AN ABSTRACT OF THE THESIS OF

MICHAEL JOHN HARRISON (Name) for the MASTER OF SCIENCE (Degree)

in MICROBIOLOGY (Major) presented on May 4, 1970 (Date)

Title: BACTERIA-PHOSPHATE INTERACTIONS IN UPPER KLAMATH LAKE SEDIMENT

Abstract approved: Redacted for Privacy

Dr. Richard Morita

Interactions between sediment bacteria and available phosphate necessary for algal production in a eutrophic lake were studied. Twenty percent of the organisms isolated solubilized phosphate forms such as $CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$, $Mg_3(PO_4)_2$ and $Al_2(PO_4)_2$. Solubilization of phosphates occurred under conditions of aerobic growth. Upon quantitative analysis, some isolates were capable of solubilizing up to 87% of the inorganic phosphate. Solubilization was associated with carbohydrate metabolism and the production of organic acids, which function as chelating agents. Organic acids that solubilize phosphates include lactic, acetic, glycolic, pyruvic, citric, glyceric, glucuronic and gluconic acids. Carbohydrates added to sediment resulted in increased microbial activity and an increase in the level of soluble phosphate. Addition of precipitating ions (Ca^{++} and Fe^{+++}) reduced the level of soluble phosphate in the sediment, but

the soluble phosphate fraction could be increased by the stimulation of microbial activity.

A cyclic liberation of phosphate was associated with death during cryptic growth. Reversible liberation of phosphate was observed when facultative and aerobic organisms were shifted from aerobic to an anoxic environment. Phosphate solubilization, liberation of phosphate with cryptic growth, and the reversible uptake and liberation of phosphate by microorganisms, with changes in aeration, may be of significance in the exchange of phosphates between the water and the sediment in Upper Klamath Lake.

Bacteria-Phosphate Interactions in Upper Klamath Lake Sediments

by

Michael John Harrison

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1970

APPROVED:

Redacted for Privacy

Professor of Microbiology and Oceanography
in charge of major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 4, 1970

Typed by Mary Jo Stratton for Michael John Harrison

ACKNOWLEDGEMENTS

I wish to express my appreciation and gratitude to:

. . . . . Dr. Richard Y. Morita for his guidance, assistance and technical advice and for the necessary provisions that made this research possible

. . . . . Dr. Robert E. Pacha for his guidance, assistance and technical advice in the early stages of this research

. . . . . Federal Water Pollution Control Administration Grant no. 16010EBB for financial assistance

. . . . . faculty and staff for academic instruction

. . . . . my fellow students for helpful suggestions and assistance

. . . . . my wife and parents whose encouragement and help have enabled this thesis to become a reality

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

BACTERIA-PHOSPHATE INTERACTIONS IN UPPER KLAMATH LAKE SEDIMENTS

INTRODUCTION

Upper Klamath Lake, located in the Modoc-Oregon geomorph, has a drainage area of 3,810 square miles. The geography of the area includes the southeastern portion of the Cascade Mountain Range and several small mountains and plateaus. Within a large basin lies the lake at a mean altitude of 4,410 feet (45). The entire lake is located in Klamath County, Oregon just north of Klamath Falls. The lake functions as an irrigation reservoir and for the generation of electrical power at its outflow on the Link River. Some recreational use is made of the lake by the more than 49,000 inhabitants of the county.

Upper Klamath Lake is a naturally enriched lake which yields high nuisance blooms of blue-green algae. This phenomenon of eutrophication can be attributed to the waters flowing into the lake from rivers, streams, springs, agricultural drainage and another large lake, Agency Lake. No appreciable amounts of sewage or industrial wastes are added to the lake or its tributaries. Under preliminary investigation of these tributaries, it was found that there was no single source of nutrients in the drainage basin (23).

The Botany Department of Oregon State University began an intensive study of the nuisance blooms in 1957. The conclusions drawn were that the blooms were predominantly composed of

Aphanizomenon sp., and that no apparent control existed (27). Subsequently, in 1962, a committee of biologists concluded that (a) chemical treatment of the algae in the lake would be uneconomical and dangerous; (b) algal control by introduction of biological agents has no scientific basis; (c) due to magnitude, harvesting of the algae would be impossible; (d) opportunities for curtailing algal nutrients are limited; (e) control of nuisance blooms by artificial light reduction would be uneconomical (23). Due to progressive improvements in technology, conclusion (d) must not be eliminated as a possible solution to the problem. The present goal of the Upper Klamath Lake project is to examine the lake in an attempt to control nutrient levels.

From March 1965 to April 1966, several hydrological and chemical studies were undertaken by the Federal Water Pollution Control Administration (23). A hydrology study used 13 U.S. Geological Survey Stream Gauging Stations and flow monitoring equipment on 90 percent of the irrigation waters and 80 percent of the agricultural drainage water. A total of 445 water and sediment samples were collected for chemical assay.

In examining the contribution of various sources of water to the total inflow of the lake, the Williamson and Wood rivers contribute the majority of the water (64.2 percent). Streams (1.1 percent), canals (7.8 percent), agricultural drainage (5.1 percent), springs (17.4 percent) and precipitation (4.4 percent) represent the remaining inflow

The Wood River flows into Agency Lake, which subsequently flows into the Upper Klamath Lake through a marshy passage. Ground seepage from springs represents the other major contributor to the water budget. It must be pointed out that the contribution of the springs was estimated and may actually be higher.

The role of the springs, agricultural drainage and rivers in the study of the lake becomes important when the chemical data is examined. The contribution of the rivers to the chemical inflow is substantial due to the volume of water that is added. Although the springs contribute a small portion of the water when compared to the rivers, their chemical contribution is substantial with 24.9 percent of the total phosphorus in the lake coming from the springs. Levels of phosphorus (26.5 percent), nitrogen (20.1 percent) and sulfate (21.5 percent) are high in agricultural drainage due to fertilizers. With its inflow to the water budget of only 5.1 percent, the chemical contribution of these constituents from the agricultural drainage represents one-fifth of the total supply to the lake.

By combining flow rates and chemical data, a chemical budget for the lake was established. During the year from March 1965 to April 1966, calcium (55 tons), iron (304 tons), nitrogen (1,860 tons), phosphorus (204 tons), silica (12,927 tons) and sulfate (109 tons) were retained by the lake. The net yearly gain of phosphorus and nitrogen in the lake is significant. Some of the nitrogen is undoubtedly lost due

to denitrification but the phosphate is retained. The phosphorus, as phosphate, may be bound to the sediment particles, exist as insoluble precipitates, or be complexed in benthic life. The high silica retention by the lake can be attributed to inorganic precipitation or absorption and to the diatoms, particularly Asterionella formosa and Melospira sp. (28).

Miller and Tash (23) concluded that if the flow rates remained constant and the water quality does not change, the supply of nitrogen, silica and phosphorus in the upper one inch of sediment is as great as that quantity of nutrients which would flow into the lake in the next 60 years. Metals which are retained in the lake are calcium, aluminum and iron. All three of these elements are capable of precipitating phosphates from the water. The phosphate may also be held as an anion complexed with sediment particles. An interchange of nutrients would support massive algal blooms for prolonged periods of time. This would be especially true if the nutrients in the sediment and the water were in equilibrium. The role played by microorganisms in the sediment with regards to the phosphate picture in a eutrophic lake was undertaken in order to assess their importance.

LITERATURE REVIEW

One of the definitions for eutrophication is the production of organic matter exceeding that which can be oxidized by respiration, predation, and bacterial decomposition (44). For a lake to become eutrophic, nutrients must be present in unlimited amounts (40). Since the process of nutrient addition establishes the rate of eutrophication, which in turn reflects changes in the water quality, it is necessary to derive information related to nutrient turnover.

Solubilization, and uptake or release of phosphates due to microorganisms was investigated. Solubilization of phosphates by microorganisms was first studied by Sackett, Patten and Brown (34) in 1908. They set rock phosphates, bone meal, $Ca_3(PO_4)_2$, $Ca_2(HPO_4)_2$, and $CaCO_3$ in agar and found that certain organisms produced clearing zones around colonies growing on nutrient agar plus 2 percent glucose. Solubilization was found to be influenced by the nature of the carbon and nitrogen sources (16).

Waksman (42) postulated that phosphate solubilization in soil could occur in three ways: 1) direct metabolism perhaps through the formation of some enzyme or interaction with some synthetic substances; 2) by the action of carbon dioxide and organic acids produced by bacteria; and 3) by the action of inorganic acids formed in the metabolism of autotrophic nitrifying and sulfur oxidizing bacteria. In 1948, Gerretson (7) showed that bacteria capable of solubilizing

insoluble phosphate forms influenced phosphate uptake by plants.

Numerous investigations into the effects of phosphate dissolving bacteria in the rhizosphere of plants were conducted (6, 7, 16, 17, 25, 34, 38, 39). Katznelson and Bose (16) found that most bacterial isolates from wheat roots were capable of solubilizing phosphates under aerobic conditions using glucose and alanine as substrates. Solution of apatite by microorganisms from the rhizosphere was due to the production of organic acids yet there was no correlation between the degree of solubilization and pH (39). Molds, isolated from seeds, were also capable of solubilizing phosphates (33). Working with submerged soils used for the cultivation of rice, Raghu and MacRae (32) were able to show the existence of phosphate solubilizers under aerobic and anaerobic conditions. Most isolates were recovered under aerobic conditions and the anaerobes were primarily fungi.

Mehltretter, Alexander and Rist (22) studied the effects of various organic acids on the sequestration of metallic phosphates. Duff, Webley and Scott (6), Louw and Webley (21), and Katznelson, Peterson and Rouatt (17) all indicated that compounds such as 2-ketogluconic acid, a fairly common product of glucose metabolism by aerobic bacteria, were capable of solubilizing numerous forms of calcium phosphate. 2-ketogluconic acid is an effective chelator of calcium and exhibits some action on magnesium.

