* | 19.6166 | .000 | 357.231 | 510.630 |
| | Upland PPase P + MRP | -4.1408 | 19.6166 | 1.000 | -80.840 | 72.559 |
| | Upland GPase P + MRP | -28.1800 | 19.6166 | 1.000 | -104.880 | 48.520 |
| | Depositional PPase + MRP | -188.8108* | 19.6166 | .000 | -265.510 | -112.111 |

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|---|---|---|---|---|---|---|
| | Depositional GPase P + MRP | -222.9942* | 19.6166 | .000 | -299.694 | -146.295 |
| | Depositional NPase P + MRP | -200.4608* | 19.6166 | .000 | -277.160 | -123.761 |
| Depositional PPase + MRP | Upland PPase P | 747.1417* | 19.6166 | .000 | 670.442 | 823.841 |
| | Upland GPase P | 723.1000* | 19.6166 | .000 | 646.400 | 799.800 |
| | Upland NPase P | 751.2667* | 19.6166 | .000 | 674.567 | 827.966 |
| | Depositional PPase P | 788.1583* | 19.6166 | .000 | 711.459 | 864.858 |
| | Depositional GPase P | 753.9583* | 19.6166 | .000 | 677.259 | 830.658 |
| | Depositional NPase P | 776.4917* | 19.6166 | .000 | 699.792 | 853.191 |
| | Control PPase P | 741.3167* | 19.6166 | .000 | 664.617 | 818.016 |
| | Control GPase P | 735.3250* | 19.6166 | .000 | 658.625 | 812.025 |
| | Control NPase P | 723.9833* | 19.6166 | .000 | 647.284 | 800.683 |
| | Upland Mehlich-3 P | 757.5583* | 19.6166 | .000 | 680.859 | 834.258 |
| | Depositional Mehlich-3 P | 690.5083* | 19.6166 | .000 | 613.809 | 767.208 |
| | Control Mehlich-3 P | 787.2942* | 19.6166 | .000 | 710.595 | 863.994 |
| | Upland NaOH-EDTA TP | 140.9333* | 19.6166 | .000 | 64.234 | 217.633 |
| | Depositional NaOH-EDTA TP | -382.3917* | 19.6166 | .000 | -459.091 | -305.692 |
| | Control NaOH-EDTA TP | 591.6250* | 19.6166 | .000 | 514.925 | 668.325 |
| | Upland NaOH-EDTA incubated P | 222.6250* | 19.6166 | .000 | 145.925 | 299.325 |
| | Upland NaOH-EDTA P | 228.1833* | 19.6166 | .000 | 151.484 | 304.883 |
| | Depositional NaOH-EDTA incubated P | -8.4250 | 19.6166 | 1.000 | -85.125 | 68.275 |
| | Depositional NaOH-EDTA P | 2.0917 | 19.6166 | 1.000 | -74.608 | 78.791 |
| | Control NaOH-EDTA incubated P | 691.8000* | 19.6166 | .000 | 615.100 | 768.500 |
| | Control NaOH-EDTA P | 691.3333* | 19.6166 | .000 | 614.634 | 768.033 |

