ABSTRACT

Title of thesis:	PHYSICAL AND POTENTIOMETRIC CONSTANT OF FERROUS AND FERRIC PHYTATE APPLIED TO ORGANIC PHOSPHATE TRANSPORT IN POORLY DRAINED SOIL

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Inositol phosphates are metabolically derived organic phosphates that increasingly appear to be an important sink and source of phosphate in the environment. Inositol hexa*kis* dihydrogen phosphate or phytic acid is the most common inositol phosphate in the environment. Iron is abundant in many terrestrial systems. Mobility of phytic acid iron complexes are potentially pH and redox responsive. Ferric and ferrous complexes of phytic acid were investigated by proton nuclear magnetic resonance spectroscopy, enzymatic dephosphoralation and potentiometrically in solution. The redox potential and concentration of iron were measured in a soil column containing a benchmark poorly drained soil from Maryland (Elkton). Ferrous phytate was found to form quickly and persist for a longer period then ferric phytate. Dissociation constants were 1.113 and 1.186 and formation constants were 0.899 and 0.843 for ferric and ferrous phytate respectively. Enzymatic dephosphoralation recoveries supported the magnitude of the kinetic and equilibrium rate constants.

PHYSICAL AND POTENTIOMETRIC CONSTANT OF FERROUS AND FERRIC
PHYTATE APPLIED TO ORGANIC PHOSPHATE TRANSPORT IN POORLY
DRAINED SOIL.

By

Lynne Heighton

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Introduction

Phosphorus, one of the macro nutrients necessary for all life is present in most

cells between 2-4% on a dry weight basis (Karl, 2000). The role of phosphorus

ecologically is unchallenged, but for a variety of reasons its biogeochemical cycle is not

completely understood. The integral part that phosphorus plays in biological processes

complicates identification of organic phosphorus species, their environmental

concentration and their persistence in the environment. The flux of inorganic phosphates

to organic phosphorus species is biologically driven and ubiquitous. In many fresh water

systems phosphorus concentration are too small to be accurately measured. Often surface

waters participating in photosynthesis are isolated from phosphorus rich bed sediments

by thermo clines. In such instances measuring the phosphate ion organic phosphate flux

generated by biological activities is below instrumental detection limit (Hudson, 2000).

Hudson, 2000 found that in measurements of 56 North American lakes that

phosphate levels were two to three times lower then previously thought. Phosphate

concentrations of 50 pM or less were capable of sustaining an efficient and thriving

microbial community. Hudson, 2000 and Karl, 2000 in separate papers interpreted these

findings as trace phosphate levels in fresh water systems are the historical norm and rapid

microbial turnover times are ecologically more important then the quantity of phosphate

present in the system.

Clark et al, 1998 found that by using ^{31}P nuclear magnetic resonance and

tangential-flow ultrafiltration in a marine environment thattwo major classes of high

molecular weight phosphorus compounds were associated with dissolved organic matter,

phosphorus esters and phosphonates. In marine surface waters the phosphorus esters

were depleted when compared to the phosphonates but as the dissolved organic matter moved down the water column ratios stabilized and were approximately equal at depths. They further found that phosphorus was depleted from dissolved organic matter and that the carbon to phosphorus and nitrogen to phosphorus ratios increased indicating that phosphorus is the limiting nutrient in oligotrophic marine surface waters and that rapid cycling is ecologically more important then the quantity of phosphorus in the system.

Mono-phosphates have been found to be the primary organic species in soils but diesters and phosphonates are also found in soils or added to soil as animal manures, bio-solids or pesticides (Clark et al, 1998; Brinch-Pedersen et al, 1998). Inorganic phosphorus is a mineral bound element with very little atmospheric transport contribution to its cycle. A sometimes notable exception is atmospheric transport of phosphorus as particle associated dust (Brinch-Pedersen et al, 2002). Mineral phosphorus is accessed by the plant community as phosphate ion. Strategies to dissolve mineral phosphate stores involve weathering of parent rock containing phosphorus and acidification of plant root zones by plants and fungi able to excrete organic acids from their roots thus dissolving phosphate containing mineral stores (Brady and Weil, 2002).

The primary transport processes of the phosphorus cycle involve non-point source movement from soil systems to surface waters. The major non-point source transport mechanisms are believed to be overland flow processes. Erosion driven movement of sediment bound phosphorus and flood water containing dissolved phosphates are believed to contribute the majority of phosphorus to surface waters (Butler and Coale, 2005; Sims et al, 1988). Other non-point source transport processes involve leaching of phosphorus in soil pore water to surface waters. Factors controlling phosphorus

movement into the dissolved phase include the oxidation reduction potential, the amount and type of clay present in the soil and the fraction of phosphorus present as an organic phosphorus species (Young and Ross, 2001; Turner and Haygarth, 2001; Sims et al, 1998; Anderson, 1980; Cosgrove, 1977).

Undoubtedly, anthropological activities have played a major role in transporting phosphorus from mineral deposits to surface waters. The unprecedented acceleration of food production can in part be contributed to phosphorus mining and redistribution as fertilizer. Crops utilize about 10% of the mineral fertilizers applied to them; the remainder is thought to be complexed in the soil and to have differing degrees of availability depending on factors such as soil organic content, clay content, pH and temperature (Cosgrove, 1977). The concentration of livestock production to relatively small geographical areas has lead to concurrent increases in manure production. It is economically unfeasible to transport the manure away from these centers of agricultural livestock production and the quantity of manure generated frequently exceeds the cropping needs of the area leading to stockpiling and composting of manures rich in organic phosphorus (Brinch-Pedersen et al, 2002; Sims, 1998; Gburek et al, 1996).

The parameters that govern fate and transport of phosphorus in soil systems are the subject of great scrutiny due to the impact of phosphorus on surface water quality. Because of the work done by Karl, Hudson, Clark and others trace levels of phosphorus in aquatic environments is likely the healthy norm in many if not all aquatic ecosystems. Understanding the entire biogeochemical cycle of phosphorus not just the primary transport processes is essential to management practices that will ensure food production and concurrently protect water quality.

Although, phosphorus transport by subsurface leaching is not thought to be the primary transport mechanism in soil systems, it is likely an important contributor in many geographic areas. Investigation of phosphorus movement by subsurface leaching in areas that are stockpiling manures or have historically over fertilized for phosphorus, have a flat landscape that does not rapidly drain, contains some clay, and readily support reducing conditions may be important contributors of phosphorus to surface waters.

Organic phosphorus has been a traditionally neglected pool in the phosphorus budget. Understanding the fate and transport of organic phosphorus is a critical component to the understanding its environmental role and impact on the phosphorus cycle. The monophosphate phytic acid is acknowledged to be the major form of organic phosphorus in soil systems and manures (Jayasundera, S et al. 2005; Hansen et al. 2004; Turner et al, 2003). Phytic acid is not responsive to oxidation reduction reactions itself but it will form complexes with metals that are redox responsive.

The goals of this thesis were to investigate phytic acid by using published acid disassociation constants (pKa) to generate the fractional species change due to changes in pH. Phytic acid formation constants for ferric and ferrous iron were also derived from proton nuclear magnetic resonance spectra (H-NMR). The magnitudes of the H-NMR derived constants were supported by measuring the phosphate recovered from the enzymatic dephosphoralation of phytic acid using the enzyme phytase. Ferrous and ferric iron speciation was measured potentiometrically and used to calculated the redox potential in a poorly drained soil column collected from a flat landscape that has been in a traditional corn soybean crop rotation, and has less then 10% iron oxide containing clay.

Chapter I: Speciation of Phytate is pH Dependent

Inositol phosphates are metabolically derived organic phosphates that increasingly appear to be an important sink and source of phosphate in the environment (Jayasundera, S et al. 2005), (Hansen et al. 2004), (Turner et al. 2001).   Inositol hex*kis*phosphates exhibit twelve acid protons in solution. Acid dissociation constants have been published for myo-inositol hex*kis*phosphate or more commonly called phytic acid or phytate (Champagne, 1988). A fractional speciation diagram was generated from this data (Figure 1). The pH driven acid/base speciation changes of phytic acid is important to understanding its ability to complex metals in soil and aqueous ecosystems. Metal-ligand interactions are known to change solubility and mobility in response to pH (Strumm and Morgan, 1996; Morel and Hering, 1993). Phytic acid acting as ligand is likely participating in such reactions.

Figure 1. Speciation of myo-inositol hexa*kis* dihydrogen phosphate (phytic acid) from acid dissociation constants (pKa).
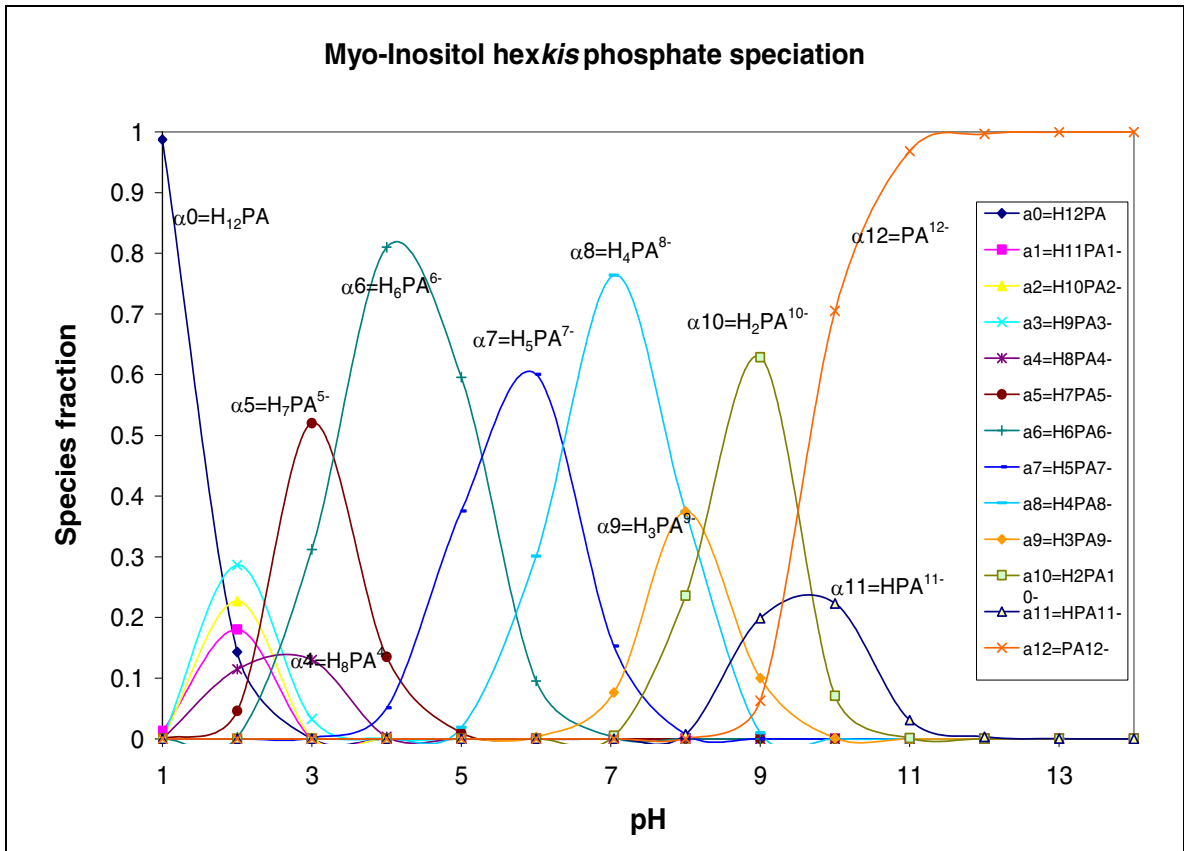
Methods

An excel spread sheet was used to calculate and graph the pH driven fractional species change of phytic acid (Strumm and Morgan, 1996; Morrel and Hering, 1993). The calculation was preformed at one unitintervals from pH 1 to 14. Each species (α) fraction was generated by the disassociation of a sequentially lost proton. The acid dissociation constants K govern the pH of the proton loss. The number designation associated with each fraction (α) represents the protons removed from the molecule and identifies each fraction.

$$\alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 + \alpha_4 + \alpha_5 + \alpha_6 + \alpha_7 + \alpha_8 + \alpha_9 + \alpha_{10} + \alpha_{11} + \alpha_{12} = 1$$

α_0 = fraction of species in the form $H_{12}A$

α_1 = fraction of species in the form $H_{11}A^-$

.

.

αj = fraction of species in the form HjA

The general form of speciation for a polyprotic acid H^nA is

$$\alpha_0 = \frac{[H^+]^n}{D}$$

$$\alpha_1 = \frac{K1[H^+]^{n-1}}{D}$$

.

.

$$\alpha j = \frac{K1K2\ldots..Kj[H^+]^{n-j}}{D}$$

where $D = [H^+]^n + K1[H^+]^{n-1} + K1K2[H^+]^{n-2} + \ldots\ldots + K1K2K3\ldots\ldots Kn$

Results and Discussion

Results demonstrate that for any environmentally relevant pH, at least one fifth of phytate molecules will not have the same charge as the most abundant phytate species. For example pH 6, the major species of phytate (H_4PA^{-6}) has a net charge of -6, but (H_3PA^{-5}) which has a -5 charge and (H_5PA^{-7}) which has a -7 charge will also be present. The average charge from both the 20% (H_3PA^{-5}) and the 20% (H_5PA^{-7}) remains -6, but at the molecular level, 40% of phytate will have the physical chemical properties of either (H_3PA^{-5}) or (H_5PA^{-7}), and not of (H_4PA^{-6}).

At pH 7 phosphate buffers are nearly an equimolar ratio of monobasic ($H_2PO_4^{-1}$) and dibasic (HPO_4^{-2}) phosphate. The phosphate speciation does not change between pH 5 and 7: only the relative abundance between these two species changes. For similar reasons, phytate speciation changes with pH predictably for the each of the twelve acid dissociation constants (pK_as) of phytate. Between pH 5 and 7 four species of phytate account for nearly all the phytate present. However, unlike phosphate, the relative amounts of all four species, not just two, change systematically as one alters pH. The co-existence of multiply charged species at any ecologically relevant pH is an essential part in understanding phytate ligand solubility and organic P mineralization. The strength and affinity of any cation associated with a phytate species can be expected to change proportionately to the unit anion charge. Binding will not necessarily prefer the most abundant phytate species and multiple species of phytate can simultaneously support multiple cationic ligands. The multiply charged species of phytate present at any one pH often precludes stoichiometric complexation of phytate with single metal cations. At pH 6 (H_4PA^{-6}) can form a neutral complex with two Al^{+3} cations, but any Al^{+3} complexes with (H_3PA^{-5}) or (H_5PA^{-7}) will simultaneously be charged.

The pH dependence of phytate associations with trace metals, sesquioxides, colloidal clays, and fluvic and humic material has been reported (Stewart et. al. 1987). However, the composition of such phytate-ligand complexes will always be heterogeneous. Competitive binding will always occur among the multiple phytate species for any particular ligand present. A ligand can more preferentially bind to less abundant species of phytate with a higher net charge than to a more abundant phytate species with a lower net charge. Elucidation of the mechanisms and hierarchy of

complexation requires identification of the distribution of charge on individual phytate sites with changes in speciation.

Enzymatic dephosphorylation occurs via two currently recognized forms of phytase, 3-phytase (E.C.3.1.3.8), which is microbial in origin and is most active at pH 2.5 and 5.5 and 6-phytase (E.C. 3.1.3.26), which is produced by some plants and is most active at pH near 5 (Pallauf, 1997). Optimal enzymatic activity could be due to pH dependent properties of the enzyme. A simpler explanation is that phytase-3 or -6 binds preferentially to specifically charged sites on the phytate molecule and that the charges on the phytate are strongly pH dependent. From Figure 1, H_8PA^{-4} and H_9PA^{-5} are the most abundant forms at pH 2.5; H_4PA^{-7} is the most abundant form at pH 5 to 5.5. The H_4PA^{-7} species must have at least one phosphorus site on the molecule with a –2 charge and the P site with the –2 charge could be the first site of enzymatic dephosphorylation. Reactions at pH 2.5 may be more complex, but any molecular mechanism to explain why 3-phytase is different from 6-phytase needs to adequately address phytate speciation.

Equimolar ratios of transition metal-phytate complexes have been shown to inhibit enzymatic dephosphorylation rates (Dao, 2003). The phytate complexation and phytase enzyme activity occur concurrently and correspondingly the activation or deactivation that can occur from mineral complexation. At equimolar ratios of a transition metal and phytate, cation binding to the –2 anionic site would makes that site less available for enzymatic activity.

Speciation is innately part of the fate and transport of phytate. Computer models that predict the fate of organic phosphorus in soils are seriously incomplete if only one form of organic phytate is assumed to be present. Between pH 6 and 7.5, five species of

phytate are present and as much as 60% of the phytate will not have the same molecular charge as the predominant molecular species. Analyses of phytate in soil systems designed to extract specific phytate species will measure only a fraction of the phytate species actually present in soil. Moreover, because each phytate molecule contains six phosphorus atoms, an error of about 8% in quantifying the amount of soluble phytate extracted would equal a 50% error in the measurement in the contribution of organic phosphorus from phytate to the total phosphate pool. Un-extracted phytate species can be a significant reservoir that is routinely undefined or misidentified in analysis of soil phosphorus. Accurate environmental mass balance and kinetic calculations for organic and inorganic phosphorus in soils is incomplete unless speciation of phytate is explicitly part of the accounting.

Chapter II: Kinetic and Equilibrium Constants of Phytic Acid, and Ferric and Ferrous

Phytate Complexes derived from Nuclear Magnetic Resonance Spectroscopy

The fate and transport of phosphorus (P) is an emergent problem affecting

environmental resources. Long time land application of P enriched manure has been

implicated in the saturation of available P binding sites in many terrestrial, wetland and

sediment systems. Transport of soluble- or particle- associated P by overland flow and

possibly by subsurface leaching has increased eutrophication in waterways on the east

coast of the United States, Europe and increasingly in Asia (Sims et al.1998).

The P cycle is not completely elucidated and resists conforming to reliable mass

balance calculations. Inclusion of organic forms of P in mass balance calculations may

yield more satisfactory results. The most prevalent form of organic P is Myo-inositol-1,

2, 3, 4, 5, 6-hexakis dihydrogen phosphate or more commonly phytic acid (PA) (Bar-

Yosef et. al.1993). Phytate is the non-protonated anion. Phytic acid contains six mono-

phosphate groups on a six carbon ring (Figure 2).

