PHOSPHORUS LIBERATION BY AQUATIC MICROORGANISMS

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LIST OF ABREVIATIONS

P	phosphorus
OA	organic acid
ATP	adenosine tri-phosphate
HAP	hydroxyapatite
FAP	fluorapatite
PSM	Phosphate solubilizing microorganisms
AIWW	Atlantic Intra-coastal Water Way, NC
Bradley	Bradley Creek, NC
Waccamaw	Lake Waccamaw, NC

ABSTRACT

The conventionally accepted route for cycling of inorganic phosphorus (P) was previously thought to be the long-term processes of abiotic physical and chemical weathering and subsequent physical exchange reactions with minerals. The ability of microorganisms to influence the liberation of labile P, from otherwise insoluble or sparingly soluble sources, in aquatic environments has been shown here to be capable of supporting appreciable biomass. The causal agents bringing about P liberation within the microcosm systems employed in this investigation were organic acids, principally gluconic acid, and [H^+] ions. The effects of chelation by organic acids as opposed to dissociation purely as a result of protonation were addressed and biologically inoculated microcosms yielded significantly more labile P than would be anticipated by the corresponding change in pH alone. Maximal rates of liberation of P of up to 320 µmol P g^{-1} day^{-1} were observed. Acid production and the subsequent liberation of otherwise refractory forms of P appeared to be an active process as it was not solely dependent on biomass, implying that organic acid production was either selective or that microbial population shifts occurred as a response to P limitation.

INTRODUCTION

The Role of Phosphorus

Through statistical reasoning and the application of the Redfield ratio, a ratio describing the average macro-nutritional composition of phytoplankton, it was posited that organisms have the ability to modify their environment. The central role of phosphorus (P) in the limitation of biological processes was demonstrated through the quantification of the ability of nitrogen cycling to satisfy planktonic biological requirements and the application of Liebig's law of the minimum. It was reasoned that the relative abundance of carbon and the efficiency of biogeochemical cycling of nitrogen are sufficient to satisfy biological requirements and that P by virtue of its slow cycling and lack of availability becomes the limiting factor (Redfield 1958). The relative contribution of P and nitrogen is however variable between different systems.

Phosphorus is a vital component in a range of biological structures and processes. It is an integral part of the molecules in all living organisms such as adenosine tri-phosphate (ATP), phospholipids, RNA and DNA. In its various forms P is indispensable in almost all biological pathways, such as photosynthesis and metabolism. Smith (1984) addressed skepticism about the limiting nature of P by the assessment of N:P ratio distributions in marine locations characterized by particularly low nutrient through put. The limited loss of nutrients from the system resulted in the incorporation of biologically driven nutrient characteristics into the sediment. Comparison of sea water and sediment nutrient proportions demonstrated that the majority of nitrogen in these locations was biologically supplied, showing that nitrogen supplied through biological cycling is both significant and rapid enough to meet demand.

It is commonly supposed that hydrological erosion of the volcanic deposits, where P originates, and the consequent uptake by early plants introduced P to the biosphere. Elemental P

is not found as a freely occurring element owing to its thermodynamic characteristics. Phosphates (principally PO_4^{3-}), however, account for some 0.1% of the Earth's crust. Biological cycling of P represents a steady recycling of organic phosphates through biological degradation of organic material, labile P in the form of soluble reactive P accounts for only a small fraction of the total P pool. The majority of phosphates tend to accumulate in sediments, incorporated within minerals or adsorbed to mineral surfaces. Global, particularly oceanic, sediments therefore represent major sinks for P. The accepted route by which lost P becomes newly available to biological processes is through physical and chemical weathering (Hanrahan 2004). Cycling on the scale of millions of years removes phosphates from the biosphere, sequestering them through sedimentation and re-introducing them to the oceans by physical and chemical erosion and addition through alluvial and eolian deposition (Figure 1).

The sequestration of labile forms of P such as orthophosphates has also been shown to have effects on much shorter scales. Increased salinities and pH in freshwater lakes, arising from terrestrial input, promotes the precipitation of aluminum hydroxides, colloidal compounds which coagulate with organic carbon, adsorb otherwise soluble forms of P. This process increases the sorption of P to the sediment. Aluminum hydroxides unlike ferrous Compounds, which also precipitate in forms that remove P from the water column, are not affected by redox potential (Kopáček et al., 2000).

The Role of Carbonate Sediments

Aquatic sediments form major sinks for labile P. The formation of P complexes with metals precipitating insoluble phosphates is a principal pathway by which labile forms of P may be sequestered from the biosphere. These principally refractory phosphates can represent a

significant fraction of total phosphorus (TP) (Ingall et al. 1990, van der Zee et al. 2004). The major species in which inorganic phosphorus (IP) occurs are aluminum phosphates, iron phosphates and calcium phosphates. Biogenic sediments, largely calcium carbonate, have also been shown to act as sites for enhanced P adsorption (Lopez 1994). This occurs as a result of complex geochemical processes dominated by the equilibrium driving free P to precipitate as insoluble calcium phosphates such as hydroxyapatite (HAP) and fluorapatite (FAP) where P concentrations are elevated (Slomp et al. 1996) or ferrous and aluminum oxides in systems where redox potentials are high. The P associated with carbonates is incorporated in the mineral matrix, not just surface adsorbed. As such the liberation of P from such sources requires the dissolution of the matrix to take place and is not achieved simply through changes to redox potential.

Authigenic carbonate FAP is a major sink for P and is not restricted to areas of particularly high P input. As a consequence coastal margin sediments represent major sinks for P (Ruttenberg, 1993, Filipelli, 1997, van der Zee et al. 2004).

Ambient concentrations of P in continental margin sediments in the range of 8 to 108 µmol P g^{-1} and localized rates of sedimentation from 80 to 8,000m my^{-1} have been described (Fillipelli 1996).

The Role of Microorganisms

The ability of microorganisms to liberate P from various mineral matrices has received recent consideration. Much previous literature however, primarily considers terrestrial evidence. In addition, the role of microbial solubilization in making phosphates available for plant uptake has become a focus of current research (Peix et al. 2001). Certain fungi and bacteria are found

associated with the rhizospheres and root nodes of many terrestrial plants. The presence of these microorganisms enhances the performance of the plant host, apparently by solubilizing otherwise refractory soil phosphates. The association of certain microorganisms with increased productivity of agricultural crops under certain regimes of nutrient limitation has long been observed (Stevenson, 1986). It has been realized in particular that the presence of certain complementary organisms produced a major response under a regime of P limitation (Barriuso Maicas et al., 2002, Peix et al., 2001). Babana and Antoun (2002) showed that inoculation of wheat crops with *Pseudomonas sp.* or the fungus *Aspergillus niger* and rock phosphate significantly increased crop productivity to a significantly greater extent than rock phosphate alone.

Other research efforts are currently concentrated in developing genetic techniques in order to probe for and modify phosphate solubilizing microorganisms (PSM) (Reyes et al., 2001; Peix. 2002). This phenomenon has become a point of interest due to its economic implications. Crop seeding with microbes is currently employed in some regions to enhance crop success, particularly as an alternative in developing countries where chemical P fertilizers are a less economically viable option.

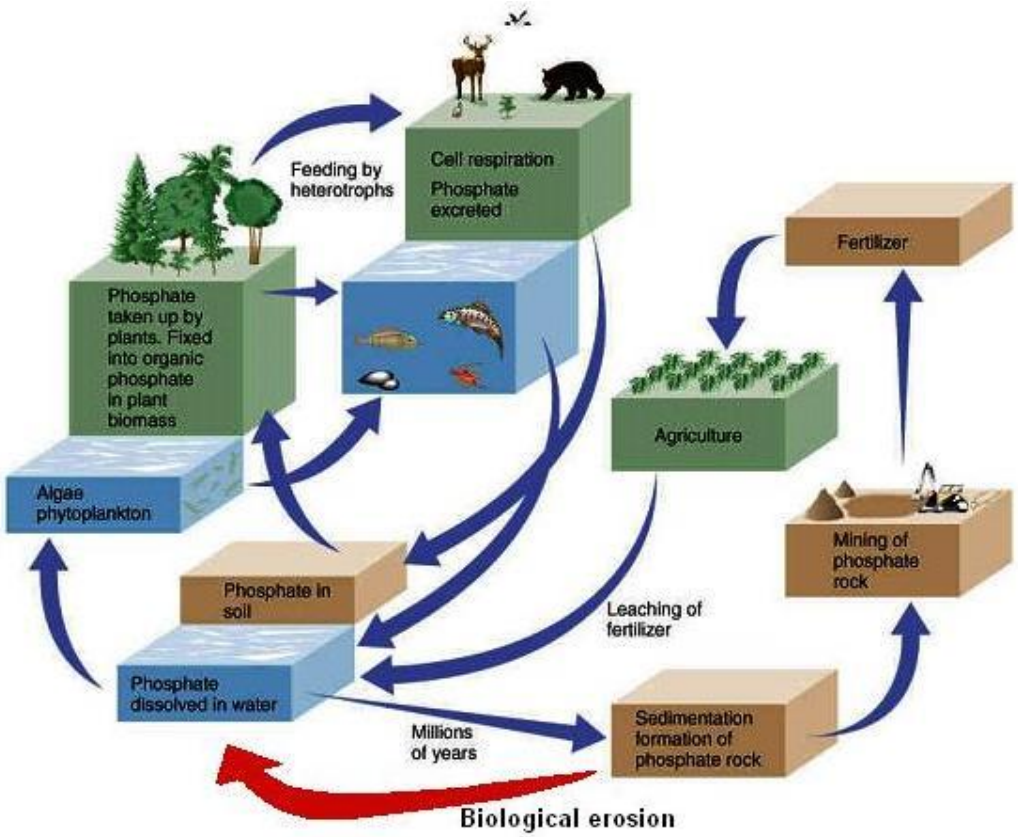


Figure 1. The phosphorus cycle

Schematic summary of the P cycle with the hypothesized process of microbial erosion and consequent liberation of soluble forms of P (adapted from Carosella 2004)

Barroso and Nahas (2004) described P limitation in Brazilian soils. Phosphorus fractionation analysis showed that the majority of phosphates formed complexes with iron, aluminum and calcium. Four hundred and eighty one species of fungi were isolated and of these 33 were shown to solubilize inorganic phosphates under culture conditions. Rates of calcium phosphate solubilization of up to 0.079 mg P ml^{-1} growth medium day^{-1} were reported for *Penicillum radicums* (Whitelaw et al. 1999) and rates of over 0.111 mg P ml^{-1} growth medium day^{-1} were described for a range of organisms by Barros and Nahas (2004). Cerezine et al. (1988) investigated the solubilization of FAP by *Aspergillus niger*. Soluble phosphate levels in solution were shown to increase with fungal growth. Maximal concentrations of soluble phosphate in solution were reached after 11 days of incubation.

The soil types that are characterized by P limitation display varied salinities and often display high calcium or metal ion concentrations. Phosphates form strong complexes with other ions and mineral surfaces under these conditions, effectively sequestering P from the biosphere and locking it into sediments. As such, marine and fresh water sediments also represent sinks for P (Figure 1 and Figure 2). The problem facing organisms extracting inorganic phosphates from aquatic sediments is to avoid the tendency of calcium, present in carbonate sediments for example, to remove P from solution by precipitation or absorption and to overcome the high pH and buffering effect of marine environments. Consequently P-bearing minerals are sparingly soluble at the typical buffered pH found in marine and brackish water (approximately pH 8.1 for oceans).

Reduced pH in such environments will, in general, increase the solubility of certain minerals and as such the opportunity for P liberation. Organic acid (OA) produced by

microorganisms may allow low pH micro-sites to be developed and as such localized acceleration of dissolution and P liberation processes.

Other analogous phenomena have been reported, many of which are commercially relevant applications for microbes in the solubilization of various minerals, specifically the isolation of metals, such as iron and copper, from their ores (Cummings and Kuiack, 1992).

Promod et al. (1987) investigated the occurrence of phosphate solubilizing bacteria in the sediments and water column in backwater regions of Cochín, India. A positive correlation between the number of phosphate solubilizing bacteria and both the concentration of adsorbed phosphate in the sediment and soluble phosphates in the water column over a one year period was demonstrated. Two potent groups *Pseudomonas sp.* and *Vibrio sp.* were incubated with a phosphate source of tri-calcium phosphate. Maximal soluble phosphate concentration was observed after 72 hours and correlated to peak biological growth.

Other Evidence of Microbial Influence

There are a number of indications that microbial extraction of P from insoluble mineral matrices may be occurring in aquatic environments. Microboring is a well studied process due to its ecological and commercial significance. Chazzotes et al. (1995, 2002) developed and employed techniques that enable the analysis of the relative effects of macro and micro faunal and floral contribution to biological weathering on the coral reefs of Moorea, French Polynesia. Through these experiments it has been demonstrated that microboring communities are very active in the weathering of carbonate sediments, representing the primary biological agent of erosion for the first two months of exposure of experimental substrates. Biological weathering rates of up to 0.6kg $CaCO_3$ m^{-2} yr^{-1} have been described (Chazottes et al., 1995). The bio-

turbation brought about by this process also serves to substantially increase the surface area available for dissolution reactions to occur.