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|---|---|---|---|---|---|---|
| | Control PPase labile Po | 717.1000* | 27.7421 | .000 | 608.630 | 825.570 |
| | Control GPase labile Po | 730.8250* | 27.7421 | .000 | 622.355 | 839.295 |
| | Control NPase labile Po | 710.7250* | 27.7421 | .000 | 602.255 | 819.195 |
| | Upland PPase labile Po | 725.1250* | 27.7421 | .000 | 616.655 | 833.595 |
| | Upland GPase labile Po | 679.5500* | 27.7421 | .000 | 571.080 | 788.020 |
| | Upland NPase labile Po | 738.9500* | 27.7421 | .000 | 630.480 | 847.420 |
| | Depositional PPase labile Po | 790.1000* | 27.7421 | .000 | 681.630 | 898.570 |
| | Depositional GPase labile Po | 781.7250* | 27.7421 | .000 | 673.255 | 890.195 |
| | Depositional NPase labile Po | 788.2500* | 27.7421 | .000 | 679.780 | 896.720 |
| | Control PPase P + MRP | 640.0417* | 19.6166 | .000 | 563.342 | 716.741 |
| | Control GPase P + MRP | 634.0833* | 19.6166 | .000 | 557.384 | 710.783 |
| | Control NPase P + MRP | 622.7417* | 19.6166 | .000 | 546.042 | 699.441 |
| | Upland PPase P + MRP | 184.6700* | 19.6166 | .000 | 107.970 | 261.370 |
| | Upland GPase P + MRP | 160.6308* | 19.6166 | .000 | 83.931 | 237.330 |
| | Upland NPase P + MRP | 188.8108* | 19.6166 | .000 | 112.111 | 265.510 |
| | Depositional GPase P + MRP | -34.1833 | 19.6166 | 1.000 | -110.883 | 42.516 |
| | Depositional NPase P + MRP | -11.6500 | 19.6166 | 1.000 | -88.350 | 65.050 |
| Depositional GPase P + MRP | Upland PPase P | 781.3250* | 19.6166 | .000 | 704.625 | 858.025 |
| | Upland GPase P | 757.2833* | 19.6166 | .000 | 680.584 | 833.983 |
| | Upland NPase P | 785.4500* | 19.6166 | .000 | 708.750 | 862.150 |

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|---|---|---|---|---|---|
| Depositional PPase P | 822.3417* | 19.6166 | .000 | 745.642 | 899.041 |
| Depositional GPase P | 788.1417* | 19.6166 | .000 | 711.442 | 864.841 |
| Depositional NPase P | 810.6750* | 19.6166 | .000 | 733.975 | 887.375 |
| Control PPase P | 775.5000* | 19.6166 | .000 | 698.800 | 852.200 |
| Control GPase P | 769.5083* | 19.6166 | .000 | 692.809 | 846.208 |
| Control NPase P | 758.1667* | 19.6166 | .000 | 681.467 | 834.866 |
| Upland Mehlich-3 P | 791.7417* | 19.6166 | .000 | 715.042 | 868.441 |
| Depositional Mehlich-3 P | 724.6917* | 19.6166 | .000 | 647.992 | 801.391 |
| Control Mehlich-3 P | 821.4775* | 19.6166 | .000 | 744.778 | 898.177 |
| Upland NaOH-EDTA TP | 175.1167* | 19.6166 | .000 | 98.417 | 251.816 |
| Depositional NaOH-EDTA TP | -348.2083* | 19.6166 | .000 | -424.908 | -271.509 |
| Control NaOH-EDTA TP | 625.8083* | 19.6166 | .000 | 549.109 | 702.508 |
| Upland NaOH-EDTA incubated P | 256.8083* | 19.6166 | .000 | 180.109 | 333.508 |
| Upland NaOH-EDTA P | 262.3667* | 19.6166 | .000 | 185.667 | 339.066 |
| Depositional NaOH-EDTA incubated P | 25.7583 | 19.6166 | 1.000 | -50.941 | 102.458 |
| Depositional NaOH-EDTA P | 36.2750 | 19.6166 | .999 | -40.425 | 112.975 |
| Control NaOH-EDTA incubated P | 725.9833* | 19.6166 | .000 | 649.284 | 802.683 |
| Control NaOH-EDTA P | 725.5167* | 19.6166 | .000 | 648.817 | 802.216 |
| Control PPase labile Po | 751.2833* | 27.7421 | .000 | 642.814 | 859.753 |
| Control GPase labile Po | 765.0083* | 27.7421 | .000 | 656.539 | 873.478 |
| Control NPase labile Po | 744.9083* | 27.7421 | .000 | 636.439 | 853.378 |
| Upland PPase labile Po | 759.3083* | 27.7421 | .000 | 650.839 | 867.778 |

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|---|---|---|---|---|---|---|
| | Upland GPase labile Po | 713.7333* | 27.7421 | .000 | 605.264 | 822.203 |
| | Upland NPase labile Po | 773.1333* | 27.7421 | .000 | 664.664 | 881.603 |
| | Depositional PPase labile Po | 824.2833* | 27.7421 | .000 | 715.814 | 932.753 |
| | Depositional GPase labile Po | 815.9083* | 27.7421 | .000 | 707.439 | 924.378 |
| | Depositional NPase labile Po | 822.4333* | 27.7421 | .000 | 713.964 | 930.903 |
| | Control PPase P + MRP | 674.2250* | 19.6166 | .000 | 597.525 | 750.925 |
| | Control GPase P + MRP | 668.2667* | 19.6166 | .000 | 591.567 | 744.966 |
| | Control NPase P + MRP | 656.9250* | 19.6166 | .000 | 580.225 | 733.625 |
| | Upland PPase P + MRP | 218.8533* | 19.6166 | .000 | 142.154 | 295.553 |
| | Upland GPase P + MRP | 194.8142* | 19.6166 | .000 | 118.115 | 271.514 |
| | Upland NPase P + MRP | 222.9942* | 19.6166 | .000 | 146.295 | 299.694 |
| | Depositional PPase + MRP | 34.1833 | 19.6166 | 1.000 | -42.516 | 110.883 |
| | Depositional NPase P + MRP | 22.5333 | 19.6166 | 1.000 | -54.166 | 99.233 |
| Depositional NPase P + MRP | Upland PPase P | 758.7917* | 19.6166 | .000 | 682.092 | 835.491 |
| | Upland GPase P | 734.7500* | 19.6166 | .000 | 658.050 | 811.450 |
| | Upland NPase P | 762.9167* | 19.6166 | .000 | 686.217 | 839.616 |
| | Depositional PPase P | 799.8083* | 19.6166 | .000 | 723.109 | 876.508 |
| | Depositional GPase P | 765.6083* | 19.6166 | .000 | 688.909 | 842.308 |
| | Depositional NPase P | 788.1417* | 19.6166 | .000 | 711.442 | 864.841 |
| | Control PPase P | 752.9667* | 19.6166 | .000 | 676.267 | 829.666 |
| | Control GPase P | 746.9750* | 19.6166 | .000 | 670.275 | 823.675 |
| | Control NPase P | 735.6333* | 19.6166 | .000 | 658.934 | 812.333 |

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| | Upland Mehlich-3 P | 769.2083* | 19.6166 | .000 | 692.509 | 845.908 |
| | Depositional Mehlich-3 P | 702.1583* | 19.6166 | .000 | 625.459 | 778.858 |
| | Control Mehlich-3 P | 798.9442* | 19.6166 | .000 | 722.245 | 875.644 |
| | Upland NaOH-EDTA TP | 152.5833* | 19.6166 | .000 | 75.884 | 229.283 |
| | Depositional NaOH-EDTA TP | -370.7417* | 19.6166 | .000 | -447.441 | -294.042 |
| | Control NaOH-EDTA TP | 603.2750* | 19.6166 | .000 | 526.575 | 679.975 |
| | Upland NaOH-EDTA incubated P | 234.2750* | 19.6166 | .000 | 157.575 | 310.975 |
| | Upland NaOH-EDTA P | 239.8333* | 19.6166 | .000 | 163.134 | 316.533 |
| | Depositional NaOH-EDTA incubated P | 3.2250 | 19.6166 | 1.000 | -73.475 | 79.925 |
| | Depositional NaOH-EDTA P | 13.7417 | 19.6166 | 1.000 | -62.958 | 90.441 |
| | Control NaOH-EDTA incubated P | 703.4500* | 19.6166 | .000 | 626.750 | 780.150 |
| | Control NaOH-EDTA P | 702.9833* | 19.6166 | .000 | 626.284 | 779.683 |
| | Control PPase labile Po | 728.7500* | 27.7421 | .000 | 620.280 | 837.220 |
| | Control GPase labile Po | 742.4750* | 27.7421 | .000 | 634.005 | 850.945 |
| | Control NPase labile Po | 722.3750* | 27.7421 | .000 | 613.905 | 830.845 |
| | Upland PPase labile Po | 736.7750* | 27.7421 | .000 | 628.305 | 845.245 |
| | Upland GPase labile Po | 691.2000* | 27.7421 | .000 | 582.730 | 799.670 |
| | Upland NPase labile Po | 750.6000* | 27.7421 | .000 | 642.130 | 859.070 |

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| | Depositional PPase labile Po | 801.7500* | 27.7421 | .000 | 693.280 | 910.220 |
| | Depositional GPase labile Po | 793.3750* | 27.7421 | .000 | 684.905 | 901.845 |
| | Depositional NPase labile Po | 799.9000* | 27.7421 | .000 | 691.430 | 908.370 |
| | Control PPase P + MRP | 651.6917* | 19.6166 | .000 | 574.992 | 728.391 |
| | Control GPase P + MRP | 645.7333* | 19.6166 | .000 | 569.034 | 722.433 |
| | Control NPase P + MRP | 634.3917* | 19.6166 | .000 | 557.692 | 711.091 |
| | Upland PPase P + MRP | 196.3200* | 19.6166 | .000 | 119.620 | 273.020 |
| | Upland GPase P + MRP | 172.2808* | 19.6166 | .000 | 95.581 | 248.980 |
| | Upland NPase P + MRP | 200.4608* | 19.6166 | .000 | 123.761 | 277.160 |
| | Depositional PPase + MRP | 11.6500 | 19.6166 | 1.000 | -65.050 | 88.350 |
| | Depositional GPase P + MRP | -22.5333 | 19.6166 | 1.000 | -99.233 | 54.166 |
| Upland Mehlich-3 P | Upland PPase P | -10.4167 | 19.6166 | 1.000 | -87.116 | 66.283 |
| | Upland GPase P | -34.4583 | 19.6166 | .999 | -111.158 | 42.241 |
| | Upland NPase P | -6.2917 | 19.6166 | 1.000 | -82.991 | 70.408 |
| | Depositional PPase P | 30.6000 | 19.6166 | 1.000 | -46.100 | 107.300 |
| | Depositional GPase P | -3.6000 | 19.6166 | 1.000 | -80.300 | 73.100 |
| | Depositional NPase P | 18.9333 | 19.6166 | 1.000 | -57.766 | 95.633 |
| | Control PPase P | -16.2417 | 19.6166 | 1.000 | -92.941 | 60.458 |
| | Control GPase P | -22.2333 | 19.6166 | 1.000 | -98.933 | 54.466 |
| | Control NPase P | -33.5750 | 19.6166 | 1.000 | -110.275 | 43.125 |
| | Depositional Mehlich-3 P | -67.0500 | 19.6166 | .213 | -143.750 | 9.650 |
| | Control Mehlich-3 P | 29.7358 | 19.6166 | 1.000 | -46.964 | 106.435 |