Other organic acids that solubilize phosphates were studied by

Sperber (39). Citric, lactic, glycolic, succinic, gluconic, and oxalic acids all produced solubilization of calcium phosphates. Johnston (15) discovered that some of these acids, as well as malic, tartaric, and α hydroxymonobasic acids solubilized iron and aluminum phosphates. Johnston further indicated that the structure of the organic acid affected the degree of solubilization more than changes in pH (14). If aliphatic acids were α or β substituted with a hydroxyl moiety, solubilization was increased.

Organic acids have been shown to exist in soils (43). Under waterlogged or submerged conditions, or during conditions of temporary anaerobiosis in normally aerated soils, organic acids, particularly lactic, may be produced (39). Batelle-Northwest (3), an independent research organization, has studied the content and forms of phosphate in Upper Klamath Lake sediment. Their data indicates that the phosphate exists in calcium, aluminum and ferric forms in the lake.

Since the lake shifts between aerobic and anaerobic conditions at periods during the year and there is mixing in the upper layers of the sediment due to wind action, this could influence the phosphate levels in the water. Shapiro (35) demonstrated that cultures of bacteria and mixed microorganisms are capable of releasing a large proportion of their phosphorus to the medium in a matter of hours when kept under anoxic conditions. The reaction is reversible if an aerobic environment is re-established. The release and uptake occurs at the

same rate and is an uptake in excess of what the cell requires for growth.

In studying the phenomenon further, Levin and Shapiro (20) found that uptake was a biological process associated with oxidative phosphorylation since uptake was inhibited by 2,4-dinitrophenol. The uptake and release was also temperature dependent and increased levels of carbon promoted the amount of biological uptake of phosphate under aerobic conditions. This uptake of inorganic phosphate under aerobic conditions and release under anoxic conditions could occur in the water as well as the upper layers of the sediment.

Both solubilization and the uptake and release of phosphate can function in the exchange of inorganic phosphate between the water and the sediment. Pomeroy, Smith and Grant (30) have shown that a two-step ion exchange between the water and sediment, plus an exchange between interstitial microorganisms and water exists. The exchange of phosphate involves a combination of a sorptive process and a biologically controlled exchange. The physico-chemical exchange reaction is an initial surface sorption that occurs at a rapid rate, followed by incorporation into the clay lattice of the sediment. The biological process involves transport of inorganic phosphate across the cell membrane where it is held in a pool. This pool of phosphate may be released with mixing of the surface sediment.

These data were supported by Phillips (26) and Phillips and Hayes

(10), which demonstrated that sediment treated with antibiotics produced a greater loss of P^{32} from the sediment. At equilibrium, the level of P^{32} in water was greater when bacteria were active in the water and sediment. For a eutrophic lake, nutrients must exist in equilibrium since they are in excess (40). It is conceivable that the microorganisms in the sediment play a vital role in recycling of inorganic phosphates for use by the phytoplankton in a eutrophic lake.

MATERIALS AND METHODS

Formation of Phosphate Salts

All phosphate precipitates with the exception of $Al_2(PO_4)_2$ were prepared by combining ten percent aqueous solutions of various reagents. The precipitates were formed according to the following reactions:

$$K_2HPO_4 + CaCl_2 \longrightarrow CaHPO_4 + 2KCl$$

$$2K_3PO_4 + 3CaCl_2 \longrightarrow Ca_3(PO_4)_2 + 6KCl$$

$$2K_3PO_4 + 3MgCl_2 \longrightarrow Mg_3(PO_4)_2 + 6KCl$$

$$K_2HPO_4 + FeCl_3 \longrightarrow FePO_4 + 2KCl + HCl$$

$Al_2(PO_4)_2$ was prepared by washing reagent grade aluminum phosphate with distilled water ten times, followed by drying at 45 C for 24 hours.

Two types of precipitate were formed while preparing $CaHPO_4$. The type formed depended on the order of mixing of the two reagents. A gelatinous form was obtained when K_2HPO_4 was added to $CaCl_2$, whereas, a precipitate which readily dropped out of solution was formed when the two reactants were poured simultaneously into a common container. This latter form was more desirable and this simultaneous mixing was used to form all of the precipitates.

During the early attempts to isolate bacterial phosphate solubilizers, the precipitates were formed in the medium by first adding the phosphate reactant followed by the precipitating agent. This

method was discontinued because it resulted in pH fluctuations and high ion concentrations in the medium. By precipitating the phosphates first, followed by washing and drying, surplus ions were removed and addition of the phosphate precipitates as an aqueous slurry had no effect on the pH of the medium.

Once the precipitate was formed, it was filtered and washed ten times with distilled water. The precipitates were dried for 24 hours at 45 C, ground to a powder, and stored in an airtight vial until needed. The precipitates formed may not be as simple as those indicated by the stoichoimetric equations. However, the precipitates gave different phosphate forms which gave a range for testing solubilization.

Phosphate Assay Procedure

The assay procedure for orthophosphate, total soluble phosphate, and total phosphate was developed by Strickland and Parsons (41). The principle of the method involves treating a sample containing phosphate with a composite reagent containing molybdic acid, ascorbic acid, and trivalent antimony. The absorbance of the reduced heteropoly acid is measured spectrophotometrically at 885 nm. The range extends from 10 to 450 mg for 100 ml of solution, employing a one centimeter optical cell. The assay was run using a Beckman DU-2 Spectrophotometer.

The standard solution was prepared by adding 2.194 g of KH_2PO_4 (oven dried) to one liter of distilled water. This gave a concentration of 0.5 mg of phosphate expressed as phosphorus per ml. Appropriate dilutions of the stock standard were made to give a working standard. The range for a phosphate standard curve was 0.05 milligrams per liter to 0.5 milligrams per liter. With samples having high phosphate levels, dilutions were made to yield an assay concentration in this range.

Orthophosphate refers to the inorganic phosphate ion and may be one or combination of the forms present in the phosphoric acid equilibria. It is determined by filtering 100 ml of water sample (or an appropriate dilution of a more concentrated sample) through a Millipore filter (HA 0.45μ).

Total soluble phosphate includes filterable phosphates, both organic and inorganic forms. The sample was filtered in the same manner as the orthophosphate sample. A digestion process was incorporated to convert all the soluble phosphates to orthophosphate. Digestion was done by adding one ml of 6 M sulfuric acid and 0.4 g of potassium persulfate to 100 ml of the sample. The sample was brought to a boil and boiled vigorously for 30 minutes. A longer time was required for samples high in organic content. The sample was then cooled, filtered if necessary, and adjusted to pH 7.0 with dilute ammonium hydroxide. The sample volume was adjusted to 100

ml with distilled water and color development followed the same procedure as orthophosphate.

Total phosphate is all of the phosphate in the sample, both particulate and soluble. The sample is not filtered. Digestion and color development is the same as for total soluble phosphate.

Media for Cultivation of Organisms

For routine bacterial counts, the spread plate technique (5) was used employing Difco tryptone-glucose-yeast extract medium (TGY). The pH of all media in this study was adjusted to pH 7.0 unless otherwise stated. All media was autoclaved 20 minutes at 121 C. The TGY agar was stored in bottles until ready for use. A TGY broth was also prepared.

All microbial dilutions were made using sterile 0.2 percent peptone water at neutral pH in 99 ml or 9.0 ml quantities.

Once an organism was isolated, it was transferred to a culture maintenance agar (CMA) of the following composition: peptone (Difco), 2.5 g; beef extract (Difco), 1.5 g; yeast extract (Difco), 2.0 g; glucose, 1.0 g; K_2HPO_4, 1.0 g; agar (Difco), 15 g; and 1000 ml of tap water. The CMA was neutralized and sterilized like the TGY medium. For preservation of cultures, CMA agar slants were prepared. Organisms were inoculated, allowed to grow for 48 hours at 15 C and then placed at 4 C for storage.

Sediment extract and sediment extract agar were used for the phosphate liberation studies. The sediment extract was prepared according to the recommendations of Harris and Keeney (9). The sediment was collected, allowed to settle overnight, and the surface water was drawn off. The sediment was autoclaved for two hours at 121 C, cooled overnight, and the extract was collected by centrifugation. The sediment extract was then diluted to the desired concentration and sterilized for 20 minutes at 121 C. Sediment extract agar was prepared by adding 20 g of agar (Difco) to 1000 ml of 50 percent (v/v) sediment extract.

Initial isolation of phosphate solubilizing microorganisms was made on the medium of Katznelson et al. (17). This medium was composed of 50 percent sediment extract, 1 percent glucose (w/v), and 2 percent agar (Difco) (w/v). It was sterilized in 300 ml quantities at 121 C for 20 minutes, cooled to 50 C, and 15 ml of sterile K_2HPO_4 (10 percent w/v) and 30 ml of $CaCl_2$ (10 percent w/v) was added. Some problems with pH adjustment were encountered. The medium was modified by adding pre-precipitated phosphates from sterile washed slurries. The basic medium was a 50 percent sediment extract agar and the added carbohydrate source varied with the needs of the experiment. This medium was designated as phosphate agar.

Incubation Conditions

Organisms isolated from the lake were cultivated at 15 C. Some fluctuation in temperature did occur but it never exceeded 18 C or dropped below 14 C. Some of the studies conducted were done at room temperature. Organisms were stored at 4 C after visible growth was detected at 15 C. All of the isolates were capable of growing at 5 C, 15 C, and 20 C. Period of incubation varied with the experiment. For anaerobic conditions, incubation was done in an anaerobic jar which was purged with nitrogen gas. Anaerobic conditions were detected by using the methylene blue indicator (24).

Isolation of Phosphate Solubilizers

Original isolation of phosphate solubilizers used the medium and procedure of Katznelson et al. (17). Initial aerobic and anaerobic solubilization studies also utilized this procedure. Subsequent isolation and testing for solubilization was done using the phosphate agar containing 1 percent glucose and varying phosphate precipitates.

Sediment samples were collected, placed in five gallon jugs, and allowed to settle overnight; the water on top of the sediment was then drained off. Ten one ml samples of sediment were taken at various depths, composited, and duplicate serial dilutions were made. The dilutions were inoculated onto the phosphate agar using a spread plate technique.

Isolations were also conducted using various carbon sources and phosphate precipitates. In each case, the carbon source was added to the phosphate agar at 1 percent (w/v) concentration. Organisms that were previously isolated on Katznelson's medium or the phosphate agar were also tested for their ability to solubilize various phosphates using different carbon sources.

Identification Scheme

Identification of the organisms was conducted using the scheme of Shewan, Hobbs and Hodgkiss (36). The scheme is presented in Figure 1. Motility tests were made using the hanging drop technique of Skerman (37), the flagella staining method of Leifson (19) and by using motility agar deeps composed of beef extract (Difco), 3 g; peptone (Difco), 5 g; agar (Difco), 5 g; and 1000 ml of tap water. Degradation of carbohydrates was tested by using the oxidative-fermentative (O/F) medium of Hugh and Leifson (12). This O/F carbohydrate medium was prepared by adding sterile sugar solutions to the basal medium to give a concentration of one percent by volume. The tubes were inoculated in duplicate for each sugar. One set of tubes had a vaspar (50 percent Vaseline and 50 percent paraffin) seal to exclude the air. Pigment of the organism was tested by growth on TGY agar and Pseudomonas F agar (Difco). The Pseudomonas F agar stimulated the production of water soluble fluorescent pigments.

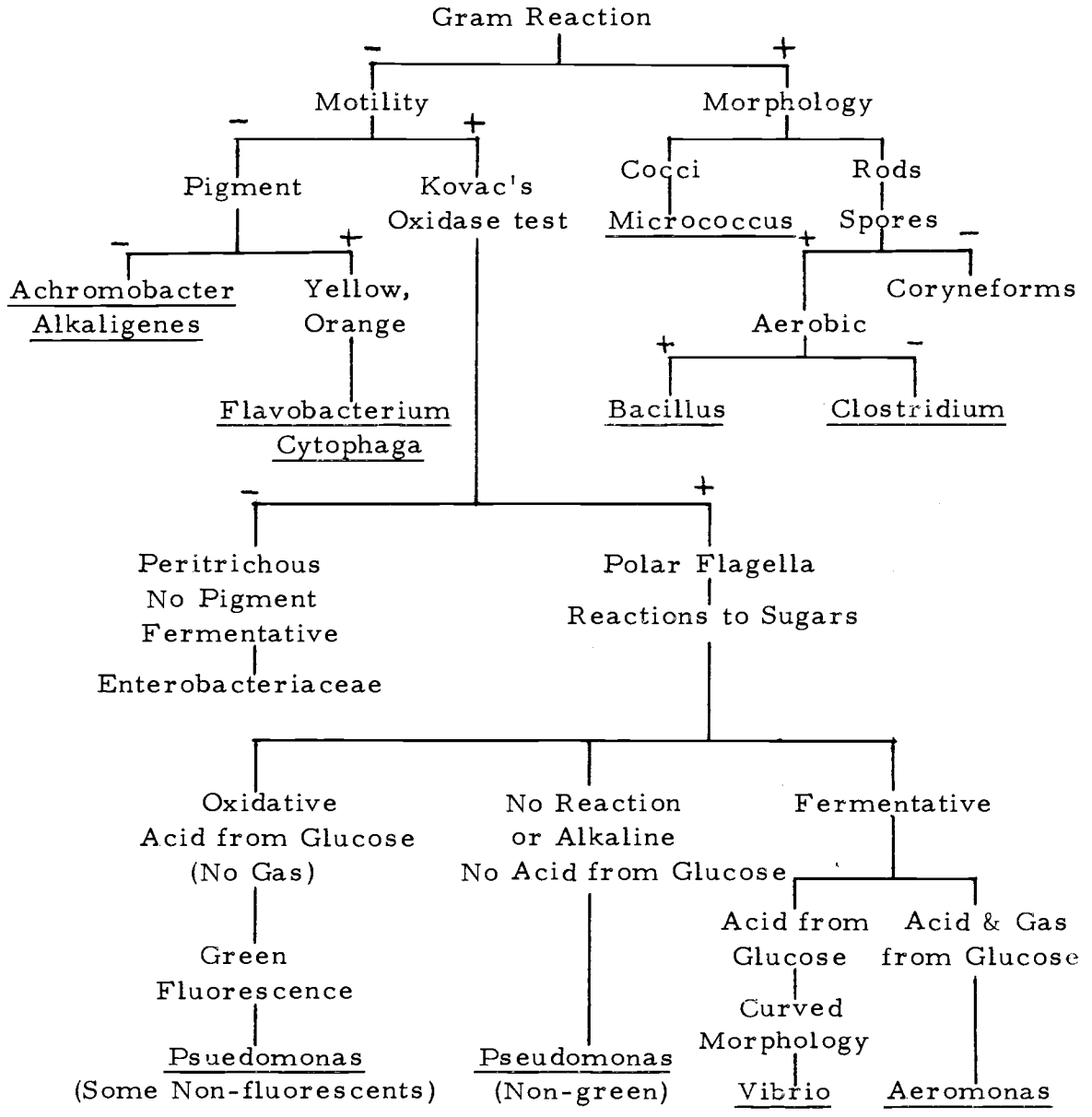

Figure 1. Identification scheme for isolates from Upper Klamath Lake sediments.

The Kovacs' oxidase test (18) was used to separate the Enterobacteriaceae from the Psuedomonadaceae. Colonies of organisms to be tested were removed from the agar using a sterile wire loop and smeared onto a strip of filter paper (Whatman No. 1) which was impregnated with one percent tetramethylparaphenylene diamine hydrochloride. The test is positive for the oxidase enzyme system if the smear of cells turns purple in five to ten minutes.

Quantitative Determination of Phosphate Solubilization

$CaHPO_4$ was dispensed in 100 mg quantities (the first experiment run used 200 mg of $CaHPO_4$ but this produced high levels of soluble phosphate in the control tubes) into screw cap tubes which were then heat sterilized at 110 C overnight. To these tubes, five ml of a previously sterilized liquid medium of the following composition was added: sediment extract 1:2 (v/v), 250 ml; yeast extract (Difco), 1.0 g; peptone (Difco), 1.0 g; glucose, 5.0 g; $CaCl_2$, 0.1 g; $FeCl_3$, 0.01 g; $MnCl_2$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $(NH_4)_2SO_4$, 0.5 g; and K_2HPO_4, 0.4 g.

Organisms were inoculated into this medium and incubated on a gyrotory shaker at 15 C for 14 days. The tubes were then centrifuged at 3000 x g for ten minutes and the supernatant was passed through a Millipore membrane filter (HA 0.45μ). Total soluble phosphate was then run on the filtrate.

Effect of Aeration on Available Phosphate

Since Upper Klamath Lake shifts from aerobic to an anaerobic environment, the effect of this shift on available phosphate was examined *in vitro*. A liquid medium composed of 800 ml of 25 percent sediment extract and 200 ml of TGY broth was placed in a two liter container having a sampling port (capped with a serum bottle cap) near the bottom. The medium was continually stirred by a water driven magnetic stirrer. The water functioned as a coolant, keeping the medium at a 15-19 C temperature range. Sterile air or N_2 was bubbled through the medium by using a fritted glass sparger. To maintain a detectable level of phosphate, ten mg/l of K_2HPO_4 was added to the system.

Organisms were inoculated under aerobic conditions as a mixed culture (sediment sample) or as a 48 hour pure culture. Growth to stationary phase was followed using optical density at 660 nm. The instrument was a Bausch and Lomb Spectronic 20.

Once stationary growth was obtained, the culture was shifted to anaerobic conditions by bubbling with sterile N_2. Samples were aseptically removed through the serum bottle cap. The samples were centrifuged at 3000 x g for ten minutes and filtered through Millipore filters (HA 0.45μ) to remove cell debris. Orthophosphate concentration was then determined on the samples. To test the reversibility of the system, air was again bubbled through the medium and assay for

orthophosphate was continued.

Phosphate Solubilization Due to Products of Microbial Growth

The ability of various end products and intermediates of carbohydrate metabolism to solubilize phosphate was tested. The assay procedure involved adding organic acids to penicillin assay cylinders that were placed on agar containing precipitated $CaHPO_4$. The concentration of the acids added was 0.056 M, which represents the conversion of one percent glucose to the acids. Acids tested for the ability to solubilize phosphate were citric, acetic, succinic, fumaric, malic, oxalacetic, formic, glycolic, lactic, glutamic, butyric, gluconic and hydrochloric acid which functioned as a control. The acid (approximately 0.5 ml) was added to the cylinder and incubated for 48 hours. Solubilization was indicated by a clearing zone.

Cultures of AH7, BH12 and Pseudomonas aeruginosa were tested for their ability to solubilize phosphate by virtue of their metabolic activity on glucose. The medium used was that of Rose (33). It was a defined medium composed of glucose, 10 g; asparagine, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; KCl, 0.5 g; and one ml of a 1:200 dilution of trace elements (1). The trace elements solution contained MgO, 10 g; $CaCO_3$, 2 g; $FeCl_3 \cdot 6H_2O$, 5.4 g; $ZnSO_4 \cdot 7H_2O$, 1.44 g; $MnSO_4 \cdot 4H_2O$, 1.11 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $CoSO_4 \cdot 7H_2O$, 0.28 g; H_3BO_3, 0.062 g; $Na_2MoO_4 \cdot 2H_2O$, 0.49 g;

concentrated HCl, 50 ml; and distilled water to make one liter of solution.

One liter of Rose's defined medium was placed into a two liter container of the same type used in the aeration studies. The medium was aerated and stirred in the same manner. Periodic samples were collected aseptically. A total viable count was run on each sample using the pour plate technique with TGY agar. Plates were counted after five days at 15 C. The sample drawn from the container was divided into two 50 ml fractions. Both fractions were filtered, one being assayed for total soluble phosphate and the other added aseptically to a bottle containing $CaHPO_4$. This bottle was then heated to 90 C for 30 minutes to inactivate any enzymes in the filtrate. The bottles were incubated for five days at room temperature. Total soluble phosphate was then run on the filtrate after incubation. The amount of phosphate solubilized was calculated. Sterility checks were conducted at the end of this five day incubation using TGY broth.

A major source of carbon in the lake is supplied from the algae. In order to correlate phosphate solubilization with the utilization of carbohydrates, it was necessary to identify carbohydrates associated with the algae. A mass of algae was collected and returned to the lab that same day. The water was removed from the algae by filtration using Whatman No. 1 paper. The collected algae had a wet weight of 10 g. The algae was resuspended into 30 ml of distilled water, cells

were broken by using a Biosonik III Sonicator, and the slurry was then frozen until it was chromatographed.

The chromatographic technique was that of Hough and Jones (11). The solvent for the descending paper chromatography was ethyl acetate:pyridine:water (10:4:3). The paper was Whatman No. 1 which was washed before use with distilled water. The numerous standards were one percent solutions of the carbohydrates. They were spotted onto the chromatogram using capillary tubes and dried by warm forced air.

The algal preparation was thawed, cell debris spun down at 3000 x g for 20 minutes, and the supernatant was spotted onto the chromatogram in the same manner as the standards.

The chromatograms were developed for ten hours in a chromatocab that was equilibrated with the solvent for 24 hours. The chromatograms were developed in the dark at 25 C. After ten hours, the chromatograms were removed and air dried under a hood. The spots were detected by using an ammoniacal silver nitrate reduction of the carbohydrates.

The dried chromatograms were dipped into a solution made from adding 0.1 ml of saturated $AgNO_3$ to 20 ml of acetone. Water was added dropwise to effect solution of the $AgNO_3$. The chromatograms were in contact with the reagent for four minutes. The paper chromatograms were then sprayed with 0.5 N NaOH in aqueous ethanol

This solution was prepared by diluting 40 percent NaOH (w/v) with ethanol. Once sprayed, the reaction was allowed to proceed for 10-15 minutes. The chromatograms were subsequently soaked for ten minutes in 6 N NH_4OH, washed for 30 minutes in tap water, dried, and the $R_{glucose}$ values were calculated.

Organic acids associated with the sediment, water and from pure cultures of AH7, CH6 and BH12 were also determined by paper chromatography. The organic acids identified were based on their R_f and R_{lactic} values. The procedure used was that of Buch, Montogomery and Porter (4).

This method gives a qualitative determination of organic acids present. The method of Buch et al. utilizes color and fluorescence reactions to separate acids having close R_f values. The method involves spotting known standards (0.1 percent solutions spotted with capillary tubes) and test solutions in multiples of five so that after the chromatogram was run, the paper could be cut into five different strips for the various reactions. Each reaction utilized a different spray reagent for development of the spot.

The solvent used in the system was 1-pentanol - 5 M aqueous formic acid which was mixed in equal volumes and shaken for three hours to mutually saturate both phases. The water phase was added to the bottom of the chromatocab and the paper chromatogram was hung in the chromatocab for three hours for equilibration. The organic

phase was then added to the trough and the chromatogram developed for 15 hours. The solvent front was marked and the paper was air dried for three hours before being cut into five segments. Chromatograms were run at room temperature and in the dark.

The defined medium of Rose (33) was used to cultivate the microorganisms. Two liters of medium was centrifuged at 3000 x g for 20 minutes to remove the cells of a five day culture. The supernatant was then evaporated to dryness on a steam table. For recovery of organic acids from water, the same procedure was used. The water tested was collected during the algal bloom and stored frozen until concentrated.

The sediment was also collected during the algal bloom and frozen. Five gallons of sediment was concentrated, at the rate of 300 ml a run, by lyophilization. The dry powder was kept refrigerated until needed. Recovery of organic acids from the dry sediment, water and bacterial supernatant, followed the procedure of Wang, Cheng and Tung (43).

Organic acids can exist free in soil or sediment, or in the form of esters and other compounds that yield organic acids on hydrolysis. Free organic acids, and those which might be adsorbed on soil colloid as anion, or be present as insoluble salts or in soil lipid are detected. The treatment used at pH 11 is strong enough to free but not hydrolyze most organic compounds (43).

Cryptic Growth

Cryptic growth was examined under two conditions. A study was run on sediment collected during late spring while a bloom was occurring, and cryptic growth was studied with pure cultures of sediment isolates. This last study was designed to follow phosphate fluctuations.

For the first study, the following protocol was established. Two five liter flasks were used which contained three liters of sediment. Over the sediment was placed three cm of filter sterilized Upper Klamath Lake water. This gave a column of sediment that was approximately 15 cm deep. Sampling was done weekly by collecting ten one ml samples at varying depths and locations. These samples were then composited, stirred, and one ml was used for the dilution series. The spread plate technique was used with TGY agar and incubation was for seven days at 15 C, the same temperature that the flasks of sediment were held. Each dilution was done in triplicate and the data represent an average. To eliminate possible counts due to phototrophs, light was excluded from the flasks and the TGY agar plates. The sediments were from Buck Island and Howard Bay.

Pure cultures of CH6 and BH12 were grown in TGY broth at room temperature on a gyrotory shaker for 48 hours. Cells were harvested by membrane filtration, divided, and inoculated into the medium used for the quantitative determination of phosphate solubilization. Two flasks of the medium, each containing 1000 ml, were inoculated for

each organism. One flask contained 1 percent glucose while the other flask in each set was devoid of added glucose. Assay for orthophosphate was done daily by removing ten ml of medium from each of the four flasks.

Plate counts were also run daily using the spread plate technique and TGY agar. Incubation was for 48 hours at 20 C.

Sediment Supplementation Studies

Formaldehyde was capable of eliminating biological activity in the sediment at a level of 0.5 percent (v/v). Pomeroy, Smith and Grant (30) also used formaldehyde for biological inactivation of marine bay sediments. It was necessary to treat the sediments in advance of the experiment to be performed by adding one percent (v/v) formaldehyde to the sediment and shaking for five days on a gyrotory shaker. A sterility test was performed at this time by plating 0.1 ml of sediment, by the spread plate technique, onto sediment agar. Formaldehyde treated sediment served as a control.

Sediment and water from Upper Klamath Lake was collected off Buck Island. Sampling was done in September of 1969 during the algal bloom. The water was centrifuged and filter sterilized (Millipore HA 0.45μ) and held at 4 C. The sediment was also held at 4 C until used.

Acid washed jars were used to hold the sediment. The original

jar lid was used after inserting a rubber ring to effect a tight seal. In the lid, a one inch hole was cut and inserted in the hole was a plastic cylinder, held in place with epoxy glue. The neck was then stoppered with cotton. The jars were sterilized with ethylene oxide. The sediment was then added aseptically to the jars.

Sampling for chemical assay of interstitial water was performed drawing off sediment at various depths in the jar followed by centrifugation at 3000 x g for 20 minutes and assay of the supernatant for total soluble phosphate. The pH of the supernatant was also taken. Biological sampling was done by removing ten one ml samples from the sediment from varying depths and stirring them together. One ml of the composited sample was plated in triplicate on sediment extract agar.

When carbohydrates were added, they were incorporated in liquid form to give a percentage of the volume. All additives were filter sterilized with the exception of the phosphates which were autoclaved as a slurry. After addition to the sediment, the sediment was shaken for one hour at high speed on a gyrotory shaker. Each jar, containing 12 cm of sediment (approximately three liters), was overlaid with one cm of lake water. Incubation was in the dark at 15 C. All of the plates from the biological assay were incubated under these same conditions.

Duplicate jars with each of the following additions were set up:

1% glucose, 1% glucose plus 1% formaldehyde, 1% starch, 1% starch plus 1% formaldehyde, 1% mannose, 1% mannose plus 1% formaldehyde, 1% sylose, 1% xylose plus 1% formaldehyde, 10% cell free algal extract, 10% cell-free algal extract plus 1% formaldehyde, sediment with no additions, sediment plus 1% formaldehyde. In another experiment, glucose at one percent levels was added on the first, eighth, twelfth and sixteenth day to duplicates as well as to the formaldehyde treated control.

The effect on the levels of soluble phosphate when precipitated phosphate was added to the medium was also measured. One hundred mg of $CaHPO_4$ was mixed with the sediment by stirring. Duplicate sets of each of the following additions were used: $CaHPO_4$ plus 1% glucose, $CaHPO_4$ only, $CaHPO_4$ plus glucose plus 1% formaldehyde. The same procedure was repeated using $FePO_4$ in place of $CaHPO_4$.

The final experiment of the series involved the addition of Ca^{++} and Fe^{+++} ions in an attempt to remove soluble phosphate. Subsequent addition of glucose was used to release the newly bound phosphate. The ionic solution was prepared by dissolving 500 mg of $FeCl_3$ and $CaCl_2$ in 100 ml of distilled water. The chloride ion was removed by precipitating with a stoichiometric quantity of $AgNO_3$. The solution was added to three liters of sediment and mixed by shaking for eight hours.

Phosphate variation, cell counts and pH were measured daily. On the sixth day of incubation, one percent glucose was added and total soluble phosphate followed.

RESULTS AND DISCUSSION

Phosphate solubilizers are those microorganisms capable of producing clearing zones in agar impregnated with a phosphate precipitate. All of the bacteria isolated as phosphate solubilizers were aerobic or facultative aerobes. Facultative aerobic organisms which were cultivated under anaerobic conditions produced no clearing zones, unless exposed to the air for at least 48 hours following growth under anaerobic conditions. It appears that phosphate solubilization is a function of aerobic metabolism in the sediments of Upper Klamath Lake. Table I lists the 31 isolates recovered from the sediment and gives their cultural characteristics used to classify them by the scheme presented in Figure 1.

The solubilizers isolated on each of the three samplings represented 20 percent of the total counts (18 percent in September 1968; 20 percent in March 1969; and 23 percent in June 1969). One Nocardia sp. was recovered (BH 9) but no molds capable of solubilizing phosphates were found in the sediment. The bacteria were all Gram negative and the majority of the isolates were motile.

Twenty isolates were selected for a quantitative measure of phosphate solubilization using glucose and $CaHPO_4$. The results are presented in Table II. Since incubation was for 14 days, it was expected that maximum growth was obtained by each organism. The amount of phosphate solubilized would be indicative of the organism's

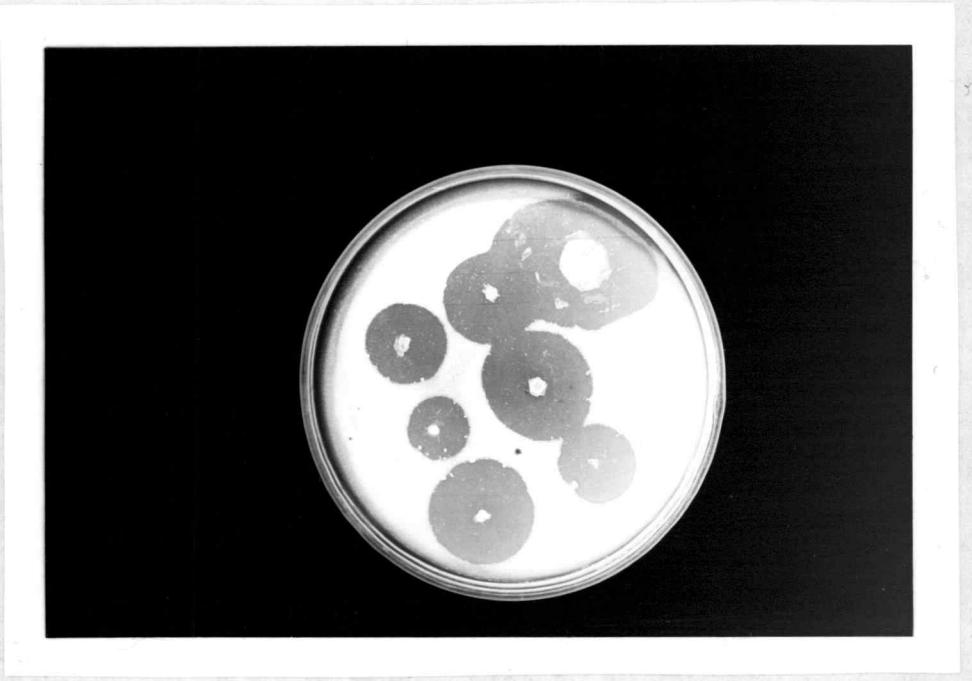

Figure 2. Photograph of phosphate solubilization as indicated by clearing zones on phosphate agar.

Table I. Cultural characteristics and identification of isolates.

Organism	Gram Reaction	Motility	Oxidative or Fermentative	Kovac's Oxidase	Pigment	Identity
AH 1	-	POLAR	NR	POS	F	Pseudomonas
AH 2	-	POLAR	NR	POS	F	Pseudomonas
AH 3	-		FR			Achromobacter
AH 4	-		NR			Achromobacter
AH 5	-		OX			Alkaligenes
AH 6	-		NR			Achromobacter
AH 7	-	POLAR	NR	POS	F	Pseudomonas
AB 8	-	POLAR	NR	POS	F	Pseudomonas
AB 9	-	POLAR	OX	POS	F	Pseudomonas
AB 10	-	POLAR	NR	POS	F	Pseudomonas
AH 11	-	POLAR	OX	POS	F	Pseudomonas
AH 12	-	POLAR	NR	POS	F	Pseudomonas
AH 13	-		NR			Achromobacter
AB 14	-	POLAR	OX	POS	F	Pseudomonas
AH 15	-	POLAR	OX	POS	F	Pseudomonas
BH 1	-		NR			Achromobacter
BH 2	-		NR		ORANGE	Flavobacterium
BH 3	-		NR		YELLOW	Cytophaga
BH 4	+	PERITRICHOUS	NR		BROWN	Bacillus
BH 5	-		NR			Achromobacter
BH 6	-	GLIDING	NR		YELLOW	Cytophaga
BH 7	-		FR			Achromobacter
BH 8	-		NR			Achromobacter
BH 9	-		FR		WHITE	Nocardia
BH 12	-		NR		WHITE	?

(Continued on next page)

Table I. (Continued)

Organism	Gram Reaction	Motility	Oxidative or Fermentative	Kovac's Oxidase	Pigment	Identity
CH 1	-		FR			Achromobacter
CH 2	-		NR			Achromobacter
CH 3	-		NR	POS	F	Pseudomonas
CH 4	-	POLAR	FR	POS		Aeromonas
CB 5	-		NR			Achromobacter
CH 6	-		FR			Achromobacter

NR = No reaction
OX = Oxidative utilization of carbohydrates (eight tested)
FR = Fermentative utilization of carbohydrates (eight tested)
F = Fluorescent pigment formed on Pseudomonas F agar (Difco)

Organism Identification Code:

First Letter
A = isolated from sediment on September, 1968
B = isolcated from sediment on March, 1969
C = isolated from sediment on June, 1969
Second Letter
B = sediment was collected from off Buck Island
H = sediment was collected from Howard Bay

Numbers indicate isolate number.

Table II. Quantitative phosphate solubilization by selected isolates.

Isolate	Phosphate solubilized Total soluble PO_4-P mg/5 ml	% Phosphate solubilized
AH 7	61	61.0
AB 8	48	48.0
AB 10	53	53.0
AH 11	78	78.0
AH 12	1	1.0
AH 13	51	51.0
AH 14	1	1.0
BH 2	51	51.0
BB 3	13	13.0
BH 4	1	1.0
BH 6	3	3.0
BH 8	6	6.0
BH 9	24	24.0
BH 12	45	45.0
CH 1	8	4.0
CH 2	3	1.5
CH 3	26	13.5
CH 4	13	6.5
CB 5	16	8.0
CH 6	50	25.0

Incubation for 14 days at 15 C.
Data represent the average of three determinations per organism.

ability to produce components that solubilize phosphate by chemical interaction.

Organisms from both Howard Bay and from Buck Island were capable of solubilizing large quantities of $CaHPO_4$. The range of solubilization ran from a low of one percent to a high of 78 percent of the $CaHPO_4$ supplied. The ability of these organisms to yield high levels of soluble phosphate may be of significance in maintaining a level of inorganic phosphate in the water.

Also of significance in maintaining levels of inorganic phosphate is the environment of the organism. With mixing (due to wind action), the upper layers of the sediment are stirred up, placing sediment bacteria in contact with water. Fluctuations in dissolved oxygen would also be expected, resulting in shifts from an aerobic environment to a condition of anoxia. Under anoxic conditions, microorganisms from the sediment liberate inorganic phosphate.

Figure 3 shows the effect of changes in aeration on a sediment inoculum and pure cultures of BH 12, AH 7 and CH 6. Levels of orthophosphate are slightly higher than those found in lake water or sediment due to the composition of the growth medium. This higher level of phosphate should not affect the results obtained since levels of inorganic phosphate in Upper Klamath Lake are in excess of that required to maintain growth (23). Shapiro (35) observed this same phenomenon in sewage which was high in phosphates.

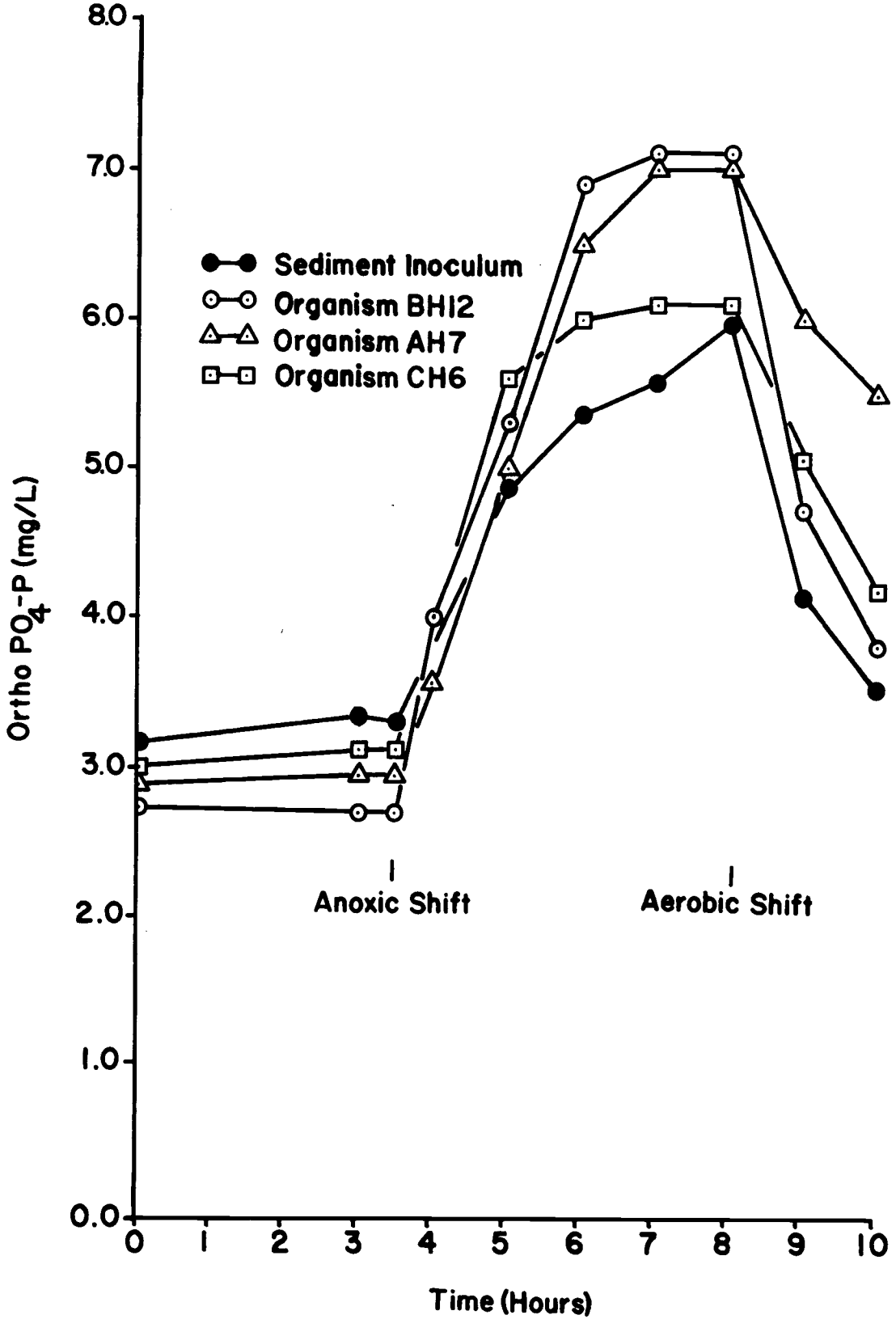

Figure 3. The reversible effect of aeration on available phosphate.

The results due to anoxia are reversible and occur more rapidly in the experiment due to the differences in solubility of the purging gas (N_2) and the air. If the curves in Figure 3 are plotted over a longer time, orthophosphate in the medium returns to the level that it was prior to the anoxic shift. For organisms BH 12, AH 7 and CH 6, a leveling off of inorganic phosphate excretion was obtained after 2.5 hours of anoxia. This plateau was not observed with the mixed culture from the sediment inoculum, presumably because the organisms in the mixed population were excreting inorganic phosphate at different rates.

Levin and Shapiro (20) determined that this reversible uptake and release of phosphate could be inhibited by 2,4-dinitrophenol and that the uptake of inorganic phosphate was in excess of the organism's requirements for the compound. 2,4-dinitrophenol blocks oxidative phosphorylation. Under aerobic conditions and in the absence of the inhibitor, inorganic phosphate forms high energy intermediates in the electron transport system. In the presence of inhibitor, phosphorylation is blocked and the cell takes up only enough phosphate for substrate level phosphorylation. The fact that bacteria take up excess phosphate and store it internally explains the rapid rate of uptake and release with changes in aeration.

Under anoxic conditions in the lake, phosphates would be liberated by the bacteria in the water as well as in the upper layers of

the sediment. Since Aphanizomenon blooms decrease the levels of oxygen in the water, anaerobic conditions would develop, resulting in a discharge of phosphates. In addition, the lake has a high organic content which the bacteria degrade and further deplete the oxygen. As the bloom declines and the lake becomes aerobic, phosphates resulting from the dying blue-green algae or from zooplankton would be absorbed by bacteria. A cycle can therefore be established for phosphates based on the degree of aeration in the lake.

Numerous organic acids were tested for their ability to solubilize phosphates. The data is presented in Table III. These organic acids were selected since they are intermediates or end products of carbohydrate metabolism. Acids of the Tricarboxylic Acid Cycle were effective in solubilizing $CaHPO_4$ as indicated by the size of the zone of solubilization. Citric acid was the most active, while succinic and fumaric acid were poor solubilizers. It is interesting that the addition of a carboxyl group at the α carbon of succinic acid, forming oxalacetic acid, more than doubles its solubilizing capacity. The same is true when a hydroxyl group is added to the α position of acetic acid forming glycolic acid. Lactic acid also contains an α hydroxyl group and is a strong solubilizer of $CaHPO_4$. Gluconic acid contains five hydroxyl groups and is slightly stronger than lactic acid in its solubilizing capabilities. No such relationship can be found to relate length of the carbon chain or the number of carboxylic acid groups to the ability

Table III. Solubilization of phosphates by organic acids spotted onto agar containing $CaHPO_4$.

Organic acid (0.056 M)	Diameter of solubilized zone (mm)
Acetic	17
Butyric	9
Citric	23
Formic	13
Fumaric	10
Gluconic	23
Glutamic	0
Glycolic	35
Lactic	22
Malic	15
Oxalacetic	19
Succinic	8
HCl control	18

Zones were measured after 48 hours at room temperature.

to solubilize phosphate. Johnston (14) and Mehltretter et al. (22) found similar results with organic acids functioning as metallic chelators.

When grown in the defined medium of Rose (33) in the presence of glucose, bacterial isolates from the sediment produced some product during growth that was capable of solubilizing phosphate. Organisms BH 12, AH 7 and Pseudomonas aeruginosa were grown in the medium and reached stationary growth after 72 hours. Daily samples were collected and cell free filtrates were tested for their ability to solubilize $CaHPO_4$. The results appear in Figure 4. The data indicate that some product is formed which solubilizes $CaHPO_4$. AH 7 produces more soluble phosphate than BH 12, which agrees with the quantitative

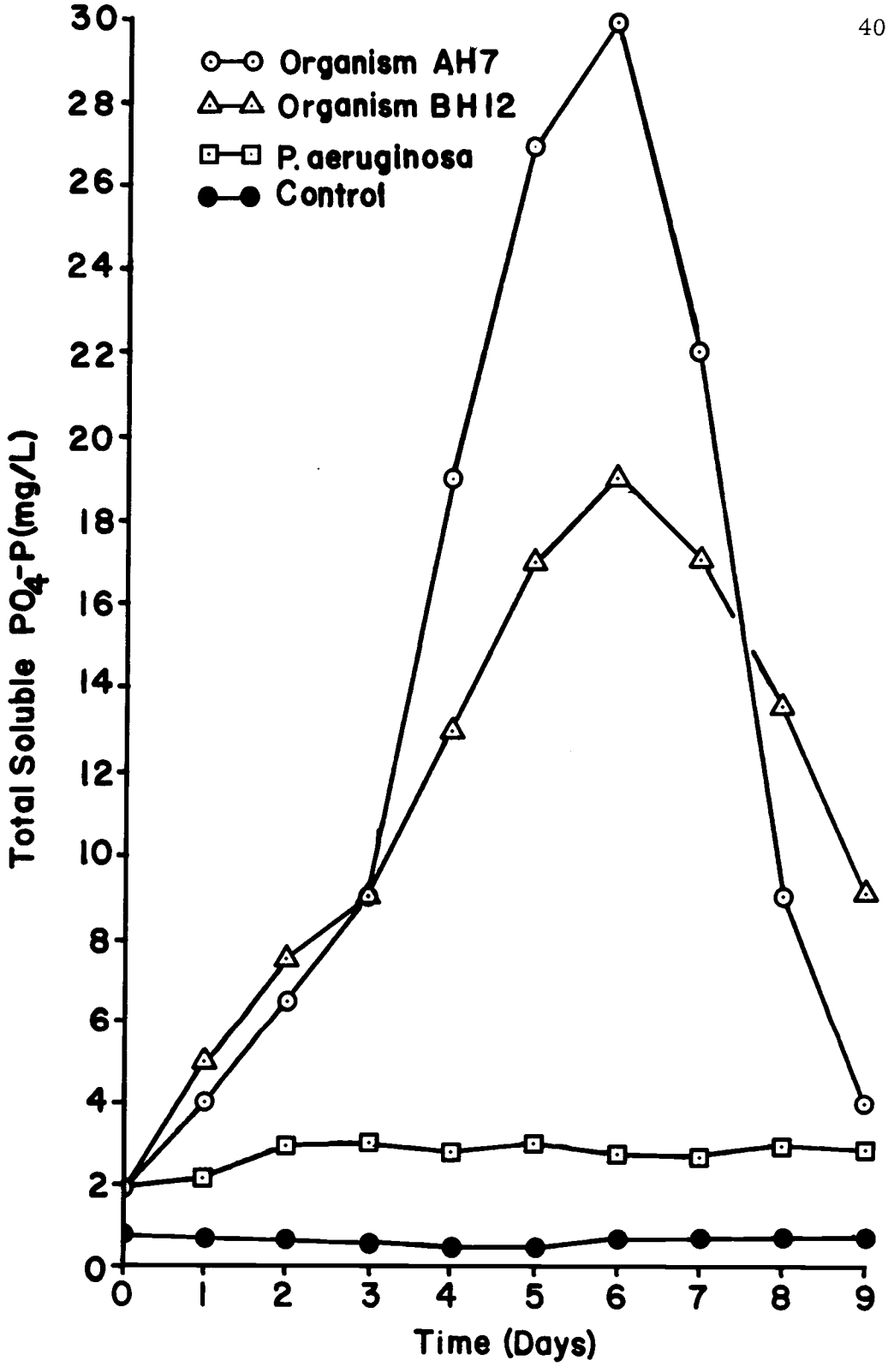

Figure 4. Solubilization of $CaHPO_4$ by culture filtrates taken at various times during cell growth.

data in Table II. The Ps. aeruginosa used was an organism that produced no detectable levels of acid from glucose.

In investigating the role of carbohydrate metabolism in phosphate solubilization, the simple sugars associated with the blue-green algae were identified. Chromatographic data for reducible sugars appear in Table IV. Only two spots were detectable for the algae. This was probably due to the low concentration of other sugars or to the presence of non-reducing carbohydrate polymers. The two spots were identified as glucose and glucuronic acid. The glucuronic acid may not actually be present since the spot also contained some algal pigment. This pigment may have been complexed with another sugar or have had a reducible carbohydrate as part of its composition. The presence of glucose provided support for the results obtained in in vitro studies. Although not analyzed, the polysaccharides of the Aphanizomenon can also be hydrolyzed enzymatically by certain bacteria to produce simple sugars, especially pentosans. Recognizing that there is a large mass of algae, some of the amino acids resulting from algal protein can also be enzymatically acted upon by bacteria to produce various organic acids.

The ability of microbial action on various carbohydrates to bring about solubilization of five different phosphate forms was tested. The data are summarized in Table V. The organisms grew on all of the phosphate agar plates, regardless of the carbon source. Growth on

Table IV. Identification of sugars from the algae.

Carbohydrate	$R_{glucose}$	Carbohydrate	$R_{glucose}$
Glacturonic	0.10	Mannitol	0.95
Glucuronic	0.13	Glucose	1.00
Raffinose	0.32	Mannose	1.04
Inositol	0.38	Arabinose	1.12
Lactose	0.56	Sorbose	1.15
Maltose	0.64	Fructose	1.16
Sucrose	0.70	Xylose	1.30
Cellobiose	0.78	Ribose	1.44
Galactose	0.83	Algae	1.00
Dulcitol	0.86	Algae	0.13

Descending paper chromatography using Whatman No. 1 paper and an ethyl acetate:pyridine:water (10:4:3) solvent.

phosphate agar without added carbohydrate functioned as a control, with no phosphate being solubilized.

Of the carbohydrates tested, glucose and xylose produced the greatest range of solubilization. Of the phosphates tested, the order of decreasing solubilization due to microbes is $CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$, $Mg_3(PO_4)_2$, $Al_3(PO_4)_2$.

Organic acids were recovered from the water, sediment and from pure cultures of AH 7, BH 12 and CH 6. R values and color reactions are given in Table VI. The data represent triplicate runs of each organic acid. The color reactions used by Buch et al. (4) were helpful in separating organic acids with close R values.

The water contained lactic, pyruvic and citric acids. These acids were probably associated with the algae, since the sample was

Table V. Solubilization of phosphates by bacteria using various carbohydrates.

Organism	Carbohydrate					
	Xylose	Mannose	Sucrose	Glucose	Arabinose	Cellobiose
AH 1	1, 2, 3, 4	-	-	1, 2, 3, 4, 5	-	-
AH 2	1	-	-	1	-	-
AH 7	1, 2, 4, 5	-	-	1, 2, 3, 4, 5	-	-
AB 8	1, 2, 3	-	-	1, 2, 3	-	-
AB 9	1, 2, 3, 4	-	-	1, 3	-	-
AB 10	1, 2	-	-	1, 2, 4, 5	-	-
AH 11	5	-	-	1, 4	-	-
AH 12	1, 2, 4	-	-	1, 3, 4	-	-
AH 13	1	-	-	1, 2, 5	-	-
AB 14	1, 4	-	-	1, 2, 4, 5	-	-
AH 15	1, 2, 3	-	-	1, 3, 5	-	-
BH 1	-	-	-	1, 4	-	1, 2, 4
BH 2	5	-	-	1, 2	-	-
BB 3	-	-	-	1, 3	-	-
BH 4	-	1, 2	-	1, 2, 4	-	-
BH 6	-	-	-	1, 2, 5	-	-
BH 8	-	-	-	1, 2	-	-
BH 9	-	1	-	1, 4	-	-
BH 12	-	-	-	1, 2, 3, 5	-	-
CH 1	1, 2, 3, 4, 5	-	1, 2, 3, 4	1, 2, 3, 4, 5	-	-
CH 3	-	-	-	1, 2	-	-
CH 4	1, 2, 4	-	-	1, 2, 4	-	-
CB 5	-	-	-	1	-	-
CH 6	1, 2, 3, 4, 5	-	-	1, 2, 3, 4, 5	1, 2, 4	1, 2, 4

1 is $CaHPO_4$; 2 is $Ca_3(PO_4)_2$; 3 is $Mg_3(PO_4)_2$; 4 is $FePO_4$; 5 is $Al_2(PO_4)_2$.
Incubation was for 14 days at 15 C.

Table VI. R values of organic acids on paper chromatograms and results of color reactions for identification.

Organic Acids	R Values (Bromophenol blue)		Silver nitrate + ammonium hydroxide		Acetic anhydride + pyridine		Ammonium vanadate		Ceric ammonium nitrate	
	R_f	R_{lactic}	daylite[1]	UV[1]	daylite[1]	UV[2]	daylite[1]	daylite[2]	daylite[1]	UV[2]
Gluconic	0.02	0.03	yellow	F[3]	-	-	-	gray	decolorized	+
Aspartic	0.03	0.05	white	-	-	-	-	-	-	+
Glutamic	0.05	0.08	yellow	F	yellow	F	yellow	-	decolorized	+
Sulfamic	0.08	0.12	white	F-blue	-	F	yellow	-	-	++
Glucuronic	0.11	0.17	yellow	F	yellow	F	-	gray	decolorized	-
Ascorbic	0.12	0.18	black	dark	yellow	F	gray	gray	decolorized	+
Tartaric	0.15	0.23	white	F	-	F	red	red	decolorized	+
Oxalic	0.15	0.23	streaks		streaks		streaks		streaks	
Glyceric	0.23	0.35	yellow	F	-	-	-	gray		
Phosphoric	0.23	0.35	-	-	-	-	-	-	-	-
Citric	0.33	0.50	pink	F	-	F	yellow	gray	decolorized	-
Malic	0.41	0.63	yellow	F	-	-	yellow	gray	decolorized	++
Acetic	0.43	0.66	white	F	-	-	yellow	gray	-	-
Glycolic	0.49	0.75	white	F	-	-	yellow	gray	-	-
Ketoglutaric	0.54	0.83	white	F-pink	-	F	yellow	gray	decolorized	-
Maleic	0.54	0.83	white	F-white	-	-	yellow	-	-	-
Malonic	0.61	0.94	yellow	F-yellow	-	-	yellow	-	decolorized	++
Succinic	0.63	0.97	white	white	-	-	yellow	-	-	-
Lactic	0.65	1.00	white	F	-	-	yellow	gray	decolorized	+
Pyruvic	0.78	1.20	white	F	-	-	-	-	-	-
Fumaric	0.88	1.31	gray	F	-	-	yellow	-	-	-

1 - observed when dry
2 - observed after 20 hours
3 - F = fluorescent; - = no reaction; + = weakly positive spot; ++ = strongly positive spot

Table VI. (Continued)

Organic acids	R values (Bromophenol blue) R_f	R_{lactic}	Silver nitrate + ammonium hydroxide daylite[1]	UV[1]	Acetic anhydride + pyridine daylite[1]	UV[2]	Ammonium vanadate daylite[1]	daylite[3]	Ceric ammonium nitrate daylite[1]	UV[2]
Water	0.34	0.52	pink	F	-	F	yellow	gray	decolorized	+
Water	0.65	1.00	white	F	-	-	yellow	gray	decolorized	+
Water	0.78	1.20	white	F	-	-	-	-	-	-
Sediment	0.02	0.03	yellow	F	-	-	-	gray	decolorized	+
Sediment	0.50	0.77	white	F	-	-	yellow	gray	-	-
Sediment	0.65	1.00	white	F	-	-	yellow	gray	decolorized	+
AH 7	0.02	0.03	yellow	F	-	-	-	gray	decolorized	+
AH 7	0.50	0.77	white	F	-	-	yellow	gray	-	-
AH 7	0.65	1.00	white	F	-	-	yellow	gray	declorized	+
BH 12	0.44	0.68	white	F	-	-	yellow	gray	-	-
BH 12	0.50	0.77	white	F	-	-	yellow	gray	-	-
CH 6	0.02	0.03	yellow	F	-	-	-	gray	decolorized	+
CH 6	0.11	0.17	yellow	F	yellow	F	-	gray	decolorized	-
CH 6	0.50	0.77	white	F	-	-	yellow	gray	-	-
CH 6	0.65	1.00	white	F	-	-	yellow	gray	decolorized	+

1 - observed when dry
2 - observed after 20 hours
3 - F = fluorescent; - = no reaction; + = weakly positive spot; ++ = strongly positive spot

taken during the bloom. The pyruvic acid spots were weak but still detectable by the color reagents. The sediment contained lactic, glycolic and gluconic acids as well as several others that could not be detected satisfactorily due to their weak spots on the chromatogram.

When cultivated on glucose, organism AH 7 produced gluconic, glycolic, and lactic acids. Organism BH 12 produced acetic and glycolic acid, and organism CH 6 produced gluconic, glucuronic, glycolic and lactic acid. All of these acids produce solubilization of $CaHPO_4$ as indicated by the data in Table III.

When Upper Klamath Lake sediment was incubated for several weeks, the bacterial counts began to oscillate in a manner similar to cryptic growth. Postgate (31) indicated that organisms growing under stress will survive by growing off the lytic products of dead organisms. Cryptic growth in Howard Bay and Buck Island sediment is shown in Figure 5. Phosphate fluctuations in the aqueous fraction of the sediment were not significant enough to be associated directly with fluctuations in microbial growth.

Using a large inoculum of BH 12 and CH 6, cryptic growth was observed in pure culture. The data are depicted in Figures 6 and 7. Phosphate levels in the medium were high enough so that fluctuations in viable counts produced a corresponding change in the level of inorganic phosphate. Duplicates of each organism were prepared, with one set growing in the medium containing one percent glucose. The

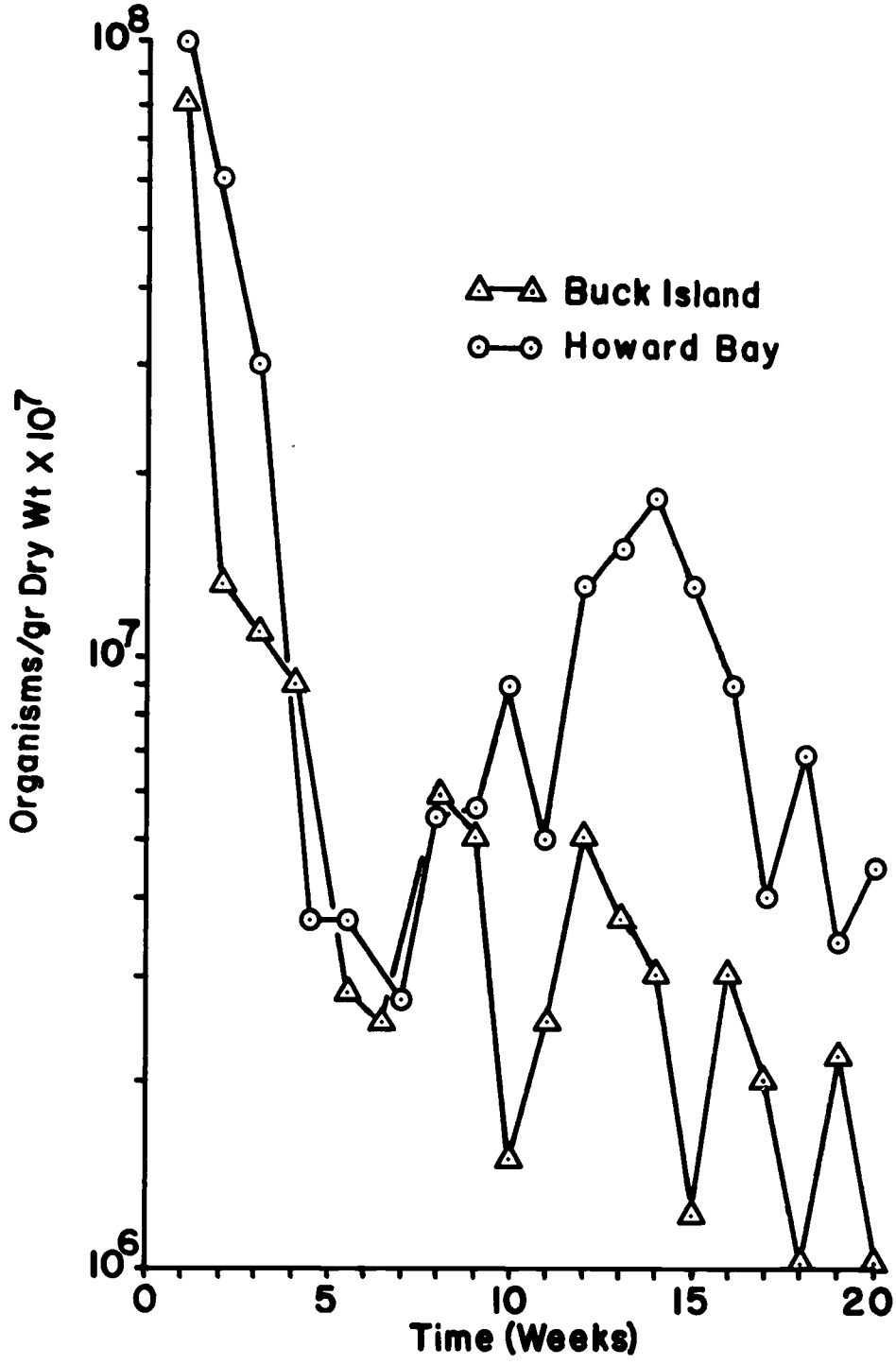

Figure 5. Cryptic growth of organisms in sediment collected during the algal bloom.

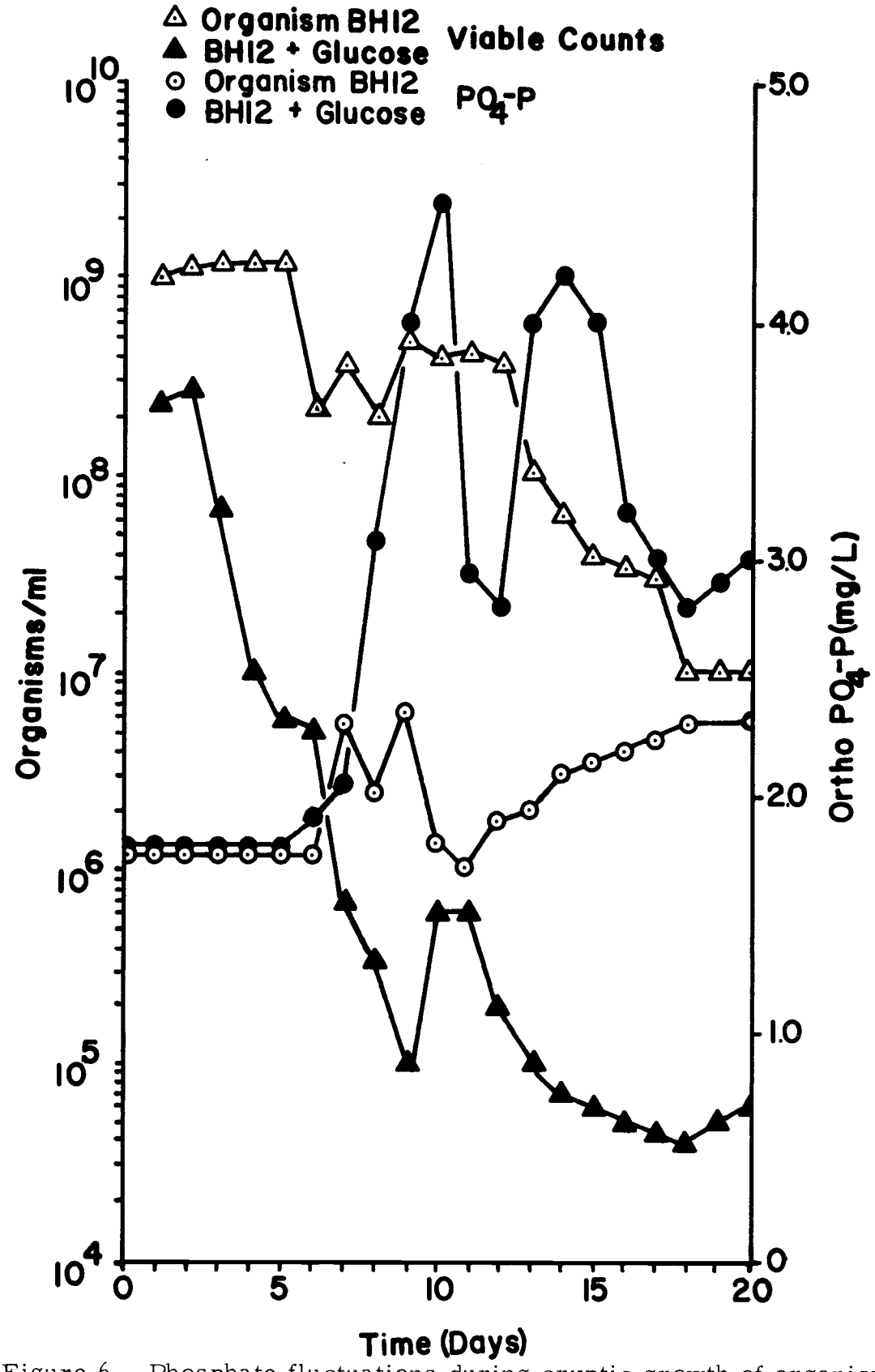

Figure 6. Phosphate fluctuations during cryptic growth of organism BH 12.

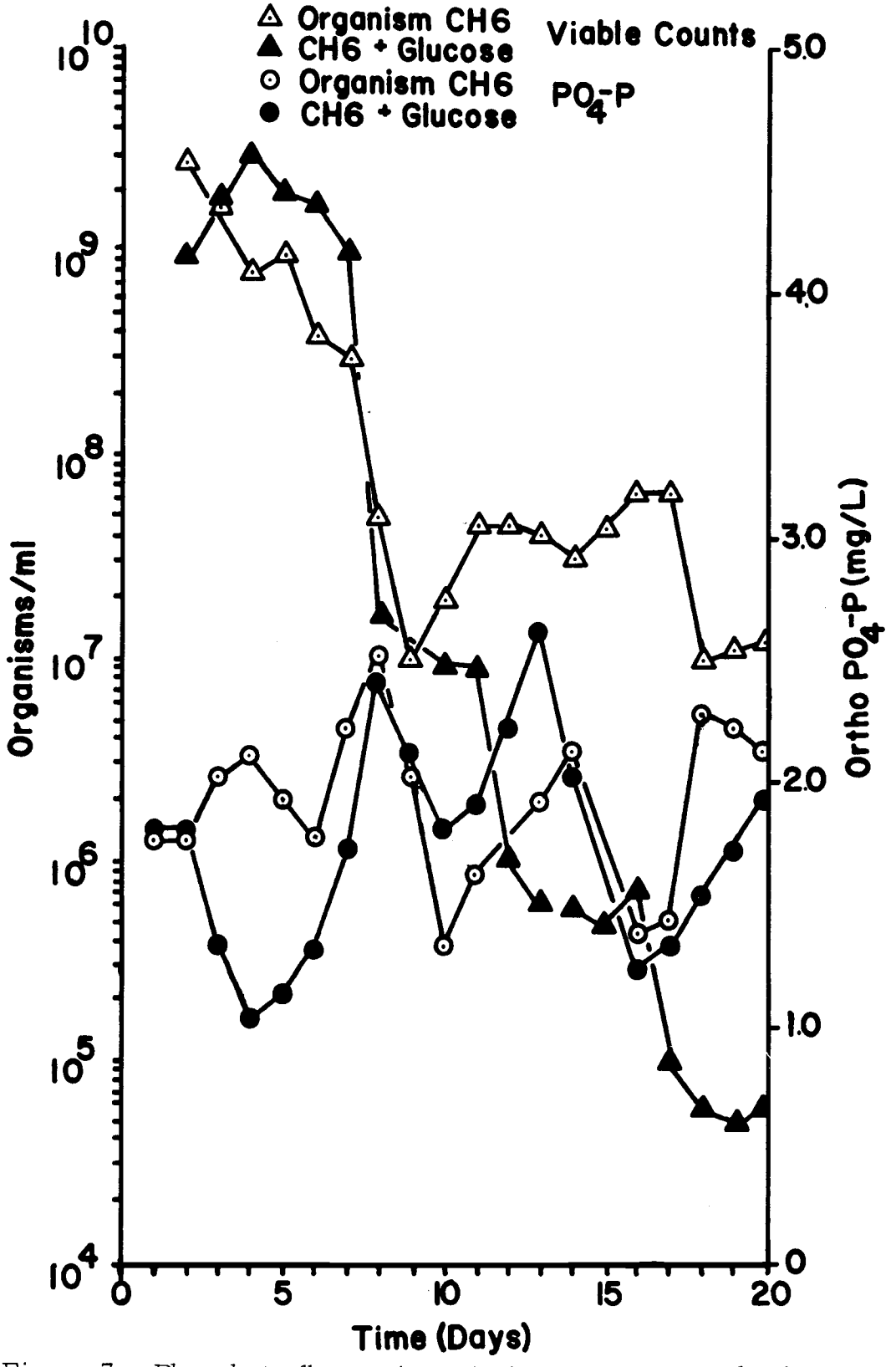

Figure 7. Phosphate fluctuations during cryptic growth of organism CH 6.

organisms growing in the presence of glucose produced greater fluctuations than the organisms growing in the absence of glucose.

Cryptic growth would produce a means to perpetuate the species and under these conditions, phosphate would be released into the sediment and water.

The addition of various carbohydrates to sediment caused changes in the level of total soluble phosphate in the aqueous fraction of the sediment. The pH, changes in bacterial counts and changes in phosphate are graphed in Figures 8-13. