A Study of Methods to Release Phosphorus from Polyphosphate Bodies in Various Microbes.

by

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ABSTRACT

A Study of Methods to Release Phosphorus from Polyphosphate Bodies in Various Microbes.

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John Hagan Brown

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Biological phosphorus removal is an accepted phenomenon applied to reduce phosphorus concentrations from wastewaters-activated sludge process with alternating aerobic/anaerobic conditions and application of a suitable chelator. The aerobic-anaerobic processes have been successfully used to enhance biological phosphorus removal (EBPR) as reported by Mino *et al.* (1987), and Smolder *et al.* (1994a & b) in recent years. Other findings in the laboratory reported by Jensen, Sicko-Goad, and Ayala (1977), and McGrath (1998) have indicated that under aerobic conditions some heterotrophic organisms store polyphosphate intracellularly. The phosphorus serves as an energy source during periods of starvation. The objective of this study was to investigate the release of phosphorus from the polyphosphate bodies of various microbes.

The uptake and release of phosphorus by pure culture of *Synechococcus leopoliensis, Plectonema boryanum, Saccharomyces cerevisiae*, and *Rhodotorula rubra* were investigated under aerobic, anaerobic conditions, different pH ranges, and the application of EDTA, and time duration (days). The total phosphorus released by these microbes varied greatly under the various experimental conditions.

The effect of pH on the aerobic release of phosphorus by these phosphorus-accumulating organisms (PAO) in this investigation was performed at pH 7.2, 8.5, and 9.0 (normal growth conditions). Cells reached their normal growth 14-17 days and percent of phosphorus released by the cells at various pH ranges, 7.2, 8.5, and 9.0, and time period 4, 6, and 8 days was low, 25.29%, 30.49%, and 33.40% by *S. leopoliensis*. At the same token, during the anaerobic conditions, there was more P release (54.14%, 76.50%, and 86.85%) respectively. When 0.25M EDTA was added, the average release was 81.33%. *P. boryanum* released 31.08%, 32.23%, and 33.66%. During the anaerobic condition, it released 72.27%, 83.02%, and 95.24%. *S. cerevisiae* released 10.41%, 10.87%, and 11.08% aerobically. Anaerobically, 54.75%, 68.93%, and 87.54%. *R. rubra*, aerobically released 11.56%, 12.07%, and 25.22%. Anaerobically, P released was 36.69%, 53.27%, and 69.04%. The greatest amount of P release was in the anaerobic condition by all the microbes. When EDTA was added to the culture in the aerobic conditions for the same time period and the same pH values P release was 76.07%, 79.68%, and 88.71% by *S. leopoliensis*. 77.76%, 79.70%, and 91.04% by *P. boryanum*. *S. cerevisiae* released 65.97%, 75.19%, and 90.35%. *R. rubra* released 66.17%, 75.05%, and 86.51%. These results are discussed in relation to anaerobic conditions, pH variations, time (days), and addition of EDTA studies of phosphorus release from polyphosphate bodies from various microbes. This uptake and release of P by these microbes in response to high pH values, anaerobic conditions, and with the addition of

EDTA provide evidence to the importance of phosphorus during growth and development and in their response to environmental stress.

To Mr. Colin George MacDonald

&

To my daughter, Rachel Alyssa

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INTRODUCTION

Phosphorus is an important element, and indeed essential to life itself. Used extensively by mankind, it has made a major contribution to agricultural and industrial development. The release of phosphorus to surface waters, however, and its consequent contribution to eutrophication has also led to increasing concerns about water quality. Policies are, therefore, being implemented throughout the world to reduce the levels of phosphorus entering surface water by the implementation of technologies to remove phosphorus from domestic and industrial wastewater.

The development of phosphorus removal techniques has been an ongoing process since the 1950s and there are no environmental or technological reasons why these cannot be developed further to facilitate removal of phosphorus. At present there are two established methods of phosphorus removal (1) chemical precipitation, and (2) biological removal (Greenberg *et al.*, 1995).

Greenberg *et al.*, (1995) proposed that sludge could take up phosphorus at a level beyond its normal microbial growth requirements. Batch experiments carried out by Srinath *et al.*, (1959) showed that vigorous aeration of activated sludge could cause the concentration of soluble phosphorus in mixed liquor to decrease rapidly to below 1-mg l^{-1}. Then in 1965, Levin and Shapiro reported on enhanced biological phosphorus removal (EBPR) using activated sludge, with over 80% being removed by vigorous aeration of the sludge without the addition of chemicals. This was termed “luxury uptake”, and further experiments using 2-4 binitrophenol to inhibit phosphorus uptake confirmed that the removal was biological. Biological removal has received attention since forty years (Levin and Shapiro, 1965).

In a conventional activated sludge plant, bacteria use only enough phosphorus to satisfy their basic metabolic requirements, resulting in typical removal rates of 20-40% (Brett *et al.*, 1997). In treatment plants that are designed to remove phosphorus in wastewater sludge, an environment is generated for the proliferation of bacteria that accumulate phosphorus in excess of normal metabolic requirements. In biological phosphorus removal systems, certain bacterial strains accumulate large quantities of polyphosphate (polyP) intracellularly. When subjected to anaerobic conditions some of the stored polyP in the bacterial cells is utilized as an energy source, but and much phosphate is released into the external medium. The phosphorus in the external medium can be measured by several different methods. In this research the Hach Phosphorus Test Kit (Reactive or Total) has been used to ascertain the amount of phosphorus released by polyphosphate bodies (PPBs) in both aerobic and anaerobic conditions (Figure 1).

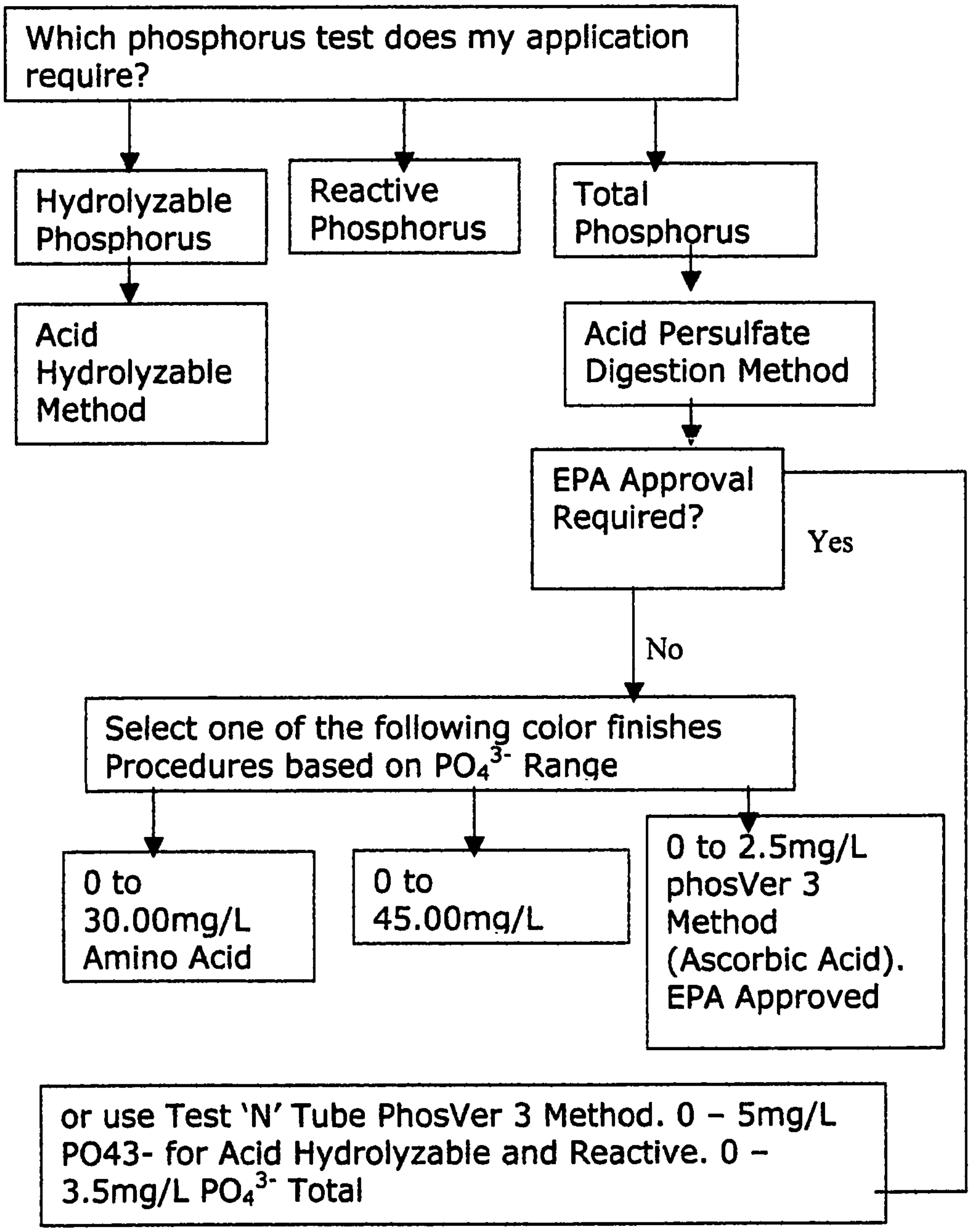
Many microbes such as *Acinetobacter* spp., *Microlunatus phosphovorus, Lampropedia* spp., *Rhodocyclus* spp., *Plectonema boryanum, Synechococcus leopoliensis, Nostoc commune, Sacchromyces cerevisiae* and *Rhodotorula rubra* have been shown to accumulate polyphosphates. Among these microbes, *Synechococcus leopoliensis, Plectonema boryanum, Sacchromyces cerevisiae*, and *Rhodotorula rubra* have been selected for this study.

To control eutrophication phosphate removal from wastewater is required before wastewater is discharged to the receiving water bodies. The accepted explanation for biological removal of phosphorus is that of the anaerobic-aerobic contacting results in a competitive substrate utilization and selection of the phosphorus-storing microorganisms. Some researchers have suggested that the release of the phosphate anaerobically is due largely to the pH. The higher the pH, the more phosphate is released (Helness and Odegaard, 1999). They further suggested that ethylenediamine-tetraacetic acid disodium (EDTA), hydrochloric acid (HCl), and 2,2'-bipyridine can be used successfully to release phosphorus from microorganisms. The pH ranges vary from 7.0 to 9.5. Boers (1991) suggested that the most favorable pH for removing phosphorus the most is 9.5 and that this is termed "supersaturation" of phosphorus. Thus, the various articles have shown the feasibility of obtaining the “supersaturation” pH by “air stripping” (removal of air which is a mixture of gases) a phosphate-enriched anaerobic supernatant.

Activated sludge processes with alternating anaerobic and aerobic conditions, pH ranges, and chelators have been successfully used. It has been designated by a number of studies as the "enhanced biological phosphate removal" (EBPR) (Mino, 1999). All the above studies were carried out with mixed batch microbes from activated sludge.

No exhaustive study has been conducted using individual organisms on the release of phosphorus from polyphosphate bodies. The work that reported here is an attempt to elucidate the conditions, under which phosphorus will be released from polyphosphate bodies in the various microbes using the above factors.

Figure 1. How to Select the Correct Phosphorus Procedure



Review of pertinent literature

Enhanced Biological Phosphorus Removal (EBPR)

Enhanced biological phosphorus removal (EBPR) is an economical and environmentally friendly method for removing phosphate (P) from sewage (Barnard, 1974). Enhanced P removal is attributed to the activities of (poly) phosphate accumulating bacteria (PP bacteria). In the process, an anaerobic stage is typically conducted before an aerobic stage. In the anaerobic stage the PP bacteria degrade intracellular polyphosphate, which liberates energy, used in the uptake of organic substrates such as glucose. The substrate taken up is stored as intracellular organic polymers such as polyhydroxyakanoates (PHAs), which are subsequently oxidized in the aerobic stage to yield energy for the regeneration of the polyphosphate (PP). Thus, the PP bacteria successfully compete for organic substrate with other bacteria in the process.

Phosphorus in wastewater is transferred into a biomass (sludge) and is finally removed from the process through the sludge, and the sludge is used as fertilizers on the farms by the farmers (Wentzel, *et al.*, 1985; Comeau *et al.*, 1986; Mino *et al.*, 1987; Smolders *et al.*, 1994a,b).

It has been demonstrated in some full-scale wastewater treatment plants that the Enhanced Biological Phosphorus Removal process is able to reduce phosphorus concentrations to very low levels (Oldham, 1985; Ketchum *et al.*, 1987).

Previously, in electron microscopic studies of the cyanobacteria *Nostoc pruniform* (Jensen, 1968), *Plectonema boryanum* (Jensen et al., 1982a; Jensen *et al.*, 1977; Sicko-Goad, and Jensen, 1976), and *Anacystis nidulans* (Lawry and Jensen, 1979) the accumulation of polyphosphate bodies under various conditions of cultivation was investigated. During these investigations special emphasis was placed on the accumulation of polyphosphate bodies by cyanobacteria under conditions approximating those of inland water bodies, i.e. conditions of phosphorus and sulphur starvation. According to Jensen and co-workers (1977), waters in such areas are known to contain about 0.01 micrograms phosphorus per liter and this creates conditions of phosphorus starvation for the microorganisms. When large amounts of industrial and domestic detergents, in particular sodium tripolyphosphate ($Na_5P_3O_{10}$), enter inland water bodies, an intensive "fluorescence" of cyanobacteria occurs leading to contamination of drinking water. This has become quite a serious problem in recent years, and many laboratories around the world are endeavoring to solve this problem. Furthermore, the discharge of phosphates into bodies of waters can lead to an increased growth of algae (eutrophication).

Essential requirements for EBPR are (1) periodic alternation of anaerobic and aerobic conditions, and (2) sufficient readily degradable substrates in the anaerobic phase. So far only activated sludge systems have been implemented for EBPR. It has been shown, however, that EBPR can also be achieved in laboratory grown microbes. It is the goal of this study to further develop an understanding of phosphorus release from various microbes using various cultures of specific species of microbes. The long-term goal of my research is to use the methods developed in phosphorus removal to study both the sequestering and the release of heavy metals from microbes.

There have been many methods employed around the world to remove phosphorus from activated sludge. At present the main types of phosphorus removal from wastewater effluents are chemical precipitation using alum or lime, and biological removal. These methods of removal do not recycle phosphorus as a truly sustainable product because it is removed along with various other waste products. The development of phosphorus removal by crystallization has provided a way of removing phosphorus in a form that has the potential to be a raw material for the phosphate industry such as struvite and hydroxyapatite. Through combining biological phosphorus removal and crystallisation a number of benefits in terms of process economics can be made. By treating the effluent initially with a biological process a concentrated phosphorus effluent stream is produced.

Levin and Shapiro (1965) reported on enhanced biological phosphorus removal using activated sludge, with over 80% being removed by vigorous aeration of the sludge without the addition of chemicals. This was termed "luxury uptake" and, using 2-4 dinitrophenol to inhibit phosphorus uptake, they confirmed that the removal was of a biological nature. Biological assimilation is now receiving increasing attention for a number of reasons, principally:

(i) The cost of flocculates is increasing, as are the concentrations of phosphorus in some areas of the world. Thus, the cost of chemical phosphorus removal could become prohibitively high.

(ii) The addition of aluminum and ferric salts as coagulants has, in some cases, resulted in unacceptable concentrations of these cations in the final effluent.

(iii) The additional sludge production from the addition of chemicals is avoided along with its consequent sludge disposal problems.

(iv) Sludges from biological phosphorus removal would be higher in available phosphorus and could make better agricultural fertilizers.

In a conventional activated sludge plant, bacteria only use enough phosphorus to satisfy their basic metabolic requirements, resulting in typical removal rates of 20-40% (Brett *et al.*, 1997). In a plant designed to remove phosphorus, an environment is generated for the proliferation of bacteria that accumulates phosphorus in excess of normal metabolic requirements. Within biological phosphorus removal systems, certain bacterial strains accumulate large quantities of inorganic phosphates intracellularly and sequester them in the form of polyphosphate bodies. When subjected to anaerobic conditions involving the absence of both nitrates and oxygen, these bacteria are able to take up short-chain fatty acids as a food stock- especially acetates. The stored polyphosphate in the bacterial cells is utilized as an energy source and much of the phosphate is released into the supernatant. The acetates are stored as poly-*B*-hydroxybutyrate inside the cells until they reach the aerobic zone. At that point the poly-*B*-hydroxybutyrate is metabolized providing energy within the cell for the uptake of all available orthophosphates. The effect of this reaction is the net uptake of phosphorus from the solution.

Many studies on algae, according to Jensen and co-workers (1982a) and other researchers, (Dawes and Senior, 1973a,), are able to accumulate polyphosphate. The conditions favoring this accumulation, however, differ greatly (Dawes and Senior, 1973a,b). The polyphosphate content according to Dawes is said to be low during rapid growth, but increases when a nutrient imbalance causes the growth rate to decline. Some microorganisms that possess polyphosphates do not display this pattern of accumulation and degradation characteristics of reserve materials, but accumulate the polyphosphate during exponential growth (Friedberg and Avigad, 1968).

In the literature two descriptions are given for the uptake of inorganic phosphate by microorganisms: (1) the "overplus" phenomenon, and (2) a "luxury" uptake. In the “overplus” phenomenon cells previously subjected to phosphate starvation undertook a rapid and extensive accumulation of polyphosphate. The phenomenon was termed "Polyphosphate Uberkompensation" by Liss and Langen (1962) and was translated as "polyphosphate overplus." The "luxury" uptake involves a larger phosphate uptake by growing bacteria than is necessary for their normal cell metabolism. Levin and Shapiro first reported this in 1965. Batch experimental studies were conducted to qualitatively assess the luxury uptake mechanism, and relevant parameters for removal of phosphates from municipal wastewaters by activated sludge. Thus, high phosphorus removal, in excess of growth requirements indicated luxury uptake by the sludge microorganism.

A study to define the luxury uptake mechanism for phosphorus removal at the Bonnybrook Sewage Treatment Plant located in Calgary, Alberta, Canada. Their results reported herein supported the Luxury Uptake Phenomenon and its biological nature.

Electron Microscopy Studies

As pointed out by Rosenberg (1966), many organisms possess cellular inclusions, which stain metachromatically with certain basic dyes. These inclusions have been termed as metachromatic volutin or Babes-Ernst granules (Harold, 1966). The term "volutin" was first used by Meyer (1904) who noticed an accumulation of distinctive granules in *Spirillum volutans*. It was Wiame (1947a,b; 1949), and Schmidt *et al.* (1946) who identified volutin granules as deposits of inorganic polyphosphates. However, polyP bodies (or granules) have long been confused with other cytoplasmic inclusions. Two controversies developed based on microscopic studies and histochemical staining. The early electron microscope studies showed that many bacteria contained granules which were highly electron scattering and had smooth and sharply defined margins as in *Mycobacteria* (Lembke and Ruska, 1940, Knasysi *et al.*, 1951, and Mud *et al.*, 1956).

From electron microscope studies involving the localization of polyphosphate granules in *Plectonema boryanum* cultured in media containing the normal content of phosphorus, as well as under conditions of phosphorus starvation followed by a subsequent "phosphate overplus" medium enriched with phosphorus, Jensen (1990) drew the following conclusions: (1) under conditions of "phosphate overplus," polyphosphates accumulated in the region of the nucleoplasm as electron dense bodies and (2) in certain cells, polyphosphate bodies formed near thylakoids containing chlorophyll ***a***, and performed phosphorylation reactions.

Polyphosphates also accumulate in the vacuoles of yeast and other eukaryotes like polyphosphate bodies in prokaryotes during growth on high-phosphate medium following phosphate starvation (Jensen *et al.*, 1977).

Polyphosphate metabolism in *Escherichia coli* was studied during transient changes in phosphate availability in order to determine its role in phosphate storage. *E. coli* was grown under phosphate-limiting conditions until the cells reached their stationary phase, and then they were shifted to phosphate-rich growth conditions. During the shift from phosphate starvation to phosphate surplus conditions, PPBs increased dramatically in size and in number during the first 60 minutes, and then decreased (Kulaev and Vagobov, 1983).

Sicko-Goad *et al.*, (1975) and Baxter and Jensen (1980a) combined electron microscopy with X-ray energy-dispersive microanalysis to reveal the elemental nature of these granules. This method enabled them to determine *in situ* that not only phosphorus, but magnesium, sulphur, calcium and potassium were also present in the bodies.

Baxter and Jensen, (1980a,b) showed that under ordinary cultivation conditions phosphorus is present in polyphosphate granules of the cyanobacteria *Plectonema boryanum* as well as appreciable amounts of potassium and lesser amounts of calcium and magnesium.

Bacteria such as *Acinetobacter calcoacetcus* are capable of storing large amounts of phosphorus as polyphosphate bodies (Buchan, 1981). Phosphate starvation (Baxter and Jensen 1980a) stimulates rapid phosphate uptake upon the resuspension of *Plectonema boryanum* and *Escherichia coli* in phosphate rich media.

Other investigators, such as Medveczky and Rosenberg (1971), have shown an increased polyphosphate accumulation in *Aerobacter aerogenes* when grown at a temperature of 23° C and pH of 7.0. During a study of the uptake of phosphate by microorganisms in activated sewage treatment, it was discovered that the organisms took up phosphates during the aeration step, but lost it to the medium rapidly in the anaerobic step. In fact, various methods have been devised to remove phosphorus from wastewater using activated sludge (Stratful *et al.*, 1999). The generally accepted theory for biological phosphorus removal is that of anaerobic-aerobic contacting, which results in a competitive substrate utilization.

It has been suggested by a number of studies that the biological models used to explain the characteristic aerobic and anaerobic biochemical transformations occur in enhanced biological phosphorus removal (EBPR) have been formulated largely from measurements with mixed culture activated sludge systems. Organisms that accumulate phosphorus in the form of polyphosphate have been selected in these systems. Various researchers have also suggested that the transformations occurring under anaerobic conditions are thought to be critical for the selection of polyphosphate accumulating organisms (PAO) in the anaerobic/aerobic system (Bond *et al.*, 1998).

Most investigations of anaerobic phosphate release have not mentioned the physical nature of polyphosphate bodies (PPBs); virtually no data are available on the PPBs in most studies. Medveczky and Rosenberg (1971), however, suggested that the release of phosphate anaerobically was due largely to the pH. The higher the pH, the more phosphate is released into the supernatant.

Chemicals studies with ethylenediamine-tetraacetic acid (EDTA), hydrochloric acid (HCl), and 2,2-bipyridyl, can also be used to release phosphorus from microorganisms (Helness and Odegaard, 1999). Researchers who used these Chelators in order to obtain the release of phosphorus by microorganisms in the anaerobic / aerobic conditions suggested them to be a more efficient method of releasing phosphorus from microbes. Some who used the pH range suggested that it is the most efficient method (Helness and Odegaard, 1999). Both methods of phosphorus release in anaerobic liquids show a necessity to limit the feedback of phosphorus released in sludge handling or anaerobic digestion to improve performances of biological P removal.

The appropriate pH range to remove phosphorus is often considered to be in the range of pH of 9.5 (Boers, 1991) and is termed the "supersaturation" of phosphorus. Boers, (1991), showed the feasibility of obtaining supersaturation pH by removing oxygen and other gases present excluding nitrogen, a phosphate-enriched anaerobic supernatant.