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| | Upland NaOH-EDTA TP | -616.6250* | 19.6166 | .000 | -693.325 | -539.925 |
| | Depositional NaOH-EDTA TP | -1139.9500* | 19.6166 | .000 | -1216.650 | -1063.250 |
| | Control NaOH-EDTA TP | -165.9333* | 19.6166 | .000 | -242.633 | -89.234 |
| | Upland NaOH-EDTA incubated P | -534.9333* | 19.6166 | .000 | -611.633 | -458.234 |
| | Upland NaOH-EDTA P | -529.3750* | 19.6166 | .000 | -606.075 | -452.675 |
| | Depositional NaOH-EDTA incubated P | -765.9833* | 19.6166 | .000 | -842.683 | -689.284 |
| | Depositional NaOH-EDTA P | -755.4667* | 19.6166 | .000 | -832.166 | -678.767 |
| | Control NaOH-EDTA incubated P | -65.7583 | 19.6166 | .251 | -142.458 | 10.941 |
| | Control NaOH-EDTA P | -66.2250 | 19.6166 | .237 | -142.925 | 10.475 |
| | Control PPase labile Po | -40.4583 | 27.7421 | 1.000 | -148.928 | 68.011 |
| | Control GPase labile Po | -26.7333 | 27.7421 | 1.000 | -135.203 | 81.736 |
| | Control NPase labile Po | -46.8333 | 27.7421 | 1.000 | -155.303 | 61.636 |
| | Upland PPase labile Po | -32.4333 | 27.7421 | 1.000 | -140.903 | 76.036 |
| | Upland GPase labile Po | -78.0083 | 27.7421 | .666 | -186.478 | 30.461 |
| | Upland NPase labile Po | -18.6083 | 27.7421 | 1.000 | -127.078 | 89.861 |
| | Depositional PPase labile Po | 32.5417 | 27.7421 | 1.000 | -75.928 | 141.011 |
| | Depositional GPase labile Po | 24.1667 | 27.7421 | 1.000 | -84.303 | 132.636 |
| | Depositional NPase labile Po | 30.6917 | 27.7421 | 1.000 | -77.778 | 139.161 |

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| | Control PPase P + MRP | -117.5167* | 19.6166 | .000 | -194.216 | -40.817 |
| | Control GPase P + MRP | -123.4750* | 19.6166 | .000 | -200.175 | -46.775 |
| | Control NPase P + MRP | -134.8167* | 19.6166 | .000 | -211.516 | -58.117 |
| | Upland PPase P + MRP | -572.8883* | 19.6166 | .000 | -649.588 | -496.189 |
| | Upland GPase P + MRP | -596.9275* | 19.6166 | .000 | -673.627 | -520.228 |
| | Upland NPase P + MRP | -568.7475* | 19.6166 | .000 | -645.447 | -492.048 |
| | Depositional PPase + MRP | -757.5583* | 19.6166 | .000 | -834.258 | -680.859 |
| | Depositional GPase P + MRP | -791.7417* | 19.6166 | .000 | -868.441 | -715.042 |
| | Depositional NPase P + MRP | -769.2083* | 19.6166 | .000 | -845.908 | -692.509 |
| Depositional Mehlich-3 P | Upland PPase P | 56.6333 | 19.6166 | .604 | -20.066 | 133.333 |
| | Upland GPase P | 32.5917 | 19.6166 | 1.000 | -44.108 | 109.291 |
| | Upland NPase P | 60.7583 | 19.6166 | .430 | -15.941 | 137.458 |
| | Depositional PPase P | 97.6500* | 19.6166 | .001 | 20.950 | 174.350 |
| | Depositional GPase P | 63.4500 | 19.6166 | .327 | -13.250 | 140.150 |
| | Depositional NPase P | 85.9833* | 19.6166 | .009 | 9.284 | 162.683 |
| | Control PPase P | 50.8083 | 19.6166 | .826 | -25.891 | 127.508 |
| | Control GPase P | 44.8167 | 19.6166 | .956 | -31.883 | 121.516 |
| | Control NPase P | 33.4750 | 19.6166 | 1.000 | -43.225 | 110.175 |
| | Upland Mehlich-3 P | 67.0500 | 19.6166 | .213 | -9.650 | 143.750 |
| | Control Mehlich-3 P | 96.7858* | 19.6166 | .001 | 20.086 | 173.485 |
| | Upland NaOH-EDTA TP | -549.5750* | 19.6166 | .000 | -626.275 | -472.875 |
| | Depositional NaOH-EDTA TP | -1072.9000* | 19.6166 | .000 | -1149.600 | -996.200 |
| | Control NaOH-EDTA TP | -98.8833* | 19.6166 | .000 | -175.583 | -22.184 |

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|---|---|---|---|---|---|---|
| | Upland NaOH-EDTA incubated P | -467.8833* | 19.6166 | .000 | -544.583 | -391.184 |
| | Upland NaOH-EDTA P | -462.3250* | 19.6166 | .000 | -539.025 | -385.625 |
| | Depositional NaOH-EDTA incubated P | -698.9333* | 19.6166 | .000 | -775.633 | -622.234 |
| | Depositional NaOH-EDTA P | -688.4167* | 19.6166 | .000 | -765.116 | -611.717 |
| | Control NaOH-EDTA incubated P | 1.2917 | 19.6166 | 1.000 | -75.408 | 77.991 |
| | Control NaOH-EDTA P | .8250 | 19.6166 | 1.000 | -75.875 | 77.525 |
| | Control PPase labile Po | 26.5917 | 27.7421 | 1.000 | -81.878 | 135.061 |
| | Control GPase labile Po | 40.3167 | 27.7421 | 1.000 | -68.153 | 148.786 |
| | Control NPase labile Po | 20.2167 | 27.7421 | 1.000 | -88.253 | 128.686 |
| | Upland PPase labile Po | 34.6167 | 27.7421 | 1.000 | -73.853 | 143.086 |
| | Upland GPase labile Po | -10.9583 | 27.7421 | 1.000 | -119.428 | 97.511 |
| | Upland NPase labile Po | 48.4417 | 27.7421 | 1.000 | -60.028 | 156.911 |
| | Depositional PPase labile Po | 99.5917 | 27.7421 | .135 | -8.878 | 208.061 |
| | Depositional GPase labile Po | 91.2167 | 27.7421 | .291 | -17.253 | 199.686 |
| | Depositional NPase labile Po | 97.7417 | 27.7421 | .162 | -10.728 | 206.211 |
| | Control PPase P + MRP | -50.4667 | 19.6166 | .837 | -127.166 | 26.233 |
| | Control GPase P + MRP | -56.4250 | 19.6166 | .613 | -133.125 | 20.275 |
| | Control NPase P + MRP | -67.7667 | 19.6166 | .195 | -144.466 | 8.933 |