Tribollet and Payru (Tribollet and Payru, 2001) working in the same region, studied the effects of microbial weathering in the biogenic mineral matrix of the coralline algae *Hydrolithon onkodes*. It was noted that in live corals infestation originated from the coralline substrate below. Although movement was generally positively phototaxic, boring showed little directionality (Fig. 3). In these studies the principal species involved in boring were the cyanobacteria, *Hyella caespitose, Plectonema terebrans, Mastigocoleus testarum* and the siphonalean chlorophyta; *Ostreobium queketti*. In all studies *Plectonema terebrans* is reported as both the pioneer species and the biggest contributor to substrate erosion. In addition to a range of fungi, a number of the phosphate solubilizing microorganism (PSM) groups identified in the terrestrial studies discussed earlier are cyanobacteria.

The investigation discussed herein aimed to address the question of microbial influence over the liberation of P from insoluble sources in aquatic sediments and as such their contribution to the P cycle. Aquatic ecosystems are frequently limited by P (Figure 2) and despite the attention the role of PSM has received, the presence of an analogous process occurring in aquatic environments remains inadequately considered.

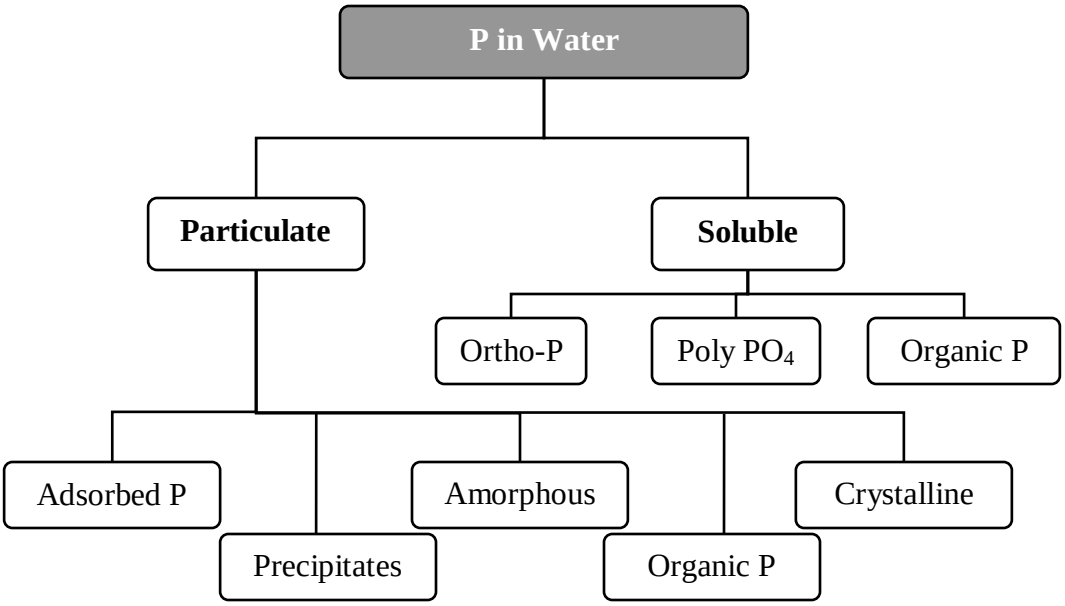


Figure 2. Phosphorus fractionation in aquatic environments

Schematic summary of the principal forms in which P occurs in aquatic environments

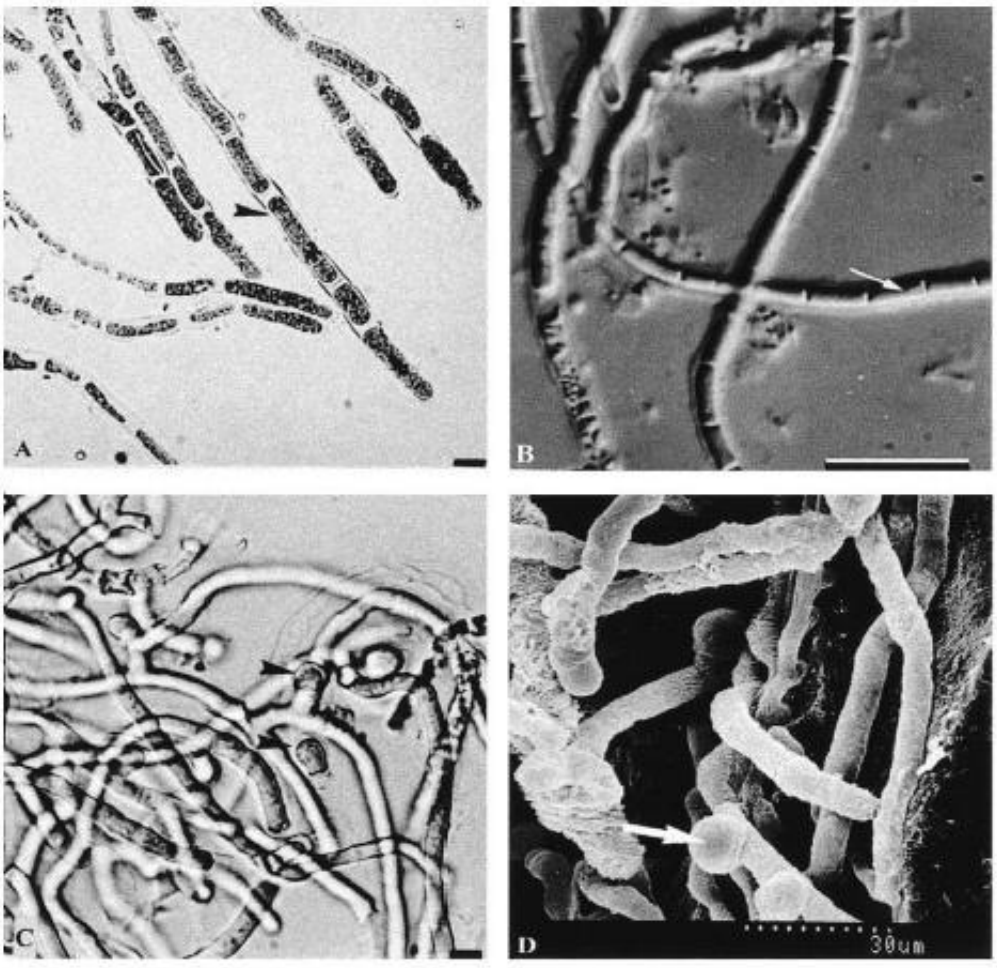


Figure 3. Light microscopy and scanning electron microscopy images of endolithic microorganisms in dead and live crusts of *Hydrolithon onkodes*.

A. Filaments and cells of the cyanobacterium *Hyella caepitosa*. B. Filaments and cells of the cyanobacterium *Plectonema terebrans*. C and D. Filaments and heterocysts of the cyanobacterium *Mastigocoleus testarum* (Tribollet and Pauyri, 2001).

HYPOTHESES

This project aimed to address the central hypothesis that certain microorganisms are able to promote the liberation of P from otherwise insoluble or sparingly soluble sources in aquatic environments through the consideration of the following hypotheses:

H_1: The rate of liberation of labile P will increase as biomass increases.

H_2: The rate of liberation of labile P will increase as the acidity of the system increases.

H_3: The rate of liberation of labile P will be greater when substrates are exposed to microbial populations that characterize lower ambient P concentrations.

H_4: The rate of liberation of labile P will be greater from more soluble substrates.

H_5: The rate of liberation of labile P will increase as OA concentration in the system increases.

H_6: OA concentration will increase as biomass increases.

H_7: OA production will be greater where substrates have lower potential dissociation constants.

METHODS

A microcosm, bottle incubation approach, adapted from methods employed in terrestrial studies, was employed with the objective of resolving the liberation of labile phosphates from experimental substrates as a result of exposure to naturally occurring suites of microorganisms.

Water and sediment samples with their naturally occurring complement of microflora and fauna were retrieved from the sediment/water interface, using a 30mm by 100mm plastic core tube. Locations were selected in order to characterize a range of ambient nutrient regimes, thus allowing some consideration of the influence of ambient nutrient conditions on microbial activity. Two local sites leased by the University of North Carolina Wilmington were used, Bradley creek (Bradley), a site with relatively high nutrient loading, and an oyster bed on the Atlantic Intracoastal Water Way (AIWW) with lower ambient nutrient levels (Mallin et. al., 2006). Lake Waccamaw (Waccamaw), North Carolina provided an oligotrophic freshwater location.

The following substrates and sediments were used as P sources for the microcosm bottle experiments; calcium HAP ($Ca_5[(PO_4)_3OH]$) reagent grade obtained from Sigma Aldrich, FAP ($Ca_5(PO_4)_3F$) obtained from CF industries Inc. in addition to natural substrates from the sites where flora were collected. Mehlich-3 extracting solution was used as a wash for the P to ensure the removal of all adsorbed, associated or other soluble forms of labile P before the microcosm incubation experiments (Mehlich 1984).

Microbial assemblages isolated from core samples were incubated with the experimental P substrates and growth media. The method employed was an adaptation of terrestrial culture analysis procedures (Cunningham and Kuaick, 1992; Barroso and Nahas, 2004; Whitehead et al., 1998, Illmer and Schinner 1995). The TP content of substrates was determined using perchloric

acid digestion and a molybdenum blue colorimetric approach (Olsen and Summers 1982, Parsons et al. 1984).

The growth medium employed was designed to satisfy all the nutrient requirements of the microorganisms present in the microcosms with the exception of P. The Growth media employed comprised the following; sucrose (1M), KCl (19.3 μM K), Na_2MoO_4 (0.7 μM Mo), H_3BO_3 (32 μM B), $CuSO_4 \cdot 5H_2O$ (0.8 μM Cu), $FeCl_3$ (6.2 μM Fe), $MnCl_2 \cdot 4H_2O$ (1.0 μM Mn), $ZnCl_2$ (21 μM Zn) and $CaCl_2$ (1.8 μM Ca), NH_4Cl (9.4 mM N) (adapted from Whitelaw et al. 1999). Synthetic sea water comprising 23.8 mM $NaCO_3$ and 2 mM $CaCO_3$ (Strickland and Parsons 1972) was used to make up the growth medium as opposed to distilled water. This provided a pH and salinity more representative of a natural scenario as well as an excess source of calcium. The P substrate was added to the growth medium at a rate of 1g P substrate L^{-1} growth media for the experimental substrates, HAP and FAP. Natural sediments were added at a rate of 50g L^{-1} growth media due to their lower P contents.

Sediment from the core samples was suspended in 1L of de-ionized water and then filtered (0.45μm glass filter) in order to isolate fungi, bacteria and microalgae. The filter was then added to 200ml of growth media in the inoculated assays. A sterile filter was added to controls assays. Plastic tissue culture flasks (250ml) were used to incubate the inoculated growth media and controls. Control assays (with no biological inoculation) with each P source (HAP, FAP and natural sediments) and one biological inoculation were run for each location (Bradley, AIWW and Waccamaw). In addition an inoculated control with no P substrate was carried out for each location. Two aliquots were taken from each assay every 48 hours over a 10-day period for the analysis of chemical and physical parameters.

Chemical and Physical Analysis

Every 48hours a 5ml aliquot was taken and diluted to 50ml. The diluted aliquot was then analyzed for soluble reactive P and biomass. The second 5ml aliquot was taken for the determination of pH using a silver electrode pH meter. The Molybdenum blue reagent method (Strickland and Parsons 1972) was used to quantify soluble reactive P. Biomass was quantified by measuring the absorbance of the homogenized media at 885nm. These absorbance values were calibrated against the concentration of adenosine triphosphate (ATP), which represented a proxy measure of living biomass. The luciferin-luciferase chemoluminesence approach of Strickland and Parsons 1972 was adapted to quantify the ATP in solution. High phase liquid chromatography (HPLC) was used to identify and quantify organic acid species in solution. An isocratic flow at 0.5 ml/min of 0.1 % phosphoric acid with a Supelcogel C610H column, 30 cm x 7.8 mm was employed at a constant temperature of 30^{o}C. Detection was at 210 nm using a deuterium lamp. Standards used were obtained from Sigma. PeakSimple software was used to denote peak retention times and perform standard curves for quantifying standards.

Abiotic Experiments

To further delineate the relative contributions to P liberation by chelation due to OAs versus instantaneous dissociation as a result of protonation a set of abiotic assay were undertaken. These assays were set up as for non biological control assays following the method described above. These bottles were not inoculated with microorganisms and aseptic technique was used throughout the experiment. The bottle microcosms were then adjusted to a known initial starting pH (pH 4, pH 5 or pH 8) by the addition of hydrochloric acid, representing an

inorganic [H^+] ion donor. In addition, oxalic acid was added, to pH 8 growth media, up to a concentration of 1mM, representing an organic [H^+] ion donor. Samples were then retrieved immediately from all bottles to obtain a time 0 value and then periodically over a 5 day period. Reactive P in solution and pH were measured.

Statistical Analysis

Mann-Whitney tests, Kruskal-Wallis independent samples tests and statistical regression were used to isolate significant relationships in the data set obtained. This allowed for adequate consideration of data sets with a non-normal distribution. Where regression analysis was performed a Kolmogorov-Smirnov test was used to determine normal distribution of the data.

Preliminary Experiments

Preliminary tests, showed that a range of OAs produced significant increases in dissolved inorganic phosphorus (DIP) when exposed to carbonate sands (Figure 4) and that oxalic acid occurs in measurable quantities in some aquatic sediments (Lake Waccamaw limestone and Florida sand).

As a supporting diagnostic measure, plates of the growth media described above with the addition of 15g L^{-1} agar were inoculated with samples taken from the sediment water interface following the sampling method described above. P liberation was observed by the production of a clearing zone in the growth media forming a halo around the microbial colonies. A range of insoluble P sources were supplied. Calcium phosphate, rock phosphate standard, ground coral sand, ground Lake Waccamaw sediment and ooilites were used as well as no P addition controls.

The plates were streak inoculated from water samples that were collected at the sediment-water interface at local locations. Samples from all sites showed very low growth in the no-P controls. Although ground sediments also showed low growth, calcium phosphate plates showed appreciable growth of two species, with pronounced colonies formed. The low growth with the ground sediments as P substrates was likely due to particle size inducing settling of sediment to the bottom of the plate during setting of agar. Due to the insoluble state of the calcium phosphate the growth medium was opaque. The colonies growing on this medium produce a clearing zone as a result of the solubilization of the insoluble calcium phosphate. This clearing halo expanded with time and colony growth (Figure 5).

A preliminary lab assay following the method described above was carried out on sediment water interface samples taken from the AIWW (Wrightsville Beach, North Carolina). A biologically inoculated incubation was carried out with a calcium phosphate ($CaHPO_4$) P source in addition to a non-inoculated control. A marked difference was observed between the inoculation and control in terms of both reactive P concentration in solution and biomass, measured in terms of turbidity (absorbance at 885nm). Peak reactive P in solution was reached after 2 days, as was biomass (Figure 6).

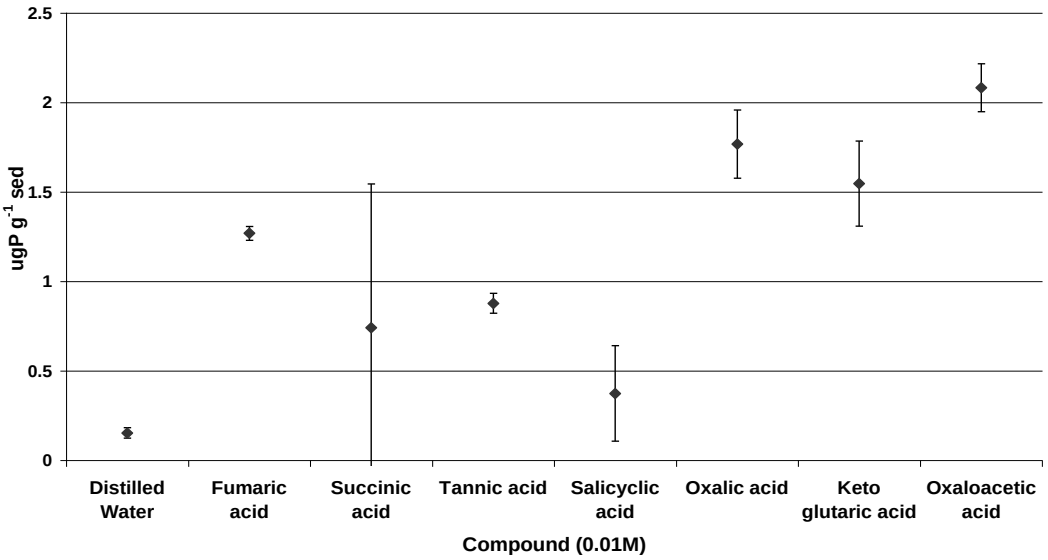


Figure 4. Phosphorus liberation capabilities of organic acids

The concentration of P in solution where carbonate sediments have been exposed to a range of organic acids for 48 hours (preliminary results Gill 2005)

Figure 5. Growth of fungal and bacterial colonies in inoculations of agar

Growth media containing calcium carbonate, showing clearing zone around colonies as a result of the solubilization of calcium carbonate

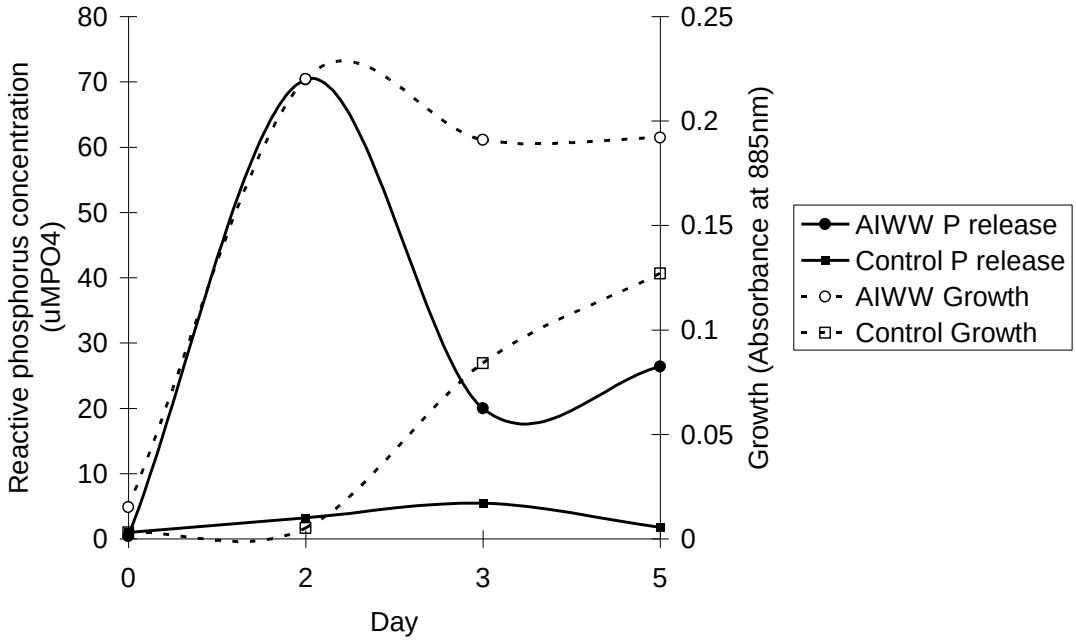


Figure 6. Phosphorus release and microbial growth

Concentrations of reactive P and biomass over a 5 day incubation of microorganisms taken from the AIWW

RESULTS

The Effect of Biomass on Phosphorus Liberation

The concentration of soluble P in the system increased as biomass, measured as ATP (µmol ATP µmol initial substrate P^{-1}), increased.

The concentration of ATP increased exponentially over the incubation period in assays where there was both a biological inoculation and a source of P. The presence of microorganisms in the microcosm bottle systems had a very significant effect on the liberation of soluble P (*Mann-Whitney: $p<0.00001$, $w=10400$, $n=144$*).

A significant positive correlation was observed between biomass and the concentration of soluble P in solution (Figure 7). It was useful to consider substrates as either 'Experimental'; HAP and FAP, or 'Natural'; AIWW, Bradley and Waccamaw sediment, due to the different scales of productivity that were supported by the different substrates.

The TP content of the substrates used was determined by the perchloric acid digestion method, as described in the methods section, and showed variation among the sampling locations (Table 1).

Figure 8 presents the regression of P liberation (net P accumulation in solution) against biomass for the assay where only HAP or FAP were the sources of P. The concentration of ATP and as such the biomass in the system showed a significant positive correlation with the proportion of labile P liberated from HAP.

% P liberated = 94300 ATP (HAP) + 1.38 (*$F=429$, $df = 71$, $p<0.0001$, $r^2=0.86$*)

Where FAP was the source of P the following equation explained the relationship between P liberation and biomass.

% P liberated = 92500 ATP (FAP) - 0.07 (*$F=263$, $df = 71$, $p<0.0001$, $r^2=0.79$*)

Sediments taken from the AIWW also yielded soluble reactive P when exposed to microbial populations. The relationship between P liberation and the biomass present in the system is presented in Figure 9. The following linear equation explained the relationship between the liberation of labile P and biomass.

%P liberated = 62300 ATP (AIWW) - 1.44 ($F=20.2$, df 35, $p<0.0001$, $r^2=37.3$)

Sediments from Bradley also yielded reactive soluble P when biomass is produced in the system. The following linear equation explained the relationship between the liberation of labile P and biomass.

% P liberated = 50500 ATP (Bradley) - 1.13 ($F=43.9$, $df=35$, $p<0.0001$, $r^2=56.4$)

The biomass associated with microbial populations did not show any significant effect on the rate of P liberation when Waccamaw sediments were the source of P (Figure 9).

Substrate Type	TP (μ mol g^{-1})	S.E. ±
HAP	5970	-
FAP	5950	-
AIWW	31.4	6.61
Bradley	24.4	1.89
Waccamaw	17.3	2.50

Table 1. Total phosphorus content of phosphorus substrates

TP content of the P substrates employed, natural substrates were determined by perchloric acid digestion (Strickland and Parsons 1972). HAP and FAP TP contents were calculated from molecular weights.

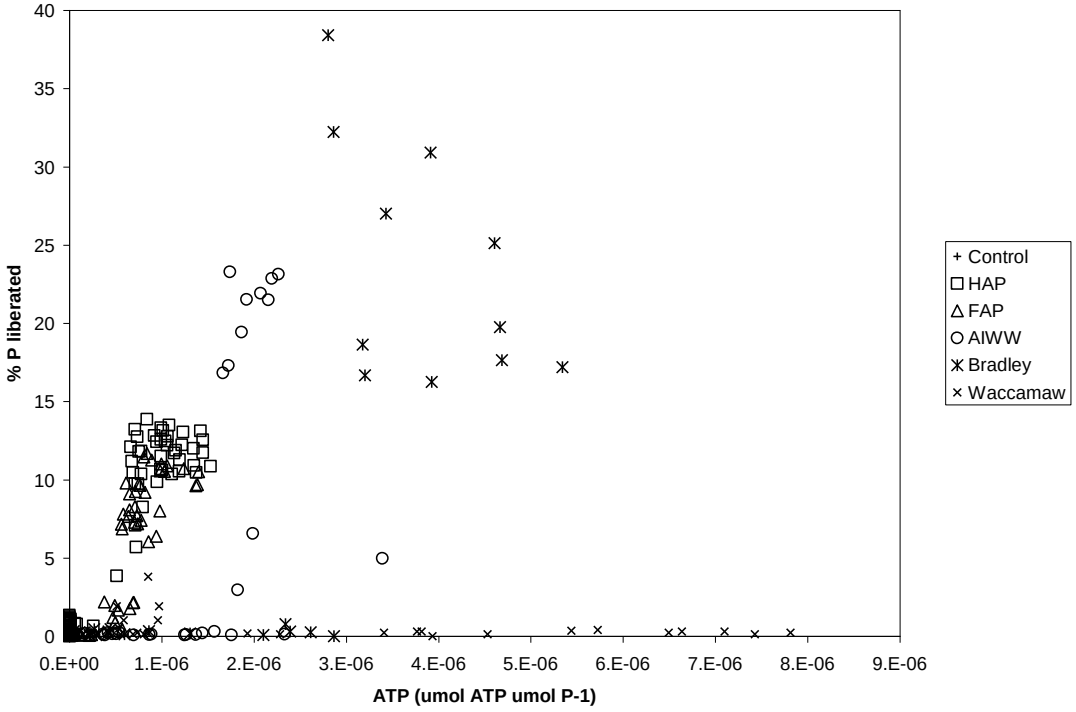


Figure 7. The effect of biomass on phosphorus liberation

The relationship between P liberation, as a percentage of the total available P and the concentration of ATP, µmol ATP µmol initial substrate P^{-1}, in the system

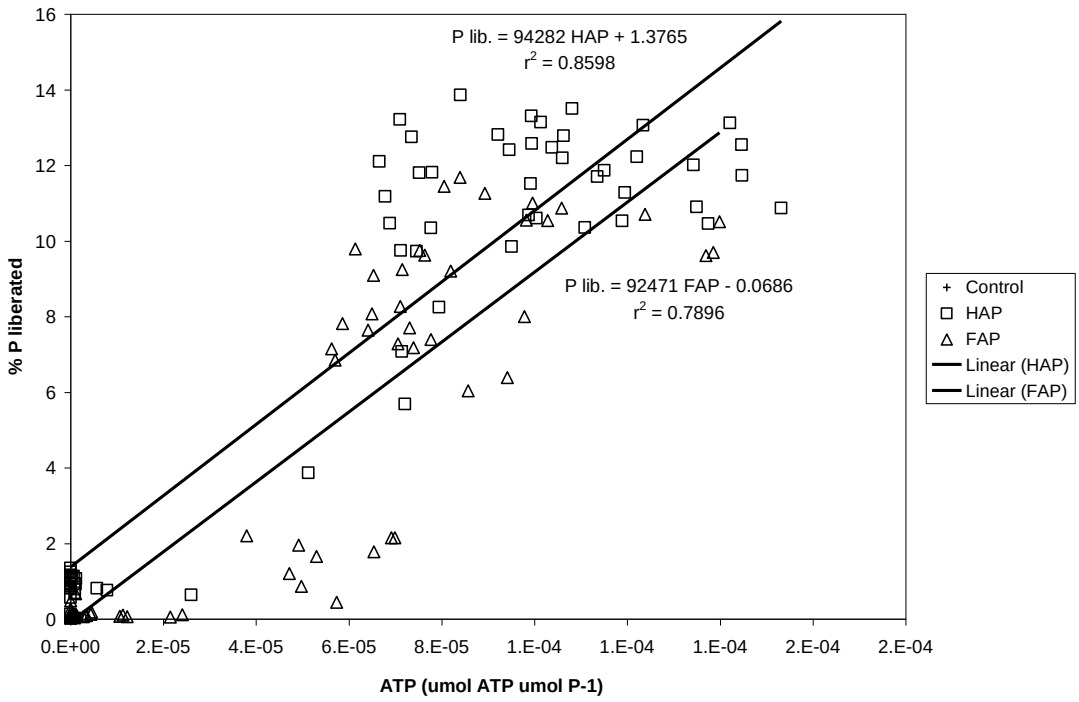


Figure 8. The effect of biomass on phosphorus liberation associated with experimental substrates

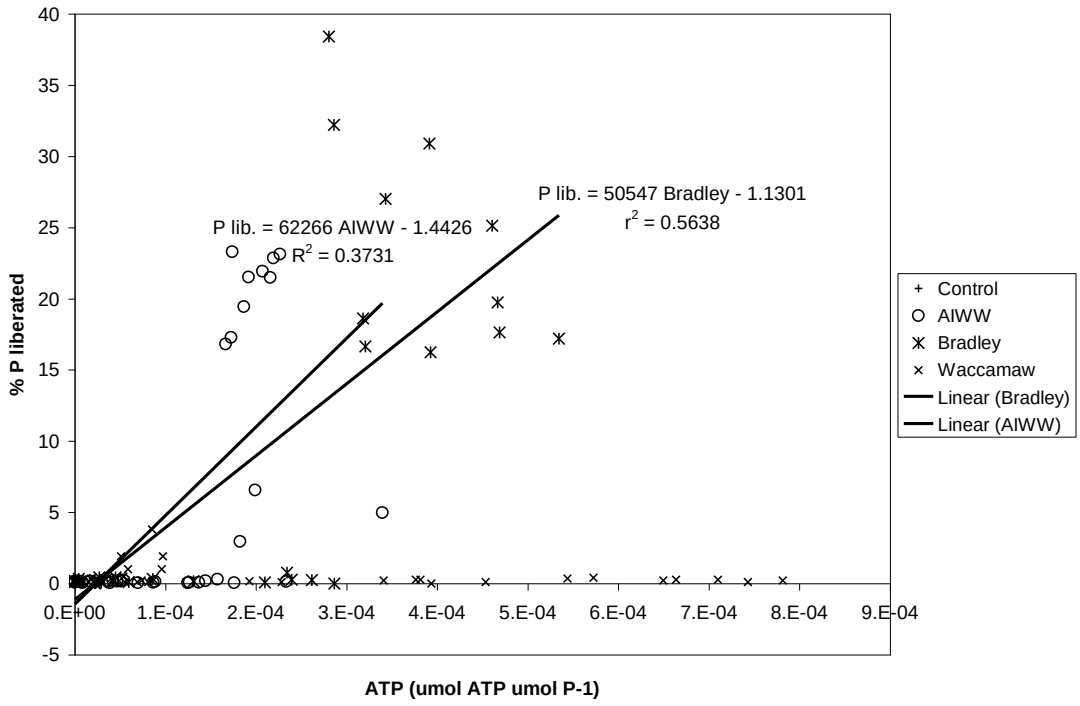


Figure 9. The effect of biomass on phosphorus liberation associated with natural sediments

The Effect of [H+] Concentration on Phosphorus Liberation

One of the principal causal agents in the dissociation of labile P from insoluble compounds was postulated to be the concentration of [H^+] ions in solution or the acidity of the solution. The rate of P liberation increased as the acidity, measured as micromoles of [H^+] ions (μM H^+), in the system increased.

Figure 10 presents the relationship between acidity and the liberation of labile P as a percentage of TP. In this figure, as for the consideration of biomass, a distinction between Experimental and Natural substrates was observed. The large disparity in P concentrations arising from natural as opposed to experimental substrates likely accounts for the different rates of P liberation observed, among other possible factors. HAP and FAP were chemically defined, but the P forms present in the natural sediments are unknown.

Further consideration of the relationship between labile P liberation and [H+] ion concentration showed that, when the rate of P release arising from each substrate was considered individually, the concentration of [H+] ions in solution explained the majority of the variation in labile P concentration for each of the P substrates except Waccamaw sediments.

HAP: % P liberation = 0.47 HAP [H+] + 3.80 ($F=87.59$, $df=71$, $p<0.0001$, $r^2=0.55$)

FAP: %P liberation = 0.5295 FAP [H+] + 0.6228 ($F=563.23$, $df=71$, $p<0.0001$, $r^2=0.88$)

AIWW: 1.0241AIWW [H+] + 0.04 ($F=249$, $df=35$, $p<0.0001$, $r^2=0.89$)

Bradley: %P liberation = 1.12 Bradley [H+] + 0.76 ($F=53.8$, $df=35$, $p<0.0001$, $r^2=0.60$)

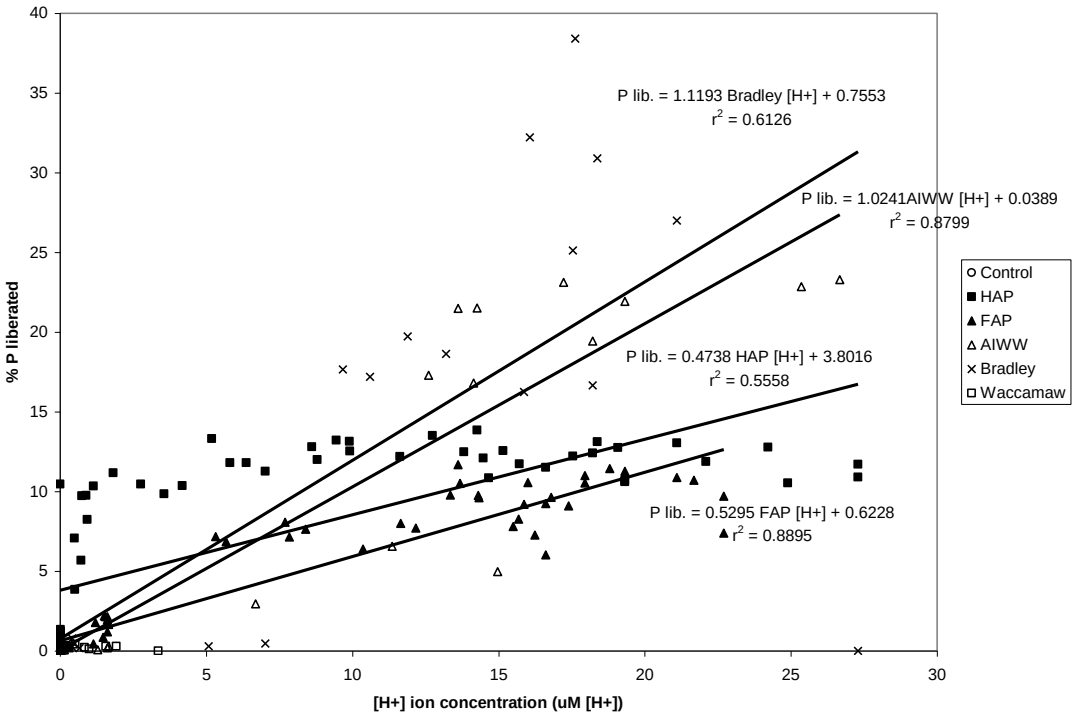


Figure 10. The effect of acidity on phosphorus liberation by substrate

The Relationship between Acidity and Biomass

Acidity was seen to vary not only as a function of biomass. Acidity increased to different extents, for the same concentration of biomass, where different substrates were used. This appears to demonstrate either active selection in microbial acid production or a competitive adjustment to the population composition, as opposed to acidity passively increasing as a function of increasing numbers of organisms. A significant positive correlation was observed between biomass and acidity, in terms of $[H^+]$ ion concentration, in the system (Figure 11). The following equations explained significant variation of the patterns observed in the data set. The units in the following equations are uM [H+] and umol ATP umol P^{-1}:

uM [H+] = 163000 ATP (FAP) - 0.95 ($F=101$, $df=53$, $p<53$, $r^2 = 0.66$)

[H+] = 128000 ATP (HAP) - 1.21 ($F=45.6$, $df=53$, $p<0.0001$, $r^2 = 0.47$)

[H+] = 67100 ATP (AIWW) - 0.05 ($F=11.2$, $df=17$, $p<0.004$, $r^2 = 0.41$)

[H+] = 31909 ATP (Bradley) + 1.6545 ($F=8.017$, $df= 17$, $p<0.012$, $r^2 = 0.33$)

[H+] = 829 ATP (Waccamaw) + 0.22 ($F=0.99$, $df=17$, $p<0.34$, $r^2 = 0.06$)

The relationship between acidity and biomass in the assays where Waccamaw sediments provided the source of P was not significant.

The Effect of Microbial Population

A Kruskal – Wallis independent samples test showed significant differences ($x^2=32.2$, $df=3$, $p<0.0001$) in the liberation of labile P between assays inoculated with different microbial

populations where HAP and FAP were the P substrates, the results of which are summarized in Table 2.

The microbial population recovered sampled at the AIWW location did on average liberate a greater percentage of the refractory P than the population recovered from the Bradley location. These two locations were more similar in factors such as salinity and tidal flow characteristics than the Waccamaw location. Waccamaw as such may be characterized by very different populations and may be better considered apart from the other two locations.

The variation in of P liberation between assays arose from the variation in liberation rates in the early stages of the incubation. In particular, day 2 samples taken from the assays using Bradley sediments yielded less labile P than those using AIWW sediments.

This experiment, as such, did not provide evidence to directly support the hypothesis that the rate of liberation of labile P will be greater when substrates are exposed to microbial populations that characterize lower ambient P concentrations.

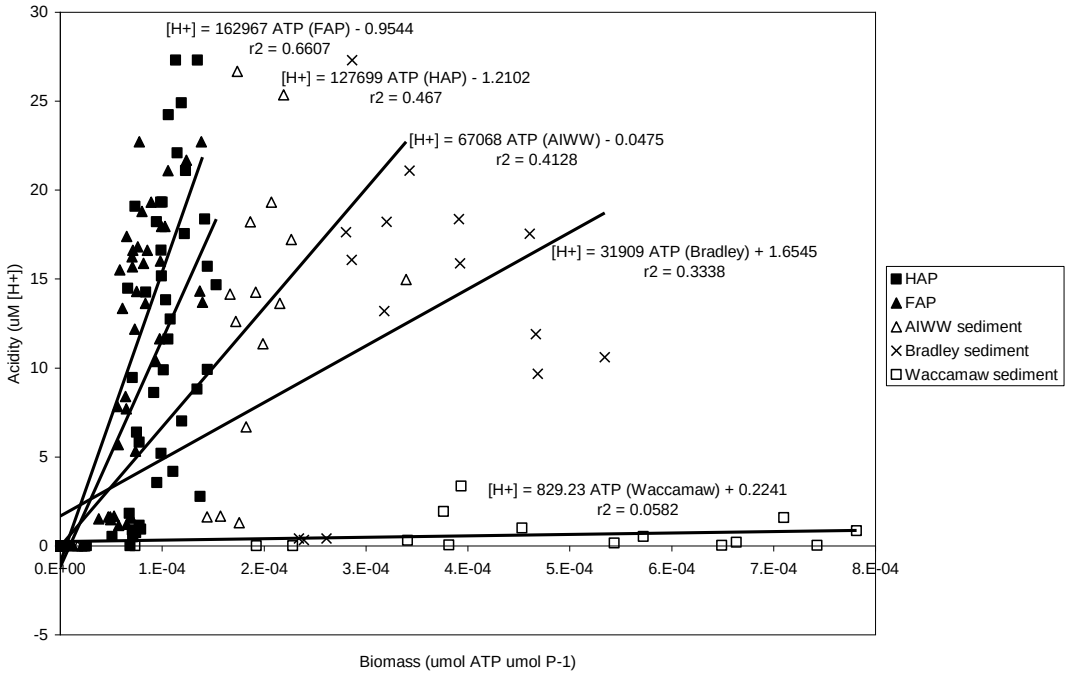


Figure 11. The relationship between acidity and biomass

Population	Median % P liberation	SE ±	n
AIWW population	10.40	4.59	18
Bradley population	9.74	4.77	18
Waccamaw population	3.01	4.61	18

Table 2. Phosphorus liberation by different microbial populations

The Effect of Substrate Solubility

The rate of liberation of labile P was significantly from more readily soluble. Within the confines of the investigation this could only be determined with respect to the experimental substrates HAP and FAP, as the potential P pools in natural sediments were both too patchy in distribution and varied in speciation to allow for confident assumptions to be made regarding dissociation constants or solubility of the forms in which P would likely be present in the sediment.

The assays in which HAP or FAP provided the sources of P were characterized by significantly different rates of P liberation (*Mann-Whitney: $P<0.00001$, $w=6440$*). The standard solubility constants at 25^{o}C and the median % P liberation associated with the two substrates are summarized in Table 3.

Regression Model

The parameters considered above were combined to develop a model in order to explain to a greater extent the variation in the data set obtained in this investigation. When the data from assays where both experimental substrates and natural sediments provide the sources of sediment in addition to no-P controls was considered, the following multiple regression equation explained 67% of the variation in the data set.

%P liberation = 0.76 - 0.17 P substrate + 0.67 [H+] ions + 8780 ATP (*$F= 214$, $df=323$, $p<0.0001$, $r^2=0.67$*)

Figure 13 summarizes the relationship between the parameters P liberation, acidity and biomass.

Due to the variation in natural substrates, in particular the disparity observed where Waccamaw sediments provided the source of P, a model was developed based on the data

obtained from the assays where HAP or FAP provided the P source in addition to the no-P control assays. The resulting multiple regression equation explained 87% of the variation in the data set.

%P liberation = 3.50 - 1.84 P substrate + 0.17 [H+] ions + 70600 ATP (*F=316, df=143, p<0.0001, r^2=0.87*)

Organic Acids

The concentration of OA in the system was seen to have a significant effect on the rate at which P was liberated. The concentration of reactive soluble P increased as the acidity arising from OA concentration in the system increased.

Production of OA followed the same sigmoidal pattern as P liberation over time. Total OA production reached a maximum of 3.09mM when microorganisms collected from the AIWW location were provided HAP as a P source, 4.46mM with FAP and 4.27mM with AIWW sediment. As observed for % P liberation and [H^+] ion concentration, maximum concentrations of OA were reached at days 4 to 6 after which they appeared to stabilize (Figure 17).

Compound	Formula	Solubility Constant (K_{sp} at 25°C)	Median % P liberation
HAP	$Ca_5(PO_4)_3OH$	1.0×10^{-36}	9.80
FAP	$Ca_5(PO_4)_3F$	1.0×10^{-60}	1.43

Table 3. Solubility of HAP and FAP

The rates of solubility reported here are solubility rate constants at 25°C under abiotic conditions.

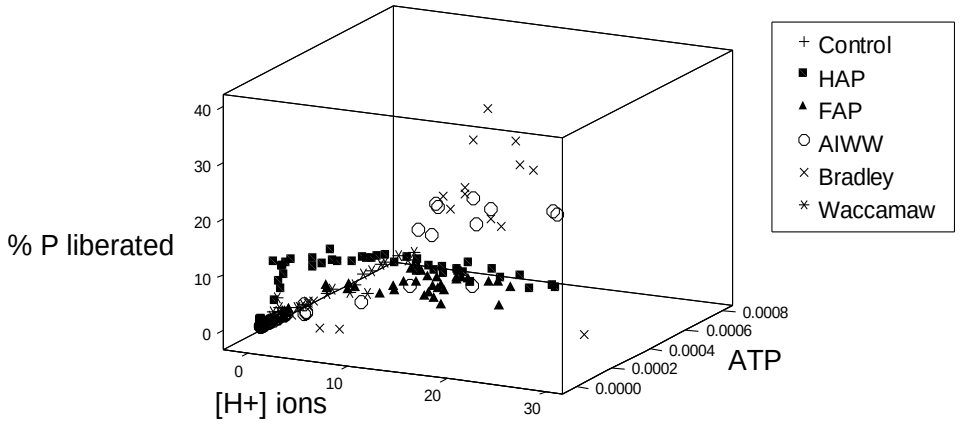


Figure 12. The effect of biomass and acidity on phosphorus liberation

Acidity is measured as uM [H+] and Biomass as umol ATP umol P^{-1}.

The Effect of Organic Acid Production on Phosphorus Liberation

A significant positive correlation was observed between OA, in terms of the potential $[H^+]$ ion concentration calculated from the acid dissociation rates and the concentration of the acid mix present, and the liberation of soluble reactive P (Figure 13).

%P liberation = 0.37 OA (HAP) + 4.81 ($F=22$, $df=15$, $p<0.0001$, $r^2=0.61$)

%P liberation = 0.21 OA (FAP) + 2.15 ($F=9.30$, $df= 17$, $p<0.008$, $r^2=0.37$)

%P liberation = 0.53 OA (AIWW) + 0.53 ($F=5.05$, $df=17$, $p<0.04$, $r^2=0.24$)

The Effect of Organic Acid Production on $[H^+]$ ion Concentration

The principal organic acids determined by HPLC were acetic acid, gluconic acid and succinic acid. Table 4 presents the acid dissociation constant and empirical formula for these acids. Total potential contribution of $[H^+]$ ions from OA in the system was calculated, using pKa values and absolute concentrations of OA in the system. A significant positive correlation was observed between the acidity in the system, measured as pH, and the calculated potential donation of $[H^+]$ ions from OA (Figure 14).

The following regression equations explained a significant portion of the variation in total $[H^+]$ ion concentration of the system, measured as pH.

[H+] = 0.70 OA (HAP) + 1.31 ($F=17.9$, $df=15$, $p<0.001$, $r^2=0.56$)

[H+] = 0.39 OA (FAP) + 2.62 ($F=8.26$, $df=17$, $p<0.008$, $r^2 = 0.36$)

[H+] = 0.55 OA (AIWW) - 0.09 ($F=9.16$, $df=17$, $p<0.01$, $r^2=0.34$)

The majority of the variation in total OA concentration arose from variation in the microbial production of gluconic and succinic acid. Little variation was observed in acetic acid production (Table 5).

The Effect of Biomass on Organic Acid Production

A significant positive relationship between OA concentration, postulated to be a causal agent in P liberation, and the concentration of biomass in the system was observed (Figure 15).

OA = 122000 ATP (HAP) + 2.69 (*F=10.59, df=15, p<0.006, r^2 = 0.43*)

OA = 160000 ATP (FAP) + 11.0 (*F=7.84, df=17, p<0.01, r^2 = 0.33*)

OA = 97900 ATP (AIWW) + 4.18 (F=52.6, df=17, p<0.0001, r^2 = 0.77)

When the production of OA was considered per unit of biomass (µM H^+ µmol ATP^{-1}) the rate of OA production per organism was observed to vary significantly when different substrates comprised the source of P (Figure 16) (*Kruskal-Wallis: x^2=31.0, df=2, p<0.0001*).

Organic Acid	pKa (25^{o}C)	Empirical Formula
Acetic Acid	4.76	$C_2H_4O_2$
Gluconic Acid	3.64	$C_6H_{12}O_7$
Succinic Acid	4.16	$C_4H_6O_4$

Table 4. Acid dissociation constants of organic acids identified

Acid dissociation constants from Perrin (1972) and Hagberg et al. (2002).

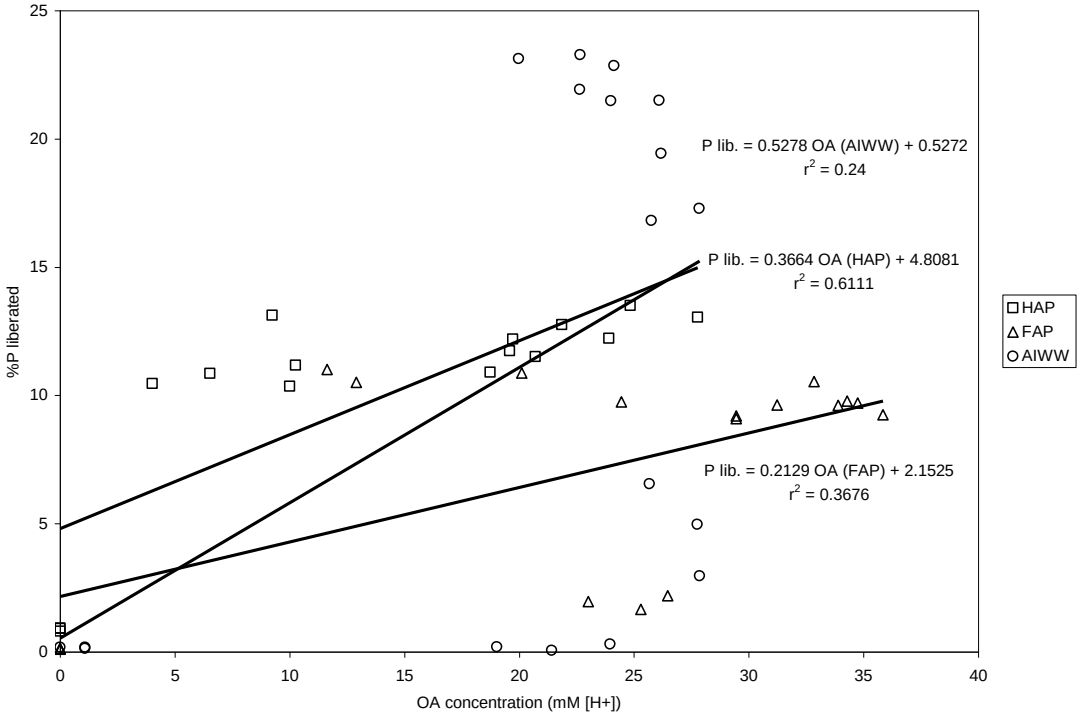


Figure 13. The relationship between organic acid concentration and phosphorus liberation

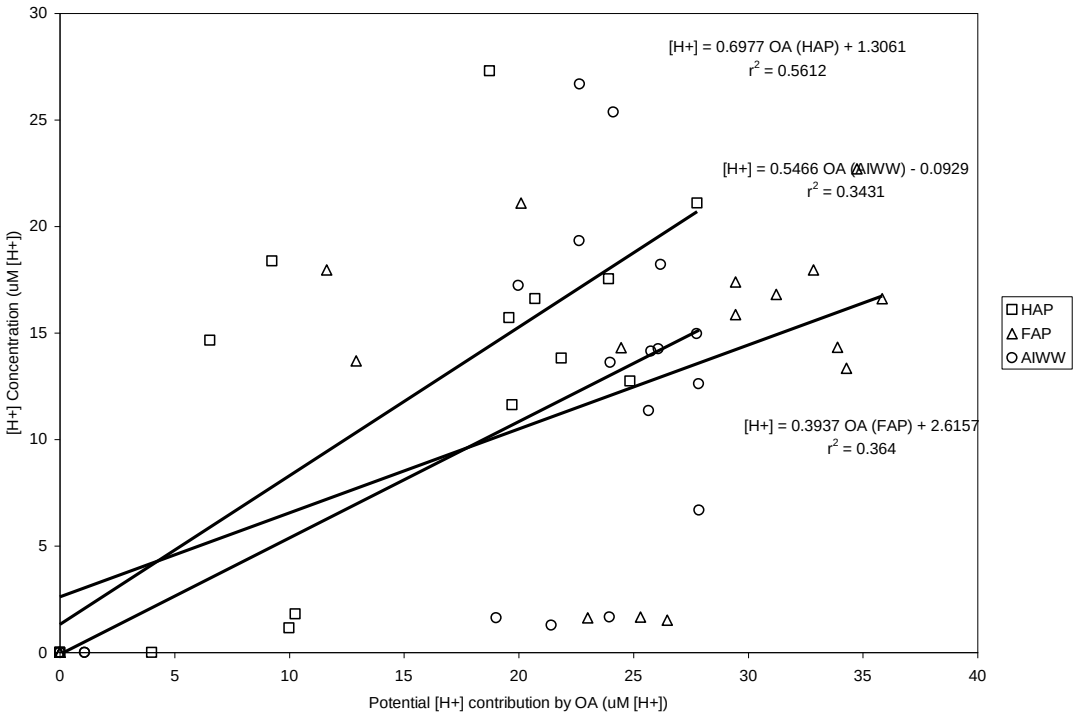


Figure 14. Relationship between potential contribution of [H^+] from organic acid and acidity of the system

The Effect of Substrate Solubility on Organic Acid Production

When the rates of P liberation and OA concentration are considered for each substrate independently a significant difference in the rate of OA production is observed between substrates of varying solubility and P content (*Kruskal –Wallis:* $x^2=8.85$, $df=2$, $p<0.01$). Variation in the rate of OA production over time was observed where substrates of different solubility and P contents were used (Figure 17). Where HAP provided the source of P the population biomass increased exponentially and then stabilized. A similar pattern was also observed in the concentration of OA.

Assays in which FAP provides the source of P however show a similar trend of increase and stabilization, but biomass did not reach the same concentration as that associated with HAP, whereas OA production was greater. In addition, where AIWW sediment provided the P source very low biological production was observed, but OA production, though not as great as that associated with other assays, remained relatively high.

Abiotic Assays

Abiotic bottle microcosm experiments at different pH showed that oxalic acid was associated with a significantly greater release of labile P than hydrochloric acid even at comparable [H^+] ion concentrations (*Mann-Whitney:* $p<0.0001$, $w=6990$) (Figure 18).

P Substrate	Percentage Contribution to Total Organic Acid Concentration		
	Gluconic acid	Succinic acid	Acetic acid
HAP	25.0	45.2	11.0
FAP	47.7	28.3	7.40
AIWW	54.0	26.1	14.4
Overall	42.9	32.7	10.9

Table 5. Relative contribution of acid species, produced by microbes growing on different phosphorus sources, to total organic acid concentration

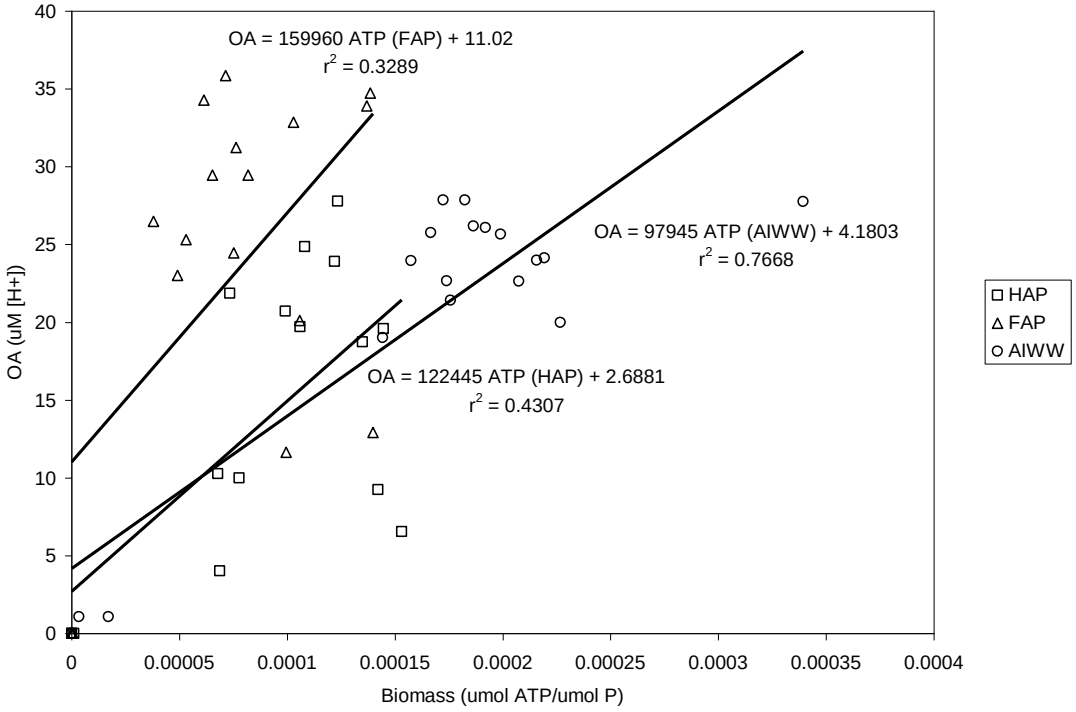


Figure 15. The relationship between biomass and organic acid production

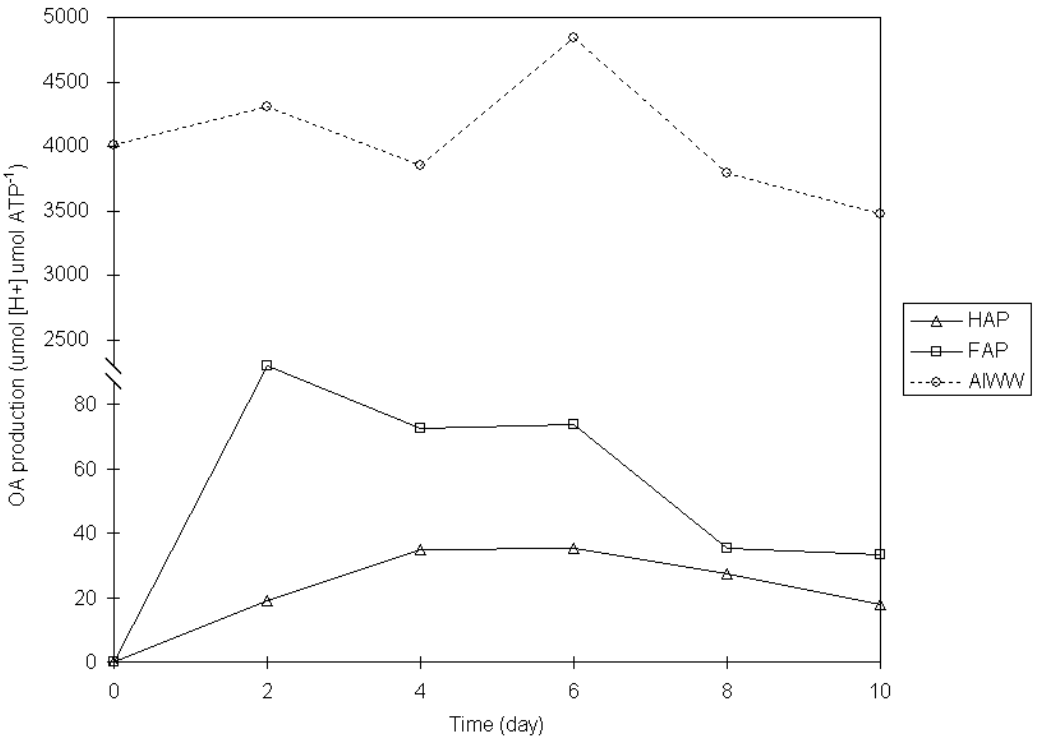


Figure 16. Organic acid production per unit biomass over time

A scale break is included on the y axis, to allow the incorporation of the OA production arising associated with AIWW sediments.

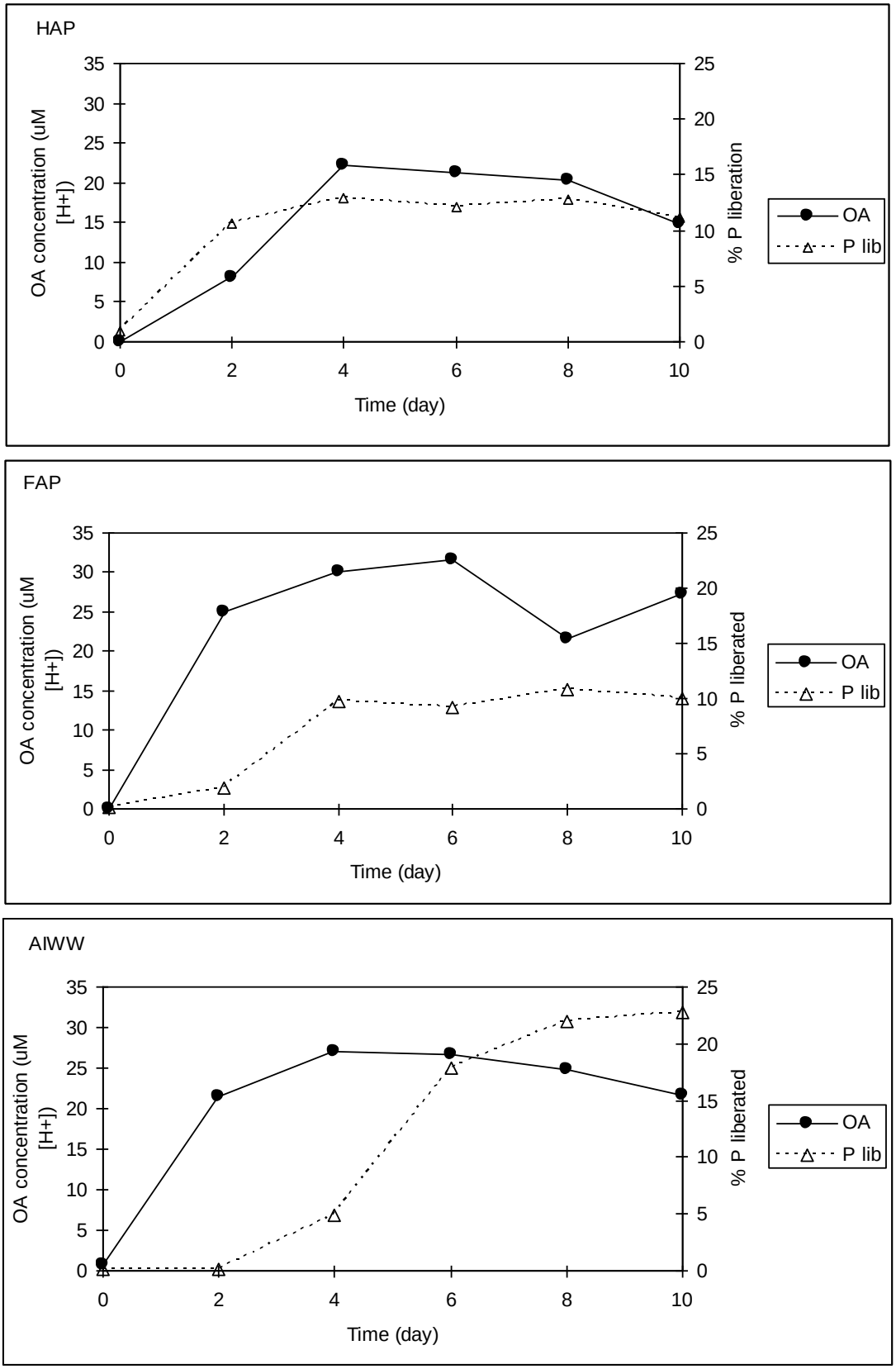


Figure 17. Organic acid concentration and phosphorus liberation over time

In order to account for the portion of P dissolution occurring merely as a function of the presence of [H+] ions, the % P liberated at time t=0, that being immediately after addition of substrate to the media, was plotted against the natural log of the initial concentration of [H+] ions (Figure 19).

HAP: % P liberation = 0.51 ($\ln[H^+]$) + 3.72

FAP: % P liberation = 0.51 ($\ln[H^+]$) + 3.74

AIWW: % P liberated = 0.12 ($\ln[H^+]$) + 0.55

The resultant equation could then be used to determine liberation of P likely to arise from OA production concentration alone (measured as OA associated [H+] production calculated from absolute concentrations of OA and the relevant acid dissociation constant Ka), for a given substrate (Figure 20). The microcosms inoculated with microorganisms yielded significantly more P than would be accounted for by passive dissociation due to [H+] ion concentration alone (*Mann-Whitney: $p<0.003$, $w=29600$*).

Microbial respiration alone supplied $[H^+]$ ions due to carbonic acid production. The calculation of potential proton donation however, suggests in stochoimetric terms that a percentage of P liberation may have been brought about by the increase in OA concentration in the system.

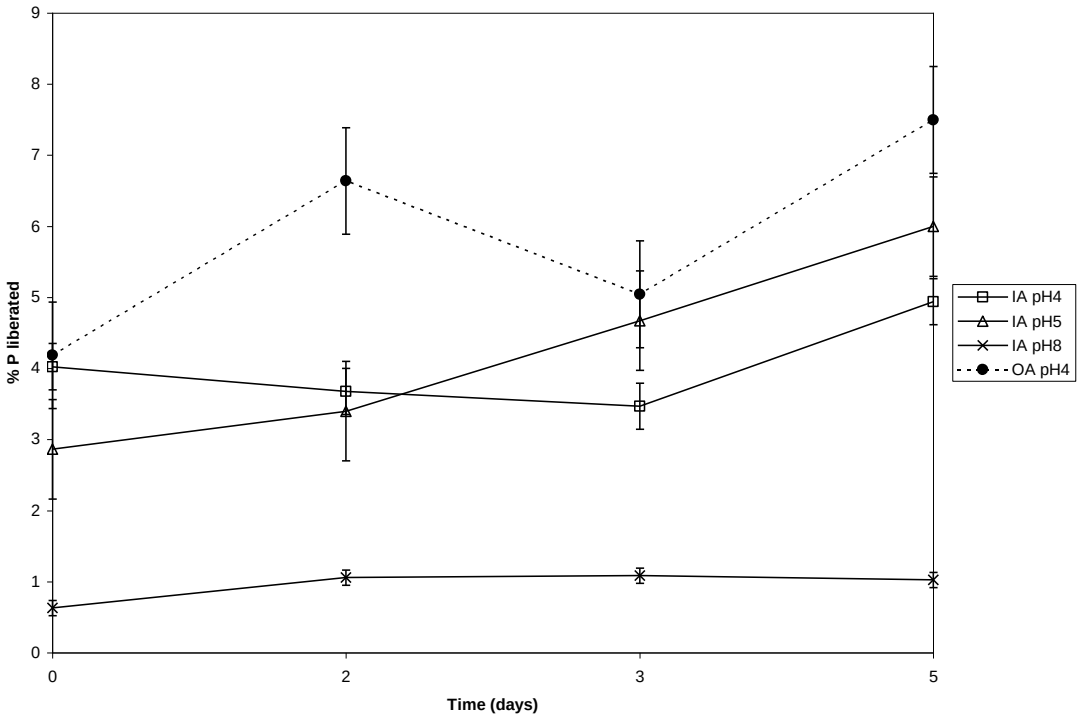


Figure 18. Abiotic phosphorus liberation over time

The liberation of P over time from HAP and FAP associated with inorganic acid (IA) and organic acid (OA).

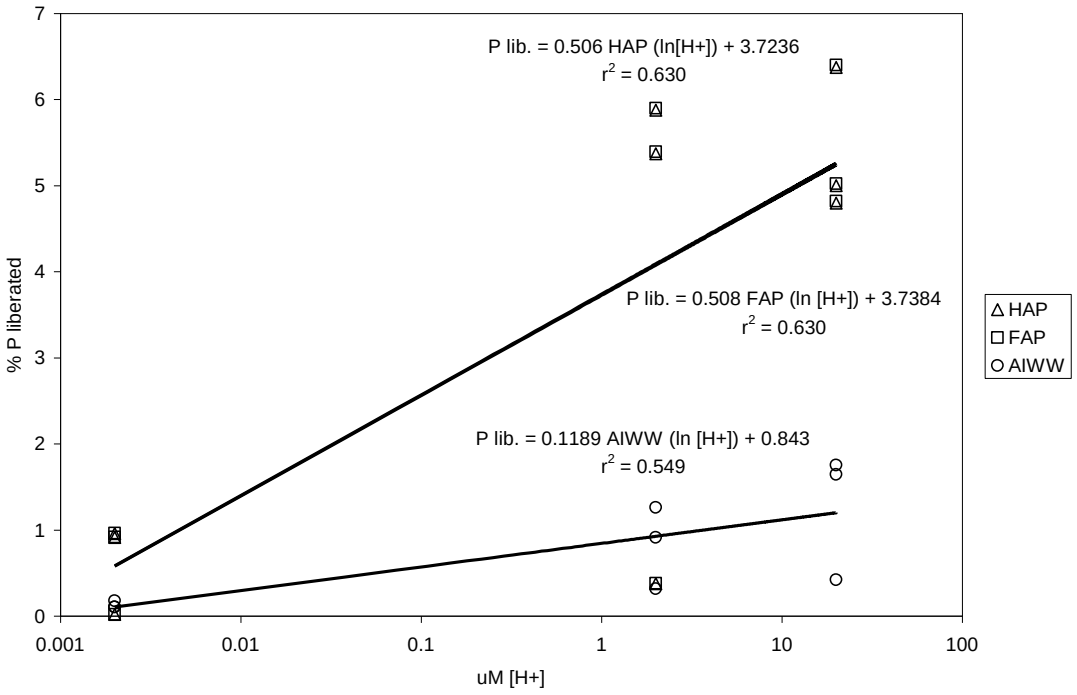


Figure 19. Instantaneous phosphorus dissociation as a function of acidity

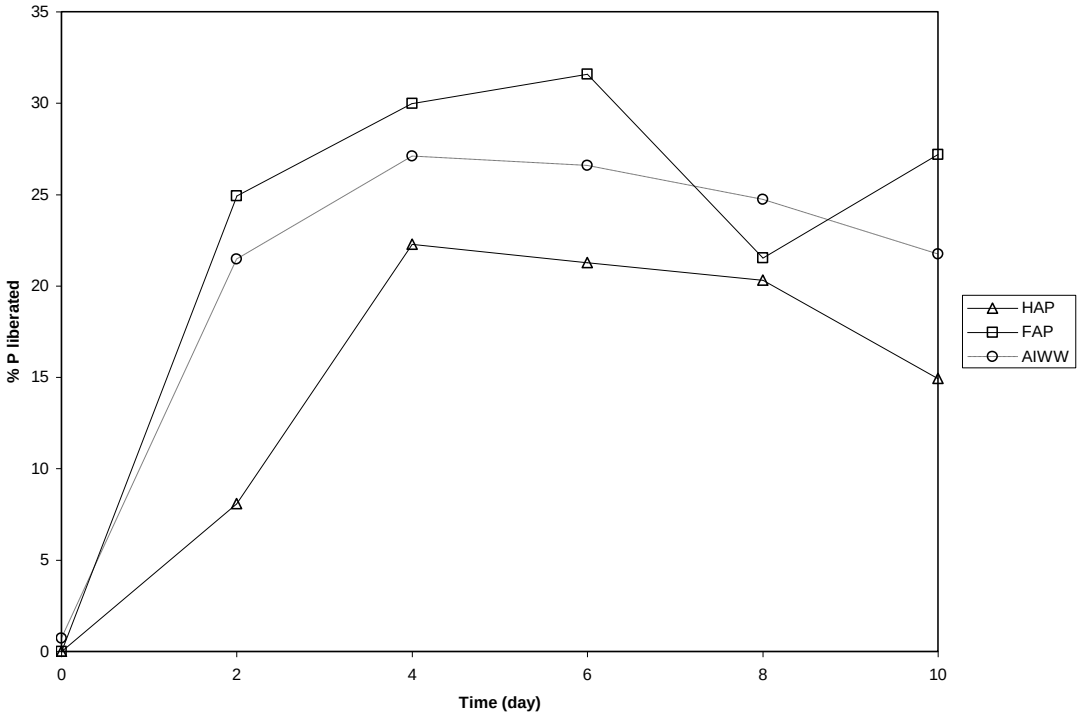


Figure 20. Phosphorus liberation associated with observed organic acid production

The liberation P here is presented as a percentage of the initial P pool. Liberation of P was calculated by applying the rate of liberation of P as a function of $[H^+]$ ion concentration, as determined in figure 19, to the observed production of OA in terms of total contribution to $[H^+]$ ion concentration.

DISCUSSION

The capacity of microorganisms to promote the liberation of labile forms of P from otherwise refractory sources was demonstrated in this investigation.

The flora from three study locations were able to bring about the release of P from both high P content experimental substrates, which would otherwise remain insoluble or sparingly soluble, as well as from the sediments which occur naturally at the study locations.

The results observed were differentiated between the experimental and the natural substrates due to the large range in the initial concentrations of potentially available P. The experimental substrates, HAP and FAP, had a P content some two orders of magnitude greater than that occurring in the natural sediments, after washing with Mehlich-3 extractant (Table 1). As such, it proved instructive to consider the effects of microorganisms on the two types of P substrates separately.

The Mehlich-3 method is known to extract all of the soluble and sparingly soluble P forms and even a portion of the insoluble phosphates (Mehlich 1984). The elimination of a portion of the insoluble P would have a marked effect in experiments where concentrations of potentially available P are already low. Consequently, within the confines of the microcosm approach, the P liberation rates described here are conservative. Furthermore, a significant portion of the P liberated from substrates will not have been sampled. The portion of labile P that was biologically sequestered would not have been resolved by the molybdenum blue method employed (Strickland and Parson 1972). The high bio-productivity achieved in the bottle experiments would have taken up an appreciable portion of what labile P may have been available. This effect is of particular relevance to the assays where natural sediments provided the source of P. Though low or non-significant P liberation was associated with natural

sediments, relative to controls or the high yields observed in assays incorporating experimental substrates, P release must have occurred even in these assays to account for the significantly higher biomass as compared to the controls where no source of P was included. The rate of P liberation would have been low enough that P remained the limiting factor in the system and as such would be quickly biologically sequestered and therefore removed from the system.

Population Composition

The selection of study locations aimed to address a range of ecosystems. AIWW and Bradley represented coastal marine habitats. Bradley was considered to be subject to higher ambient nutrient loading in the water column than the relatively oligotrophic AIWW site (Mallin et. al., 2006). The inclusion of the Waccamaw site allowed for the consideration of a freshwater system. Microbial populations that characterized the AIWW oyster reef study site were associated with the greatest yield of labile P from the experimental substrates liberating 10.41% (SE ± 4.59) of the initial pool of potentially available P (Table 2). The locations from which flora were sampled had a significant influence over the rate at which P liberation occurred in the microcosm. Comparison of the rate of growth and P liberation over time for the different populations demonstrated that this variation in labile P yield was not simply a consequence of an extended lag phase in the populations that characterize ecosystems with greater nutrient availability (Figure 21 and Figure 22), i.e. Bradley and to some extent Waccamaw. Though generally nutrient poor, Waccamaw has been shown to receive a significant influx of P through the degradation of the Waccamaw limestone (Cahoon et al. 1993).

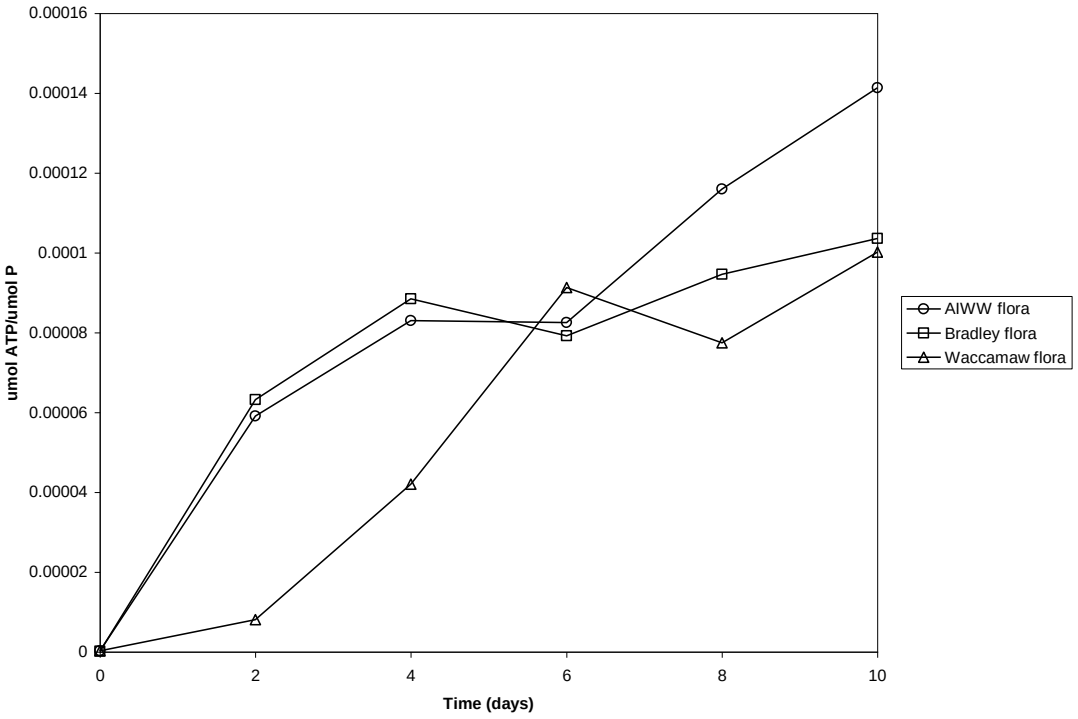


Figure 21. Microbial biomass associated with experimental substrates

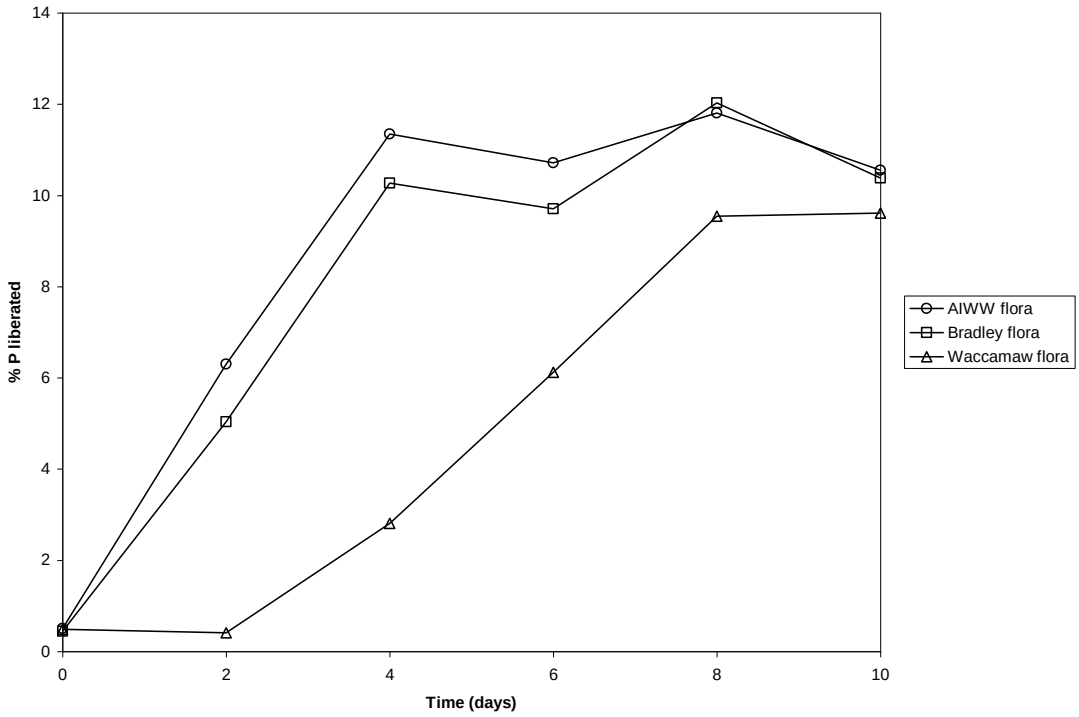


Figure 22. Phosphorus liberation arising from microbial populations associated with experimental substrates

The composition of the microbial population appeared to be the most important factor in regulating the rate of P liberation. During the early stages of the incubation period, though biomass increased quickly, there was a lag in P release associated with the microbial populations from the more nutrient available locations. As such, the relatively few organisms with P liberation capabilities would become favored and subsequently begin to dominate, through competitive advantage, where populations are sampled from nutrient replete locations. The populations characterizing nutrient depleted locations however, would already be rich in organisms better adapted to utilizing refractory sources.

The abundance of microorganisms capable of liberating P, and their relative contribution to population biomass, has been observed to vary in relation to the availability of labile P in the sediments. Barroso and Nahas (1994) showed that of 481fungi isolated from Brazilian terrestrial habitats only 33 were capable of liberating refractory inorganic forms of P in culture. Of the 33, 14 were described as promoting a high or very high rate of P solubilization, defined as greater than 1000µg PO_4^{3-} ml^{-1}. The greatest contribution by P liberators to the microbial populations was observed in populations isolated from pasture soil, which also represented the highest content of insoluble phosphates. Tropical rain forest and forest patch soils presented the next most abundant P liberators respectively. It was shown that the range and size of P fractions influenced the number of fungi and their ability to solubilize insoluble or sparingly soluble phosphates.

Mechanisms of Phosphorus Liberation

The principal factor driving P liberation was acidity. The concentration of $[H^+]$ ions increased rapidly in the systems where microorganisms were present. This rapid influx of

protons drives the equilibrium state in which the P compounds exist. HAP and FAP exist as insoluble or sparingly soluble compounds at high pH. As shown by abiotic experiments, a relationship exists between the solubility of HAP and FAP and the concentration of $[H^+]$ ions. The increased concentrations allow proton substitution to occur, releasing soluble phosphates as a consequence.

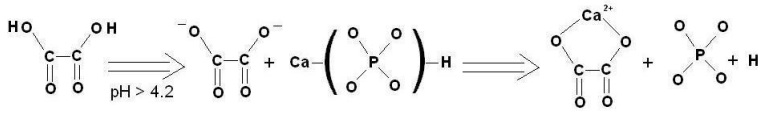
Whether the production of specific acids or the acid production effort are traits that are actively selected as a response to P limitation stress is a question that could not be resolved adequately by the techniques employed in this investigation. Certainly on a population scale it has been demonstrated that different suites of organisms have differing abilities to liberate P.

The Role of Organic Acids

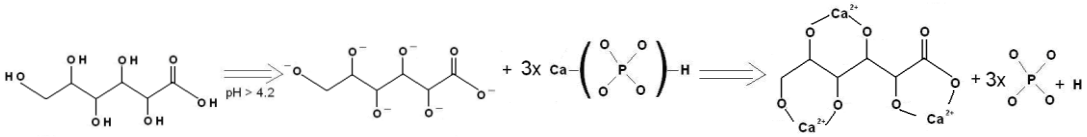
In addition to the role of $[H^+]$ ion concentration it has been observed that the acids produced by microorganisms in such systems as described above are OAs and that the nature of these acids affects the liberation of P sequestered as phosphates bound in complexes with metal ions (Barroso and Nahas 2004, Cerezine 1998, Cunningham and Kuiack 1992, Gadd 1999, Piex 2002, Whitelaw et al. 1999). Acids produced by microorganisms are principally by-products of the metabolic process (Figure 23). Through variations in the metabolic process and substrate, the mitochondrially produced OAs may vary (Gadd 1999). As such, certain organisms are more effective acid producers and consequently should be more efficient liberators of otherwise refractory P.

The two or more electron donor groups in these molecules allow the acid to form one or more rings with metal ions (Figure 24). As such, the chelation properties associated with the OAs produced by microorganisms help to make P available to the biosphere. Although a simple

increase in [H^+] ions can temporarily liberate P from metal ions by proton substitution, the presence of a chelating agent, in this case an OA, can form complexes with the liberated metal ions, preventing them from re-sequestering soluble ortho-phosphate (Equation 1 – oxalic acid and Equation 2 – gluconic acid).



Equation 1 Chelation of calcium phosphate by oxalic acid.



Equation 2 Chelation of calcium phosphate by gluconic acid

The abiotic experiments described in this investigation facilitated further consideration of the importance of the role of OA. The yields of labile P observed could not be accounted for entirely by the rate of P liberation as a result of instantaneous dissociation due to concentration of [H^+] ions. Generally the liberation of P that would be anticipated to arise solely due to an increase in [H^+] ions accounted for less than half the observed yield of labile P (Figure 20). The remainder of the P liberation was likely brought about by the action of OAs produced by the microorganisms in the system. Oxalic acid was observed to liberate P to a greater degree than an equivalent pH change brought about by an inorganic proton source (Figure 18).

The contribution of OA production to the [H^+] ion concentration of the system per unit biomass was seen to vary as a function of P substrate (Figure 16). As such, the ability of organisms to vary the rate of production of OA in response to P limitation was demonstrated.

There is evidence in the literature that the production of OAs is a response to nutrient limitation. *P. chrysosporium* was shown not to produce oxalate when not nutrient stressed (Kuan

and Tien, 1993). Oxalate production has also been observed to increase in the presence of carbonate and bicarbonate in *Paxillus involutus* (Lapetsie et al., 1987). This is likely to be a response to the influence of carbonate and bicarbonate in reducing pH and as such limiting the availability of P from low solubility sources.

The presence of calcium oxalate associated with microorganisms as well as in many natural habitats such as terrestrial soils and aquatic sediments is well documented (Graustein and Phillip Sollins 1977). The occurrence of calcium oxalate crystals associated with the hyphae of oxalic acid-producing fungi in particular (Figure 25) indicates the likely role of oxalic acid in the chelation of calcium, and consequently the liberation of the P, which often occurs in complex with that calcium, in the natural environment (Figure 26).

The presence of a similar process occurring in aquatic environments and that the action of microbial [H^+] ion and OA production is capable of overcoming the buffering characteristics of high salinity ecosystems was demonstrated by this investigation. It is likely that this process occurs in micro-niches. Dramatic changes in pH, redox potential and other parameters are observed in aquatic sediments. Microorganisms should be able to significantly alter the conditions within pockets of sediment, or in particular within boring casts.

When no P substrate was included no bio-production was observed, as compared to systems that incorporated some form of refractory P substrate where an appreciable level of bio-production was observed. Furthermore when an identical system was incubated with no biological influence, insignificant concentrations of P were detected.

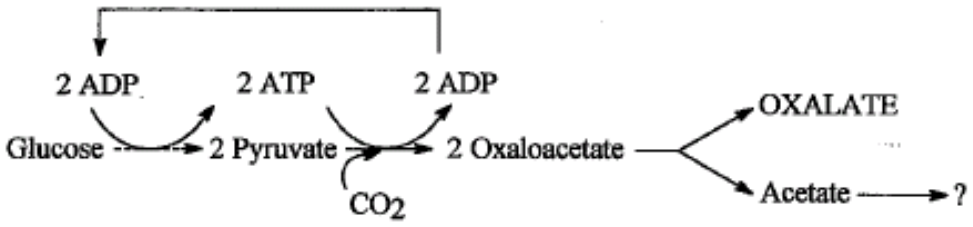


Figure 23. Organic acid biosynthesis

(adapted from Gadd 1999)

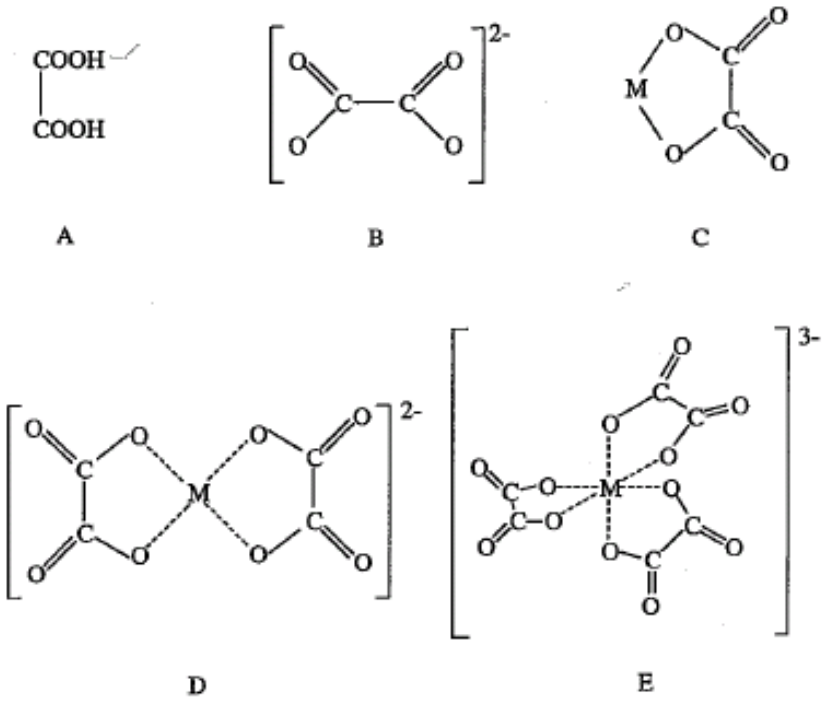


Figure 24. Metal complex formation by oxalic acid

(A) oxalic acid; (B) oxalate; (C) bidentate metal (M) complex; (D) Complex formation with metals which form planar four-coordinate complexes, e.g. Cu^{2+}; (E) complex anion formation with metals which form octahedral six-coordinate complexes, e.g. Al^{3+}, Fe^{3+}. (adapted from Gadd 1999)

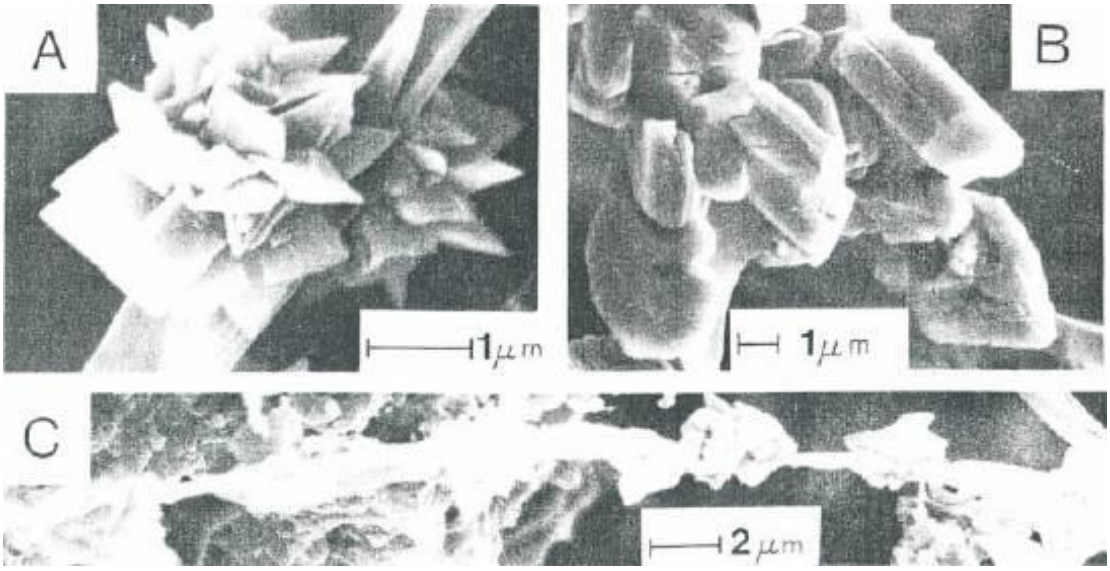


Figure 25. Calcium oxalate crystals associated with the hyphae of oxalic acid producing fungi.

(A) Calcium oxalate crystals adhering to a hypha of the fungus *H. crassum.* (B) Calcium oxalate crystals adhering to fungal hyphae. (C) Hyphae of *H. crassum*. The crystals are attached to the hyphae only in the interstitial space, the hypha in contact with the solid phase is bare. (adapted from Graustein and Phillip Sollins 1977).

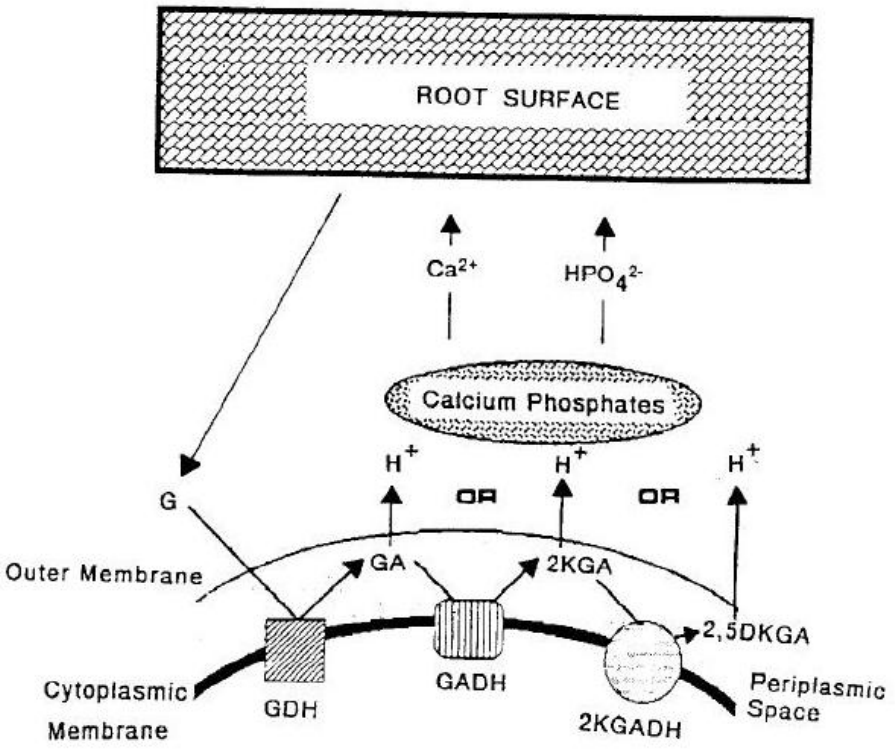


Figure 26. Microbially induced phosphorus release at the rhizosphere

Glucose is metabolized producing organic acids as by-products. P_i is released from the mineral substrate by proton substitution with Ca^{2+}. G; glucose, GA; gluconic acid, 2KGA; 2-ketogluconic acid, 2,5DKGA; 2,5-diketogluconic acid, GDH; glucose dehydrogenase, GADH; gluconate dehydrogenase, 2KGADH; 2-ketogluconate dehydrogenase (Goldstein 1995).

CONCLUSIONS

The processes described herein may not directly contribute significant quantities of labile P on the macro scale. However, these micro scale processes represent the basis for significantly greater bio-productivity than would otherwise be anticipated based on measurable labile nutrient quantities. This investigation suggests therefore that both bio-production and the true nature of nutrient limitation within aquatic sedimentary habitats are significantly under estimated.

Though the rates of P liberation determined in microcosm experiments and reported herein are of limited direct application to natural systems, the need to properly quantify the effects of this process in a range of natural environments in order to obtain robust estimates of P liberation rates has been demonstrated. This would enable the proper inclusion of, as yet unconsidered, potentially available P when estimating nutrient regimes and biogeochemical cycles.

P liberation from insoluble sources in aquatic environments would represent an as yet inadequately considered pathway in the P cycle (Fig. 1) and a source of aquatic biomass. There may also be a range of implications for the management of nutrient loading effects in aquatic systems.

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