Figure 2. Structure of Myo-inositol-1,2,3,4,5,6-hexa*kis* dihydrogen phosphate or more commonly phytic acid ($C_6H_{18}P_6O_{24}$)
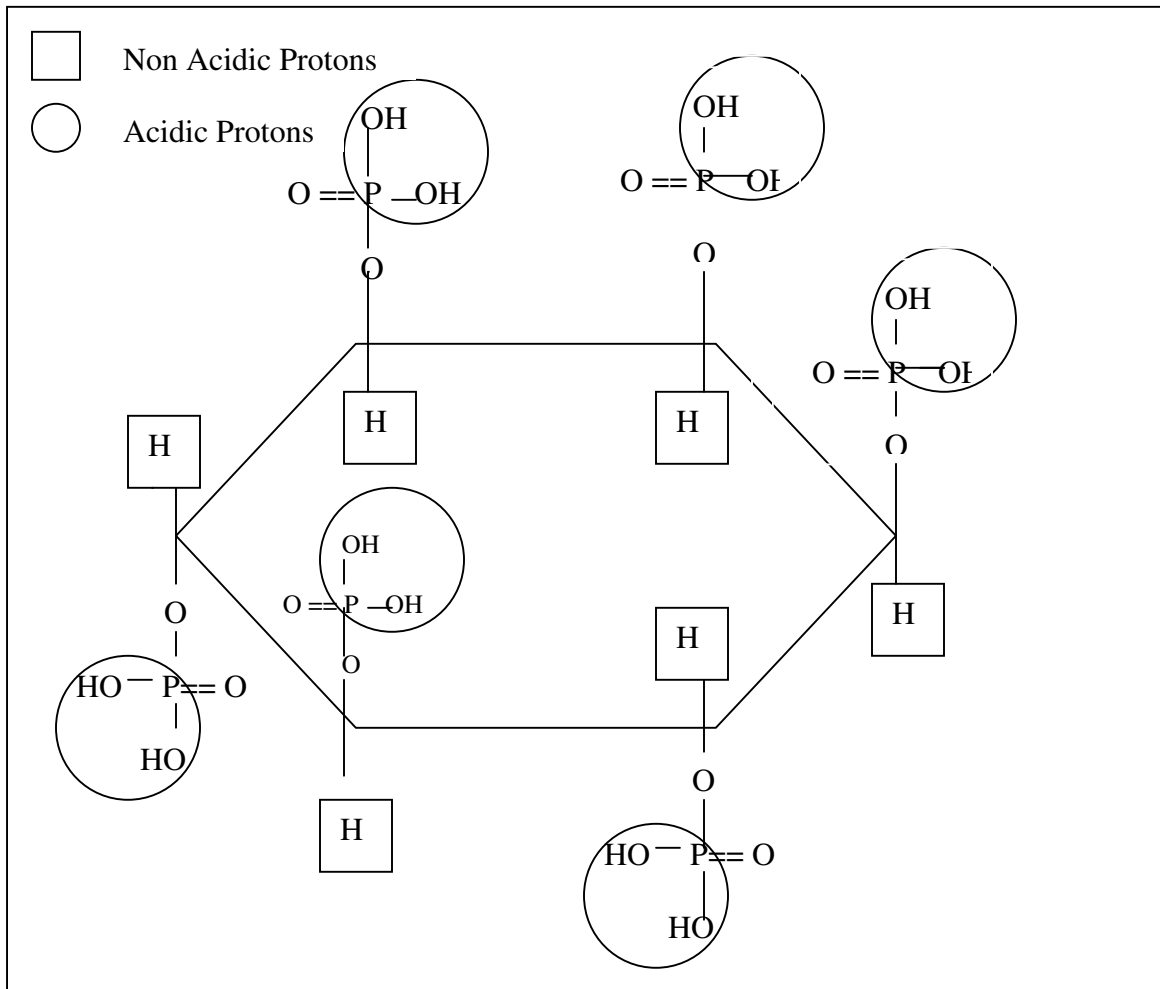
The environmental impact of the organic pool is biologically driven by uptake of soluble phosphate and repackaging as an organic phosphate. Phytic acid and its pentaphosphate analog represent 60% of the organic phosphate in soil (Bar-Yosef et al. 1993). Diesters such as nucleotides and phospholipids occur at much lower concentrations of 5% and <1% respectively. The diester compounds are derived from decomposing plant and animal material (Bar-Yosef et al.1993). Diesters are highly labile and rapidly recycled by biota. The monoester phytic acid has been identified as a phosphate storage molecule

involved in the germination of seedlings (Brinch-Pedersen et al. 2002). Monoesters such as phytic acid are less likely to be bio-available than diesters because of their high surface to charge ratio. Aluminum and ferric complexes of phytic acid have been found to inhibit enzymatic dephosphorolation, indicating a potential to persist in soil systems (Dao 2003).

Phosphorus levels in soil are dependent on the mineral composition of the soil, the anthropological inputs of fertilizer, decomposing detritus and outputs of crop removal or erosion. Concentrations of phosphorus in soil range 300 to 4700 mg Kg^{-1}. The organic fraction of the total phosphate concentration can vary from 20 to 80% in surface soil horizons (Brady and Weil 2000). Iron oxide and aluminum oxides form strong recalcitrant complexes with phosphates that in the case of iron are susceptible to oxidation reduction reactions as well as pH dependent equilibria (Stewart and Tiessen 1987).

Available iron and aluminum oxide reaction sites in soil systems may be saturated in some instances especially in non-tropical soils that have moderate levels of oxides (Brady and Weil 2002; Brinch-Pedersen et al. 2002). Historical increases in agricultural production were fueled by increases in fertilization rates of phosphates as well as other nutrients. Transport of phosphate from upland terrestrial to aquatic systems may be related to the capacity of a soil system to complex phosphate with iron and aluminum oxides. Equilibrium constants for inorganic phosphates are well characterized. Solubilization of iron phosphate complexes is known to respond to changes in the oxidation reduction status. Equilibrium, kinetic and solubility constants as well as oxidation reduction potentials for many organic phosphates such as phytic acid and its complexes are unavailable in the literature.

Phytic acid can be enzymatically dephosphorolated by phytases from plant, microbial and animal sources. *Aspergillus ficcum* is a soil microbial component that produces 3-phytase (E.C. 3.1.3.8). This phytase acts on the C_3 phosphate group preferentially but eventually dephosphorolates all six phosphates on the PA molecule (Pallauf and Rimbach 1997).

Phytic acid and phytases are supplied naturally and also anthropologically, entering terrestrial systems in manure and as plant decomposition by-products. Phytic acid is found at high concentration in small grain producing plants. As much as 70% of dry weight mater in germinating seedlings is phytic acid (Brinch-Pedersen et al. 2002). The high content of phosphate per molecule of phytic acid makes it a logical choice for a livestock phosphate supplement. Where phytases are not produced by the animal, they may be supplied in the feed as for poultry, or the phytic acid molecule can be enzymatically dephosphorolated before feeding, as in swine livestock production (Brinch-Pedersen et al. 2002). Phytic acid has been found in bovine, swine and poultry manure (Brinch-Pedersen et al. 2002). It is clear that the addition of phosphate to the diet increases growth rate but the efficiency of digestion may not be improved and higher levels of phosphate may occur in manures of animals fed on a phytic acid, phytase supplement. The fractionation of the inorganic and organic pools of phosphate in manure, soil and water is an area that should be examined (Sims, 1994; Brinch-Pedersen et al. 2002).

Iron is a common component in many soils on the east coast of the United States, making it a potentialmetal -phytic acid complex of interest. Determination of kinetic,

equilibrium and solubility constants for complexes of phytic acid will contribute to the understanding of the fate and transport of phytic acid and its role in the phosphorus cycle.

The objective was to use proton nuclear magnetic resonance to derive kinetic (k) and equilibrium constants (K_d), (K_f) for ferric and ferrous phytate. In addition, the relative magnitude of the kinetic constants was supported by the concentration of phosphate produced by enzymatic dephosphorolation of phytic acid and ferric and ferrous phytate.

Materials and Methods

Nuclear Magnetic Resonance

Phytic acid in an aqueous solution of 50 % D_2O was pH adjusted to 4.0, 6.0 and 8.0 and monitored on a 300 MHz proton nuclear magnetic resonance spectroscopy (H-NMR). Complexes of ferric and ferrous iron where then monitored at pH 4.5. The ferrous complex was protected from oxidation by nitrogen gas. The NMR tube was permanently sealed by melting the open end of the tube with a blow torch.

Kinetic and Equilibrium Calculations from NMR Spectra

Kinetic constants (k) for hydrogen, ferric and ferrous complexes of phytate were derived by monitoring the spectral shift of each complex with proton nuclear magnetic resonance spectroscopy (H-NMR). The twelve protons associated with the six phosphate groups have acid dissociation constants that participate in trace metal chelation but do not produce the H-NMR spectra being monitored. The six non-acidic protons on the carbon ring respond to the trace metal chelated with the phytate molecule; producing a spectral shift that is a direct measurement of the kinetic rate constant for the metal-phytate complex..

The spectral shifts were measured in ppm hertz (One hertz equals a reciprocal second). The kinetic formation constant was measured as the change in location of the highest peak on the x axisx 10^{-6} s^{-1}.

$$nCation + Anion \overset{k}{=} Cation\text{-}Anion\ Complex \qquad [1]$$

The cation was hydrogen, ferric or ferrous iron. The anion was phytate. The thermodynamic constants (K_f) and (K_d) were derived from the ratios of the hydrogen and metal kinetic constants when the activities of the reactants are in equilibrium (Levitt, 2003).

$$K_f = k_{metal}/k_{hydrogen} \qquad [2]$$

$$K_d = k_{hydrogen}/k_{metal} \qquad [3]$$

Enzymatic Analysis

Ferric and ferrous complexes of phytic acid were incubated at pH 4.5 and 24^oC with 0.03 units/ml of *Aspergillus ficcum* (E.C.3.1.3.8) purchased from Sigma. Phytic acid and metal-phytate complexes were prepared in mM ratios of PA to Iron at concentrations ratios of 1:0, 1:0.6, 1:0.3 and 1:0.1. Incubation occurred over a time course of 15, 30, 60, 90, 1080, 1440 and 2880 minutes. The highest concentration treatment of ferric and ferrous phytate was governed by the solubility of the ferric phytate treatment. The 1mM PA and 0.6mM ferric iron were allowed to equilibrate at pH 4.5 overnight to determine visible precipitate. The solutions used for the analysis were prepared just prior to the addition of the enzyme solution.

The concentration ratios were incubated with enzyme present and enzyme absent representing samples and controls respectively. The enzyme was denatured by boiling

for 15 minutes, acidified by adding of 200ul of 1M hydrochloric acid to prevent

precipitation of ferric phosphate immediately after boiling and frozen until analysis.

Liberated phosphate ion was analyzed colormetrically by an ascorbic acid molbdate

reaction (Murphy and Riley, 1962). Controls were subtracted from matching samples.

The ferrous complexes were protected from oxidation by a blanket of nitrogen and sealed

in polyethylene tubes. Controls and sample were prepared and analyzed in triplicate.

Statistical Analysis

The program SAS 5.1 (SAS Institute 1989) was used to test for statistical

differences in phosphate concentration across treatments for the reaction times of 1080,

1440 and 2880 minutes. Analysis of covariance (ANCOVA) was used to assess

differences through time and among treatments. The reaction time 2880 minutes was

chosen to calculate the percent recovery of the 1mM phytic acid treatment. All analysis

were considered statistically significant at $p < 0.05$. The null hypothesis stated no

difference between the concentrations of phosphate recovered from each treatment. The

alternative hypothesis stated that one or more treatments have significantly different

concentration of phosphate recovered from the enzymatic dephosphoralation. A pair

wise analysis of variance was preformed for each possible pair of treatments. For k

number of treatments k (k-1)/2 different treatments were compared. The means of

treatments with significant variance were ranked in order of magnitude and a multiple

comparison test was used to determine if a difference existed between means. A

calculated q value was generated according to equation [4].

$$q = \frac{\text{mean of treatment k1} - \text{mean of treatment k2}}{\text{standard error}} \qquad [4]$$

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where standard error = square root of error mean square from the analysis of variance of the means divided by the number of samples in the treatments.

The calculated q was compared to a tabulated q. When the calculated q was found to be greater then tabulated q the null hypothesis was rejected and the treatments were found to be different. The tabulated q was based on possibility of falsely rejecting at least one null hypothesis, the error degrees of freedom from the analysis of variance and the total number of means being tested.

Results

Constants derived from Nuclear Magnetic Resonance Spectra

Changes in pH did not affect the H-NMR spectra of phytic acid making it an appropriate method for monitoring changes in kinetic rate constants (Figure 3). Spectral shifts were attributed to the cation associated with the phytate molecule. The kinetic rate constant for phytic acid, ferric phytate and ferrous phytate were 4.69, 4.21 and 3.95×10^{-6} s^{-1} respectively (Figure 4). The experimental equilibrium rate constant for the dissociation of ferric phytate (K_d) was 1.114 and the equilibrium dissociation constant (K_d) for ferrous phytate was equal to 1.186. The experimental formation constants (K_f) are 0.899 and 0.843 for ferric and ferrous phytate respectively.

Figure 3. Proton Nuclear Magnetic Resonance (1H NMR) indicates no peak shift due to pH in aqueous samples of phytic acid making NMR an appropriate tool for monitoring phytic acid metal binding constants.
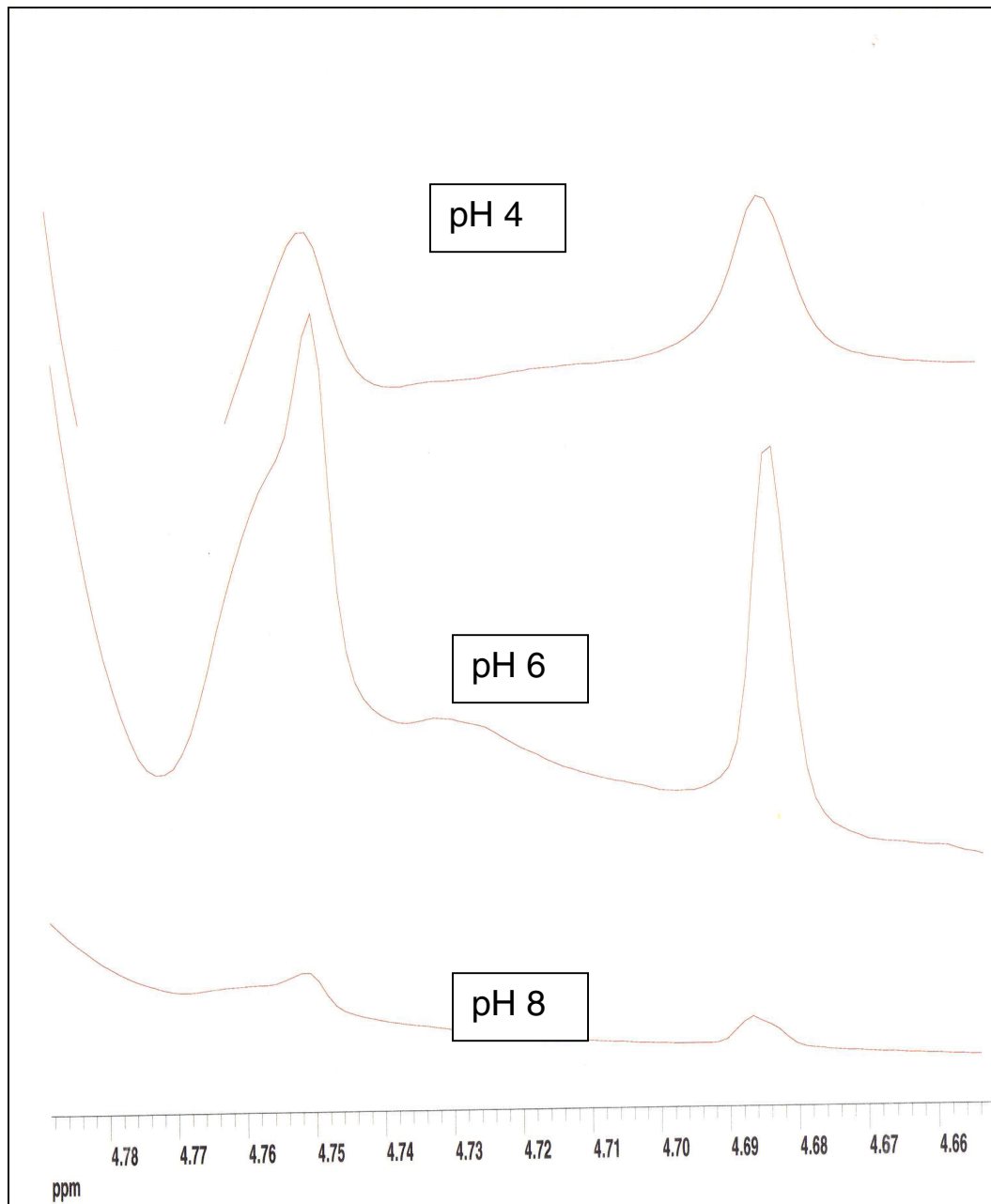
Figure 4. Proton NMR Spectra of Ferric and Ferrous Iron Complexes of Phytic Acid at pH 4.5 NMR spectra of phytic acid and phytic acid complexes of ferric and ferrous iron show shifts in spectral peak maxima. Observing the largest peak of each spectrum: changes in ppm, when converted to s^{-1} yield the kinetic rate constant for the formation of the PA-Metal complex. The phytic acid spectra show the kinetic acid disassociation constant. Phytic acid = 4.69×10^6 s^{-1}, the complex of ferric phytate = 4.21×10^6 s^{-1} and the ferrous phytate = $3.95 \times 10^6 s^{-1}$.

Ferric and Ferrous Complexes of Phytic Acid
Top Spectrum - Ferrous
Bottom Spectrum - Ferric

Ferrous phytate

Ferric phytate

4.50   4.40   4.30   4.20   4.10   4.00   3.90   3.80   3.70   3.60   3.50

ppm
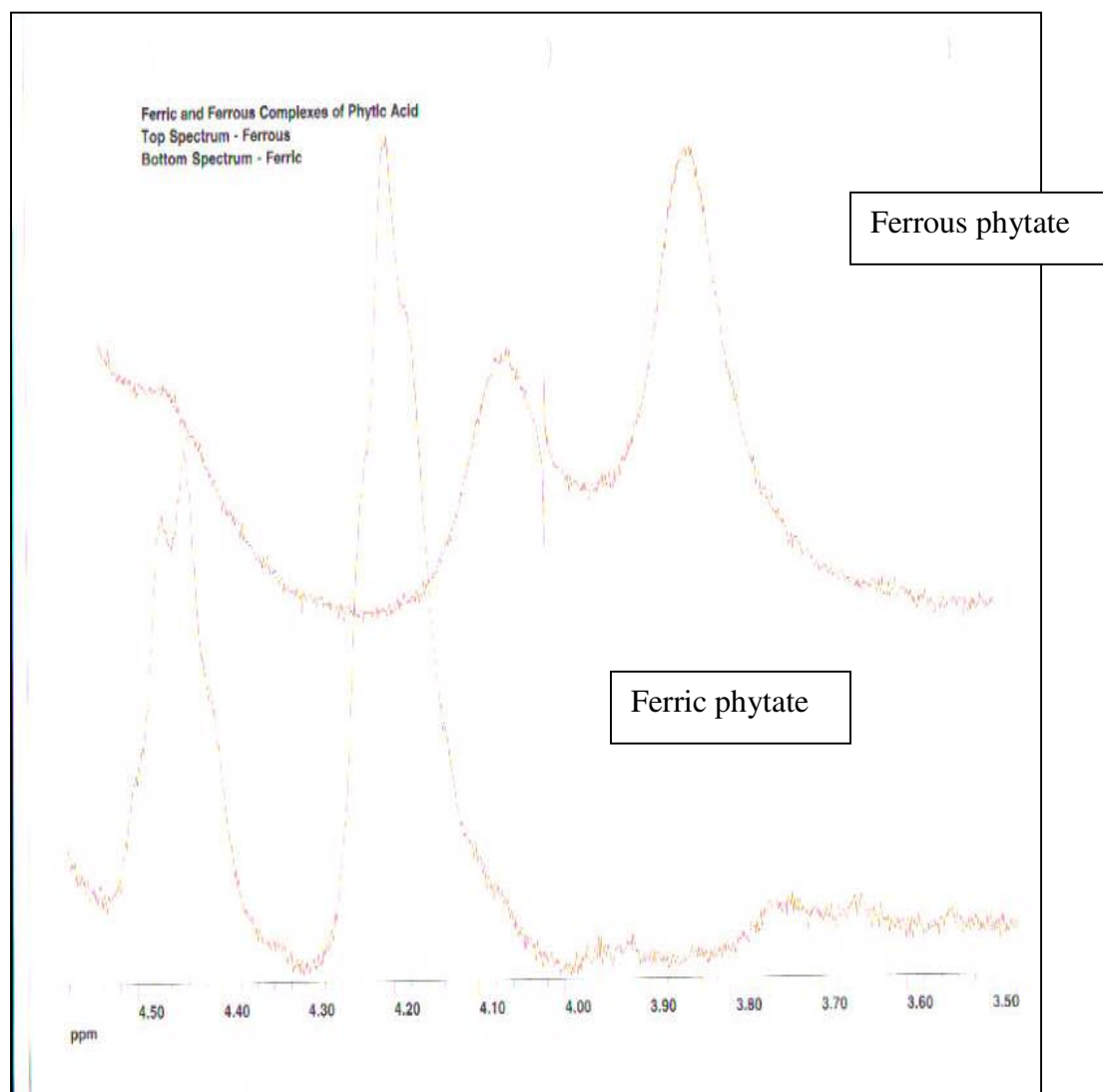
Enzymatic Dephosphorolation

Significant differences existed among all of the treatments with theexcept ion of ferric phytate with a treatment concentration 0.6mM and ferrous phytate with a treatment concentration of 0.3mM (Table 1: ANCOVA F=84.16; P<0.0001). The phosphate model was significant (ANCOVA r^2=0.989; F=343.63; P<0.0001). Treatment and Time covariant with Treatment are significant (ANCOVAF =84.16 and 12.47 respectively; P<0.0001). The independent time variable was not significant (ANCOVA F=3.45; P=0.0691). Table 1 summarizes the average (n=3) phosphate (ug/ml) liberated by the enzyme phytase over the series of incubation times and gives the percent recoveries for time=2880minutes. Phytic acid with a concentration of 1mM or 57ug/ml was 98 % dephosphoralated at the time 2880 minutes. The non enzyme controls were not reported in Table 1. The average and standard deviation of the control data points was 3 $\pm$ 1 (N=147). The percent recovery of phosphate ion from the enzymatic dephosphoralation of phytic acid must account for the 6mM of phosphate ion per 1mM of phytic acid. The molecular weight of phosphate ion is 95 g/mole. The expected concentration of phosphate ion from a 1mM solution of phytic acid was calculated as in equation [5].

$$(0.0006 \text{ mol/L PO}_4^{2-}) \times (95 \text{ g/mol}) \times (1\text{L}/1000 \text{ ml}) \times (1*10^6 \text{ ug/g}) = 57\text{ug/ml PO}_4^{2-} \quad [5]$$

Table 1. Seven treatments were prepared each contained 1mM of phytic acid. Three of the six treatments contained ferrous phytate in the mM concentrations listed. The remaining three treatments contained ferric phytate in the concentrations listed. Recoveries of enzymatically dephosphoralated phosphate ion are in ug/ml. The tukey group letters denote statistical difference. Treatments with the same letter are not statistically different.

Treatment	Concentration (mM) in each Treatment	Concentration (ug/ml) of phosphate recovered	% HPO^{2-} Recovered at T=2880	Tukey Groups
Phytic acid	1.00	56.0 ± 0.5	98	A
Ferric Phytate	0.1	38.6 ± 2	68	B
Ferrous Phytate	0.1	34.6 ± 1	61	C
Ferric Phytate	0.3	30.5 ± 2	54	D
Ferrous Phytate	0.3	19.1 ± 0.5	33	E
Ferric Phytate	0.6	17.2 ± 3	30	E
Ferrous Phytate	0.6	9.7 ± 1	17	F

Discussion

Ferric and ferrous iron form complexes with phytic acid that can persist in solution and resist dephosphoralation by the enzyme phytase. The experimental kinetic constants derived from proton nuclear magnetic resonance indicated that ferrous phytate forms faster and dissociates at a slower rate then ferric phytate although they are of the same order of magnitude.   The H-NMR derived findings are supported by the overall enzymatic dephosphoralation recoveries of phosphate over a 2-day period. The phosphate recovered from the enzymatic dephosphoralation of ferrous phytate was

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significantly less then the phosphate recovered from ferric phytate and phytic acid alone. The phosphate dephosphoralated from ferric phytate was also significantly less then the phosphate dephosphoralated from uncomplexed phytic acid.

Ferrous phytate is soluble at higher pH then ferric phytate but at atmospheric levels of oxygen is rapidly converted to ferric phytate and precipitated out of solution. In anoxic ground water, ferrous phytate has the potential to resist microbial decomposition from the enzyme phytase. This ability to persist may enable ferrous phytate to be transported by subsurface leaching. Agricultural areas that are prone to saturated soil conditions on a seasonal basis or are continually saturated and adjacent to agricultural areas receiving manure fertilizers have the potential to export phosphate as ferrous phytate.

Young and Ross, 2001 found that 13 of 14 seasonally flooded soils from the Champlain Valley in New York increased pore water phosphate concentrations over a 60 to 90 day period, additionally five of the soils exhibited increased ferrous iron concentration concurrently with phosphate pore water increases. The soils that exhibited increased ferrous iron and phosphate concentrations in pore water exhibited decreased flood water phosphate concentration indicating retention of phosphate in the pore water possibly in association with ferrous iron. In soils with no ferrous iron present the pore water to flood water phosphate concentration ratios ranged from 1.0 to 3.3 suggesting equilibrium between flood water and pore water or retention of phosphate in pore water. Young and Ross hypothesize that phosphate solubility and transport are mediated by the available phosphate status and seasonal anoxia of saturated soils. The ratio of pore water

to flood water phosphate concentration implicates subsurface leaching as a likely transport mechanism in saturated soils.

Butler and Coale, 2005 investigated four coastal plains soils in Maryland and concluded that phosphorus losses to surface waters by overland flow processes was more of a concern then subsurface leaching of phosphorus. The fraction of the phosphorus pool investigated by Butler and Coale were water extractable phosphorus and soil test phosphorus. Water extractable phosphorus is a measure of phosphate immediately available to plants and soil test phosphorus is a measure of plant available phosphorus over the growing season. Phytic acid is unlikely to fall into either of those fractions because of its potential to persist in soil systems and resist dephosphoralation. The six mono-phosphate groups associated with phytic acid would not be evident in either fraction because phosphate derived from phytic acid requires enzymatic dephosphoralation.

The conversion from ferric iron to ferrous iron is mediated by oxidation reduction (redox) reactions. Phosphorus has a valence of +5 and does not respond directly to redox processes, but iron cations associated with a phytate molecule will respond to changes in redox potential. Phytic acid acting as a ligand can change the potential of the redox process, making investigation of the iron phytate redox couple an area of interest for further research. Redox processes in many soil systems are temporal and known to reflect seasonal fluctuations of the water table. Landscapes that can support reducing soils are also highly variable spatially (Richardson et al, 2001). Export of phosphate as ferrous phytate and storage of phosphate as ferric phosphate is possible and an area of

interest in the phosphorus cycle. Investigation of iron complexes in soil systems should incorporate temporal and spatial components.

Celi et al, 1999 investigated the adsorption of phytic acid and phosphate ion on common soil clays goethite, illite and kaoliniteusi ng fourier-transform infrared (FT-IR) spectroscopy found that the mechanism for absorption was the phosphate groups on the phytic acid molecule. They inferred that phosphate groups on phytic acid form complexes with surface Fe-OH groups on the clay in an analogous manner to phosphate ion. Goethite had the greatest capacity to complex phytic acid with four of the six phosphate groups postulated to be participating in the complexation reaction. Illite and kaolinite although less effective then goethite in complexing phytic acid were found to be more effective then phosphate ion. The illite and kaolinite had two ferric hydroxyl sites on the clay complexed by the phytic acid. The results suggest displacement of phosphate ion by phytic acid in soil systems containing goethite, illite and kaolinite clays due to lowering of the bond energy of P=O group and the stabilization of multiple P-O bonds (Celi et al, 1999). The displacement of phosphate ion by organic phosphates has been reported by Anderson, 1980 and Cosgrove, 1977. The uncomplexed phosphate groups of the phytic acid clay complexes negatively change the surface of the clay and effectively decreased the size of the clay aggregates leading to greater overall clay surface area for absorption of phytic acid, incomplete mineralization, accumulation in soils and a negative impact of phosphorus bioavailability (Celi et al, 1999; Stewart and Tiessen, 1987).

Conclusion

Phosphorus fate and transport is an emergent problem impacting sensitive aquatic ecosystems. Long time land application of phosphate enriched manure has been

implicated in the saturation of available phosphate binding sites in many terrestrial, wetland and sediment systems. Phosphate binding sites in many soils are believed to be clays such as goethite, illite and kaolinite that contain exposed oxides on their surface layer. Transport of organic and inorganic forms of phosphate from terrestrial to aquatic systems is not completely understood but in the case of oxide containing clays must involve acid base or oxidation reduction reactions. Phytic acid has a large charge to mass ratio and an ability to chelate ferrous and ferric iron in solution. Iron is ubiquitous in many soils that receive phosphorus rich manure inputs making iron phytic acid complexes metal ligand reactions likely to occur in soil systems that contain iron oxides such as goethite. In solution the ferrous phytic acid complex exhibits a higher equilibrium constant and is soluble at relevant soil pH implicating subsurface leaching of ferrous phytic acid complexes as a likely phosphate transport mechanism. Ferric phytate is much less soluble than ferrous phytate at environmentally relevant pH and may be an important binding site for phytic acid in iron containing oxidized soil systems. Soil systems prone to seasonal or periodic soil pore water anoxia may support cycles of fixation and mobility of iron associated phytic acid complexes.

Inorganic phosphate is known to be to be particulate and sediment attached. It is easily transported with sediment in above ground overland flow. Ferric oxides form strong recalcitrant complexes with phosphates. Equilibrium and kinetic constants for inorganic ferric and ferrous phosphate complexes are well characterized. Complexes of ferric phosphates are susceptible to oxidation-reduction reactions as well as pH dependent equilibrium. Ferric and ferrous complexes of organic phosphates are not characterized. Determination of equilibrium constants of ferric and ferrous complexes of

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phytic acid will contribute to more accurate mass balance calculations in soils with iron content. Results suggest subsurface transport of phosphate, as a ferrous phytic acid complex from terrestrial systems to eutrophication sensitivewaterways is a possible and previously neglected pool of phosphate. Inclusion of organic forms of phosphorus in the phosphorus budget may lead to a more reliable mass balance calculation and a better understanding of the phosphorus cycle. A thorough understanding of the phosphorus cycle is an essential first step toward limiting phosphorus export to aqueous ecosystems.

Appendix I: Potentiometric measurement of ferric and ferrous species in soil
                columns containing Elkton soil series, Queen Anne County,
                Maryland

Abstract for Appendix I

Soil columns from Queen Anne County, MD were collected in order to have an applied test for proton nuclear magnetic resonance spectroscopy derived formation and dissociation constants of ferric and ferrous complexes of phytic acid. The concentrations of ferrous and ferric iron were measured potentiometrically at three levels within the column and in the column effluent with platinum working electrodes and a silver/silver chloride reference electrode in order to determine the oxidation reduction potential of goethite and ferrous iron redox couple within the column and the export of ferrous iron from the column.

Geographic areas that are likely support the export of phosphorus as ferrous phytate must be prone to development of anoxic soil pore water, contain iron oxide clays such as goethite, and have poor to moderate permeability and low slope. Correlation of landscape features or soil taxonomy to ferrous iron concentration in soil could be an indicator of phosphorus export as ferrous phytate by subsurface leaching.

Introduction

Ferrous phytate is a soluble complex at environmentally relevant pH and has been shown previously to form readily and resist microbial degradation. Ferric phytate is much less soluble but also resists microbial degradation from the soil microbe *Aspergillus ficcum*, a common soil constituent. Traditionally inorganic phosphorus as phosphate is thought to be transported in association with sediment in overland flow processes (Sims et al, 1998). The ferric ferrous redox couple is a control of phosphate ion solubility in

areas where iron is found in soil. Ferric phosphate is not highly soluble while ferrous phosphate disassociates releasing phosphate ion. Movement of reduced iron is readily observable in many soil systems as iron concentrations and depletions (Richardson et al., 2002). It could be expected that organic phosphates would behave in an analogous manner to inorganic phosphates but we have found that not to be the case based on equilibrium constants derived from nuclear magnetic resonance spectroscopy.

Organic phosphates have been thought to be highly labile and converted quickly to inorganic phosphates due to efficient dephosphoralation and recycling by the microbial community (Graf, 1986). We have shown previously that ferric and ferrous phytate resist microbial degradation. The stability and solubility of ferrous phytate implicate phosphorus transport by subsurface leeching of the organic monophosphate complex of reduced iron, ferrous phytate. Ferric phytate also resists microbial dephosphoralation; but with much lower solubility then ferrous phytate. It can possibly sequester phosphate as the oxidized ferric phytate.

Eutrophication has become a major problem in many east coast watersheds including the Delmarva Peninsula. A desire to control the outcomes of eutrophication, which by definition is an excess of nitrogen, phosphorus or both in surface water leading to excessive plant growth, oxygen depletion and in extreme cases fish kills is of intrinsic value as well as being of practical importance in maintaining clear and navigateable waterways for the traffic of goods, recreational uses such as boating and fishing and maintaining human and animal health (Hamilton et al., 1993).

Several naturally occurring factors such as the geology, topography, climate and hydrology in combination with anthropological activities such as grain crop production,

concentrated livestock production and urbanization lead to situations where eutrophication can occur. The Delmarva Peninsula is an example of such a situation. Comprised of the state of Delaware and the coastal portions of Maryland and Virginia bounded by the Chesapeake Bay it is part of the North American geological coastal plain. The Delmarva this is described as unconsolidated sediments composed of varying proportions of clay, sand and gravel (Schmidt, 1993). These sediments have not yet been compressed into solid sedimentary rock. This type of geology generally has high rates of infiltration and when combined with the low relief topography implicates ground water recharge as the primary hydrologic process in the undisturbed ecosystem. Within the ecosystem there are significant areas that do not have high rates of infiltration. Areas in agricultural production have used extensive ditching to remove water expeditiously thus expanding areas available for crop production (Schmidt, 1993).

The dominant soil order is Ultisol, which is a low nutrient soil that when used for crop production requires inputs of nitrogen, phosphorus and potassium in order support optimal plant growth. Alfisols are also found and are a high nutrient soils but they are a smaller representative proportion and frequently not available for cultivation due to location in the landscape and the position of the water table. Those parcels of land completely unsuited for agricultural uses are in many cases left to native vegetation, which was historically mixed hard wood forest or tidal marsh. The juxtaposition of well drained cultivated land, networks of drainage ditches close to sensitive waterways and an adequate supply of precipitation leads to the perfect scenario for eutrophication. (Hamilton et al, 1993)

We hypothesize that in areas capable of supporting reducing ground water and containing iron sesquioxides there is a potential for export of phosphate as ferrous phytate by subsurface leeching. A potentiostatic three electrode system, two platinum electrodes and a saturated Ag/AgCl reference electrode was used to measure the ferric and ferrous iron concentration within a soil column containing a poorly drained soil, USDA Elkton from Queen Anne County, Maryland. The effluent from the column was monitored for ferric and ferrous iron concentration by potentiostat and a standard curve measuring concentration of iron as a function of current. The Nernst equation will be used to calculate the cell potential according to equations [6] and [7] within the soil column and in the effluent.

$$E_{cell} = E_{(Fe3+, Fe2+)} - E_{(Ag/AgCl)} \qquad [6]$$

$$E_{(Fe3+, Fe2+)} = E^o + 2.3\frac{RT}{nF} \log\frac{[Fe^{3+}]}{[Fe^{2+}]} \qquad [7]$$

The oxidation reduction potential will be calculated according to equations [8] and [9].

$$Fe(OH)_{3(s)} + 3H^+ + e^- = Fe^{2+} + 3H_2O \qquad \log K = 16 \qquad [8]$$
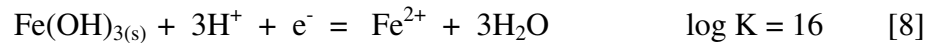
$$E_{H(ox-red)} = E^o{}_{Fe(OH)3-Fe2+} + 2.3\frac{RT}{nF} \log\frac{[H^+]^3}{[Fe^{2+}]} \qquad [9]$$

where R = Ideal gas constant in 0.082057 liter atmdeg^{-1}mol^{-1}, T = temperature (K), F = Faraday Constant (charge of 1 mole of electrons)in 96,485 C mol^{-1} and n = the number of electrons in the system.

Calculations for pE of goethite/ferrous couple and the partial pressure of oxygen pO2 are represented in equations [10], [11], [12] ,[13] and [14]

$$pE = pEo_{Fe(OH)3-Fe2+} + \log\frac{\{H^+\}^3}{\{Fe^{2+}\}} \qquad [10]$$

$$pE = 16 - 3pH + pFe^{2+} \tag{11}$$

$$O_2(g) + 4\,H^+ + 4e{-} \; = \; 2H_2O \tag{12}$$

$$pE = pEo + \tfrac{1}{4}\log pO_2 *\{H^+\} \tag{13}$$

$$\log pO2 = 4(pE - pEo + pH\;\;) \tag{14}$$

where $pEo_{Fe(OH)3\text{-}FE2+} = 16$, $pEo_{O2(g)} = 20.8$

  Ferric and ferrous phytate was be monitored in the effluent by proton nuclear magnetic resonance spectroscopy.

Materials and Methods

  In order to test this hypothesis, 3 soil columns were collected from Wye Island, in Queen Anne County, Maryland. Platinum electrodes were constructed, installed horizontally in each column at 5, 15 and 30 cm and monitored potetiometrically twice a day for 3 days. The platinum electrodes were replicated twice at the 5 cm level and 4 times at the 15 and 30 cm levels. The column effluent was monitored poltentiometrically at four hour intervals for a four day period. Ascorbic acid and peroxide were used to induce reducing and oxidizing conditions within the column. Aliquots containing 0.4g of phytic acid in 100 ml of water were added to the column at two intervals on day 2 and day 3. Three hundred ml of 0.1M ascorbic acid was added to water saturated columns on day 1. One hundred ml of 3% solution of hydrogen peroxide was added on day 4.

Platinum Electrode Construction

Platinum electrodes were constructed by cutting 12-gauge FT-1 rated solid copper wire insulated with gas and oil resistant 600V T-90 nylon into 12 inch lengths. The wire was cored with 1 mm drill bit to a depth of approximately 0.3mm with a lath. Platinum wire with a diameter of 0.0385 inches, purchased from DFG Goldsmith Chemical and Metal Corporation, Evanston, Ill. was cut into 0.6 mm lengths. The platinum was inserted into the cored copper wire and commercially available Benzomatic MAPP gas consisting of 44% methylacetylene- propadiene and 56% liquefied petroleum gas was used to sweat the union of copper and platinum and ensure contact. Epoxy was used to cover the exposed copper wire from the platinum copper interface to where intact plastic coating covered the copper wire. Polyolefin shrink tubing was used to cover the epoxy. A second coat of epoxy was applied to the open end of the shrink tubing adjacent to the platinum wire. Applications of epoxy were allowed to cure for at least 24 hours. The electrodes were cleaned with 0.1M HCl and calibrated using a potentiometer with a saturated Ag/AgCl reference electrode.

Soil Column Collection

Three 12 inch diameter, 40 cm length schedule 40 PVC pipe were driven into the ground with a sledge hammer and excavated. The columns were sealed on each end with kitchen plastic wrap and a rubber band for transport. The effluent end of the column was fitted with ¼ inch concentric polyethylene disks glued together. One circle fit within the internal diameter of the PVC pipe and the other covered the outer diameter. A ½ inch hole was drilled through the center of both disks. A ½ inch polyethylene fitting was used to drain the effluent from the columns. The polyethylene fitting and the concentric disks

were sealed with silicon glue.   The platinum electrodes inserted into the columns were also sealed with silicon glue. The columns were kept moist with water while in storage.

Soil Selection

The United States Department of Agriculture, Natural Resource Conservation Service, and Soil Taxonomy classification system lists Elkton as a fine-silty, mixed mesic Typic Ochraquult. It has been listed as benchmark soil in Maryland commonly found in Queen Anne County. It has moderately slow permeability and poor drainage and a slope of 1%. The iron content ranges from 7.9 to 9.4% as goethite. Elkton has an aluminum sesquoxide concentration of 23% and a pH of 4.8 to a depth of 88 cm. The site used to collect soil columns was in a corn soybean crop rotation and was within 400 yards of University of Maryland soil pit designated as Elkton.

Potentiogram Analysis

An EG&G Princeton Applied Research Model 362 Scanning Potentiostat and an ASEA Brown Boveri Model SE790 plotter were used to generate potentiograms. The voltage was programmed controlled and cycled from -0.2 to -1.2 V. The current was full scale at 100uA. Ferric chloride was used to prepare a linear calibration curve that measured iron concentration as a function of current. A second curve was generated in 0.1 M ascorbic acid in order to ensure no interference in the potentiometric measurements due to ascorbic acid iron ligand interactions. The y-axis of the potentiogram curves were measured with a ruler in units of centimeters for changes from baseline and a conversion factor in Ampheres/cm was used to convert length to current. The inflection point of the curve on the x-axis was measure in units of V/cm in order to identify the redox couple.

Results

Two of the three soil columns did not have sufficient flow rate to be useful. The

soil column used for the experiment had a flow rate of 4.0 ± 0.7 ml/hr. Effluent was

collected every four hours for 60 hours. The pH and the concentration ratio of the

ferric/ferrous couple were calculated for each data point (Figure 5).   The experimental

potential of the ferric/ferrous couple $E_{Fe3+,Fe2+}$ as well as the redox potential $E_{H(ox-red)}$

from the column effluent samples is presented in (Table 2). The data reflect the use of

the Ag/AgCl reference electrode which has a calculated value of 0.5722 V when the

ferric/ferrous redox couple is at equilibrium concentrations. The standard redox potential

for the ferric/ferrous couple is 0.771 V at equilibrium.

The potential of the ferric/ferrous couple was monitored at 5, 15 and 30 cm four

times over the course of 2 days (Figure 6).   A pH of 4.8 was used to calculate the

standard potential within the column according to the Nerst equation for the redox couple

goethite and ferrous ion equation [9].

Figure 5. The ratio of the concentration of ferric to ferrous species is a function of the concentration of protons.
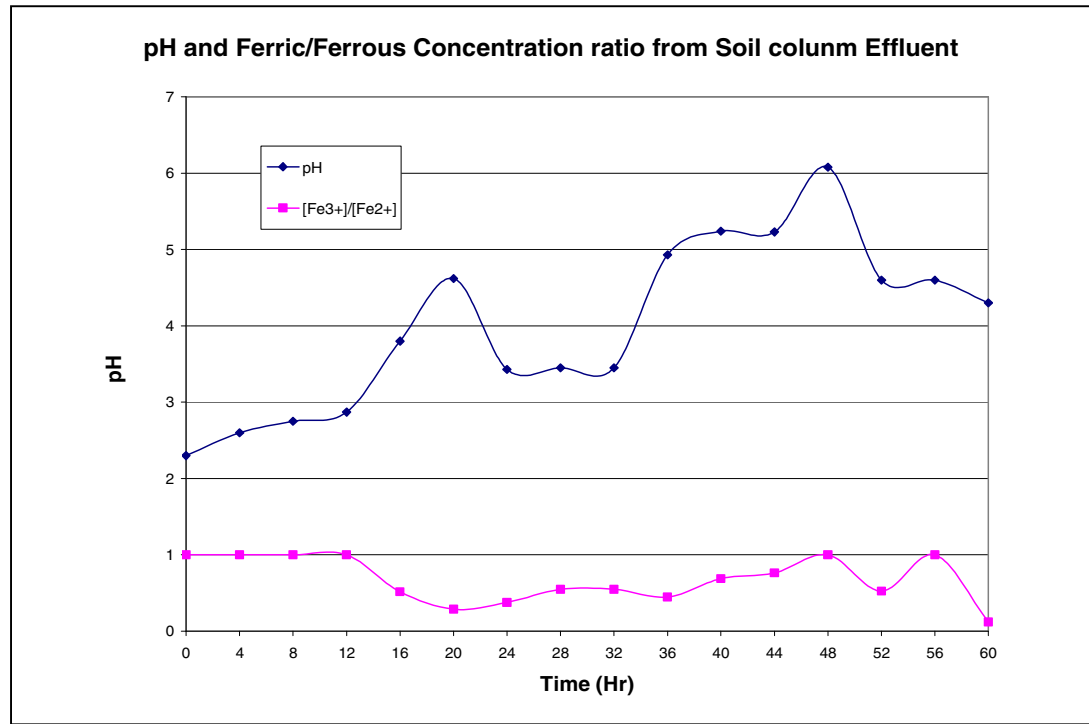

pH and Ferric/Ferrous Concentration ratio from Soil colunm Effluent

Table 2. Soil Column effluent collected every four hours over a three day period

sample ID	Ferric concentration (M)	Ferrous concentration (M)	$E_{Fe3+,Fe2+}$	$E_{H(ox-red)}$	pE	logpO2	pH
c1	2.50E-04	2.50E-04	0.5722	0.771	13.03245	-21.87018	2.3
c2	1.21E-03	1.21E-03	0.5722	0.771	13.03245	-20.67018	2.6
c3	9.22E-04	9.22E-04	0.5722	0.771	13.03245	-20.07018	2.75
c4	4.42E-04	4.42E-04	0.5722	0.771	13.03245	-19.59018	2.87
c5	1.54E-04	2.98E-04	0.5553	0.754	12.74654	-17.01385	3.8
c6	5.80E-05	2.02E-04	0.5402	0.739	12.49201	-14.75197	4.62
c7	5.80E-05	1.54E-04	0.5472	0.746	12.60952	-19.04193	3.43
c8	5.80E-05	1.06E-04	0.5567	0.756	12.77129	-18.31484	3.45
c9	5.80E-05	1.06E-04	0.5567	0.756	12.77129	-18.31484	3.45
c10	5.80E-05	1.30E-04	0.5515	0.750	12.68289	-12.74842	4.93
c11	1.06E-04	1.54E-04	0.5626	0.761	12.87068	-10.75728	5.24
c12	1.54E-04	2.02E-04	0.5652	0.764	12.91494	-10.62023	5.23
c13	1.96E-05	1.96E-05	0.5722	0.771	13.03245	-6.750183	6.08
c14	1.06E-04	2.02E-04	0.5557	0.754	12.75317	-13.78732	4.6
c15	1.06E-04	1.06E-04	0.5722	0.771	13.03245	-12.67018	4.6
c16	1.00E-05	8.20E-05	0.5183	0.717	12.12118	-17.51527	4.3

Figure 6. The oxidation reduction potential of the ferrous ferric couple at three

levels within the Elkton Soil column over a period of four days

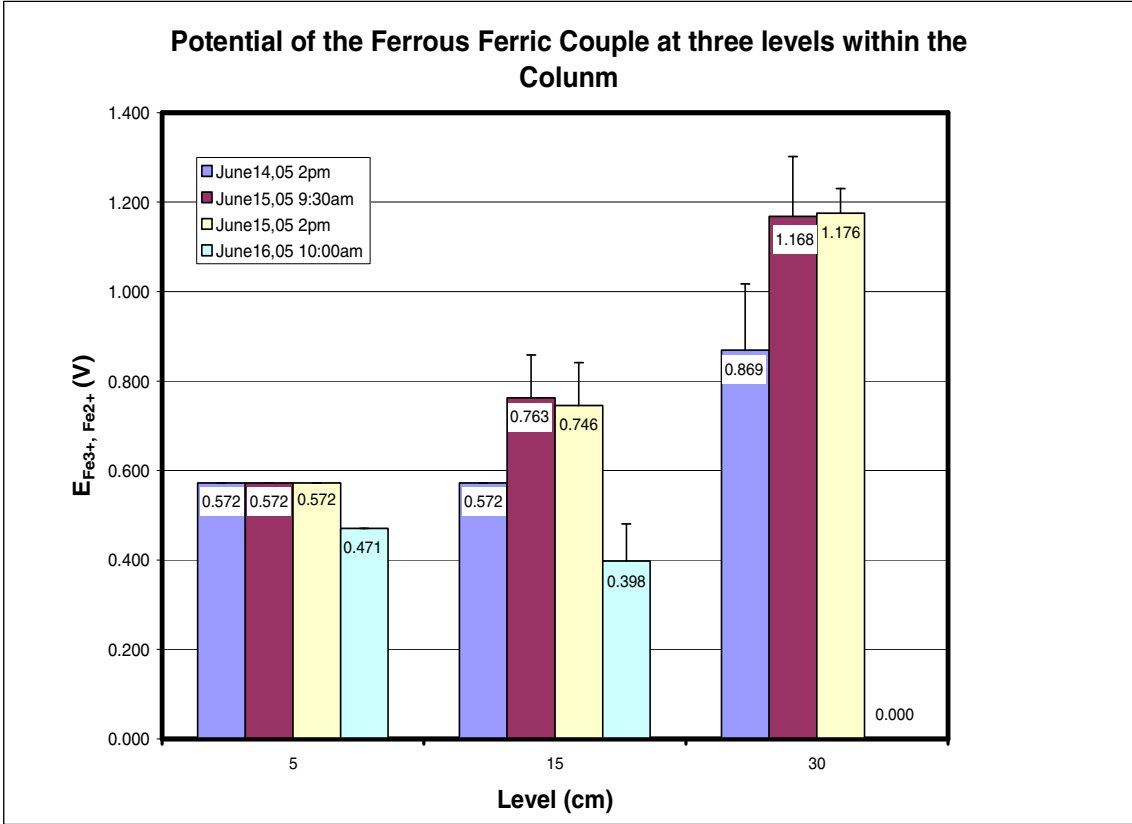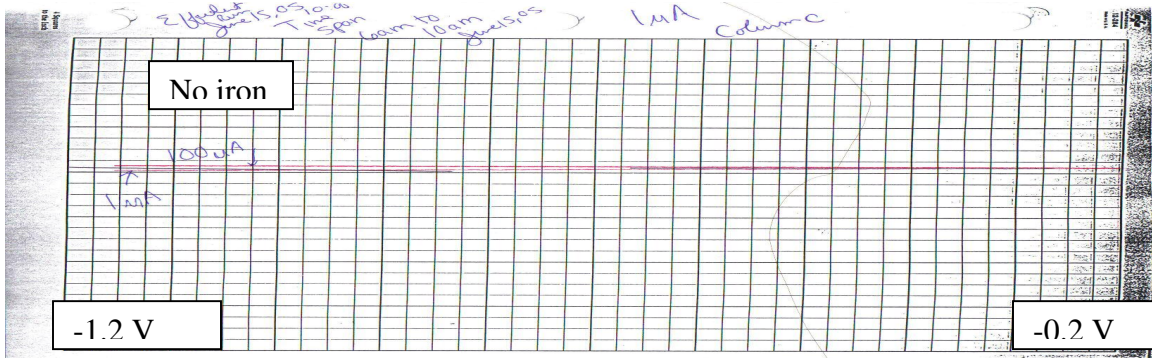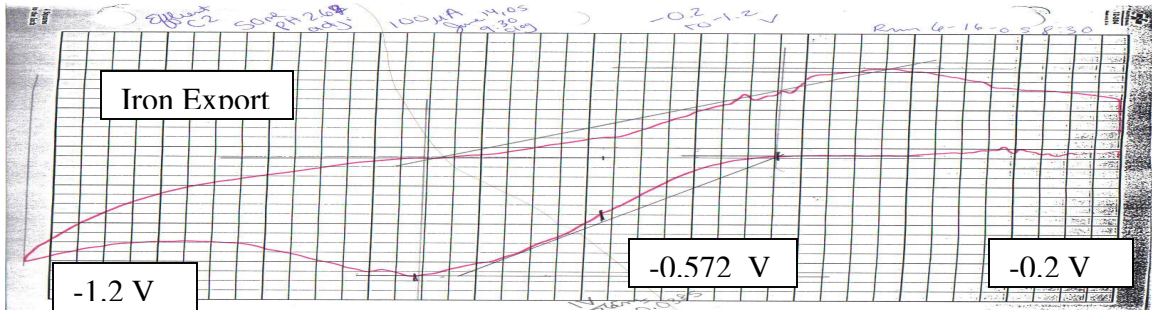Potential of the Ferrous Ferric Couple at three levels within the Colunm

Figure 7. Examples of potentiograms of column effluent using a Ag/AgCl
standard electrode. The redox potential of a hydrogen reference electrode is 0.771 V.
The Ag/AgCl is 0.1988 V. $E_{Fe3+,Fe2+}$= 0.771 V – 0.1988V = 0.572 V

The proton nuclear magnetic resonance spectrophotometer was not operational in
the time frame of this experiment. The samples will be saved, analyzed and presented at
a later date.

Discussion

Iron was exported from the Elkton soil column as the ferrous species (Figures 5
and 6). At the level of 5 cm the redox couple is in equilibrium as exhibited in figure 6.
The concentration of ferrous and ferric iron is unity and the potential is the standard
potential 0.771 V minus the adjustment for the Ag/AgCl electrode 0.1988. The potential

of the ferric/ferrous couple should and does equal 0.5722 V when in equilibrium using a saturated Ag/AgCl standard electrode. Ferrous ion is being exported with the exception of the sampling time June 16, 2005 at 10:00 am. At this time there is a decrease in ferric iron when compared to ferrous iron or there is insufficient moisture within the column interrupting the circuit between the reference electrodes and the working electrodes. The conversion from ferric to ferrous ion is occurring at the same rate at the three other sampling times at level 5 cm. At level 15 cm, the sampling times of June 14 and 15 at 2pm and 9:30 am respectively show the system in equilibrium (Figure 6). At level 15 cm and the finial two sampling times June 15 and June16 at 2pm and 10:00am the system is no longer in equilibrium as the concentration of ferrous iron is decreasing on June 15, 2005 at 2:00 pm and increasing on June 16, 2005 at 10:00 am. Ferric iron is being depleted and exported as the reduced ferrous ion from the higher levels of the column to the lower levels. The soil column level 30 cm may have been anoxic prior to the introduction of the ascorbic acid. The first two sampling times indicate ferric iron in excess of ferrous iron and iron is observable in the effluent from samples corresponding to this time (Figure 7). The June 15, 2005 has higher ferric iron then ferrous iron indicating export. The sampling time of June 16, 2005 and 30 cm depth has no iron of either species as reflected by the flat potentiogram (Figure 7). This potentiogram corresponds to oxidation of the column with hydrogen peroxide.

The curve from -0.2 to -1.2 represents the 1 electron redox conversion from ferric to ferrous iron. The curve from -1.2 to -0.2 V represents the 1 electron conversion from ferrous to ferric iron. Control of the potential ramp allows for identification of specific redox couples that would not be possible with only a voltmeter and reference electrode.

The ability of the potetiostat to measure very small amounts of iron allows for identification of very small changes in the redox couple concentration ratio. The column effluent is in a concentration range that may not interfere with detection of phytic acid as ferric and ferrous phytate.

Ferric and Ferrous phytate should be present in the effluent. If ferric or ferrous phytates are undetectable by H-NMR sequestration by aluminum sesquioxides present in the Elkton soil at a level of 23% could be the cause. If iron complexes of phytic acid are undetectable in the effluent then a further experiment in which phytic acid in excess of the absorption capacity of the aluminum oxides present in the column will be conducted.

Conclusions

In order to understand fate and transport of phytic acid it is essential to determine pH driven charge speciation of the molecule. The molecule has twelve protons that are responsive to changes in pH. Phytic acid due to its high mass to charge ratio is an excellent chelator and can participate in metal ligand interactions that make the fate and transport of several metal phytate complexes interesting environmentally. Iron is present in many soil systems making it a potentially important complex of phytic acid and one that is likely to be found at environmentally significant quantities. Phytic acid does not have an oxidation reduction potential but can form complexes that are redox responsive. We have shown that both oxidized and reduced iron complexes of phytic acid readily form in solution and others have shown that phytic acid represents a large pool of organic phosphorus in soil and manure (Jayasundera, S et al. 2005; Hansen et al. 2004; Turner et al. 2001). Ferric and ferrous iron are forming complexes with phytic acid that can persist

in solution and resist dephosphoralation by the enzyme phytase making ferrous phytate a compound with the potential to be exported from soil systems to surface waters.

The experimental kinetic constants derived from proton nuclear magnetic resonance indicate that ferrous phytate forms faster and dissociates at a slower rate then ferric phytate. The H-NMR derived findings are supported by the overall enzymatic dephosphoralation recoveries of phosphate over a two day period. In anoxic ground water ferrous phytate has the potential to resist microbial decomposition from the enzyme phytase. This ability to persist may enable ferrous phytate to be transported by subsurface leaching.

Applications of manure to pastures and cropped fields provide regular imputes of phytic acid that can be stored as ferric phytate and exported in late winter and early spring months when conditions are right. The water table is generally high due lack of evapotranspiration and soils can rapidly become reducing due to the height of the water table and or microbial activity.   Concentration of industrialized livestock production and the economic unfeasibility of moving manure to away from production sites lead to stockpiling and composting of manures in areas that can not support land application as a strategy for removal. Large stores of manure rich in organic phosphorus may increase the potential to leach organic phosphorus to ground water and eventually surface waters.

It is widely believed that the major transport processes mobilizing phosphorus from soil systems to surface waters is overland flow of sediment attached phosphorus and dissolved phosphorus in flood waters. Given the recent work by Karl, 2000 and Hudson, 2000 in separate papers indicating that rapid turnover of phosphorus not the quantity is the norm in most fresh water systems and in oligotropic marine systems and that

phosphorus is the limiting nutrient in fresh water systems then all transport mechanisms of phosphorus become important to the overall phosphorus budget.

The relative contributions of phosphorus to surface waters from overland flow processes and leaching is unlikely to be spatially uniform. Areas prone to leaching phosphorus to ground water and surface water can potentially be correlated to landscape features and soil properties. Anoxia in soil pore water would be a requirement for the transport of ferrous phytate to surface waters. Landscape features that contribute to anoxia in pore water are drainage class, organic content and slope.

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