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| | Upland PPase P + MRP | -505.8383* | 19.6166 | .000 | -582.538 | -429.139 |
| | Upland GPase P + MRP | -529.8775* | 19.6166 | .000 | -606.577 | -453.178 |
| | Upland NPase P + MRP | -501.6975* | 19.6166 | .000 | -578.397 | -424.998 |
| | Depositional PPase + MRP | -690.5083* | 19.6166 | .000 | -767.208 | -613.809 |
| | Depositional GPase P + MRP | -724.6917* | 19.6166 | .000 | -801.391 | -647.992 |
| | Depositional NPase P + MRP | -702.1583* | 19.6166 | .000 | -778.858 | -625.459 |
| Control Mehlich-3 P | Upland PPase P | -40.1525 | 19.6166 | .991 | -116.852 | 36.547 |
| | Upland GPase P | -64.1942 | 19.6166 | .301 | -140.894 | 12.505 |
| | Upland NPase P | -36.0275 | 19.6166 | .999 | -112.727 | 40.672 |
| | Depositional PPase P | .8642 | 19.6166 | 1.000 | -75.835 | 77.564 |
| | Depositional GPase P | -33.3358 | 19.6166 | 1.000 | -110.035 | 43.364 |
| | Depositional NPase P | -10.8025 | 19.6166 | 1.000 | -87.502 | 65.897 |
| | Control PPase P | -45.9775 | 19.6166 | .940 | -122.677 | 30.722 |
| | Control GPase P | -51.9692 | 19.6166 | .788 | -128.669 | 24.730 |
| | Control NPase P | -63.3108 | 19.6166 | .332 | -140.010 | 13.389 |
| | Upland Mehlich-3 P | -29.7358 | 19.6166 | 1.000 | -106.435 | 46.964 |
| | Depositional Mehlich-3 P | -96.7858* | 19.6166 | .001 | -173.485 | -20.086 |
| | Upland NaOH-EDTA TP | -646.3608* | 19.6166 | .000 | -723.060 | -569.661 |
| | Depositional NaOH-EDTA TP | -1169.6858* | 19.6166 | .000 | -1246.385 | -1092.986 |
| | Control NaOH-EDTA TP | -195.6692* | 19.6166 | .000 | -272.369 | -118.970 |
| | Upland NaOH-EDTA incubated P | -564.6692* | 19.6166 | .000 | -641.369 | -487.970 |
| | Upland NaOH-EDTA P | -559.1108* | 19.6166 | .000 | -635.810 | -482.411 |
| | Depositional NaOH-EDTA incubated P | -795.7192* | 19.6166 | .000 | -872.419 | -719.020 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Depositional NaOH-EDTA P | -785.2025* | 19.6166 | .000 | -861.902 | -708.503 |
| | Control NaOH-EDTA incubated P | -95.4942* | 19.6166 | .001 | -172.194 | -18.795 |
| | Control NaOH-EDTA P | -95.9608* | 19.6166 | .001 | -172.660 | -19.261 |
| | Control PPase labile Po | -70.1942 | 27.7421 | .861 | -178.664 | 38.275 |
| | Control GPase labile Po | -56.4692 | 27.7421 | .992 | -164.939 | 52.000 |
| | Control NPase labile Po | -76.5692 | 27.7421 | .707 | -185.039 | 31.900 |
| | Upland PPase labile Po | -62.1692 | 27.7421 | .966 | -170.639 | 46.300 |
| | Upland GPase labile Po | -107.7442 | 27.7421 | .055 | -216.214 | .725 |
| | Upland NPase labile Po | -48.3442 | 27.7421 | 1.000 | -156.814 | 60.125 |
| | Depositional PPase labile Po | 2.8058 | 27.7421 | 1.000 | -105.664 | 111.275 |
| | Depositional GPase labile Po | -5.5692 | 27.7421 | 1.000 | -114.039 | 102.900 |
| | Depositional NPase labile Po | .9558 | 27.7421 | 1.000 | -107.514 | 109.425 |
| | Control PPase P + MRP | -147.2525* | 19.6166 | .000 | -223.952 | -70.553 |
| | Control GPase P + MRP | -153.2108* | 19.6166 | .000 | -229.910 | -76.511 |
| | Control NPase P + MRP | -164.5525* | 19.6166 | .000 | -241.252 | -87.853 |
| | Upland PPase P + MRP | -602.6242* | 19.6166 | .000 | -679.324 | -525.925 |
| | Upland GPase P + MRP | -626.6633* | 19.6166 | .000 | -703.363 | -549.964 |
| | Upland NPase P + MRP | -598.4833* | 19.6166 | .000 | -675.183 | -521.784 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Depositional PPase + MRP | -787.2942* | 19.6166 | .000 | -863.994 | -710.595 |
| | Depositional GPase P + MRP | -821.4775* | 19.6166 | .000 | -898.177 | -744.778 |
| | Depositional NPase P + MRP | -798.9442* | 19.6166 | .000 | -875.644 | -722.245 |

Table 12. Homogeneous subsets for treatments. Three soils of four sampling times each: N = 36 (PASW/SPSS Statistics 18, 2010)**.**

**mg P/kg soil**

Tukey HSD[a,b]

| Treatment | N | Subset 1 | Subset 2 | Subset 3 |
|---|---|---|---|---|
| PPase P | 36 | 33.128 | | |
| NPase P | 36 | 41.419 | | |
| Mehlich-3 P | 36 | 46.880 | | |
| GPase P | 36 | 54.539 | | |
| Base P | 36 | | 484.797 | |
| Chem P | 36 | | 490.000 | |
| NaOH-EDTA TP | 36 | | | 675.278 |
| Sig. | | .422 | .999 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2073.549.

a. Uses Harmonic Mean Sample Size = 36.000.

**mg P/kg soil**

Tukey HSD[a,b]

| Treatment | N | Subset 1 | Subset 2 | Subset 3 |
|---|---|---|---|---|
| PPase P | 36 | 33.128 | | |
| NPase P | 36 | 41.419 | | |
| Mehlich-3 P | 36 | 46.880 | | |
| GPase P | 36 | 54.539 | | |
| Base P | 36 | | 484.797 | |
| Chem P | 36 | | 490.000 | |
| NaOH-EDTA TP | 36 | | | 675.278 |
| Sig. | | .422 | .999 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2073.549.

a. Uses Harmonic Mean Sample Size = 36.000.

b. Alpha = 0.05.

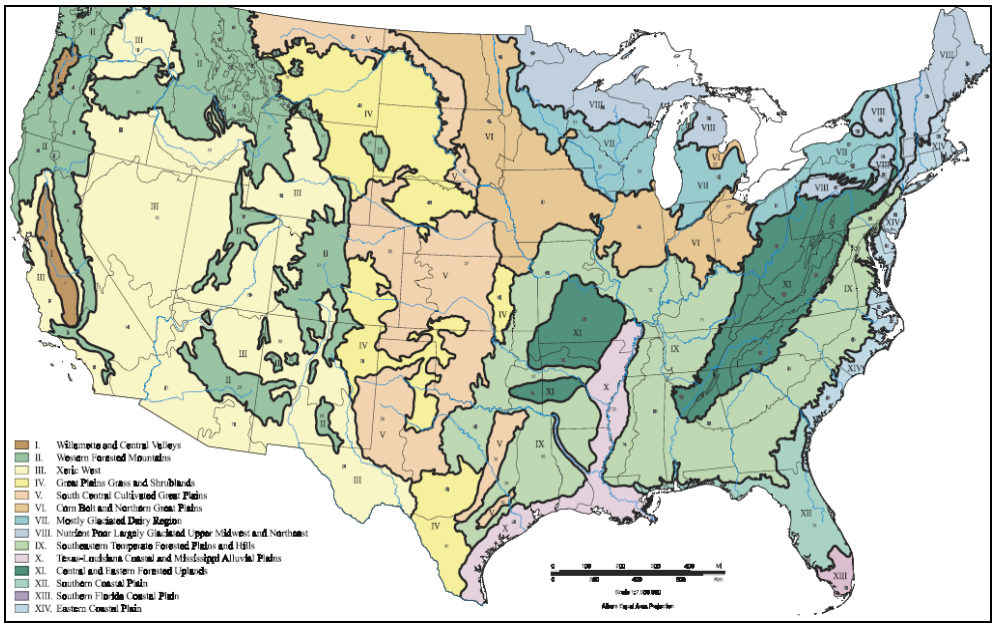


Figure 17. Aggregations of level III USEPA ecoregions for the national nutrient strategy (USEPA publication 822-B-01-012, 2001).

# VITA

Themis Stone was born in Denver, Colorado and lived in Texas, Georgia and Tennessee for a number of years. She presently lives in North Carolina and is employed by the Natural Resources Conservation Service as a Soil Conservationist. She has degrees from Hampshire College and Central Carolina Community College in Social Sciences and Sustainable Agriculture, respectively. She hopes someday to develop a solid crop rotation for her land which includes grains for Bread Uprising and forage legumes for a number of honey bees.