We feel we now we have the technology and methodology to solve these problems using cultures of single microbial isolates.

Biological Phosphorus Removal Mechanisms

An understanding of the steps involved in biological phosphorus removal provides a useful insight into the factors that can affect the performance of a biological phosphorus removal system (Ganczarczyk and Hamoda 1973, and (Michell *et al.*, 1977). The following observations by various investigations are presented as a background to the proposed mechanisms.

Fuhs and Chen (1975) examined activated sludges from the Baltimore Back River and the Seneca Falls, New York treatment plants when the plants were exhibiting high levels of phosphorus removal. They concluded that the organism associated with phosphorus removal belonged to the *Acinetobacter* genus. They subjected a pure culture to anaerobic-aerobic cycles, and noted excess phosphorus removal. They postulated that the anaerobic phase in the excess phosphorus removal system was important for the production of simple carbohydrates, such as ethanol acetate and succinate, which serve as carbon sources for *Acinetobacter*.

Contrary to later findings, they felt that the *Acinetobacter* assimilated the simple carbohydrates in the aerobic phase of the cycle. This is crucial for EBPR because volatile fatty acids (VFAs) are considered essential substrates for the polyphosphate-accumulating bacteria, as Fuhs and Chen (1975), found that a significant phosphorus release rate could be promoted by the addition of carbon dioxide during the anaerobic phase, which also lowered the pH. This was also observed by Deinema *et al.* (1985).

Other investigators have also reported observing significant levels of *Acinetobactor* in biological excess phosphorus removal systems (Buchan, 1981, Lawson and Tonhazy, 1980, and Lotter and Murphy, 1985). Lotter and Murphy (1985) also found significant levels of *Aeromonas* spp., and *Pseudomonas* spp., which are capable of polyphosphate accumulation. Hascoet and Florentz (1985) also noted the presence of *Bacillus cereus* in addition to *Acinetobacter* and Suresh *et al.* (1985) found small numbers of *Pseudomonas vesiculcris* besides *Acinetobactor* in samples cultured from anaerobic-aerobic phosphorus removing pilot plant. Brodisch (1985) noted that the removal of phosphorus in a system containing *Acinetobacter* became significant only after the development of an *Aeromonas* population. He postulated that the *Aeromonas* bacteria served the important function of producing fermentation products in the anaerobic phase for Acinetobacter.

Lotter and Murphy (1985) noted an increase of *Pseudomonas, Acinetobacter and Aeromonas* in biological phosphorus removal systems. They also suggested that these bacteria and species accomplished denitrification in anoxic zones of biological nitrogen removal systems. Osborn and Nicholls (1985) reported rapid biological phosphorus uptake during nitrate reduction in the absence of dissolved oxygen (DO) indicating that phosphorus uptake may be occurring with denitrifying bacteria. Hascoet and Florentz (1985) also reported phosphorus release in anoxic zones by *Acinetobacter,* provided that there was a relatively high level of substrate availability.

Anaerobic Conditions

A full-scale plant investigation at Palmetto Florida found that increasing the anaerobic detention time from 1.1 to 2.6 hours increased the percent total phosphorus removal from 59 to 71 percent (Hungate, 1969). Rensink, (1981) studies on *Acinetobacter* led him to investigate the change in soluble phosphorus concentrations in the anaerobic phase in conjunction with acetate. His experiments showed a 1:3 decrease in acetate concentrations and an increase in orthophosphate concentrations as a function of the anaerobic time. Various investigators have observed a decrease in soluble substrate and an increase in orthophosphate concentrations in the anaerobic zone of anaerobic-aerobic sequenced biological phosphorus removal systems. Hong *et al.* (1982) showed a soluble biochemical oxygen demand (SBOD) concentration decrease from 45 to 15 mg/l, and an orthophosphorus concentration increase from 6 to 24 mg/l in the anaerobic zone. Ekama *et al.* (1984a, b) related phosphorus release in the anaerobic zone to the presence of a soluble readily -biodegradable substrate.

Anaerobic Fermentator Zone Consideration

Further experiments have indicated that the longer the time the microbes are in the anaerobic zone the more phosphorus is released. The anaerobic zone contact time for the modified Bardenpho and anaerobic/oxygen (A/O) system has ranged from 0.9 hour for the Largo A/O facility, to 2.0 hours for modified Bardenpho facilities at the Payson and Kelowna plants (Ekama *et al.,* 1984a, b). Early full-scale plant investigations at Palmetto Florida found that increasing the anaerobic detention time from 2 to 9 hours, increased phosphorus release (Ekama *et al.,* 1984a, b).

Gases used in anaerobic work generally are carbon dioxide (CO_2), hydrogen (H_2), nitrogen (N_2), or a mixture of these gases. Normally, they are supplied in cylinders. Premixed gases are available but expensive, and it is more economical in the long run to use a gas-mixing system such as that supplied by Matheson Gas Products. It is convenient to dispense the O_2-free gas through a gassing manifold to several tubes at the same time (Balch and Wolfe 1976; Balch *et al.*, 1979). From the gassing manifold, the gas is led through thick-walled plastic or rubber tubing, which ends with 3 to 4 inches. In this experiment dry nitrogen gas was used for the anaerobic condition.

Media for the culture of anaerobically chosen microbes

It is impossible to list here the compositions of all media for the culture of microbes that could be used in this study. Many organisms require quite rich media, which, in addition to carbon and energy sources, also contain yeast extract, tryptone, vitamins, minerals and reducing agents. However, the development of chemically defined media for several heterotrophic bacteria has been successful Macy, *et al* (1984).

Solid Media

In general, solid media have the same or similar compositions as liquid media except for the addition of a solidifying agent. The most widely used solidifying agents are agar, carrageen, silica gel and Gerrite (Bryant 1972). All of these can be used to solidify media for anaerobic bacteria. The choice of a solidifying agent depends on the growth requirements. Agar is the best known and also the most widely used. It is obtained from certain red marine algae, and it consists of two polysaccharides, agarose and agaropectin, which are not degraded by most bacterial species according to Bryant (1972).

According to Balch *et al.* (1979), there are certain traces of elements suitable for the growth of microbes as indicated below.

Stock solution for growth of microbes

Chemical	Concentration. (Mg/l)
Nitriloacetic acid	1,500
$MgSO_4.7H_2O$	3,000
$MnSO_4.H_2O$	500
NaCl	1,000
$FeSO_4.7H_2O$	100
$Co(NO_3)_2.6H_20$	100
$CaCl_2$, anhydrous	100
$ZnSO_4.7H_2O$	100
$CaSO_4.5H_2O$	10
$AlK_2(SO_4)_3$, anhydrous	10
Boric acid	10
$NaMoO_4.2H_2O$	10

There are many designs for the continuous anaerobic fermentations with the possibility of cell recycling. The diagram below is an example of such designs for the studies of acetate production by fermentation of glucose with thermophilic acetogenic bacteria and for ethanol production with *Thermoanaerobacter ethanolicus* (Ljungdahl, 1983), *in* Proceedings of the 7th Symposium on Biotechnology for Fuels and Chemicals, in press; Weigel *et al.*, (1981).

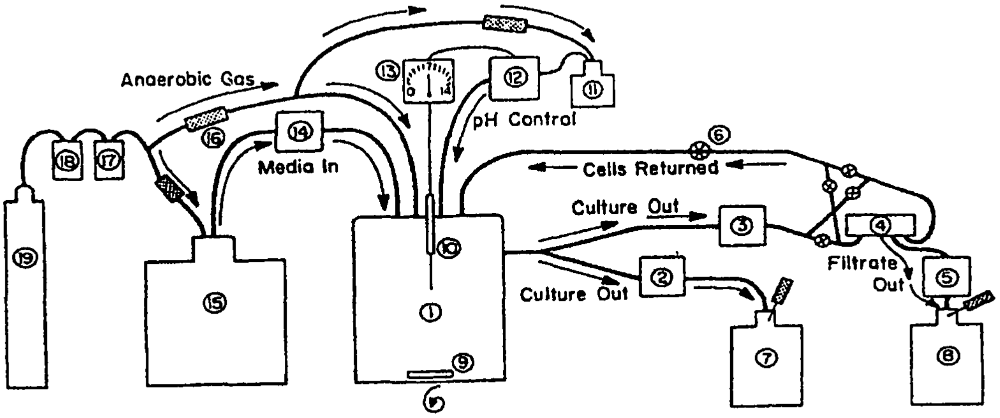


Figure 2. Anaerobic control setup system for the release of phosphorus.

The setup for continuous and cell recycling studies under anaerobic conditions (1) fermentor (model C30, New Brunswick Scientific Co., (2) cassette pump (junior model, catalog no 72-510-000 [115 V] with pumping cassette (catalog no. 72-550-000, Manostat), (3) peristaltic pump (model 1203, Harvard Apparatus), (4) hollow-fiber cartridge filter (type H1MP01-43) with hollow-fiber cartridge adapter (model DH2, no. 54077, Amicon Corp., Scientific System Division), (5) same as (2) above, (6) regulating valve (e.g., Nupro part no. B-4JR, Nupro Co.), (7 and 8) outflow container (glass carboy), (9) magnetic stir bar, (10) pH electrode (catalog no. 5573705-DL, Phoenix Electrode Co.), (11) container for NaOH (glass flask), (12) and 13) pH controller (model pH-40, New Brunswick Scientific Co.), (14) peristaltic pump (type 4912-A, LKB Instruments, Inc.), (15) medium container (glass carboy), (16) sterile cotton gas filter, (17) backflow trap (glass flask), (18) gas-purifying furnace (model S-36517) with copper granule-filled gas-purifying tube (model S-36518, sergeant-Welch Scientific Co.) (19) Gas cylinder (CO_2), and (20)-connection tubing (butyl rubber tubing, Viton or Versinic).

Kinetics of Biological Phosphorus Release

Observations indicated that the magnitude of biological excess phosphorus (P) uptake is linked strongly to the magnitude of phosphorus release in the anaerobic condition. The theory that describes the kinetics of P release is presented in terms of the readily biodegradable chemical oxygen demand (COD); (chemical oxygen demand refers to the amount of oxygen required to oxidize the organic compounds in a water sample to carbon dioxide and water): (1) in the influent, (2) the non-polyP heterotrophic mass, (3) the anaerobic, (4) the mass fraction and (5) the reactor flow regime as reported by Wentzel *et al.*, (1985). Marais *et al.*, (1983) also put forward the following behavioral characteristics for phosphorus removal: (1) P release under anaerobic conditions must be observed in order to give rise to P uptake subsequently under anaerobic conditions, which results in a net P removal for the system. (2) For any fixed process configuration, the magnitude of the excess P removal is linked directly to the magnitude of the readily biodegradable (readily assimilable) influent COD concentration, S_{bsi}; the higher the S_{bpi} the higher the P release uptake and removal. In contrast, the slowly biodegradable influent COD, S_{bsi}, appears to have little direct influence on excess P removal. (3) The greater the fraction of the total sludges mass in the anaerobic reactor f_{xa}, the greater the net P removal. (4) Nitrates recycled to the anaerobic reactor have an adverse effect on P removal; the greater the mass of nitrate recycled the lower the P removal.

Where S_{bsi} = Readily biodegradable COD available for conversion per liter influent (mgCOD/L)

f_{xa} = anaerobic mass fraction

S_{bpi} = biodegradable influent COD (mgCOD/L)

The Impact of Phosphorus on Aquatic Life

Excessive phosphorus from runoff and erosion can fertilize surface waters. In this process called eutrophication, microscopic floating algae multiply rapidly when fertilized by phosphorus. These algae cloud the water, making it difficult for larger submerged aquatic vegetation (SAV) to get enough light. The SAV may die back, reducing available habitat of aquatic animals. When the algae themselves eventually die they decompose. During decomposition dissolved oxygen (DO) is removed from the water. Lowered oxygen levels make it difficult for other aquatic organisms to survive.

Phosphorus attached to sediments derived from soil erosion may accumulate in the sediments of lakes and streams. This phosphorus may be recycled slowly, or released more rapidly when these sediments are disturbed; for example during a storm or flood. Pollution from phosphorus is, therefore a long-term problem.

As phosphorus is further increased in the body of these waters it causes the growth of the algae and dissolved oxygen is eliminated, and increasing in the concentrations of nutrients and other organic compounds (Hudson, and Marson, 1970, and Vollenweider, 1979). These investigators noted that some blue-green algae produce compounds that have been implicated in the poisoning of fish, and even livestock. Algae can also affect the treatment of water for potable supplies by blocking filters, or by producing a bad odor and taste (Vollenweider, 1979), which, in some cases, leads to the shutdown of works. As a consequence of the reduced economic value to man of phosphorus-limited eutrophic water bodies, considerable attention has been given to them in recent years, e.g. the Great Lakes area of North America (Hughes and Poole, 1991) and the Norfolk Broads in the United Kingdom.

It has been estimated that significant sources of phosphorus, both natural and human, entering freshwaters include drainage from agricultural land, excreta from livestock as well as municipal industrial effluents and diffuse urban drainage (Lee *et al.*, 1978).

Phosphorus inputs from point sources, such as municipal sewage effluents, are more amenable to control than those from non-point sources (Lee *et al.*, 1978). In order to control phosphorus, many scientists around the globe have decided to reduce its concentrations in the effluents of sewage treatment works (Phillips *et al.*, 1993). Conventional biological wastewater treatment does not remove phosphorus effectively enough to achieve these aims. Trickling filters remove approximately 15% of influent phosphorus (Vacker *et al.*, 1967), and conventional activated sludge plants remove approximately 30 to 40%. Therefore, the application of advanced wastewater treatment techniques (either chemical or biological) is required to reduce phosphorus discharges to potentially eutrophic water bodies.

Sources of Phosphorus

The major sources of phosphorus in raw wastewater are derived from human, domestic and industrial wastes; especially food processing effluents (Lee *et al.*, 1978; and Vacker *et al.*, 1967), and run-off from phosphorus-rich fertilized land. Human waste usually contains orthophosphate plus phosphorus present in other biological compounds such as nucleic acids, phospholipids and phosphorylated proteins. In the domestic and industrial wastes, phosphorus may also be present in other forms such as condensed phosphates. Often a high proportion of phosphorus originates from detergents and cleaning compounds due to the discovery 40 years ago that certain phosphate salts, in particular sodium tripolyphosphate (STPP), had outstanding properties as safe non-toxic builders in detergents. Subsequently their use has become relatively unregulated.

De Haas, *et al.* (1990) stated that 99% of the phosphorus in detergents is condensed in the form of pyrophosphates or trypolyphosphates and, that during the activated sludge process 50% of this is hydrolyzed to orthophosphate. These authors observed that prior to aerobic biological treatment, the effluent orthophosphate varied from 25% to 85% of the total phosphorus input, and the effluent orthophosphate was between 60% and 95%. In both cases and the remainder was condensed phosphate. Hence, under certain conditions, almost total hydrolysis of condensed polyphosphates can occur. Organic phosphates such as phosphomonoesters and phosphagens constitute the other main group of phosphorus species found in wastewater.

Phosphates are usually released from these compounds spontaneously or non-degradation although certain pesticides are non-degradable so that the phosphorus is unavailable to microorganisms (Pitman *et al.*, 1991). The reported concentrations of total phosphorus in different wastewaters vary greatly from, 7.5 mg liter^{-1} (Pitman *et al.*, 1991), to 34.5 mg liter^{-1} (Gerber *et al.*, 1987).

Forms of Phosphorus

Phosphorus has a complicated history. Pure "elemental" phosphorus (P) is rare. In nature, phosphorus usually exists as part of a phosphate molecule (PO_4). Phosphorus in aquatic systems occurs as organic phosphate and inorganic phosphate. Organic phosphate consists of a phosphate molecule associated with carbon-based molecule as in plant or animal tissue.

Phosphate that is not associated with organic material is inorganic. Inorganic phosphorus is the form required by plants. Animals can use either organic or inorganic phosphate. Both organic and inorganic phosphorus can either be dissolved in water or suspended (attached to particles in a water column).

The Phosphorus Cycle

Phosphorus cycles through the environment changing form as it does so. Aquatic plants take in dissolved inorganic phosphorus and convert it to organic phosphorus, as it becomes a part of their tissues. Animals get the organic phosphorus they need by eating aquatic plants, other animals, or decomposing plant and animal materials. As plants and animals excrete wastes or die the organic phosphorus they contain sinks to the bottom of lakes and ponds where bacterial decomposition converts it back to inorganic phosphorus, both dissolved and attached to particles. This inorganic phosphorus gets back into the water column when animals, human activity, chemical interactions or water currents stir up the bottom. Then plants take it up and the cycle begins again.

In stream systems the phosphorus cycle tends to move phosphorus downstream as the current carries decomposing plant and animal tissue and dissolved phosphorus. It becomes stationary only when it is taken up by plants or is bound to particles that settle to the bottom of pools.

In the field of water quality chemistry, phosphorus is described using several terms. Some of these terms are chemistry based (referring to chemically based compounds), and others are methods-based (they describe what is measured by a particular method).

The term "orthophosphate" is a chemistry-based term that refers to the phosphate molecule all by itself. "Reactive phosphorus" is a corresponding method-based term that describes what is actually being measured when a test is performed for orthophosphate.

More complex inorganic phosphate compounds are referred to as "condensed phosphates" or "polyphosphates". The method-based term for these forms is "acid hydrolyzable" and organic phosphorylated compounds.

Condensed Inorganic Phosphates.

This may be defined as "pentavalent" phosphorus compounds in which a various numbers of tetrahedral PO_4 groups are linked together by oxygen bridges (Thilo, 1962). These may further be divided into three classes (Harold, 1966) as: (a) cyclic condensed phosphates, (b) linear condensed phosphates, (See Figure 2), and (c) cross-linked condensed phosphates.

Cyclic Condensed Phosphates.

These have the general formula $M_nP_nO_{3n}$ and they are referred to as metaphosphates. When they are treated with strong alkali they are converted to their corresponding linear polymer. They can further be hydrolyzed into orthophosphates when heated with a strong acid (Harold, 1966).

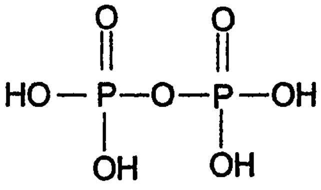
Linear Condensed Phosphates

The linear condensed phosphates have the formula $M_{n+2}\,P_n\,O_{3n+1}$. These phosphates are referred to as polyphosphates and range in sizes: chain-length of 2-pyrophosphate to chain-length of 1×10^4, Kurrol and Maddrel's salts (Harold, 1966).

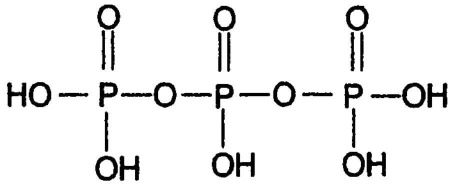
Cross-linked condensed phosphate (ultraphosphates)

These are the third class of inorganic polymers. Their branching points or their phosphate groups have three oxygen atoms, which are shared with neighboring phosphate groups.

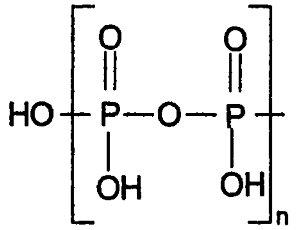
Figure 3. Structures of several condensed phosphates



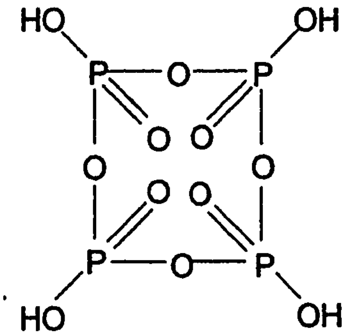
Pyrophosphoric Acid



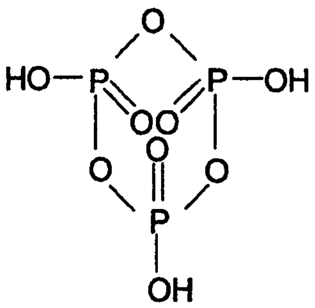
Tripolyphosphoric acid



Polyphosphoric acid



Tetrametaphosphoric acic



Trimetaphosphoric acic

After F.M. Huennekens and H.R. Whitley, 1960.

Acinetobacter spp.

Acinetobacter spp. has become the model organism for biological phosphorus removal since it was first isolated from a phosphorus-removing activated sludge plant. *Acinetobacter* species can accumulate polyphosphates, and although they are not the only organisms showing this ability, they have been found to dominate in enhanced biological phosphorus removal (EBPR) sludge plants (Fuhs and Chen, 1975; Deinema *et al.*, 1985; Streichan *et al.*, 1990). Subsequently, many researchers reported its predominance in EBPR processes based on culture-dependent methods like fluorescent antibody staining (Cloete and Steyn, 1987) and quinone profile measurements and/or fluorescent *in situ* hybridization with an oligoncleotide probe specific for *Acinetobacter* (Wagner *et al.*, 1993; Kampfer, *et al.*, 1996; Snaird *et al.*, 1997, Wagner, *et al.*, 1993). Cloete and Bosch (1994) found that the cell volume and mass of *Acinetobacter calcoacticus* increased with the growth rate, indicating that smaller cells have a reduced growth rate. It can therefore be said that the smaller cells simply accumulate phosphorus. Thus, phosphorus removal by *Acinetobacter* can be influenced by the growth rate of the cells (Cloete and Steyn, 1987; Cloete and Bosch 1994).

Microlunatus phosphovorus

In 1991 and 1995, Nakamura *et al.* isolated new polyphosphate-accumulating bacterium from a laboratory-scale EBPR process and proposed the name *Microlunatus phosphovorus* for it. *Microlunatus phosphovorus* is physiologically close to polyphosphate-accumulating bacteria (PAB) found in enriched sludges. It accumulates large amounts of polyphosphate (polyP) under aerobic conditions, which is then consumed along with the anaerobic uptake of carbon sources like glucose and casamino acids. This bacterium lacks the key metabolic characteristics. It neither takes up acetate nor accumulates polyhydroxyalkanoates (PHA). Recently, a 16S rRNA-targeted oligonucleotide probe for *Microlunatus phosphovorus* was developed and applied to quantify the bacterium in an EBPR process (Kawaharasaki *et al.*, 1999). By using a probe, *Microlunatus phosphovorus* was found to constitute about 2.7% of the total bacteria, when the percentage of PAB detected by 4,6-diamidino-2-phenylindole (DAPI) stain for polyP was about 9% of the total bacteria.

The relevance of *Microlunatus phosphovorus* to EBPR still remains to be clarified (Mino, 1999). Ubukata and Takii (1994) isolated a bacterium that morphologically and physiologically resembles *Microlunatus phosphovorus* and demonstrated that the bacterium exhibited the anaerobic utilization and aerobic accumulation of polyP only after alternating anaerobic and aerobic conditions were applied a few times. This, then, implies that the presumed enzyme system for the polyP metabolism is not constitutive, but inducible (Mino, 1999).

Lampropedia spp.

This microbe was isolated from sequence batch reactor (SBR) systems designed for EBPR and identified as *Lampropedia* spp. (Stante *et al.*, 1996, Stante *et al.*, 1997). This isolate has the ability to take up acetate and stores it as PHA with concomitant polyP degradation and P_i release under anaerobic conditions (Mino, 1999). Functionally, it possesses the key metabolic characteristics of polyphosphate-accumulating bacteria (PAB) but morphologically it has a very unique sheet-like cell arrangement that is not common in EBPR processes. It is not certain at this moment that *Lampropedia* spp. plays a significant role in EBPR.

Rhodocyclus spp.

Bond *et al.* (1995) first reported the presence of bacteria in EBPR. When the polymerase chain reaction (PCR) cloning approach was applied to two EBPR reactors 15 out of 189 isolated clones phylogenetically belonged to the group *Rhodocyclus*. Recently, fluorescent *in situ* hybridization shows that an EBPR reactor fed with an acetate-containing substrate will be dominated by the *Rhodocyclus* group up to 81% of the DAPI-stained (Hesselman *et al.*, 1999).

Thus, the staining of intracellular polyP and PHB confirms the dominant bacteria in a community that performs the key metabolism of PAB, namely acetate uptake accompanied by PHA accumulation and Pi release along with polyP utilization under anaerobic conditions. This strongly suggests that certain species within the *Rhodocyclus* group should be mainly responsible for EBPR, at least under certain circumstances.

Phosphate-accumulating Bacteria in Wastewaters

The recent development of biotechnologies dealing with the removal of nitrogen- and phosphorus-containing compounds from a mixture of industrial and municipal wastewater are due, in part, to the discovery made in the early 1970s that widespread phosphate-accumulating microorganisms can carry out intracellular accumulation of metal-containing polyphosphates (Levin *et al.*, 1972). Of particular importance for such systems with discontinuous aeration are bacteria of the genus *Acinetobacter*, which mainly use acetate as a substrate (Fuhs and Chen, 1975).

These systems may contain as many as 1 to 10 million *Acinetobacter* cells/ml of activated sludge accounting for 1-2% of the total bacterial biomass and accumulating 5-15% of the phosphorus consumed by the whole microbial community (Cloete *et al.*, 1985, Comeau, 1990, and Saralov *et al.*, 1995).

As far as other phosphate-accumulating organisms (heterotrophic and autotrophic bacteria, algae and fungi) are concerned biological water purification plants may also contain numerous representatives of the genera *Pseudomonas, Aeromonas, Escherichia, Flavobacterium, Klebsiella, Rhodococcus, Rhodotorulla rubra*, and *Synechococcus leopoliensis*. Thus, the pattern of colonization of diverse kinds of waste and drainage waters by microorganisms capable of intracellular accumulation of volutin granules requires further research.

Polyphosphate-Hydrolysis

Polyphosphates as noted require a large investment of metabolic energy. It seems conceivable that polyphosphates may also have other specific functions apart from what has been reported in the literatures. It has been suggested that in addition to providing a phosphate reservoir, polyphosphates may serve as an energy reservoir for ATP formation through polyphosphate kinase, which has been identified in bacteria and in yeast (Wood and Clark, 1988).

Pick *et al.* (1990) suggested that different microorganisms, including yeast and algae, accumulate large amounts of polyphosphates. However, the physiological role of polyphosphates is unknown. In *vivo* ^{31}P NMR studies carried out in the unicellular alga *Dunaliella salina* demonstrate that cytoplasmic alkalization induces massive hydrolysis of polyphosphates. This correlates kinetically with the recovery of cytoplasmic pH. Analysis of acid extracts of cells indicates that long-chain polyphosphates are hydrolyzed mainly to tripolyphosphates. It therefore suggests that the hydrolysis of polyphosphates provide a pH-stat mechanism to counterbalance alkaline stress.

Polyphosphate and Phosphate Pump

Microorganisms are dependent on the environment for their viability and metabolism as well as for the functioning of their cell machinery (Kulaev and Kulakovskaya, 2000). As a result, they have evolved the ability to store a number of important metabolites in the form of osmotically inert polymers. Inorganic phosphates are one such metabolite. They are stored within the cell as a high molecular weight inorganic polyphosphate (polyP$_n$, where n is the approximate number of phosphate residues in the polyP molecule).

Furthermore, it has been suggested that the structure of polyP/polyhydroxybutyrate complexes may function as a phosphate pump under the condition that a polyP chain is extended on one side of the membrane, whereas orthophosphate is split from its end on the other side (Reusch 1992, and Reusch 1999).

Localization of Polyphosphates in Microbial Cells

The main compartments of bacterial cells are the cytoplasm, cell surface, periplasm and plasma membrane. PolyP is found in all of these compartments. PolyP-containing granules are present in the cytoplasm of many bacteria (Kulaev, 1999) and (Kulaev and Vagabov 1983). PolyP granules were observed in the vicinity of the bacterial nucleoid

(Kulaev and Vagabov, 1983). Some amounts are localized in the periplasmic region of bacteria outside of the cytoplasmic membrane (Nesmeyanova *et al.*, 1974, and Rao and Torriani, 1988). PolyP is a component of the cell capsule, which for *Neisseria* spp. is loosely attached to the surface membrane. This capsular polyP represents about 50% of the polyP content in *Neisseria* cells (Tinsley *et al.*, 1993, and Tinsley and Gotschlich 1995). In the *Helicobacter pyloric* bacteria colonizing the gastric atrium, polyP was found in at least three different locations, the cytoplasm, the flagella pole, and the cell membrane (Bode *et al.*, 1993).

Phosphate Accumulation and Polyphosphate Metabolism in *Saccharomyces cerevisiae*

Inorganic phosphate (Pi) is an essential nutrient for all organisms, used in the biosynthesis of diverse cellular components, including nucleic acids, proteins, lipids, and sugars. It is therefore essential for organisms to have evolved regulatory mechanism for storage acquisition, storage and release of this molecule (Torriani-Gorini *et al.*, 1994).

Eukaryotes

Eukaryotic microorganisms possess polyP pool in all cell compartments. Their contents vary depending on cultivation conditions. The vacuoles of *Saccharomyces cerevisiae* at lower osmolarity have no polyP (Pichko, unpublished data). The content of polyP in the cytosol of *Saccharomyces cerevisiae* depends on culture age and cultivation conditions which may contain from 10% (Okorokov *et al.*, 1980) to 70% (Kulaev *et al.*, 1999). In this yeast the amount of polyP in the cytosol increases about twofold with the so-called "overplus," which occurs when the cells are transferred from a medium without phosphate to medium with phosphate (Baxter and Jensen, 1986, and Kulaev *et al.*, 1999). Therefore, the nutritional condition that can stimulate the formation of polyP is the restoration of the phosphorus supply following the phosphate starvation Liss and Langen (1962), Harold (1963), Harold (1964, 1965), and Voelz *et al.* (1966). The ability of the cells to accumulate phosphorus was first investigated by Ketchum (1939) in the marine diatom *Nitzschia closterium*. He referred to the deficiency as the phosphorus or phosphate debt, and measured this debt by the amount of phosphate absorbed from the medium by the diatom, or by direct analysis of the cells. The magnitude of the phosphorus debt was directly related to the length of time the cells grew in the light in phosphorus-free medium.

In 1944, Jeener and Brachet found that in yeast cells, basophilia associated with volutin granules decreased when the cells were grown on a phosphorus-deficient medium, and increased when the cells were transferred to medium containing phosphorus. This volutin is termed polyphosphate bodies by Jensen (1968). Wiame (1947a, b) demonstrated twice the abundance of these volutins; polyphosphate bodies (PPBs) synthesis when phosphate-starved *Saccharomyces cerevisiae* was transferred to a phosphate-rich medium.

Other researchers have studied this phenomenon using bacteria such as *Caulobacter* (Grula *et al.*, 1954), *Klebsiella aerogenes* (Duguid, 1948), *Aerobacter aerogenes* (Smith *et al.*, 1954), and *Myxococcus xanthus* (Voelz *et al.*, 1966).

Polyphosphate Functions

In bacteria the functions of polyP are associated with energy metabolism. Hsieh *et al.*, (1993) suggested that polyP in very primitive organisms could perform the functions of energy-rich compounds. These suggestions were supported experimentally in a number of works (Bonting *et al.*, 1991, Hsieh *et al.*, 1993; Kulaev *et al.*, 1983, Kuroda and Kornberg 1997, and Phillip *et al.*, 1993). In many prokaryotes polyP is a direct phosphorus donor for biochemical reactions.

Another function of polyP is the detoxification of heavy metal cations. PolyP sequesters nickel in *Staphylococcus aureus* (Gonzalez and Jensen, 1998). The cells of *Anacystis nidulans* with high intracellular polyP levels show greater tolerance to cadmium than those with smaller polyP reserves (Keyhani *et al.*, 1996). The cadmium tolerance of *E. coli* also depends on the polyP metabolism (Keasling and Hupf, 1996). The following mechanisms of polyP participation in the detoxification of heavy metals have been proposed (1) polyP sequesters heavy metals, and (2) the entry of metal cations into cells stimulates exopolyphosphate activity which releases Pi from polyP. Unlike most cultured microorganisms. Many bacteria including magnetaactic bacteria obtain these metals from natural environment. Magnetaactic bacteria are bacteria that use magnetic resonance to take up metals, present cytoplasmic inclusions rich in phosphorus (Corpe and Jensen, 1992). These inclusions contain phosphorus, magnesium, potassium, and calcium; and sometimes also sulfur, chlorine, iron, sodium (Jensen and Corpe, 1994). In previous work, Lins and Farima (1999) showed that bacteria from Itaipu Lagoon present phosphorus-rich granules that naturally accumulate Al, Fe, and Zn even though they were not obtained from a metal-contaminated environment.

Many uncultured bacteria contain polyphosphate bodies (Corpe and Jensen 1992), capsules, sheaths, and S-layers. Extensive work has been done on the accumulation of metals in the polyphosphate bodies of cultured bacteria. Titanium was found to accumulate in the polyphosphate bodies of *Anacystis nidulans* (Crang and Jensen 1975), Ba and Mn in *Plectonema boryanum* (Baxter and Jensen, 1980a,b), Cd, Co, Cu, Hg, Ni, and Zn in *Plectonema boryanum* (Jensen *et al.*, 1982a,), Cd in *Anabaena flos-aquae* (Rachlin *et al.*, 1984), Al in *Anabaena cilindrica* (Patterson *et al.*, 1985), Ni in *Staphylococcus aureus* (Gonzalez and Jensen, 1998); and Al, Cd, Cu, and Zn in *Synechococcus leopoliensis* (Goldberg and Jensen, 1999). All the experiments carried out by the individual researchers were done using high concentrations of metal salts in the culture media. It has been found that some culture media could complex with the ions, changing the metal species present (Hughes and Poole, 1991). The metal complexes are then transferred out of the cells (Keasling 1997, and Keasling and Hupf, 1996). PolyP participates in the cell envelope formation. PolyP in the cell envelope is of great importance for the negative charge on the cell surface (Ivan *et al.*, 1996, and Vagabov *et al.*, 1990). It can bind with the cationic dye 9-aminoacrydine (9AA) in the presence of thiamine, an inhibitor. By measuring the 9AA-absorption rate, one can determine variations in the polyphosphate content in the cell. It is also involved in the regulation of gene expression in bacteria. The mechanism of this involvement is still under investigation (Kim *et al.*, 1998, Kusano and Ishihama, 1997, Rao *et al.*, 1998, and Shiba *et al.*, 1997).

Polyphosphate "Overplus" Phenomenon

The function of polyP in prokaryotes as a phosphate reserve is well known. The content of polyP in the cells of microorganisms strongly depends on the phosphate content in the medium. A large amount of polyP is characteristic of the bacteria from wastewaters with high phosphate content. The polyP drops drastically under phosphate starvation and the subsequent addition of orthophosphate to the medium restores the initial phosphate level.

In eukaryotes, the accumulation of phosphate reserves as polyP is also used at phosphate starvation period of the eukaryotic microorganism. PolyP in the cytosol performs the function of phosphorus reservation in bacteria. Phosphorus in eukaryotic microorganisms is reserved as polyP in their vacuoles and the cell envelope.

MATERIALS AND METHODS

The test microbes were chosen after a series of preliminary growth tests were conducted using various culture media, and different pH levels. All the materials and methods applied equally to the four microbes. After these tests, the culture media and the pH levels were selected. The four microbes chosen were *Synechococcus leopoliensis* (UTEX B 2434), *Plectonema boryanum* (UTEX 581), *Saccharomyces cerevisiae,* and *Rhodotorula rubra. Synechococcus leopoliensis* and *Plectonema boryanum* were obtained from the Starr Culture Collection (Starr and Zeikus, 1987) and cultured in modified Fitzgerald's medium (Fitzgerald and Rohlich, 1952; Zender and Gorham, 1960) (see Appendix A). *Sacchacromyces cerevisiae* and *Rhodotorula rubra* were obtained from the American Type Culture Collection (ATCC), P. O. Box 1549, Manassas, VA 20108.

Culture conditions:

Synechococcus leopoliensis (UTEX B 2434), and *Plectonema boryanum* (UTEX 581)

All stock and experimental cultures of *Synechococcus leopoliensis* and *Plectonema boryanum* were grown in several 250ml flasks containing 13mg PO_4 per liter (1.76 mg P per liter) for 14 -17 days in Modified Fitzgerald's Medium (MFM), pH 7.2 in a Sherer controlled growth chamber (Model Cel B) at 25° C, and illuminated with 500 ft-candles of cool white fluorescent light, supplemented by two 25-watt incandescent bulbs. The chamber was set to a 12-hour light/12-hour dark cycle. Under these conditions the cells reached logarithmic growth at 14 - 17 days. Cells were harvested, washed three times with deionized water and deposited on 300 mesh copper formvar coated grids and air-dried in a 37°C chamber to be analyzed for the presence of polyphosphate bodies at the logarithmic period using a Hitachi H-7000 electron microscope.

The cells in addition were harvested and centrifuged at 3500 relative centrifugal force (RCF) for 35 minutes and the supernatant was analyzed for phosphorus using Hach Test Kit and results tabulated (Table 1, and appendix B). This testing method was used

throughout the entire experiment for the amount of phosphorus released by the various microbes used in this
experiment.

Starvation Conditions

Cells were grown in Modified Fitzgerald's Medium containing 13mg PO_4 per liter (1.76mg P/L) for 14 -17 days at 25°C, 500 ft-candles as already mentioned above, at a pH of 7.2. To induce phosphorus starvation the cultures were harvested and centrifuged at 3500 RCF for 35 minutes. The supernatant was decanted. The cells were washed three times in sterilized phosphate free medium (MFM - PO_4). At the third wash, the supernatant was tested for the presence of phosphorus and found to contain no phosphorus. The washed cells were resuspended in the same phosphate free medium (MFM - PO_4) and then placed under the original environmental conditions for a period of 5 days for optimal starvation conditions (Jensen *et al.*, 1977).

Electron Microscopy Study

Cells from the starvation period were transferred into cuvettes, washed three times with deionized water, deposited on 300 meshed copper grids, air-dried in a 37° C incubator and analyzed for the presence of polyphosphate bodies. The cells were examined with a Hitachi H-7000 transmission electron microscope (TEM) operating at 75 kV. The polyphosphate bodies (PPB) were located and negatives were taken at X 10,000 magnification. The PPBs were also visually inspected and counted to later compare with the overplus conditions.

The "Overplus" or the Rapid Phosphorus Uptake of *Synechococcus leopoliensis and Plectonema boryanum*

The Luxury storage of phosphate by *Synechococcus leopoliensis* and *Plectonema boryanum* was carried out by starving the cells for a period of 5 days as above, and then inoculated into the media of a known amount of phosphate and at different pH ranges, 7.2, 8.5, and 9.0. The known phosphate concentration in each medium was 13 mg per liter (1.76mg/L) P. The rapid uptake was monitored by taking the cells out from each pH range, centrifuging them down and testing the supernatant for the amount of phosphorus present in each medium for 0 minutes, 20 minutes, 40 minutes, and 60 minutes. These tests were done using the Hach Test Kit (High Range) (Table 2). The cells were then washed 3 times with deionized water, deposited on 300 meshed formvar-coated copper grids and air-dried in a 37° C incubator and analyzed using Hitachi H-7000 electron microscope to locate polyphosphate bodies (Figure 2). Also PPBs count was visually carried out during the electron microscopy analysis (no data shown).

Cell Count Using the Spectronic-20

Cell count was carried out from these media using the Spectrophotometer (Spectronic 20). The wavelength was set to 430 nanometers according to Hach (Hach Water Analysis Handbook, 3rd Edition) Hach Company, Loveland, Colorado, and USA, 1977. After warming up, the left knob was turned until the needle pointed to zero transmittance.

Adjusting the blank

The trap door on top of the left was opened and a cuvette with blank liquid (the analysis reagent) was inserted into the Spectronic 20 and using the front knob to adjust the absorbance to zero (100% transmission). The blank was removed and the trap door closed and re-checked for zero reading. This adjustment and sequence readings were repeated until the blank showed that the transmittance was stabilized at 100%. The twenty cuvettes that contained the cells, each was wiped clean, shaken and immediately inserted into the Spectronic 20 and read and converted to percent transmission.

Since these old instruments have a tendency to drift, it was checked and adjusted with a blank to zero absorbance after each sample measurement was read and tabulated. The percent transmission was converted to number of cells in the medium using a conversion table (table not shown). Each pH range (7.2, 8.5, and 9.0) was tested and recorded.

Determination of polyphosphate bodies under an anaerobic condition at their different pH Ranges

The cells were harvested from the various pH ranges and under the anaerobic setup conditions (were cultures were completely covered with aluminum foil, 50 gauge syringe inserted, and dry nitrogen gas pumped in) but different time periods as previously described and prepared for electron microscopy in the following manner: The cells were pelleted from the conditions of the different pH ranges and put in cuvettes, labeled according to their pH ranges, 7.2, 8.5, and 9.0, and centrifuged at 3500 RCF for 35 minutes. The supernatant was decanted and tested to ascertain the amount of phosphorus in the media. The cells were then washed three times with deionized water and deposited on formvar-coated grids as previously described. The cells were examined under the electron microscope (Hitachi H-7000) operating at 75 kV. Negatives were taken on Kodak film and developed with D-19 diluted with 1 part water at 68°F about two minutes. The negatives were enlarged 2.8 times for a final print magnification of 28,000. Cell length and width and the diameter of each PPB in each cell were measured.

Air-dried cells preparation

The air-dried method is simple, efficient, and most importantly, is a method that can prevent serious elemental changes in the preparation processes (Baxter and Jensen, 1980a). It is frequently used to prepare cells for EDX. Cells were collected from the growing cultures by centrifugation, washed three times in deionized water (diw), placed on formvar-coated (0.25%) grids and air-dried in a 37°C incubator. After being dried the cells were ready for electron microscopy study.

Determination of Cell volume occupied by PPBs

The volume of cells occupied by PPBs for all experiments was determined by using a simple computer program that calculated:

The volume of cylinder $V = \pi r^2 h$

The volume of PPB sphere $V = 4/3\pi r^3$

The Volume of ellipsoid $V = 4/3\pi ab^2$

Determination of Standard Deviation and Standard Error

The standard deviation was determined for all experiments carried out in the laboratory. The experiment was run five times. The standard deviation was calculated as follows:

1.The arithmetical mean (X_0) was determined by adding all of the individual observations ((X_i) and dividing the total by the number of individual samples (N).

2. The individual deviation from the mean ($X_i - X_0$) was calculated.
3. Each individual deviation from the mean was squared $(X_i - X_0)^2$ so that positive values were obtained.
4. The sum of the squared deviations from the mean was determined (E $(Xi - X_0)^2$.
5. The standard deviation of the samples was calculated using the following formula to compare with the computer program calculations.

$$S = \sqrt{\frac{E\,(Xi - X_0)^2}{N - 1}}$$

The standard deviation enabled me to measure the possible error of the estimate. It is also possible to measure the samples mean by an interval. The value s/n is the standard error of the samples' mean (Table 6).

Cell Count - Optical Density Method (Aerobic Phase)

A separate culture was grown as described for the 14 -17 days, which is approximately the log phase, and then taken through the starvation period for cell count under the anaerobic-aerobic condition. During the growth period, all cultures were constantly agitated. Cell growth was determined by optical density (turbidity technique) for a period of 14 -17 days (Sicko-Goad and Jensen, 1976), with a Bausch and Lomb's Spectronic 20 spectrophotometer at a wavelength of 430 nm, which was determined by dilution curves (Sorokin, 1975). The cells were put in 25 cuvettes of equal height. The spectrophotometer was adjusted to the desire nanometers (nm). Before each cuvette was read, it was shaken, cleaned with Kimwipe tissue paper, and inserted into the Bausch and Lomb Spectronic 20 spectrophotometer following the same procedure as already described. The readings were recorded for each medium of the three pH ranges, 7.2, 8.5, and 9.0.

Anaerobic condition of cells plus Resazurin

Cells were grown in the same conditions as previously described and taken them through their Overplus phenomenon to create many PPBs. The set was done also as previously described. Dry nitrogen gas was bubbled in at 10 pounds per square inch (PSI) three times a day for 45 minutes each. The dissolved resazurin was syringed out and injected into the medium containing the cell in their anaerobic set up condition. This was done through the rubber stopper. After constant stirring by the magnet and the bubbling of the dry nitrogen gas for time intervals, the cells were syringed out and visually inspected for color change, an indication of the presence of oxygen in the medium.

Resazurin Test

The anaerobic conditions of all cells plus resazurin test was carried out to make sure that during the anaerobic condition all oxygen was removed and also to make sure that oxygen was not leaking into the setup. 100mg of resazurin was dissolved in 100 ml of deionized water. 0.1 ml of this solution per 100ml of the anaerobic media was taken out and tested. Color of resazurin is naturally blue and when introduced into an oxygen-containing medium it turns pink while it turns colorless in the absence of oxygen (Jacob, 1970). The test was carried out under anaerobic condition at a very short period of time to avoid the accidental entering of oxygen. Another sample from the organisms grown under the anaerobic conditions was put in 250 ml flasks with the addition of the resazurin and allowed to stand for of two days to test for dissolved oxygen (DO).

Study of the Phenomenon of Phosphorus Release Anaerobically by *Synechococcus leopoliensis* and *Plectonema boryanum*

To study the phenomenon of the release of the phosphorus by these microbes under the anaerobic condition, fresh samples were cultured and transferred into 250 ml flasks pH values of 7.2, 8.5, and 9.0 for a time period of 4, 6, and 8 days. The flasks were completely covered with aluminum foil to eliminate light. They were tightly closed with rubber stoppers to eliminated air. Holes were previously drilled in these stoppers. Glass rods were bend into "U" shape and inserted into the flasks through the pre-drilled holes. The other ends of the glass rods were inserted into rubber tubes. The entire connection was attached to the dry nitrogen gas tank obtained from Matheson Gas Products. 932 Patterson Plank Rd. P. O. Box 85 East Rutherford, NJ 07073). A 50-gauge syringe was inserted in each of the flasks through the rubber stoppers and tightly fit to prevent entry of air.

Agitation

Agitations of the anaerobic cultures were provided by magnets in each flask and were placed on a Thermolyne stirrer type 7200 set to a speed of 5. Although, there has not been an exhaustive study of agitation conditions, some preliminary findings have been presented in the literature (Angenent and Dague, 1995). However, results on agitation intensity and mechanical agitation are, at this date not available.According to Angenent and Dague (1995), intense agitation may cause rupture (break up) of the granules.

Intermittent agitation enhances the gas-liquid separation efficiency. Mechanical agitation is another alternative to mixing the contents in the flasks. Timur and Osturk (1999) used magnetic bars in bench-scale anaerobic sequencing batch reactors. Timur and Osturk also used a mechanical mixer, providing mixing for 1 hr and a separation and/settle time efficiency of 25%.

Here, the magnets in the flasks were under constant stirring. The entire experiment was kept from further contact with light and air. The dry nitrogen gas was bubbled in at 10 pounds per square inch (PSI) three times a day for 45 minutes. Each time period, at 1, 2 through 8 days, samples were withdrawn, using a 50-gauge syringe and discharged into tubes and centrifuged briefly (10 minutes). Resolution indicating anaerobic conditions by the introduction of the dry nitrogen gas, which replaced the oxygen to keep conditions in the anaerobic state.

Molybdate-ascorbic colorimetric reaction

If the total phosphorus in the culture was less than 1mg P/L ($P < 1$), the vanadatemolybdate colorimetric reaction became unreliable. For such samples, the molybdate-ascorbic acid method (standard Methods, 1985) as described by De Haas *et al.*, (1990) was applied with one exception: orthophosphate standards (range 0.0 to 1.76 mg P/L) were carried throughout the experiment.

Phosphorus release during the different time periods (4, 6, 8 days) was determined using Hach Test Kit. The results of such experiment were then tabulated. This was carried out at a pH of 7.2, 8.5, and 9.0. The High Range Measurement was used during this experiment (appendix B).

Reversed Determination

To determine the nature of the release, I investigated its reversibility. After a period of 8 days the longest time of phosphorus release, the anaerobic condition was discontinued and air was allowed into the culture of each pH range, (aerobic condition). The Hach Test was carried out using the High Range Measurement. Cells were analyzed using a Hitachi H-7000 electron microscope to compare the volumes of polyphosphate bodies present at this reversed period with those found during the anaerobic condition period and with their normal growth.

Separate cultures were grown for 14-17 days, which approximates the mid-log phase as controls at pH 7.2, 8.5, and 9.0. The cells of these cultures were spun down and the supernatants were tested for phosphorus content using the Hach Test Kit at High Range measurement.

Cells were taken out, washed three times with deionized water, placed on formvar-coated grids and air-dried in a 37°C incubator. Negatives were taken at a magnification of 10,000 using a Hitachi H - 7000 electron microscope operating at 75 kV. The negatives were enlarged at 2.62 times for a final print magnification of X26, 920. Cell length and width and the diameter of the PPB in each cell were measured.

The Effect of EDTA on Phosphorus Release from *Synechococcus leopoliensis* and *Plectonema boryanum*

Ethylenediamine-tetraacetic acid (EDTA) disodium salt has been used for the treatment of soils contaminated with metals from mining and smelting activities and has been found to improve the metal removal efficiency from 50% to 98% (Papassiopi *et al.* 1999).

The cells were cultured, starved as previously described and transferred into nine 250 ml flasks containing media of the various pH ranges and concentration of phosphorus (1.76mg/l phosphorus). When the cells had reached their maximum uptake of phosphorus as previously determined, the EDTA was added to the culture aerobically at the concentrations: 0.025, 0.05, 0.125, and 0.25 M (Papassiopi *et al.* 1999).

Anaerobic/aerobic condition

Biomass for all experimentation was carried out in Dr. Jensen's laboratory, Lehman College, City University of New York, 250 Bedford Park Blvd. West, Bronx, NY 10468, USA, under laboratory-scale anaerobic/aerobic conditions. Cells of the four microbes were transferred after their phosphorus starvation (MFM-PO4) period into 250 mL flasks containing 1.76mg/l phosphorus (MFM+PO4) and subjected to the anaerobic conditions. Their phosphorus release was then determined using the Hach Test Kit obtained from Hach Company, Loveland, Colorado, and USA.

Rhodotorulla rubra

Rhodotorulla rubra can be isolated from different sources, such as decaying lemon. The identified strain used in this experiment was *Rhodotorula rubra, which* was used to remove phosphorus. In addition, and on the basis of results, cells grown at the optimized growth conditions were spun down at a velocity of 3500 RPM at a temperature of 25° C at pH of 7.2. Considering the kinetics of carotenoid synthesis by this yeast, the maximum rate of production is at 24 hours, cells started to grow within 1 day.

Sacchacromyces cerevisiae, Rhodotorula rubra strains, and their Polyphosphate Overplus Phenomenon

Polyphosphate Overplus (Harold, 1966) of the cultures of the microbes was performed as follows: The two strains indicated were grown in Sabouraud Dextrose (SD) media overnight. The grown cells were collected, washed three times with SD - P and resuspended in 250 ml P-free media. The culture was grown for 5 days in a 28°C chamber. Potassium phosphate (KH_2PO_4, pH 5.8) was added at 10mM final concentration, (Ogawa *et al.*, 2000) to induce Overplus polyphosphate bodies. The pH was adjusted to 7.2, 8.5, and 9.0.The cells were taken out and inoculated in these pH ranges and monitored for 1 hour, 2hours, and 48 hours for maximum uptake of phosphorus. The cultivation of the cells was under constant agitation at these pH ranges and at 28°C. The cells were taken out, washed 3 times with deionized water and deposited on formvar coated copper grids to be analyzed for the presence of polyphosphate bodies during their Overplus period.

Anaerobic Conditions of *Sacchacromyces cerevisiae* and *Rhodotorulla rubra*

To induce the anaerobic conditions, the setup was the same as was previously described for *Synechococcus leopoliensis* and *Plectonema boryanum.*

Test for Phosphorus Release by *Sacchacromyces cerevisiae* and *Rhodotorulla rubra*

The set up for the anaerobic condition for *Sacchacromyces cerevisiae* and *Rhodotorula rubra* was the same as that of *Synechococcus leopoliensis* and *Plectonema boryanum.* The starved cells were inoculated into the complete media pH 7.2, 8.5, and 9.0 (Overplus phenomenon). Cell count was conducted using a Spectronic 20 during the time of their aerobic condition. The wavelength was set at 430 nanometers (nm) as suggested by Hach (Hach Company 2001). The percent transmission was converted to Optical Density (OD), and the number of cells in the medium was determined (data not shown). The set up time for the anaerobic condition was for 4, 6, and 8 days for all pH ranges (Table 4). The set up was in parallel. All the flasks were labeled and covered with aluminum foil and again labeled according to their pH range. Nitrogen was bubbled in for the same amount of time at 10 pounds per square inch (PSI). The media were tested with resazurin to be sure complete anaerobic conditions were achieved as previously described. The contents were withdrawn at the end of each time period (days), spun down and supernatants were measured for the release of phosphorus by the microbes using the Hach Test Kit (Orthophosphate Reactive High Range). The results were tabulated.

Determination of Redox State of Media (Resazurin).

It is not within the scope of this dissertation to give details on how to measure the redox potentials of media used. Such details were described by Jacob (1970). For most anaerobic work it is necessary to know whether or not oxygen has been removed from or incidentally reintroduced into the anaerobic medium. It is important to add a suitable indicator, which should not be toxic for the microbes to be cultured. In this case, the indicator is resazurin (Balch, and Wolfe, 1976, Bryant, and Burkey, 1953, Costlow 1981, and Hungate 1969), the Resazurin that has an energy E of –51mV. It goes from blue to pink indicating the presence of oxygen to completely colorless--the absence of oxygen. Lowering the redox potential to –110mV will have greater effect on the sensitivity of the indicator.

Statistical Analysis of Cell Volumes Occupied by Polyphosphate Bodies.

The standard deviation and standard error were used to ascertain the distribution of polyphosphate bodies in each cell during the starvation period, Overplus phenomenon, and the anaerobic condition period of all the four microbes. Statistical analyses were carried out using Statview (version 2.01), and JMP (version 2.01, SAS Institute, Inc.) programs (Feldman and Gagnon 1986). The Excel program was also used to plot histograms.

Hach Test Kit, Phosphorus (Orthophosphate Reactive PhosVer 3) Ascorbic Acid Method

In the ascorbic acid test, a combined Orthophosphate Reactive PhosVer 3 prepackaged powder reagent, consisting of sulfuric acid, potassium antimonyl tartrate, ammonium molybdate, and ascorbic acid is added to the sample. This colors the sample blue in direct proportion to the amount of orthophosphate in the sample. Absorbance or transmittance is then measured after 1 minute using a color comparator with a scale in milligrams per liter. The spectrophotometer measured the amount of light absorbed or transmitted at a wavelength of 430 nanometers (nm). The use of the meter required the preparation and analysis of known standard concentrations ahead of time in order to convert the absorbance readings of the sample to milligrams per liter. The meter was also read directly as milligram per liter to compare with the conversion factor.

Standard Concentrations preparation

The standards were prepared using a phosphate (PO_4) standard solution of 3mg/L. This is equivalent to a concentration of 1mg/L Phosphorus. All references to concentrations and results from this point on in this research will be expressed as mg/L of P

Eleven standard concentrations were prepared for every sampling (supernatant) in the range of expected results. The following concentrations were prepared:

1. 0.00 mg/l
2. 0.20 mg/l
3. 0.40 mg/l
4. 0.60 mg/l
5. 0.80 mg/l

6 1.00 mg/l
7 1.20 mg/l
8 1.40 mg/l
9 1.60 mg/l
10 1.80 mg/l
11 2.00 mg/l

RESULTS

Determination of normal growth of *Synechococcus leopoliensis* and *Plectonema boryanum.*

Both *Synechococcus leopoliensis* and *Plectonema boryanum* cultures while grown under normal growth conditions began the rapid growing phase in 1-2 days after inoculation and reached a maximum yield after 14 to 17 days. For maximum yield, the growth was extended to 24 days (Table 1). Optical density (OD) was used to determine their growth pattern from day 1 up to 24 days. At the end of 24 days the cultures were tested for release of phosphorus using a Hach High Range Test Kit. The cells taken out and transferred into tubes and centrifuged. The supernatant was decanted and tested for the amount of phosphorus in milligram phosphorus per liter (mgP/L) and converted to percentage. It was found that *Synechococcus leopoliensis* released 0.2 mg/l of P and then converted to percent of phosphorus (11.36%) and *Plectonema boryanum* released 0.46 mg/L or 26.14% of phosphorus per liter of solution (Table 2). At the declining phase, the two microbes failed to produce phosphorus release. At this declining phase, a new phase of phosphorus uptake was noticed when fresh media containing phosphorus was added and normal growth resumed (data table not shown). When the cells were examined using a Hitachi H – 7000 electron microscope following the addition of the fresh media, polyphosphate bodies were located and frequently distributed on the cells surfaces. The PPBs were almost as numerous as in the overplus phenomenon.

Table 1. Growth pattern of *Synechococcus leopoliensis* and *Plectonema boryanum* while grown under normal growth conditions, pH 7.2 (1.76 mgP/L). Optical Density (OD) was used to determine the growth pattern.

Days	*S. leopoliensis*		*P. boryanum*	
	OD	% Yield	OD	% Yield
0	0.000	0.00	0.000	0.00
6	0.276	53.0	0.297	50.5
12	0.638	48.0	0.658	22.0
18	0.770	42.0	0.810	16.0
24	0.959	36.0	1.126	7.5

Figure 4. Shows the formation of PPBs by *Synechococcus leopoliensis* when fresh medium containing 1.76 mgP/L was added (reversed condition). Notice the many PPBs. X 28,000. This formation of PPB is the same as the uptake phenomenon.

The number of viable cells of *S. leopoliensis* and *P. boryanum* in the media on day 24 (Optical Density) was 36% and 7.5% respectively of the total number of cells (Table 1). When the supernatant was tested using Hach Test Kit (High Range), phosphorus released by *S. leopoliensis* was 11.36% and phosphorus released by *P. boryanum* was 26.14% of the total phosphorus (Table 2). This is an indication that the more viable cells in the medium the more phosphorus is retained by the cells as polyphosphate bodies (PPBs). The less viable cells in the medium, the more phosphorus are released in the medium suggesting that dead cells release phosphorus into the medium, as dead cells need no energy. Thus, there was more phosphorus release by *P. boryanum* with less viable cells than *S. leopoliensis* with more viable cells in the media by 4.78% (Table 2).

Table 2. The average value of phosphorus released aerobically during the normal growth period of (up to 24 days) culture of *S. leopoliensis* and *P. boryanum.* This is in relation to when cells were cultured up to only 14 or 17 days period according to their growth characteristic (Myers, 1962).

Microbe	% of phosphorus release
S. leopoliensis	11.36%
P. boryanum	26.14%

Characteristics of Microbial growth in cultures of limited volume.

Table 2 depicts the general characteristics growth pattern of a unicellular alga in a culture of limited volume. A general account of the laboratory culture of microbes has been given by Myers (1962). The most usual kind of volume of culture in experimental work is one in which a limited volume of medium containing the necessary inorganic and organic nutrients is inoculated with a relatively small number of cells and then exposed to suitable conditions of light, temperature, and aeration.

Increase in cell numbers in such a culture follows a characteristic pattern in which the following phases may usually be recognized: a lag or induction phase in which no increase in cell numbers occurs, an exponential phase, in which cell multiplication is rapid and numbers increase in geometric progression, a phase of declining relative growth, a phase in which cell numbers remain more or less stationary, and a death phase (Myers, 1962).

A lag in cell multiplication may be apparent rather than real if a large proportion of the cells inoculated are not viable. Cell numbers will then remain nearly stationary until the progeny of the cells capable of dividing reach a number comparable with the total inoculated. This has been shown to be the most important cause of the lag when *Monodus subterraneus* is subcultured after a period of dark incubation (Belcher and Miller, 1960). The most detailed study of the five phases in algae appears to be that on *Phaeodactylum tricornutum* by Spencer (1954), *Anabaena cylindrical* (Fogg, 1944) and probably with all algae. The length of the phases is dependent on the age (time) of the inoculums. All the four microbes chosen for this work were cultured and tested as mentioned above (no tests results of the other microbes were tabulated as shown in Table 2).

Effect of different pH ranges on *Synechococcus leopoliensis* and *Plectonema boryanum* at different time (days).

Synechococcus leopoliensis and *Plectonema boryanum* were taken out of their normal grown condition flasks, spun down once at a speed of 3500 RPM and then inoculated into growth media containing the appropriate ingredient (phosphorus) in different pH ranges 7.2, 8.5, and 9.0. The flasks were put in the same growth chamber and at the same growth conditions as described above. They were agitated for 24 hours and tested for the release of phosphorus by the microbes into the supernatants. At pH 7.2, 8.5, and 9.0, phosphorus released by *S. leopoliensis* were 25.01%, 30.10%, and 33.12%. At the same pH levels, phosphorus released *Plectonema boryanum* was 29.15%, 32.02%, and 33.15% respectively (Table 3 a & b). The results indicated that at a higher pH level (9.0) more phosphorus was released within this time period.

Table 3 a, b. The percent of phosphorus released by *Synechococcus leopoliensis* and *Plectonema boryanum* in 24 hours under the different pH ranges.

(a) ***Synechococcus leopoliensis***

	pH 7.2	**pH 8.5**	**pH 9.0**
Phosphorus Released	25.01%	30.10%	33.12%

(b) ***Plectonema boryanum***

	pH7.2	**pH8.5**	**pH9.0**
Phosphorus Released	29.15 %	32.02%	33.15%

As shown in table 3a, *Synechococcus leopoliensis* released 33.12% at pH 9.0, 30.10% at pH 8.5, and 25.01% at pH 7.2 (table 3a). *Plectonema boryanum* released 33.15% at pH 9.0, 32.02% at pH 8.5, and 29.15% at pH 7.2 (Table 3b). The data showed that the two microbes released higher amounts of phosphorus at higher pH levels within 24 hours of inoculation.

When the cells of *S. leopoliensis* were cultured for a period of 4, 6, and 8 days aerobically (controls) and tested for phosphorus release, results showed that at pH 7.2 phosphorus released into the media was 25.15%, 25.35%, and 25.38% respectively. At pH 8.5 was 30.22%, 30.54%, and 30.71%. At pH 9.0 % phosphorus released was 33.17%, 33.48%, and 33.54%. Average of phosphorus released at pH 7.2 was 25.29%, at 8.5 was 30.49%, and at 9.0 were 33. 40% (Table 4). Showing again that pH affects significantly the release of P by *S. leopoliensis.*

The pH was maintained daily for the duration of the experiment. Phosphorus was not completely released during this control condition as can be expected.

Samples of the media were taken from the aerobic zone of a Modified Fitzgerald Medium (MFM) laboratory system operated for 4, 6, and 8 days scale for exhibiting biological enhance phosphorus removal (BEPR). These were compared with the experimental data from the anaerobic condition and with the addition of EDTA (Figures 2 and 12). During the aerobic and anaerobic zone, samples were taken from the set up and placed in centrifuge tubes used to perform the extractions. The supernatant was decanted and tested for the release of phosphorus and the milligram of phosphorus was converted to percent of Phosphorus released into the media. The addition of phosphorus in the media was 1.76 mg P/L.

Table 4. Aerobic condition (control). Percent of phosphorus released by *Synechococcus leopoliensis* into the media at different time intervals (days). Numbers indicate percent phosphorus release into the media, (35 replicas per treatment) (1.76mgP/L)

Time (days)	**pH**		
	7.2	**8.5**	**9.0**
4	**25.15%**	**30.22%**	**33.17%**
6	**25.35%**	**30.54%**	**33.48%**
8	**25.38%**	**30.71%**	**33.54%**
Average	**25.29%**	**30.49%**	**33.40%**

Phosphorus Release by *S. leopoliensis* under anaerobic conditions

Table 5 summarizes the results in this study of the anaerobic condition of phosphorus release by *S. leopoliensis*. The process was maintained in the anaerobic condition for 8 days. The pH range was monitored and maintained in the anaerobic condition in the same growth conditions as already mentioned in materials and methods throughout the experiment, by inserting a pH meter electron through the rubber stopper into the media. The anaerobic condition was also checked using resazurin to make certain there was no accidental dissolving of oxygen (DO), as the presence of oxygen with resazurin turns the normal color blue to pink. Samples the media were periodically withdrawn by using the 50-gauge syringe, (which was permanently inserted in the flasks containing the media).

The results indicated that the higher the pH, the more phosphorus was released and the longer the microbes stayed in the anaerobic condition (8 days), the more phosphorus was also released by *S. leopoliensis* (table 5). Higher pH and anaerobic conditions both caused significant phosphorus release, as shown by a single classification ANOVA, ($P<0.0001$) (Table 9).

Further analysis by Fisher's LSD pairwise tests showed that the PPBs decreased significantly ($P<0.0001$) when the cells were grown at pH 9.0 under anaerobic conditions cultures grown for 8 days. The same analysis applied to pH 8.5, and 7.2 anaerobically for the 8 days compared to the control cells. The data were obtained from 140 tests 35 vials from each pH range and each time period.

In this anaerobic condition, experimental results indicated that the levels of phosphorus corresponded to the degradation of PPB along with anaerobic stages of the process. According to table 5, pH significantly influenced the behavior of phosphorus release and PPB degradation. Table 4 showed that phosphorus retention occurred during aerobic stages when the process was operated normally for 4, 6, and 8 days. Subsequently, the microbes did not completely release phosphorus in the aerobic stage presumably because PPB is stored as reserve energy for the cells as were observed by Jensen *et al.*, (1986), and Jensen, (1969), Ebel *et al.* (1958), Jensen (1990), and Harold, (1966).

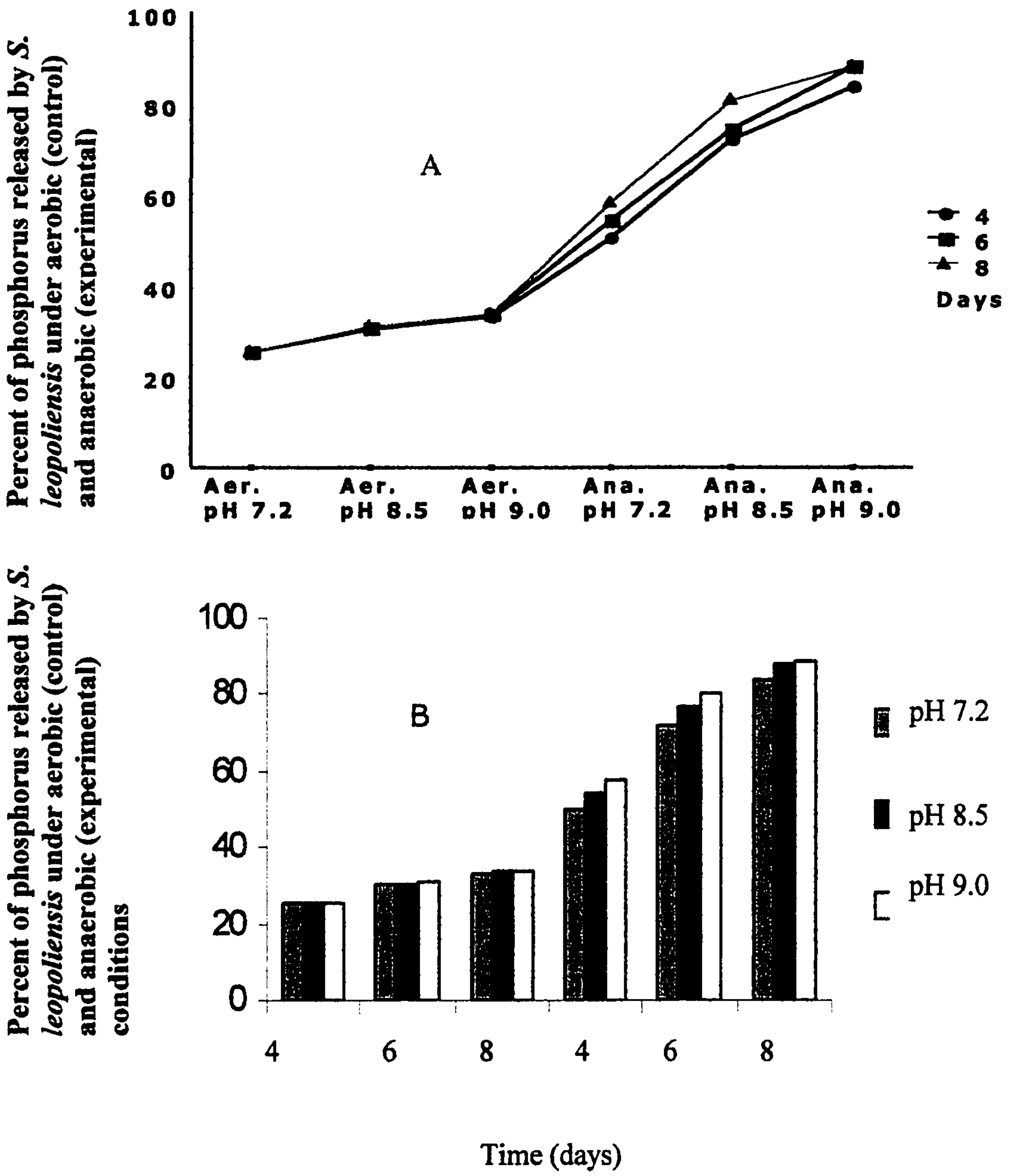
Additionally, phosphorus uptake or release behaviors apparently depend on dissolved oxygen (DO). By subjecting the microbes to anaerobic conditions allowed phosphorus to be released. Starving the microbes for a period of time from their essential nutrients and then reintroducing them to a fresh medium concentrated with phosphorus, allows them to rapidly pick up phosphorus. This is termed Overplus phenomenon as already mentioned in the introduction.

Table 5. Phosphorus released by *Synechococcus leopoliensis* at different time intervals. Numbers refer to the time in days in which the treatment was carried out. 35 replicas were analyzed to compare the amount of P released in the experimental phase against the control of the various pH ranges in the same number of days with 1.76 mg P/L in the media.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**50.23%**	**72.00%**	**83.57%**
6	**54.15%**	**77.04%**	**88.05%**
8	**58.03%**	**80.45%**	**88.92%**
Average	**54.14%**	**76.50%**	**86.85%**

Figure 5A. Represents line graphs of the release of phosphorus by *S. leopoliensis* in the media during normal growth, aerobic (aer.), and during anaerobic (ana) conditions for the cells. Figure 5B. (Histograms), is also a comparison of control (aer.) and the experimental, anaerobic (ana.) phase when the media were gassed with dry nitrogen for the same number of days. The percent of P released is compared against the total amount of P in the media (1.76mg P/L).

Figure 5. Aerobic vs. Anaerobic conditions (A & B)



The graphs represent the amount of phosphorus released by *S. leopoliensis* during the various time intervals and the pH ranges in the control and the experimental stages. The graphs were generated as a comparison between the aerobic phase (control) and the anaerobic phase (experimental stage). They showed that for 8 days in the anaerobic condition and pH 9.0, the amount of phosphorus released by *S. leopoliensis* was higher than in 4, and 6 days at either pH 7.2, or pH 8.5, as table 5 shows.

The time course of phosphorus uptake and release by the microbe into the media was monitored until the tests were completed. The tests confirmed that the released phosphorus at day 8 and pH 9.0 (Table 5) was high (86.85%) on average.

Figure 5 shows the difference between the control values of phosphorus release (aerobic) and the experimental values (anaerobic) conditions. After the extended period (day 8) of anaerobiosis, no polyphosphate granules were seen. After adding fresh medium and re-examined the cells in the usual way, massive deposits of polyphosphate bodies were found (no data shown).

When micrographs were taken with Hitachi H – 7000 during the anaerobic condition period, cells indicated the loss of polyphosphate bodies. At pH 9.0 and 8 days, S. *leopoliensis* lost all the PPBs (Figure 6). Under the anaerobic condition, the cells of *S. leopoliensis* released most of the polyphosphate bodies as phosphorus into the supernatant at 4 and 6 days time period but not as much as the 8-day period. This was an indication that time in anaerobic condition played a role in phosphorus release by the microbe. The amount of polyphosphate bodies (PPBs) present during these days were compared with the Overplus conditions by visual inspection and by counting.

This finding indicated that the accumulation of phosphorus is also related to pH levels, as well as in aerobic and in anaerobic stages. During the aerobic stage, the cells retained their phosphorus as PPBs and in the anaerobic phase, the cells released their polyphosphate bodies as phosphorus into the media. Cells were examined under the TEM and they showed no cell fraction (denaturization-no micrograph shown).

Release of Phosphorus by the 4 microbes in Pure Culture

Tables 4, 7, 10, and 12 of the 4 selected microbes indicated the amount of phosphorus released during pure culture. The pH was adjusted to 7.2, 8.5, and 9.0.No anaerobiosis was produced since these were controls. The average in the supernatant of phosphorus by cells of S. *leopoliensis* at pH 7.2 for 4 days was 25.29%, at pH 8.5 was 30.49%, and at pH 9.0 33.40%. By *P. boryanum*, at pH 7.2 was 31.08%, at pH 8.5 was 32.23%, and at pH 9.0 was 33.66%.

Table 6. Volume of cell occupied by PPB of *S. leopoliensis* in the Overplus condition containing 1.76mg P/L in media.

Time (min)	Mean	SD	SE
0	0.254	0.334	0.034
1	0.538	0.335	0.049
2	0.604	0.284	0.040
3	0.948	0.332	0.048
4	1.288	0.728	0.121
5	1.506	0.617	0.100
10	2.076	1.435	0.190
15	3.026	1.427	0.248
30	3.044	1.729	0.244
60	2.924	1.512	0.263

Percent of P released by *S. cerevisiae* at pH 7.2 was 10.41%, at pH 8.5 was 10.89%, and at pH 9.0 was 11.08%. By *R. rubra* at pH 7.2 11.56%, at pH 8.5 12.07%, and at pH 9.0 25.22% were released into the supernatant. The release or retention of phosphorus was quite variable depended on time (days) and pH levels.

During the Overplus condition, the cells picked up phosphorus. The uptake was very rapid. Beginning at 1 minute PPB increased in size and number (Figure 4). When the volume of the cells and volume of PPB were calculated it showed that after 1 minute in the phosphorus containing media, the volume of the cells occupied by PPBs rose from 0.21% to 0.54% in the starved condition. At 5 minutes the volume rose to 1.51%. At 15 minutes, 3.03% and at 30 minutes, and 3.04% (Table 6). As can be seen with these calculated values of PPBs, *S. leopoliensis* was capable of releasing phosphorus in an anaerobic condition than in aerobic condition. The cells were void of PPBs.

Figure 4 shows that there are more polyphosphate bodies in their overplus and reversed conditions than in their anaerobic period. This result, therefore, indicates that *S. leopoliensis* releases phosphorus when subjected in anaerobic condition. Furthermore, when *S. leopoliensis* was starved of its essential nutrient (phosphorus) for a period of time (in this case 5 days) and reintroduced into a medium rich in phosphorus, the microbe picks up more phosphorus in the form of PPBs.

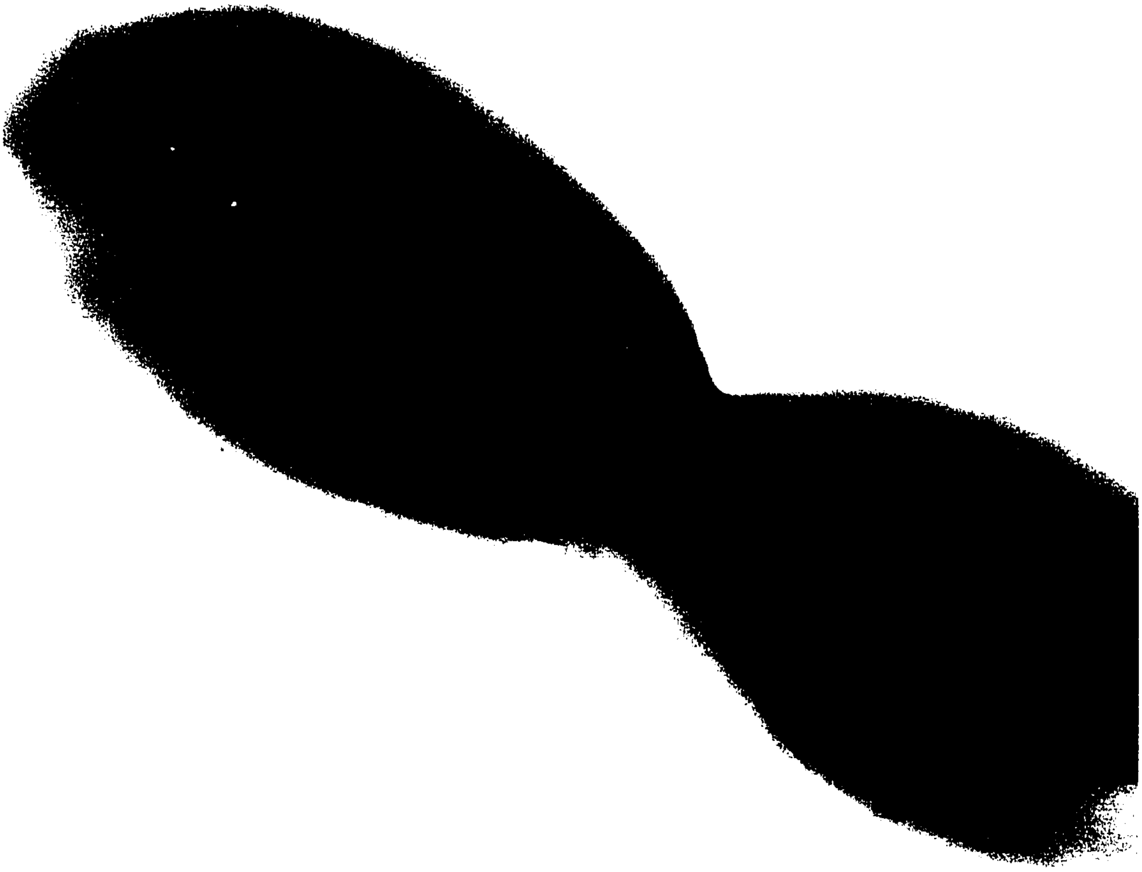


Figure 6. Micrograph of a section of *Plectonema boryanum* cell grown in the anaerobic condition at pH 9.0 for 8 days. Note the absence of PPBs. X 26,000.

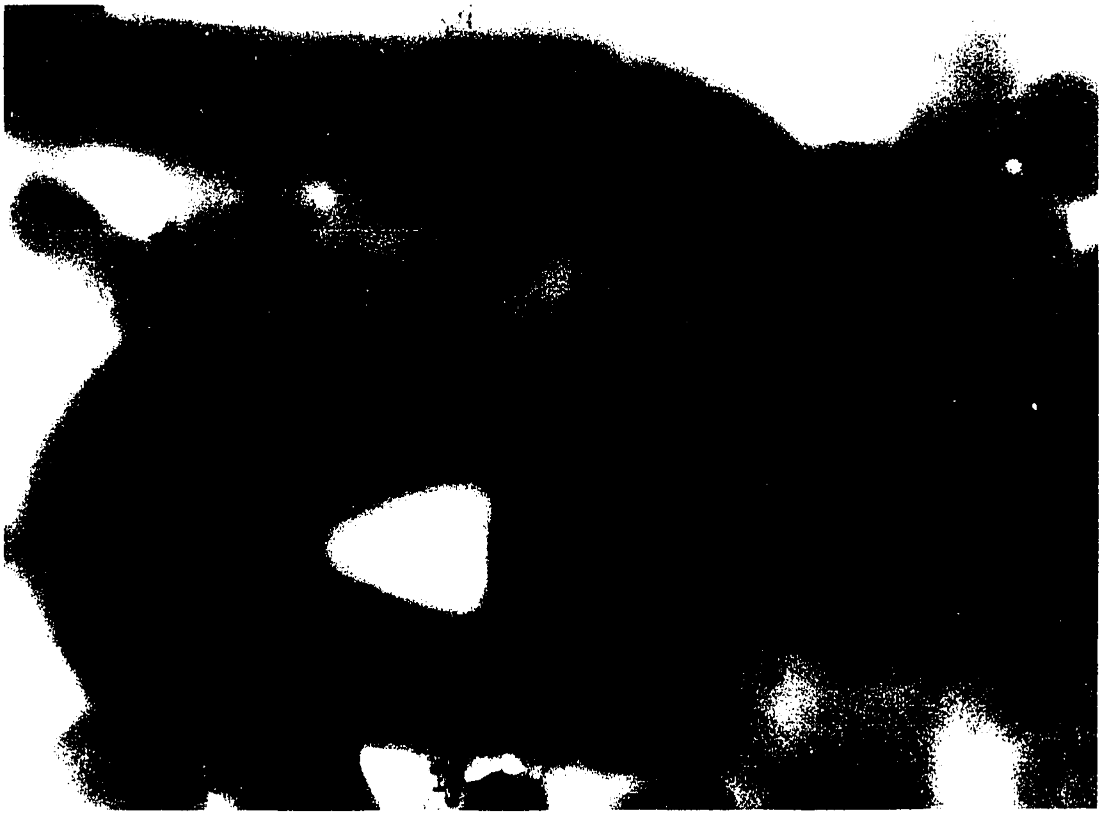


Figure 7. Micrograph of whole-unfixed cells of *Synechococcus leopoliensis* after 5 days in phosphorus free medium and adding back PO_4, pH 7.2 (Overplus phenomenon). All of the electron dense inclusions are polyphosphate bodies (PPBs). X 28,000.

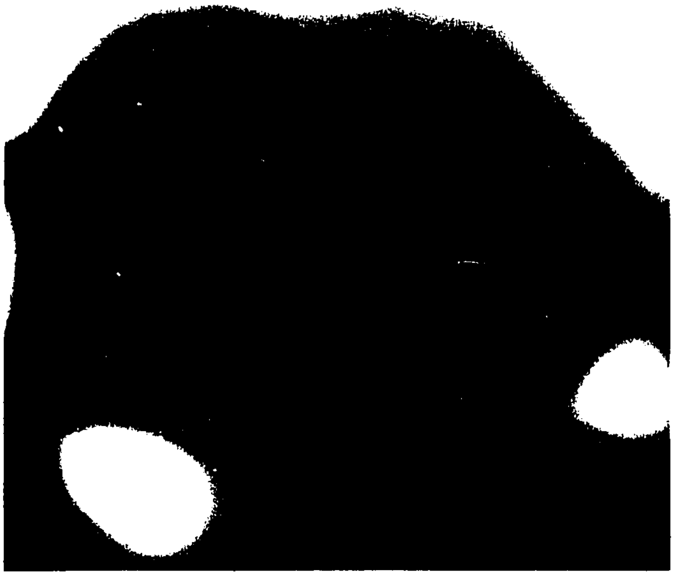


Figure 8. Micrograph of whole-unfixed cells of *Plectonema boryanum* after 5 days in phosphorus free medium, pH 7.2 (Overplus phenomenon). All of the electron dense inclusions are polyphosphate bodies (PPBs) (arrow). X 28,000.

The release of phosphorus by *P. boryanum* under anaerobic conditions.

Polyphosphate bodies decreased to a zero value by *P. boryanum* in the supernatant, as phosphorus, when subjected in the anaerobic conditions for a period of 4, 6, and 8 days at the usual pH ranges (Table 8). The PPBs were visually inspected and counted and compared against their Overplus conditions (no data shown). Figure 5 indicated the loss of PPBs and the release of phosphorus by *P. boryanum* during the anaerobic condition.

Based on figures 4 and 6, it can be inferred that the two microbes accumulated PPBs in media rich in phosphorus after they had been starved for a period of time. This phenomenon was accompanied by P uptake or a low release of phosphorus. When in the anaerobic phase, P is released in high quantity in the supernatant by the two microbes *S. leopoliensis* and *Plectonema boryanum* (Tables 5 and 8). The release of P concentration in the anaerobic condition was a critical aspect of P removal in the subsequent aerobic stage.

After *P. boryanum* was cultured and tested for the presence of phosphorus in the supernatant aerobically, results revealed the following: At pH 7.2, 29.21% in 4, 32.0% in 6, and 32.03% in 8 days. At pH 8.5, 32.17% in 4, 32.25% in 6, and 32.28% in 8 days. At pH 9.0, 33.54% in 4, 33.61%, in 6, and 33.82% in 8 days (Table 7). The average values are 31.08% at pH 7.2, 32.23% at pH 8.5, and 33.66% at pH 9.0.

Table 9 shows that when *Plectonema boryanum* was tested in the anaerobic condition, results indicate high phosphorus release (97.25%) at high pH (pH 9.0) during the 8-day period. Compared to the other microbes, *P. boryanum* seems to be more efficient to release phosphorus from its PPBs.

Table 7. Aerobic Release of phosphorus by *Plectonema boryanum* at different time (days). Numbers refer to the time in days cells were grown normally as controls. (Total amount of 1.76 mg P/L in the media).

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**29.21%**	**32.17%**	**33.54%**
6	**32.00%**	**32.25%**	**33.61%**
8	**32.03%**	**32.28%**	**33.82%**
Average	**31.08%**	**32.23%**	**33.66%**

Table 8. Anaerobic condition of phosphorus released by *Plectonema boryanum* at different time interval. Numbers refer to the time in days.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**62.09%**	**72.11%**	**92.60%**
6	**70.32%**	**87.00%**	**95.87%**
8	**84.40%**	**89.95%**	**97.25%**
Average	**72.27%**	**83.02%**	**95.24%**

Figure 9 A. Represents line graphs of the release of phosphorus by *P. boryanum* in the media during normal growth, aerobic (aer.), and during anaerobic (ana) conditions for the cells. Figure 5B. (Histograms), is also a comparison of control (aer.) and the experimental, anaerobic (ana.) phase when the media were gassed with dry nitrogen for the same number of days. The percent of P released is compared against the total amount of P in the media (1.76mg P/L). Both figures clearly show that phosphorus released during the anaerobic phase was greater than during the aerobic (control) phase.

Figure 9A. Aerobic vs. Anaerobic conditions

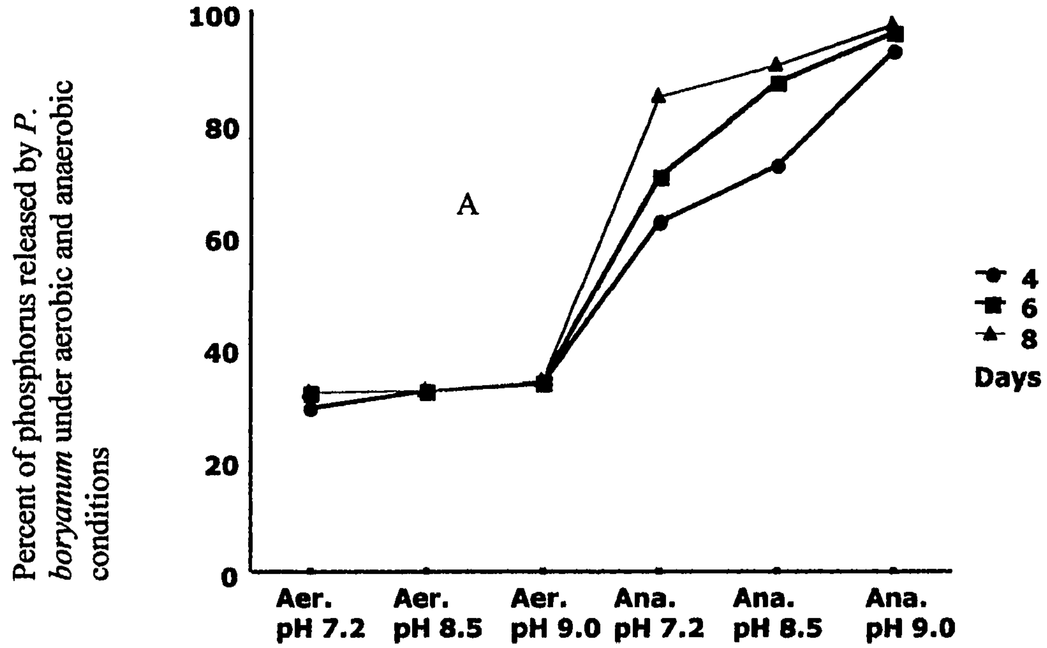


Figure 9 B. Aerobic vs. Anaerobic conditions

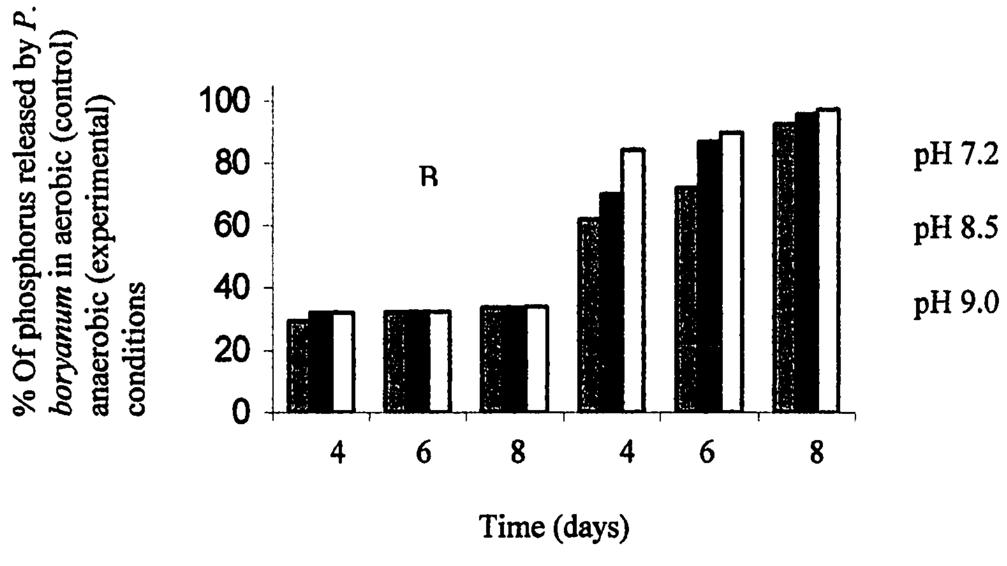


Table 8 represents the release of P in anaerobic condition. The difference between the aerobic and the anaerobic phase for 8 days at pH 9.0 was 63.59% with the anaerobic treatments releasing more phosphorus at each pH level and each time period than the control. This difference of P released is statistically significant. This stands to say that the microbe was able to release phosphorus at long time period in the anaerobic condition than the aerobic condition and at higher pH levels, influences more phosphorus release.

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Table 7. Shows that the release of P is enhanced by increased pH level and by the time. The release of phosphorus by the cells increased by nearly three-fold, from 33.66 to 95.25 at pH 9.0 in 8 days. Furthermore, the results also confirmed that the longer the cells remained in their anaerobic condition, the more phosphorus is released as can be seen in Table 8. The data obtained from the cells subjected to an anaerobic conditions and all the pH levels were analyzed using Hach Test Kit at High Range and by using single classification ANOVA to calculate the significance to compare the aerobic release of phosphorus and the anaerobic release (Table 9).

Under aerobic conditions of the microbes, as well as in anaerobic conditions, *Plectonema boryanum* released more phosphorus than the other three microbes (Table 8). When EDTA was added aerobically to all the three microbes at their perspective pH ranges, *Plectonema boryanum* again released more P than the other three microbes (Table 15) as compared to the results of the other microbes. It can therefore be said that *Plectonema boryanum* is more efficient than *S. leopoliensis, S. cerevisiae,* and *R. rubra* to be employed to release phosphorus for environmental and commercial uses.

Determination of Phosphorus Release by *Sacchacromyces cerevisiae* and *Rhodotorula rubra* in the aerobic Condition at different time periods (days).

During the aerobic growth condition of *Sacchacromyces cerevisiae* and *Rhodotorula rubra,* phosphorus release was low (Tables 10, 12), compared to *S. leopoliensis and P. boryanum.* This indicated that the microbes *S. leopoliensis* and *P. boryanum* released phosphorus more efficiently than *S. cerevisiae* and *R. rubra* when both are subjected in the same environmental growth conditions.

Table 9. Paired t-test showing the difference of phosphorous release by the microbes under different pH value at different period of time (days). Aerobic, anaerobic, and EDTA.

Number	Compared Cultures of Microbes	Mean	DF	t	P
1	*S. leopoliensis*	42.33%	8	11.396%	0. 0001
2	*P. boryanum*	51.187%	8	13.614%	0. 0001
3	*S. cerevisiae*	59.611%	8	12.448%	0. 0001
4	*R. rubra*	36.720%	8	10.485%	0. 0001
5	*S. leopoliensis*, 0.25M EDTA	51.760%	8	48.092%	0. 0001
6	*P. boryanum*, 0.25M EDTA	50.510%	8	26.652%	0. 0001
7	*S. cerevisiae*, 0.25M EDTA	66.377%	8	18.998%	0. 0001
8	*S. rubra*, 0.25M EDTA	59.627%	8	45.200%	0. 0001

Table 10. Aerobic release of phosphorus by *Saccharomyces cerevisiae* at different time intervals (time dependency test).

Days	**PH**		
	7.2	**8.5**	**9.0**
4	**10.26%**	**10.83%**	**11.07%**
6	**10.26%**	**10.86%**	**11.09%**
8	**10.71%**	**10.98%**	**11.09%**
Aver.	**10.41%**	**10.87%**	**11.08%**

Table 11. Anaerobic condition of phosphorus released by *Saccharomyces cerevisiae* at different time intervals (time dependency test).

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**52.07%**	**67.13%**	**82.02%**
6	**54.00%**	**67.93%**	**87.12%**
8	**58.19%**	**71.72%**	**93.47%**

Figure 9A. Aerobic vs. Anaerobic conditions

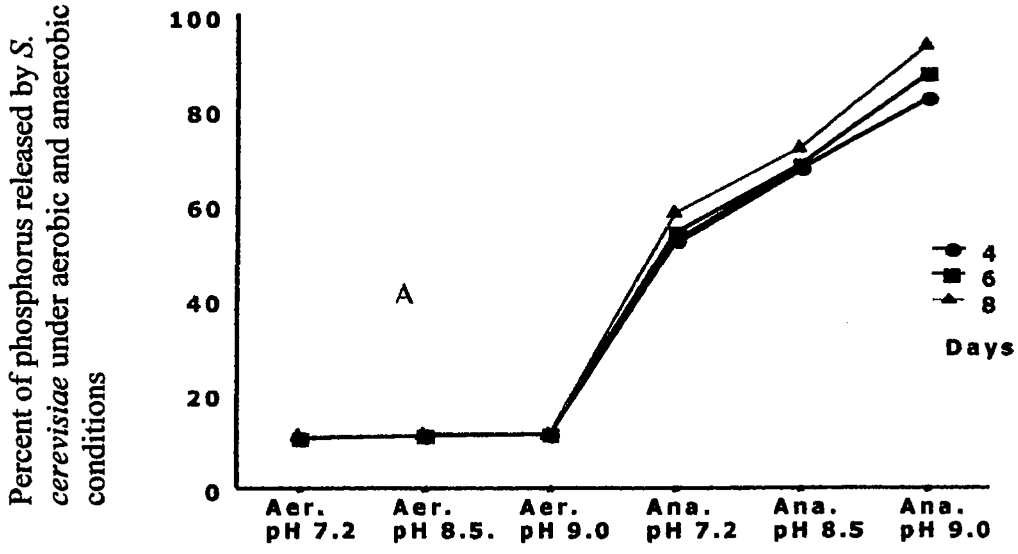


Figure 10B. Aerobic vs. Anaerobic conditions

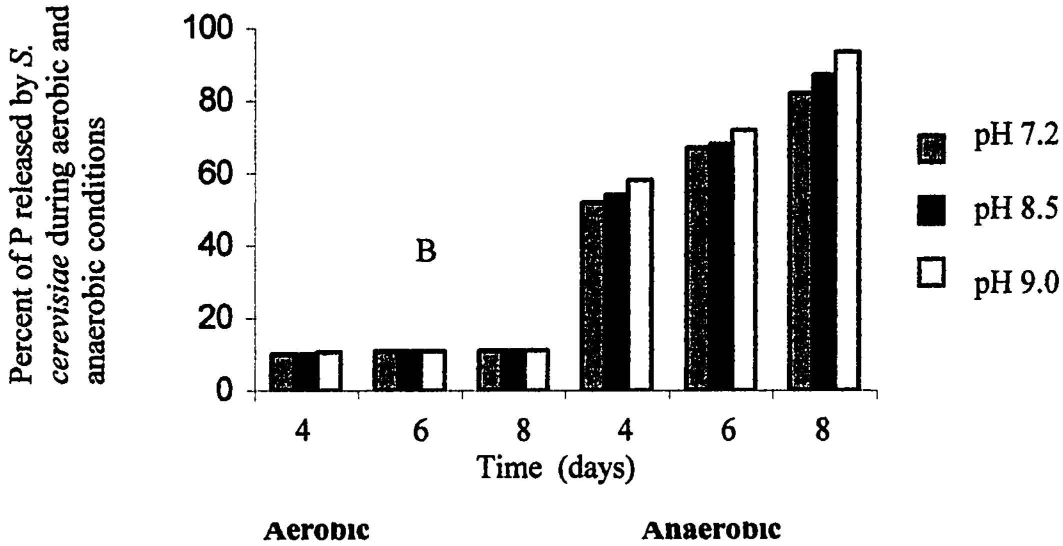


Figure 10 A and B. Show the amount of phosphorus released during aerobic (aer.) and the experimental anaerobic (ana.) conditions by *S. cerevisiae*.

To elucidate the effect of anaerobic conditions of P content in the media and the accumulation of P release by *S. cerevisiae* cells, the phosphorus content in the aerobic and anaerobic was analyzed and compared as shown in figure 8 A, and B, and tables 10 and 11. When the anaerobic cells were examined using Hitachi H – 7000, the cells were completely devoid of PPBs at the day 8 period (Figure 9) and high pH level, 9.0.

As can be seen from the phosphorus release curve at Figure 8 aerobic phase (control) and anaerobic condition, it can be said that *S. cerevisiae* is another microbe that is capable of releasing phosphorus when subjected to anaerobic condition.

Under aerobic condition (control) the release of P was much less as predicted. As can be seen, at pH 7.2 release of P in the average is 11.56%, at pH 8.5 is 12.07%, and at pH 9.0 is 25.22% (Table 10). Results indicate that *R. rubra* will not release phosphorus aerobically during long time periods or higher pHs as compared to the other microbes. This is obvious as shown in results of table 10.

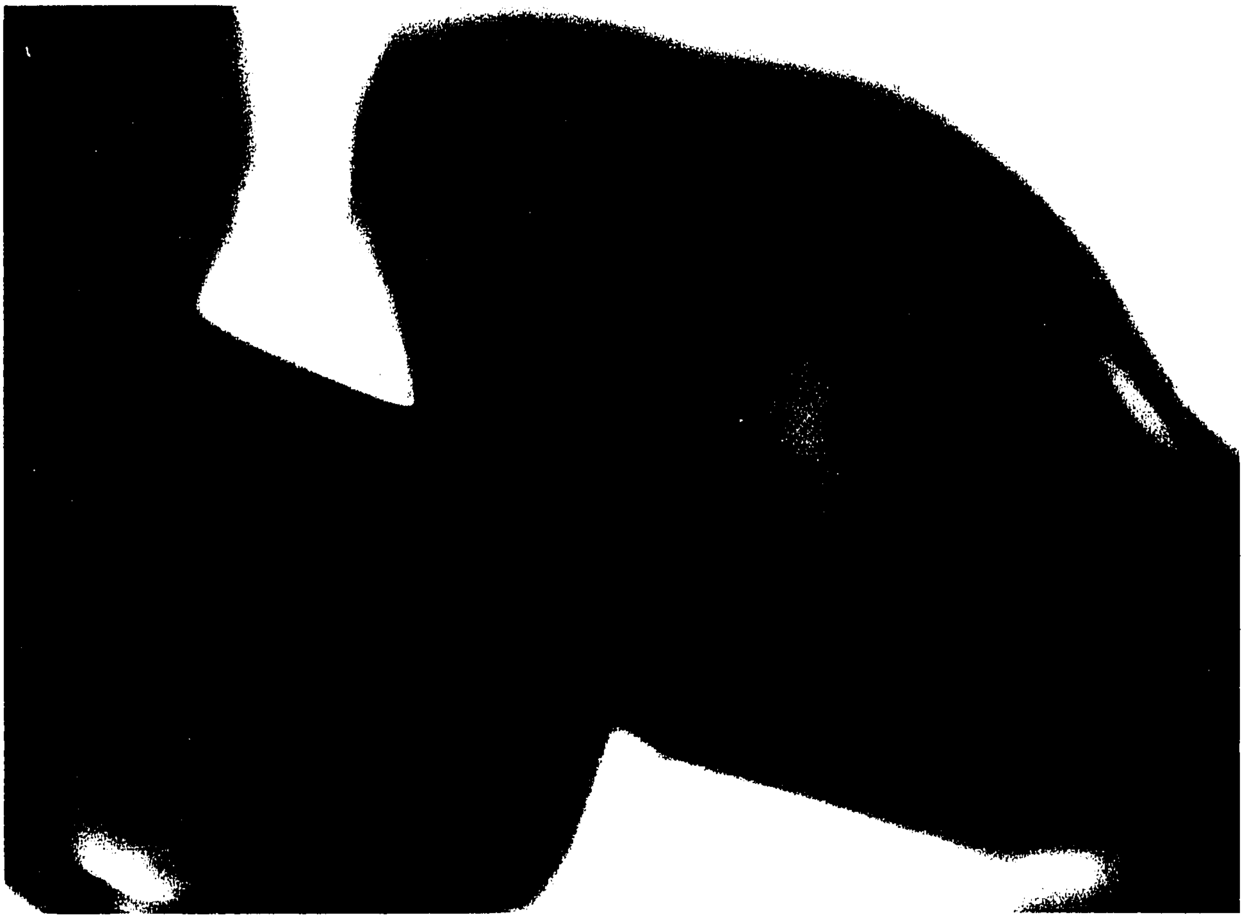


Figure 11. Electron micrograph of *S. cerevisiae* exposed to anaerobic condition for 8 days and pH 8.5. Note only one large PPB. X 28000.

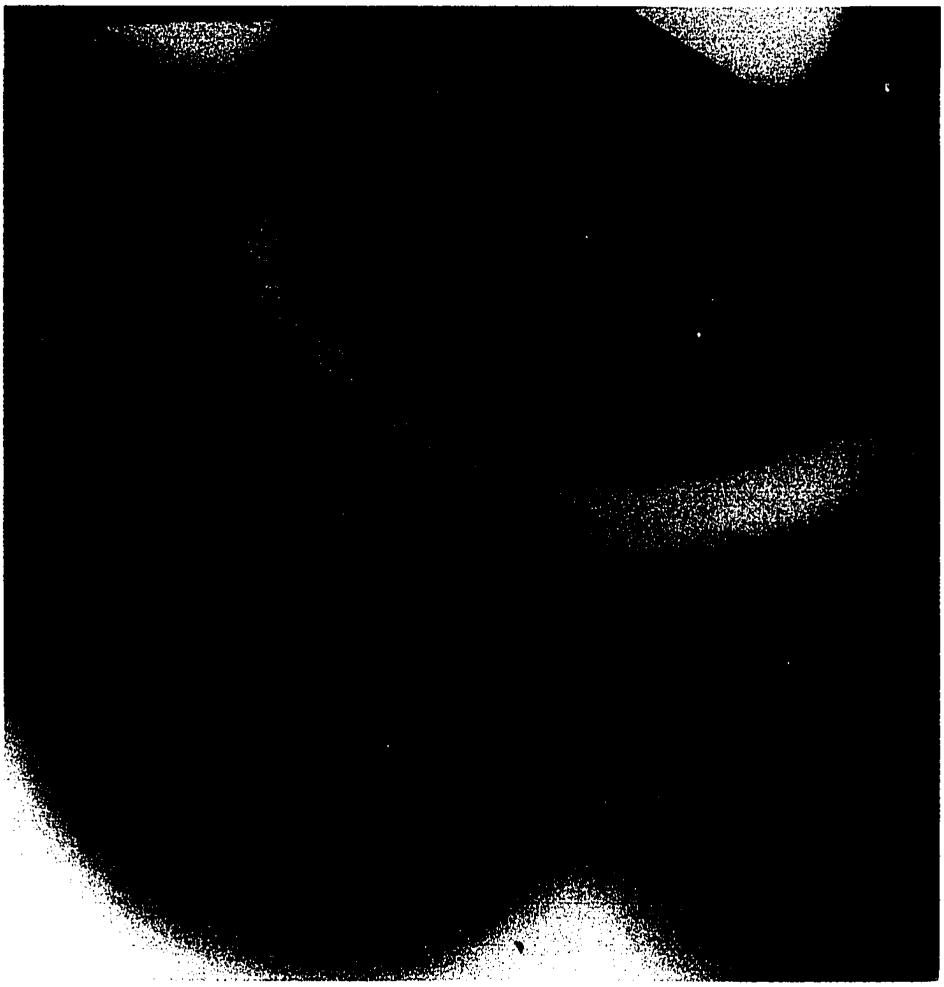


Figure 12. Electron micrograph of *S. cerevisiae* under anaerobic condition for 8 days and pH 9.0. Note the absence of PPB. All of the PPBs were released as phosphorus into the supernatant . X 28000.

Table 12. Aerobic release of phosphorus by *Rhodotorula rubra* at different time interval (time dependency test). Numbers indicate days in which test for phosphorus release (control) was conducted.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**11.51%**	**11.85%**	**25.15%**
6	**11.52%**	**12.01%**	**25.16%**
8	**11.65%**	**12.35%**	**25.35%**
Average	**11.56%**	**12.07%**	**25.22%**

Table 13 summarizes the results of the release of phosphorus by *Rhodotorula rubra* subjected under anaerobic condition for 4, 6, and 8 days and pH 7.2, 8.5 and 9.0. As can be seen at Table 13, at pH 7.2 and 4 days average release of phosphorus is 36.69%, at pH 8.5 is 53.27%, and at pH 9.0 is 69.04%. When the microbes were subjected to anaerobic conditions and pH 7.2 for 4, 6, and 8 days, the release of P was 34.00%, 37.11%, and 38.97% respectively. At pH 8.5for the same period of time, the values of P was 47.93%, 54.06%, and 57.83%, and at pH 9.0 it was 59.03%, 68.34%, and 79.76%, respectively (Table 13). This indicates that pH as well as time plays a significant role in phosphorus release.

Table 13. Anaerobic condition of phosphorus release by *Rhodotorula rubra* at different time intervals (time dependency test) and pH levels.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**34.00%**	**47.93%**	**59.03%**
6	**37.11%**	**54.06%**	**68.34%**
8	**38.97%**	**57.83%**	**79.76%**

Figures 13 A and B. Percentage of phosphorus released by *R. rubra* under aerobic and anaerobic conditions (aer: aerobic, control, Ana: anaerobic, experimental). The experiment was also carried out at pH-values of 7.2, 8.5, and 9.0 and 4, 6, and 8 days. The results are completely different from that of the other microbes in both aerobic and anaerobic condition, being low at the same pH-values and time period (days).

Figure 13A. Aerobic vs. Anaerobic conditions

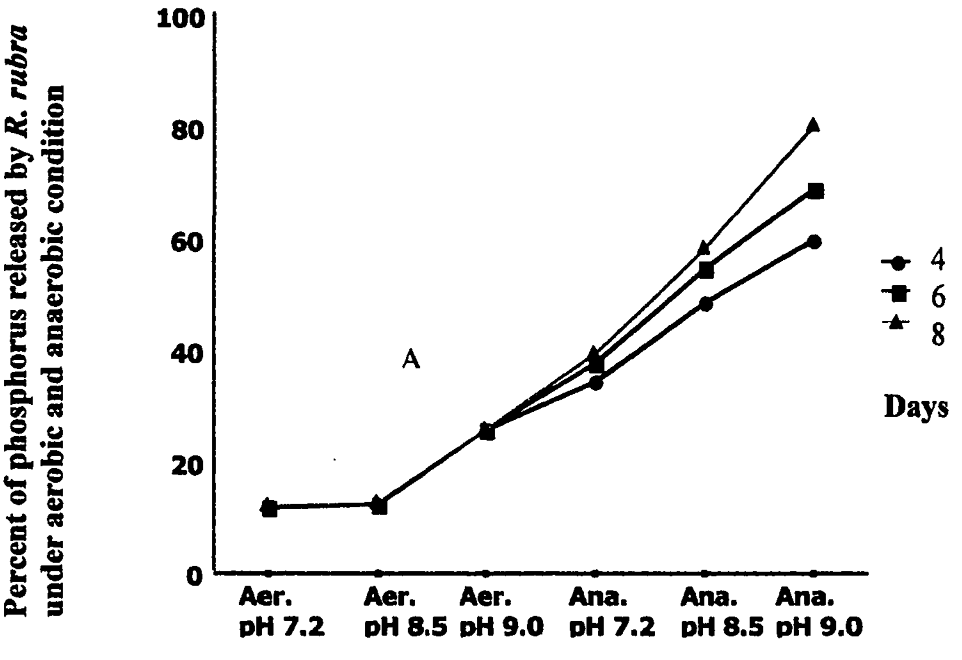
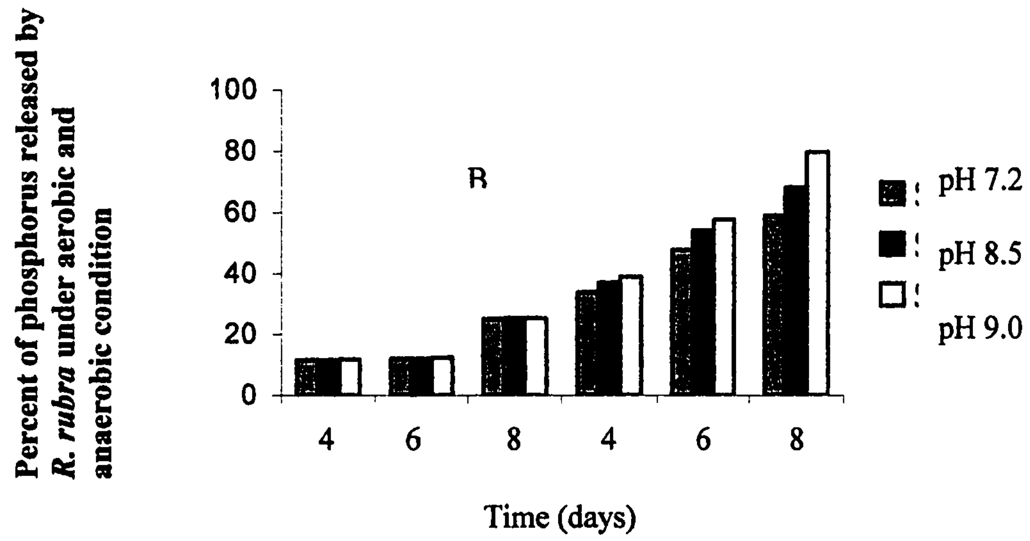


Figure 13B. Aerobic vs. Anaerobic conditions



Determination of Phosphorus Release by the microbes with addition of EDTA at different time periods (days).

The phosphorus in the media and the bacterial samples were determined at various phases of the uptake experiments under four (4) conditions: (a) without addition of EDTA, (b) after addition of 0.25M EDTA, (c) after gassing with dry nitrogen at 10 pounds per square inch (PSI) for 45 minutes three times a day, and (d) allowing it to settle for 24 hours. Treatment (b) was to determine the release of P with the addition of the EDTA and (c) was intended to induce phosphate release by establishing anaerobiosis (Fuhs and Chen 1975) and treatment (d) was intended to test for complete anaerobic conditions by adding resazurin against treatment a.

After the culture has been left for 24 hours in the anaerobic condition and tested, it was found to contain no oxygen. Complete anaerobic state was established. The anaerobic condition was monitored as it was throughout the entire experimental period by withdrawing the media from the flasks under the anaerobic conditions periodically with a 50-gauge syringe, and was tested with the resazurin. When it was satisfied that the anaerobic condition was constant, no more dry nitrogen gas was pumped in. The media were tested and found to contain a higher concentration of phosphorus in the supernatant than in the aerobic state (Tables' 3a & b). Microscopic examination revealed that the polyphosphate bodies (PPBs) had decreased in numbers and sizes but had not disappeared completely. Even though, the cells remained in the anaerobic phase for a day (24 hours) under their different pHs, phosphorus release was incomplete.

Treatment of *S. leopoliensis* with EDTA

Table 14 shows the effect of 0.25M EDTA additions on aerobically cultured cells in the various pH ranges. During the 8-day period, and under pH 9.0, the cells of *S. leopoliensis* released an average of 88.71% of phosphorus. This amount was higher than phosphorus released by *Rhodotorula rubra* with the same amount of EDTA added (Table 17) and less than *Plectonema boryanum* (table 15) or *S. cerevisiae* (table 16). Table 14 showed that good recovery of phosphorus by addition of 0.25M EDTA was obtained. The observed phosphorus release was also three times higher than the aerobic state (Tables 4 and 14).

Taking into account that the recovery of total phosphorus in these experiments was not exactly 100%, the possibility cannot be ruled out that these observations were due to experimental limitations. Alternatively, two explanations may be put forward: either EDTA addition caused rapid hydrolysis of a portion of the acid polyphosphate pool with the resultant orthophosphate binding with the EDTA in a non-acid-soluble form or else EDTA became completed with a part of the polyphosphate and converted it into a form not soluble in Modified Fitzgerald Medium.

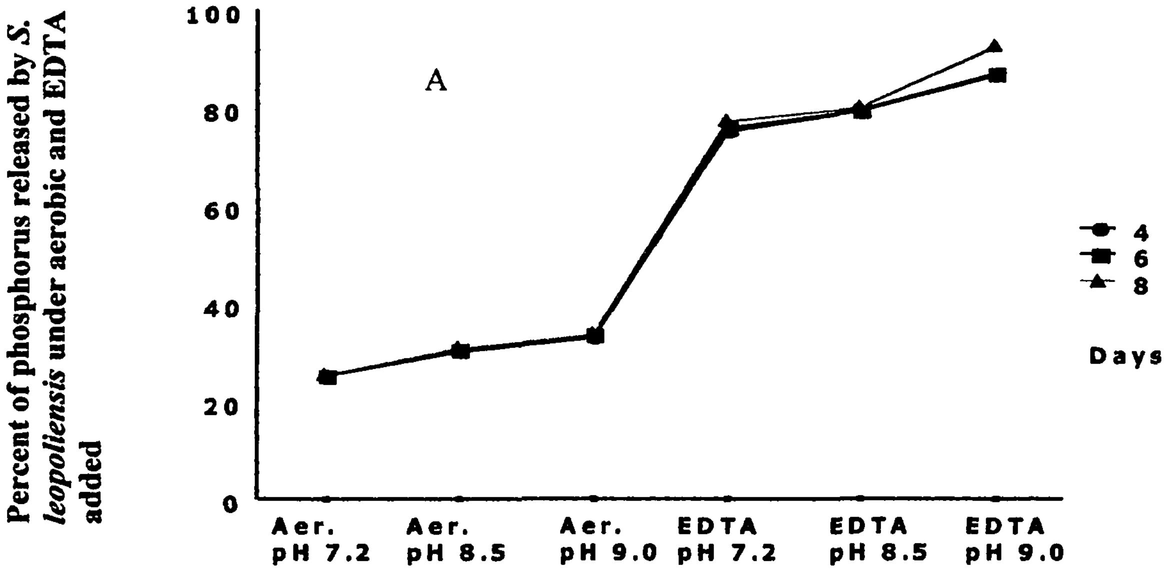
Table 14. Addition of 0.25M EDTA in the aerobic Condition caused the release of Phosphorus by *Synechococcus leopoliensis* at different time interval (time dependency test), days and pH ranges.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**75.18%**	**79.52%**	**86.71%**
6	**75.72%**	**79.55%**	**86.84%**
8	**77.32%**	**79.97%**	**92.57%**
Average	**76.07%**	**79.68%**	**88.71%**

Figures 12 A and B summarize the results in table 14. The graphs depict the amount of phosphorus released into the supernatant when 0.25M EDTA is added to the media containing the cells in their aerobic condition. Day 8 and pH 9.0 show a higher release of phosphorus than day 4 or day 6 at the same pH-values, (table 14). These also show the difference between the control (without EDTA) and the addition of EDTA. The results show that EDTA played a significant role in phosphorus release and as well as time (days) and pH ranges by cells of *S. leopoliensis*. During the time period, average P released was 76.07% at pH 7.2, 79.68% at pH 8.5, and 88.71% at pH 9.0. When the EDTA was added to the media, monitored, and maintained, the following results were obtained: at pH 7.2 in 4, 6, and 8 days, the release of phosphorus was 75.18%, 75.72%, and 77.32% respectively. At pH 8.5 in 4, 6, and 8 days the release was 79.52%, 79.55%, and 79.97% respectively. At pH 9.0 in 4, 6, and 8 days phosphorus release was 86.71%, 86.84%, and 92.57% respectively. Obviously, as pH and time durations increased phosphorus release is greater (Yall *et al*, 1979).

The results indicate that EDTA was capable of inducing bacteria to release phosphorus. Apart from the release of phosphorus, other heavy metals can also be released (Sun *et al*, 2000). The release of the metals is coupled with pH variations. Laboratory studies have shown that EDTA is effective in removing Pb, Zn, Cu, from contaminated soils and wastewaters. The removal efficiency depends on many factors, such as the strength of the EDTA and the level of pH of the medium (Elliot and Brown, 1989, Brown and Elliot, 1992, Pichtel and Pichtel, 1997, Elliot and Shastri, 1999). The result of this work is in agreement with these studies.

Aerobic vs. EDTA



Aerobic vs. EDTA

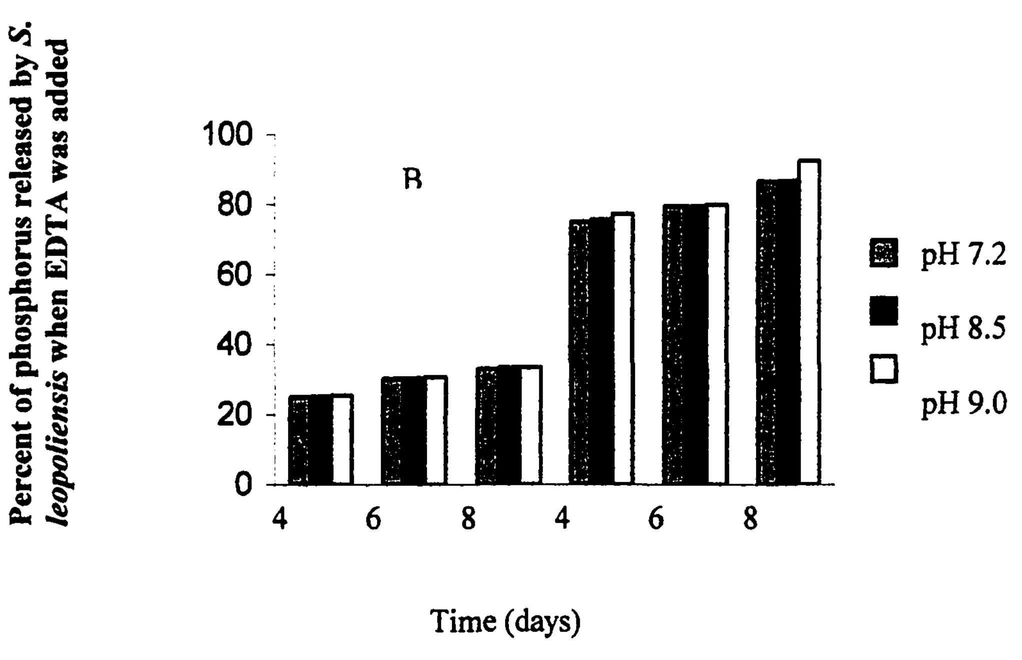


Figure 14 A and B. Graph shows the percentage of phosphorus release by *S. leopoliensis* with addition of EDTA and without EDTA (control) aerobic conditions.

The release of phosphorus by *Plectonema boryanum* with addition of 0.25M EDTA and various pH levels and time period (days).

Table 15 shows the amount of phosphorus released by *P. boryanum* in the aerobic phase with the addition of 0.25M EDTA. The results confirmed that at pH 9.0 and a period of 8 days in this condition, an average of 91.04% phosphorus was released. This amount was higher than the other three microbes. It appears obvious that *P. boryanum* is an excellent phosphorus-releasing microbe when EDTA is added and this confirmed that EDTA played a major role in the release of P by *P. boryanum*. When the PPBs were visually examined and counted the number of PPBs noticed were lesser than found in *S. leopoliensis*, *S. cerevisiae*, and *R. rubra* (no data shown).

The longer the EDTA remained in the media containing the cells of *P. boryanum*, the more phosphorus was released, as indicated in table 15. As previously described, EDTA added to a medium with higher pH values caused the cells to release more phosphorus. The release of phosphorus by *P. boryanum* with addition of EDTA was more efficient than with *S. leopoliensis* (Table 14), *S. cerevisiae* (Table 16), and *R. rubra* (Table 17).

Figure 15 compares phosphorus release between the control (without EDTA) at the same time periods and pH ranges and the addition of EDTA. This shows that in general, EDTA can enhance the release of phosphorus by microbes.

Table 15. Addition of 0.25M EDTA at a temperature of 25°C in aerobic Condition. Percent of Phosphorus released by *Plectonema boryanum* at different time intervals. The percent amount released was compared with the original phosphorus in media (1.76mgP/liter).

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**77.35%**	**79.61%**	**87.81%**
6	**77.37%**	**79.73%**	**88.90%**
8	**78.55%**	**79.77%**	**96.41%**
Average	**77.76%**	**79.70%**	**91.04%**

Figure 15 A, and B. Shows the percentage of phosphorus released by *P. boryanum* with addition of EDTA in comparison with no EDTA added (Control model vs. observed EDTA data). The figure also shows the comparison between time (days) and the pH-values in which the phosphorus is released.

Figure 15A. Control model vs. observed EDTA data

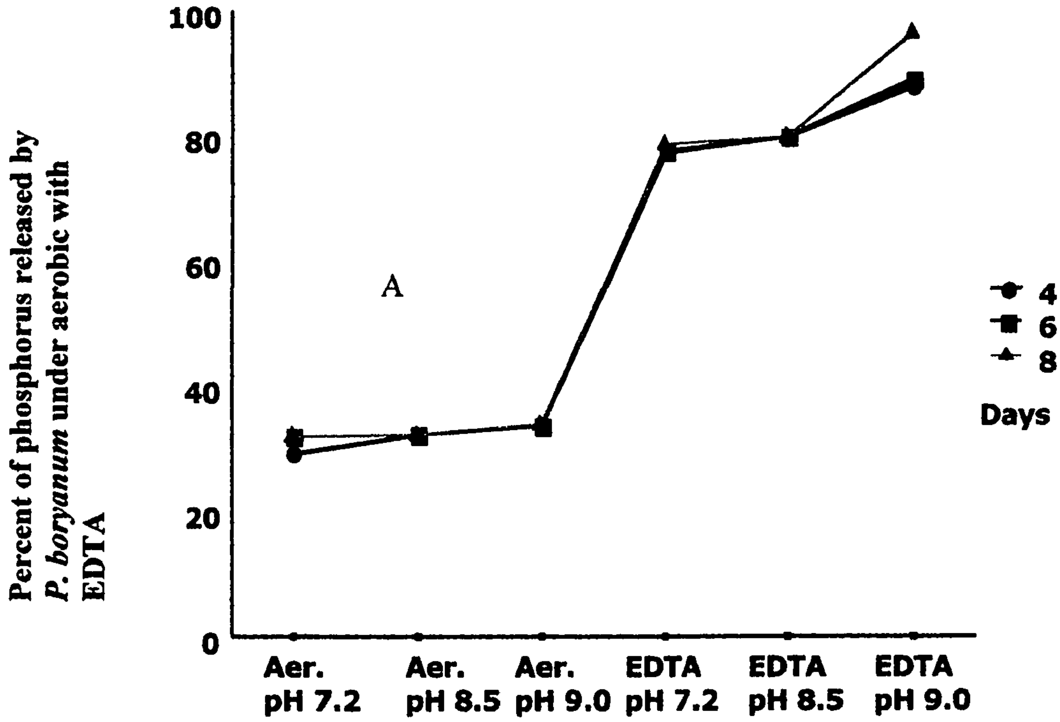
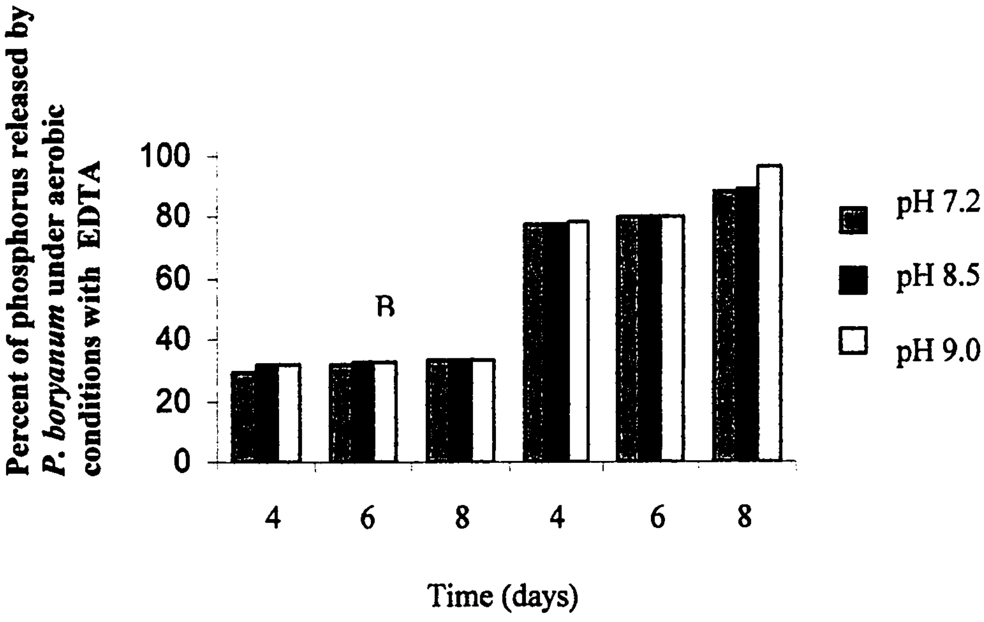


Figure 15B. Control model vs. observed EDTA data



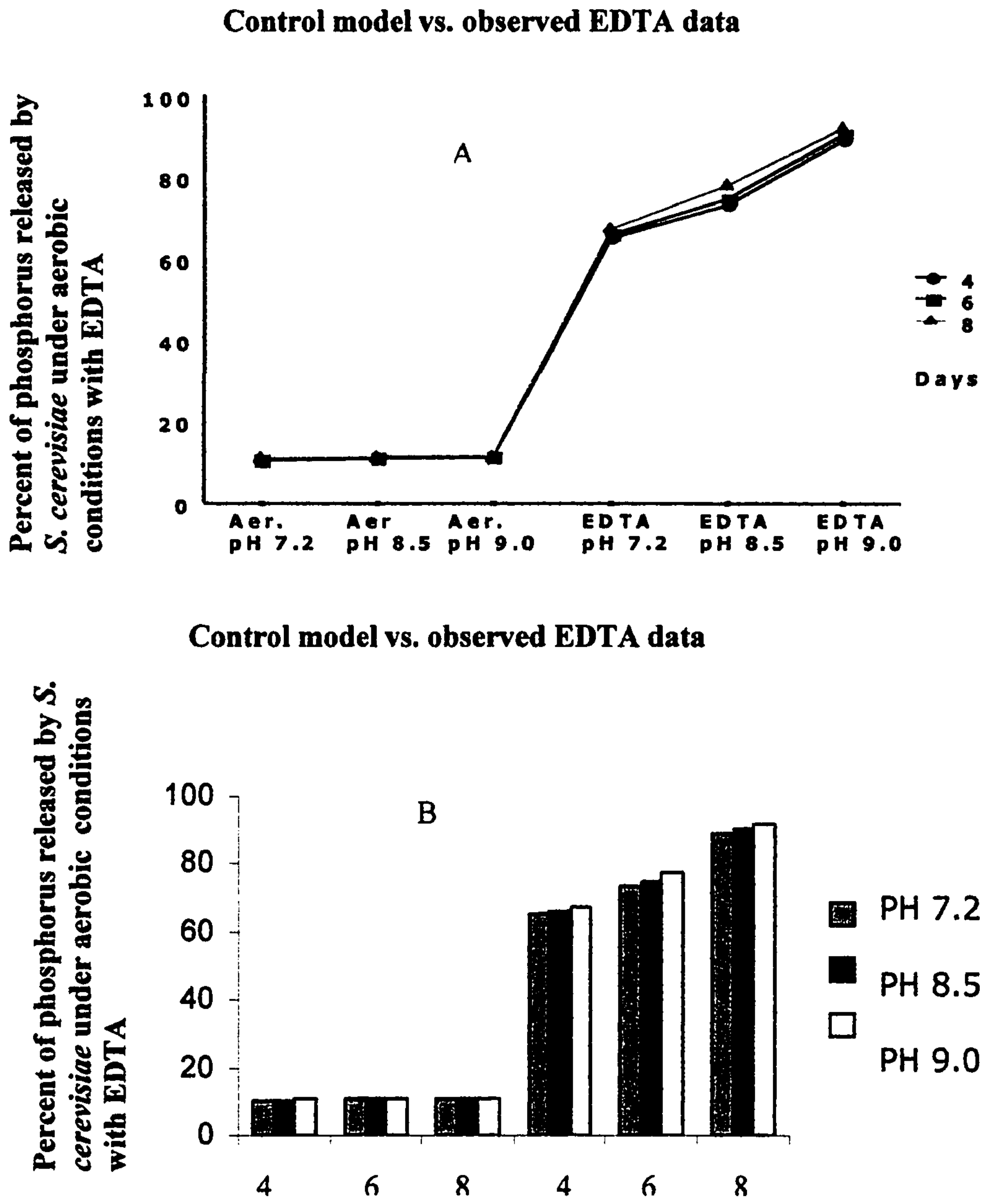
Release of P by *Saccharomyces cerevisiae* with addition of 0.25M EDTA.

Addition of 0.25M EDTA into the media of *Saccharomyces cerevisiae* increased the release of phosphorus. At high pH (pH 9.0) the cells released an average of 90.35% P (Table 16) than without EDTA (Table 10). It can be concluded that EDTA, influences the release of phosphorus by *S. cerevisiae.*

Figure 14 shows the difference of released of phosphorus from cultures treated with 0.25M EDTA and the control (Table 10). As can be seen the release of P is less in the aerobic state (control) both at shorter or longer time periods (days). The release of P was greater when EDTA was added: in 4, 6, and 8 days at pH 7.2 were 65.00%, 65.79%, and 67.13%. At pH 8.5 was 73.17%, 74.55%, and 77.85%. At pH 9.0 was 88.91%, 90.23%, and 91.91%. The experiment was carried out at temperatures of 25°C.

Table 16. Addition of 0.25M EDTA at a temperature of 25°C aerobic Condition. Caused *Saccharomyces cerevisiae* to release Phosphorus at different time interval (time dependency test-days) and with their different pH levels.

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**65.00**	**73.17%**	**88.91%**
6	**65.79%**	**74.55%**	**90.23%**
8	**67.13%**	**77.85%**	**91.91%**
Average	**65.97%**	**75.19%**	**90.35%**


Control model vs. observed EDTA data

Control model vs. observed EDTA data

Figure 16 A and B. Difference between the amount of phosphorus released by *S. cerevisiae* during the aerobic (control) conditions and the experimental conditions when 0.25M EDTA was added to the media. This proves that EDTA is capable of inducing *S. cerevisiae* to release phosphorus in aerobic stage, coupled with pH variation and time differences.

Figure 17. Electron micrograph of *S. leopoliensis* exposed 0.25M EDTA showing partial loss of PPBs to the supernatant at a pH of 7.2 for 4 days. The dense bodies are polyphosphate bodies (arrow).

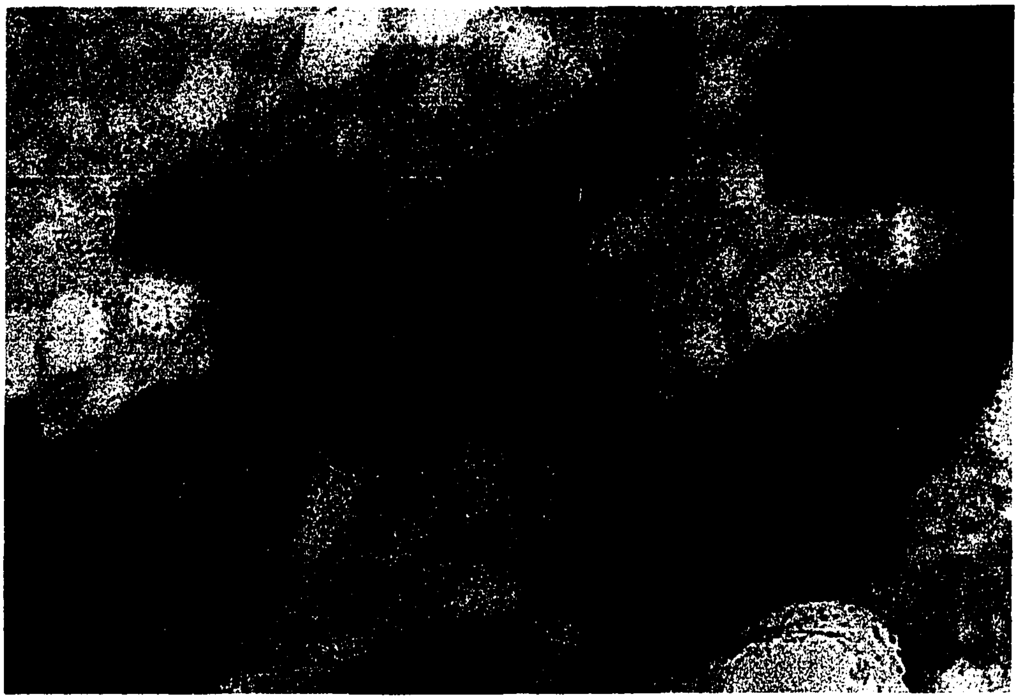


Figure 18. Electron micrograph showed partial absence of PPB in *S. leopoliensis* when 0.25M EDTA was added in the medium in the aerobic conditions for 6 days a pH of 9.0. X 22000. White spots indicate when cells were loaded with numerous bodies in the overplus conditions. The dense bodies in the cell are polyphosphate bodies (arrows).

Release of phosphorus by *Rhodotorula rubra* with addition of 0.25M EDTA to media at different pHs and time intervals (days) at 25°C

Results showed that at a temperature of 25°C and addition of 0.25M EDTA to media of different pH ranges, EDTA influenced the release of phosphorus by *Rhodotorula rubra* (Table 17). The amount of phosphorus release depended also on the level of pH and the number of days the EDTA remained in the media. For pH 7.2, average of phosphorus release was 66.17%, for pH 8.5, 75.05% and for pH 9.0 it was 86.51%.

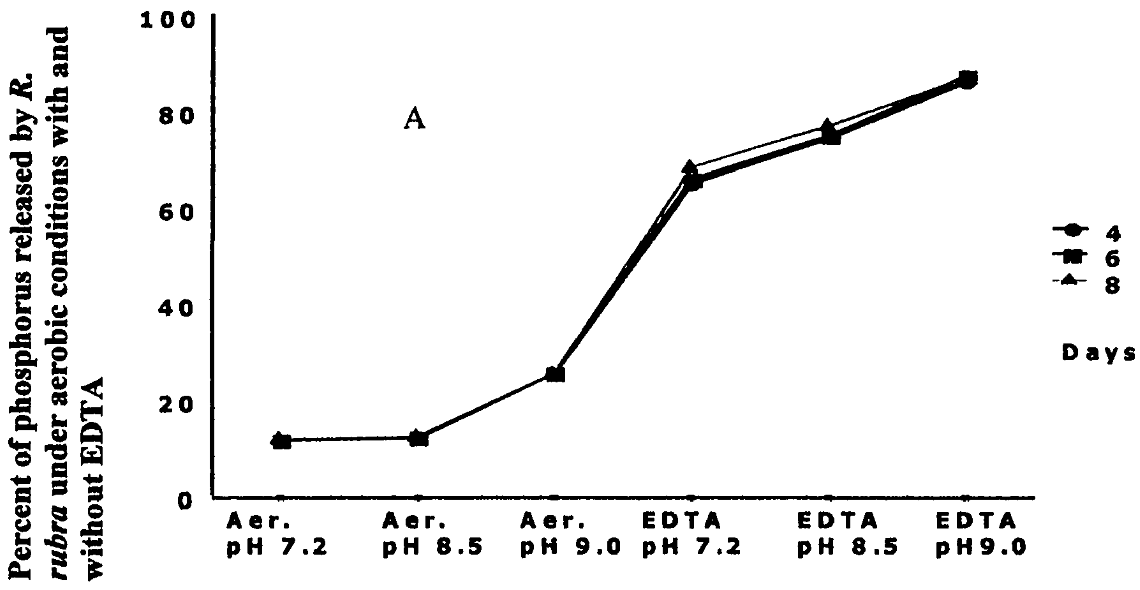
Rhodotorula rubra did not release as much phosphorus as *Plectonema boryanum*, which was 91.04% average (Table 15), or *Synechococcus leopoliensis*, which was 88.71% (Table 14), and *Saccharomyces cerevisiae*, which was 90.35% (table 16).

Figure 19 shows the release of phosphorus when 0.25M EDTA was added to the media containing the cells of *R. rubra* at 25°C against the control of the same growth condition (Table 12). The results confirmed that with addition of EDTA more phosphorus is released. Also at higher pH ranges more phosphorus was released.

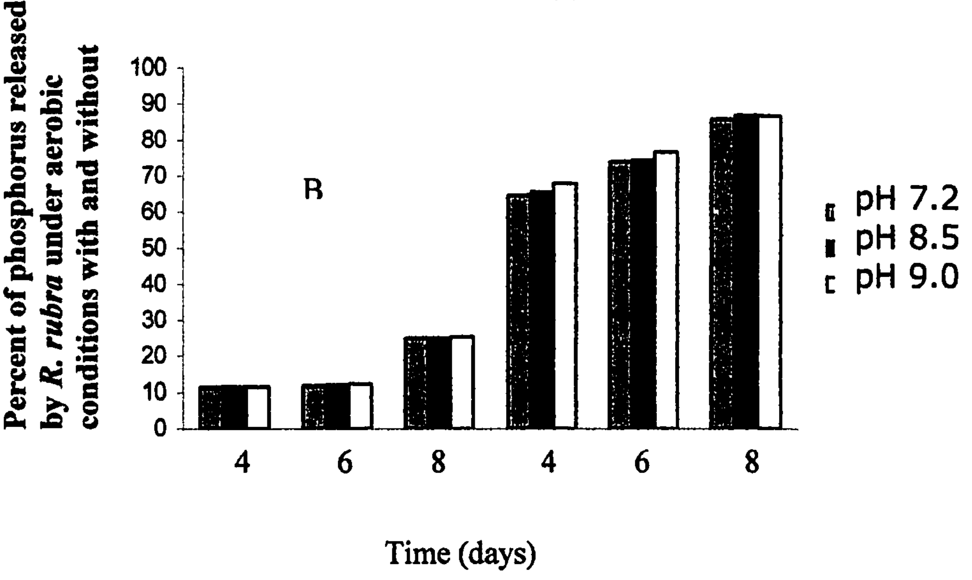
Though, there is a difference in phosphorus release with pH differences in the aerobic condition without EDTA added, the release was lesser than when the EDTA was added.

Table 17. Release of phosphorus by *Rhodotorula* rubra at different time interval when 0.25 M EDTA was added, at a temperature of 25°C. (Aerobic Condition).

Days	**pH**		
	7.2	**8.5**	**9.0**
4	**64.81%**	**74.08%**	**85.94%**
6	**65.69%**	**74.45%**	**86.86%**
8	**68.02%**	**76.61%**	**86.73%**
Average	**66.17%**	**75.05%**	**86.51%**



Control model vs. observed EDTA data



Aerobic versus EDTA

Figure 19 A and B. Show the percentage of phosphorus released by *R. rubra* with addition of 0.25M EDTA (experimental) and without EDTA (control) in their aerobic phases at a temperature of 25°C

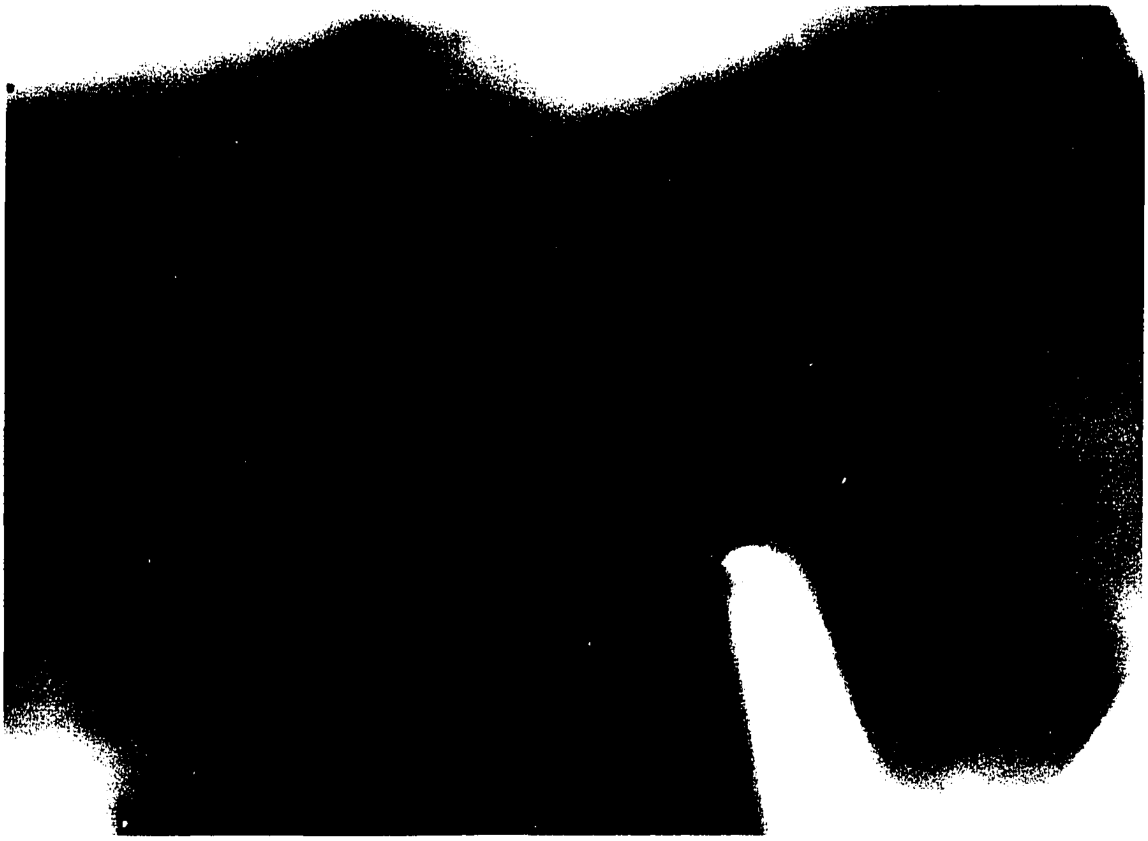


Figure 20. Electron micrograph of a section of a control *Synechococcus leopoliensis* cell grown at pH 7.2. Note the large polyphosphate bodies (PPBs). X 26,000. The dense bodies in the cell are polyphosphate bodies (arrow)

Figure 21. Electron micrograph of a section of control cells of *Synechococcus leopoliensis* cell grown at pH 7.2. Note the diminished polyphosphate bodies (PPBs) shown by arrow. X 26,000.

DISCUSSION

Effect of light on the uptake of phosphorus by the microbes

Light has been shown to stimulate the uptake of phosphorus by many microbes (Talpasayi, 1962, Kanai and Simonis, 1968, and Fitzgerald, 1970). Uptake of phosphorus in the dark environment has also been reported (Kanai and Miyachi, 1963, Batterton and Van Baalen, 1968, and Overbeck, 1962). In my experiments, all the four microbes chosen, appear to be organisms, which require light for maximal uptake of phosphorus (Figure 4). After the depletion of the phosphorus the number of PPBs in the cells was very low compared to the control cultures. When phosphorus was added to the medium and the culture was placed under the same environmental conditions (normal growth), uptake was rapid and numerous bodies were observed (Figure 4). The total increase in cell phosphorus was found to be directly proportional to light intensity. High light intensity (2000 and 4000 ft-candles) and availability of phosphorus in the medium results in high cell phosphorus levels (no data shown). When cells were inoculated in fresh medium, placed in the dark environment and examined using Hitachi H-7000 electron microscope, the uptake of phosphorus was found to be greatly reduced (no data shown).

The intensity of light on the uptake of phosphorus suggests several possibilities: (1) Energy is required for phosphorus uptake to occur. The process of photosynthesis, respiration, or a combination of both processes can supply this energy. (2) Metabolic processes and the products of photosynthesis occur: the inhibition of metabolic pathways would result in either complete or partial inhibition of the uptake of phosphorus. (3) Metabolism of storage materials in the cells through respiration. In this situation, one would expect to see an increased uptake with increasing light intensity and also phosphorus uptake in the dark with the metabolism of storage materials.

According to Blum (1966), and other scientists, such Jensen (1968), phosphorus is an energy source used by most microorganisms for survival. Blum (1966) demonstrated that phosphorus uptake by *Euglena gracilis* was inhibited by 2,4-dinitrophenol. Borst-Pauwels and Jagger (1969) found that uptake of phosphorus in *Saccharomyces cerevisiae* was inhibited by 0.1mM 2,4-dinitrophenol and 20 mM fluoride. Jungnickel (1970) found that 2,4-dinitrophenol only reduced the inhibition of the uptake of phosphate in *Candida utilis* and did not inhibit it completely. In my experiments I found that a light source would induce the uptake of phosphorus. In the anaerobic phase, phosphorus uptake was inhibited and the release of phosphorus was enhanced during the anaerobic phase. Furthermore, when EDTA was added, it inhibited the uptake of phosphorus and caused the release of the phosphorus from the polyphosphate bodies in *Synechococcus leopoliensis*, *Plectonema boryanum*, *Saccharomyces cerevisiae*, and *Rhodotorulla rubra*. These granules are the energy source for the cells (Jensen, 1968, Jensen, 1969). The results of this investigation are in agreement with the work of Jensen.

Starved and Overplus Phenomenon

Figure 1 demonstrates the changes that occur when cells are grown in phosphorus-free medium and subsequently inoculated into phosphorus-containing medium. Cells starved of phosphorus for a period of five days generally show a decrease in PPBs of the cells. The reduction in PPBs is due to the fact that the cells use the stored phosphorus. Beyond the five days, cells are likely to die of starvation, as phosphorus indicating the depletion of the phosphorus, energy for the microbe. Examination of the cultures reveals an increase in cell death during the starvation period. Cells that appear normal under the electron microscope often have small polyphosphate bodies and these polyphosphate are scattered far apart from each other. It does seem that the dead cells release phosphorus back to the medium, and the surviving cells are able to use this phosphorus for growth even at this low phosphorus levels.

Electron Microscopy Study

Cells of *S. leopoliensis, P. boryanum, S. cereviasae,* and *R. rubra,* grown in media containing the same amount of phosphorus (1.76mg/L) in aerobic and anaerobic conditions at different times (days) and pH ranges with addition of EDTA, release their phosphorus from their polyphosphate bodies into the supernatant in different concentrations (%) (Tables 4, 7, 10, and 12), (5, 8, 11, and 13), and 14, 15, 16, and 17).

Effect of high pH levels on the release of phosphorus by *S. leopoliensis, P. boryanum, S. cereviasae, and R. rubra*

The results of this study demonstrate that at low pH ranges, there is no effect on the release of phosphorus. Time (days) did not play any significant role in the release of phosphorus in the aerobic phase and during overplus condition. The rate of phosphorus released by *S. leopoliensis* in 4, 6, and 8 days at pH 7.2 was 25.29%, at pH 8.5 was 30.49 %, and at pH 9.0 33.40%; by *P. boryanum* at pH 7.2 was 31.08%, at pH 8.5 was 32.23%, and at pH 9.0 was 33.66%, and *S. cereviasae* at pH 7.2 was 10.41%, at pH 8.5 was 10.87%, and at pH 9.0 was 11.08%; and by *R. rubra* at pH 7.2, was 11.56%, at pH 8.5 was 12.07%, and at pH 9.0 was 25.22%. These results indicate that the microbes will not release significant amount of phosphorus whether in long time periods or high pH ranges, (Tables 4, 7, 10, and 12).

The effect of pH on microorganisms can obviously be demonstrated by the fact that at high pH levels it inhibits uptake of the phosphorus and degrades the polyphosphate bodies accumulated in the cells. Some laboratory experiments using wastewater to evaluate the effect of pH ranges have been reported to follow this trend (Groenestijn and Deinema 1985). As shown in the tables and graphs described in the results confirm the effect of pH on phosphorus release from PPB is confirmed. Below a pH of 7.2 a decline in phosphorus release is noticed, (no data shown) while above this range the release of phosphorus is higher.

Tracy and Flammino (1985), studying the effect of pH on the specific phosphorus uptake and release in the aerobic phase, also confirmed that release of phosphorus by the cells into the media varies with time (0-180 minutes in anaerobic/oxic (A/O) zone, termed anaerobic/aerobic). Tracy and Flamino (1985) found that total phosphorus removal in the modified Bardenpho process was improved from 42% to 92% as the pH was increased from 5 to 8. My results and their results suggest that the efficiency of biological phosphorus removal may decline significantly with a pH below 7.2 and increase with an increase in pH up to 9.0.

The release of phosphorus by *S. leopoliensis, P. boryanum, S. cereviasae, and R. rubra* under anaerobic condition

The success of the biological removal process is dependent on the exposure of the cells to anaerobic conditions, that is, both oxygen and nitrates must be absent. Under these conditions, the microbes in the solution release phosphorus. Wentzel *et al.* (1985) have shown this to be an important phenomenon, as they were able to demonstrate that the magnitude of biological excess phosphorus uptake was strongly linked to the magnitude of phosphorus release in the anaerobic zone. It is vital to the success of biological phosphorus removal that readily biodegradable substances be present in this zone for uptake by phosphorus-accumulating bacteria, of which *Acintobacter spp.* is usually the dominant species. In this research *S. leopoliensis, P. boryanum, S. cereviasae, and R. rubra* were successfully used and the release of phosphorus was greatly achieved.

In Tables 5, 8, 11, and 13, and pH ranges of 7.2, 8.5, and 9.0 the measured release of phosphorus in the anaerobic stage is summarized. The measured average release of phosphorus (54.14%, 76.50%, and 86.85% by *S. leopoliensis,* 72.27%, 83.02%, and 95.24% by *P boryanum,* 58.19%, 71.72%, and 93.47% by *S. cerevisiae,* 38.97%, 57.83%, and 79.76% by *R. rubra*) shows that anaerobiosis plays a role in phosphorus release. These microbes have a route to degrade PPB and release it as phosphorus into the media. Apparently, it appears that it is essential that oxygen not be present for phosphorus release from PPB (aerobically) by these microbes.

Dissolved Oxygen (DO) concentration in phosphorus release.

No specific studies have been reported addressing the effect of the DO concentration on biological phosphorus release from PPB of the microbes used in this research. The biological phosphorus removal mechanism suggests that the DO concentration may affect phosphorus release in the aerobic phase (Tables 4, 7, 10, and 12). The higher the DO concentration in the medium, the lower the release of phosphorus from the PPB. Ekama, and Marais, (1983) state that biological phosphorus removal will be adversely affected in biological phosphorus removal (BPR) systems unless the DO concentration is reduced or eliminated. Therefore high phosphorus release from PPB will be in the anaerobic phase.

Anaerobic phase of phosphorus release from PPB of the chosen microbes

Phosphorus release is enhanced in the anaerobic stage. Many experiments have been carried out to confirm that phosphorus can be released in greater quantities when cells are subjected in anaerobic state. Full scale-scale plant investigations at Palmetto, Florida, found that increasing the anaerobic detention time from 1.1 to 2.6 hours increased total phosphorus removal from 50% to 71%, (Stensel 1980). In a Bardenpho pilot-plant study, McLaren and Wood (1976) found that the effluent soluble phosphorus concentration in the medium increased from 1mg/L to 3mg/L after time was doubled from 2 hours to 4 hours. My experimental results are in agreement with these findings. As time (days) increased from 4 to 8 in the anaerobic phase, the amount of phosphorus release increased to nearly a hundred fold.

During the investigations using the A/O (anaerobic/aerobic) phases the nitrogen gas was periodically turned off after 45 minutes and this improved phosphorus release by the microbes. This technique of turning off the nitrogen gas was also reported by Vinconneau *et al.* (1985) in France where anaerobic conditions of wastewater facilities were turned off to increase phosphorus-releasing efficiency. The necessity for and success of longer anaerobic condition may vary depending on the strength and nature of the medium.

Another important aspect of anaerobic design and performance is to totally eliminate the entry of oxygen in the medium. Any DO present in the medium will deplete readily available substrate and will thus reduce the amount of fatty acids that will be produced for biological phosphorus removal (BPR). As can be seen in my experimental setup and results obtained, DO was totally eliminated. Compared with the control condition where DO was present, and the release of phosphorus was poor. This was also suspected, in combination with weak wastewater, of causing poor phosphorus removal and filamentous sludge growth during a portion of the operating period of a U.S. Modified Bardenpho system. Stensel (1985) found that large amount of DO input in the anaerobic zone produced poor phosphorus release. In this experiment, it was necessary to make sure that the anaerobic medium is void of DO, testing periodically with Resazurin was conducted as previously described.

The effect of EDTA on phosphorus release by *S. leopoliensis, P. boryanum, S. cereviasae, and R. rubra*

The "clean up" soils contaminated with heavy metals (HMs) in drinking water systems are one of the most difficult tasks for environmental engineering. A number of techniques have been developed that aim to remove HMs from contaminated soils and wastewaters, including *ex-situ* washing with physical-chemical methods (Anderson, 1993) and *in-situ* phytoextraction (McGrath, 1998, Salt *et al.*, 1998). In the *ex-situ* washing methods, chelating agents or acids are used to enhance biological phosphorus removal. Ethylendiamintetraacetic acid (EDTA) is the most commonly used chelate because of its strong chelating ability for different HMs (Norvell, 1991), and wastewaters. Laboratory studies have shown that EDTA is effective in removing Pb, Zn, Cu, Cd, and P from contaminated soils and wastewater systems. Removal efficiency depends on the strength of the EDTA and pH levels (Elliot and Brown, 1989, Brown and Elliot, 1992, Pichtel and Pichtel, 1997, Elliot and Shastri, 1999, Heil *et al.*, 1999, Papassiopi *et al.*, 1999).

By using EDTA a large amount of these contaminants were released with a mean value of 94%. This percent release corresponds to a higher pH range (9.0) and the strength of EDTA (0.2M/L), (Sun *et al.*, 2000).

Many microbes are capable of releasing large amounts of phosphorus with EDTA added. One such microbes is *Acinetobacter*, which is used extensively in enhanced biological removal systems (Mino, 1999). In this investigation, four different microbes are used. Such microbes are: *S. leopoliensis, P. boryanum, S. cerevisiae, and R. rubra.* Tables 14, 15, 16, and 17 show the amount of phosphorus released by the above-mentioned microbes. The amount of phosphorus was determined prior to additions of the EDTA (Tables 4, 7, 10, and 12). At low time (4 days) and low pH value (7.2) a low amount of phosphorus is released. Long time (8 days), and pH of 9, phosphorus released is higher than 4 days and pH of 7.2, 25.29% and 33.40%, 31.08% and 33.66%, 10.41% and 11.08%, and 11.56% and 25.22% in the average by these microbes. When the EDTA is added, the amount of phosphorus released is high in the same time and pH values. This is in agreement with some experiments performed by Shapiro *et al.* (1967), which demonstrates the percent of phosphorus removed from solution when 0.1M HCl was added. The removal rate was 35% at pH 5 and 65% at pH 7 in 60 minutes.

This study demonstrates that phosphorus release from polyphosphate bodies of *S. leopoliensis, P. boryanum, S. cerevisiae, and R. rubra* depends on pH variations, anaerobic-aerobic conditions, and EDTA. Under the right phosphorus concentrations, the cells accumulate high PPBs in the aerobic stage. This finding corresponds to that of Temmink *et al.* (1996) in anaerobic-aerobic batch experiments using acetate as substrate for polyhydroxyalkonoates (PHB) storage. The overplus phenomenon reveals a higher phosphorus uptake than in the normal growth conditions in this study. It implies that the microbes show dissimilar phosphorus uptake potential in different situations. Establishing the relationship between the microbes and their phosphorus uptake and release for different processes is critical for evaluating phosphorus release.

The experimental results suggest that anaerobic conditions, pH variations, EDTA and time influence phosphorus-release behavior. Results presented here demonstrate that low pH and short time (days) lead to low phosphorus release. In addition, experimental results also show that high pH levels in anaerobic condition lead to high phosphorus release.

Finally, Tables 5, 8, 11, 13 illustrate the efficiency of phosphorus release in the anaerobic stage. Tables 14, 15, 16, and 17 illustrate the effects of EDTA on phosphorus release. As the tables (anaerobic, and EDTA) reveal, the required time for nearly complete phosphorus release is 8 days or even more. Based on the above results, it could be concluded that the anaerobic stage should be well controlled and under adequate levels and the EDTA be the right concentrations to release phosphorus in anaerobic-aerobic processes.

The need for phosphorus recovery

Historically, phosphorus has been inexpensive and seemingly inexhaustible. Its dramatic effect on soil fertility and hence agricultural production has ensured that its use has been liberal. Phosphorus addition to soils around the world has, for many years, far exceeded agronomic requirements with the agricultural community regarding phosphate fertilizers. With such a large amount of phosphate in the Earth's crust, why should this be a concern? Even making no allowance for global population increase, phosphate reserves are being rapidly depleted. The quality of commercial phosphate rock is declining inexorably and it is vital to recover the phosphorus in our water system, rivers, lakes and ponds.

Recovering phosphorus and returning it to arable or other productive land is of major importance in this work in the long run. Another reason for this is primarily to avoid environmental problems such as eutrophication which is caused by nutrients from wastewater otherwise ending up somewhere else or problems associated with mining, fertilizer production and in the long term limited amount of minable phosphate minerals in the natural world. The investigation shows, however, that there is strong reason to adopt this approach including the recovery of other nutrients as well, such as sulphur, nitrogen, and potassium, in addition to phosphorus.

The costs for recovering phosphorus and other nutrients will probably increase considerably compared to the current level regardless of which system are developed. The costs are, however, difficult to estimate, both regarding the measures required to achieve acceptable phosphorus, and regarding the development and introduction of preparation and phosphorus extraction systems. This study and the materials and methods used are economical and less costly and can be used in large-scale phosphorus removal systems.

APPENDICES

Appendix A

Composition of Culture medium-Modified Fitzgerald (Fitzgerald *et al.*, 1952)

	Mg/liter
$NaNO_3$	124
$K_2HPO_4.3H_2O$	13
$MgSO_4.7H_2O$	25
$CaCl_2.2H_2O$	36
$NaCO_3$	20
$NaSiO_3.9H2O$	58
Ferric Citrate	3
Citric Acid	3
Gaffron's minor element solution	0.04ml.

pH is adjusted to 7.2, 8.5, and 9.0 by adding 1N HCl or 1N NaOH.

Gaffron's solution:

	G/liter
H_3BO_3	3.10
$MnSO_4.4H_2O$	2.23
$ZnSO_4.7H_2O$	0.287
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.088
$CuSO_4.5H_2O$	0.125
$Co(NO_2)_2.6H_2O$	0.146
$Al_2(SO_4)_3KSO_4.24H_2O$	0.474
$NiSO_4(NH_4)SO_4.6H_2O$	0.198
$Cd(NO_3)_2.4H_2O$	0.154
$Cr(NO_3)_3.7H2O$	0.037
$V_2O_4(SO_4)_3.16H_2O$	0.035
$Na_2WO_4.2H_2O$	0.033
KBr	0.119

KI	0.083

Appendix B

Measurement of experimental samples.

A: High-Range Test (0-50 mg/L)

- Remove the color comparator the Long Path Viewing Adapter
- Rinse two viewing tubes with deionized water.
- Fill a viewing tube to the first (5-mL) line with deionized water. This is the blank.
- Place this tube in the top left opening of the color comparator.
- Rinse the plastic dropper several times with the sample to be tested.
- Fill the dropper to the first (0.5 mL) mark with the sample. Put this sample in the second viewing tube.
- Add deionized water to the first (5-mL) line on the second tube.
- Swirl to mix.
- Add the contents of one Phos Ver 3 Phosphate Reagent Powder Pillow to the second tube.
- Swirl to mix. Waite at least one minute for full colors to develop. If phosphate is present, a blue-violet color develops. Complete the test and read the result within five minutes of the addition of the powder.
- Place the second tube in the top right opening of the color comparator.
- Hold comparator up to a light source such as the sky, a window or a lamp. Look through the openings in front.
- Rotate the color disc until the color matches in the two openings.
- Read the mg/L phosphate in the scale window. ***Note***: Divide the mg/L phosphate value to obtain the mg/L phosphorus.

Appendix C

Anaerobic condition.

A: Connection of the dry Nitrogen Gas to the experimental samples.

- Flasks containing the different pH level media were fit with rubber stoppers
- 50 gauge syringes were inserted in the flasks through the rubber stoppers
- Rubber tubes were connected to the flasks and the nitrogen tank with a switch, which was used to turn the gas on and off.
- The gas was pumped in at 10 pounds per square inch (PSI) for 45 minutes a day.
- Each time period (4, 6, and 8) days, media were withdrew from the flasks, spun down, decanted and supernatant tested for release phosphorus of by the microbes as outlined in appendix B.

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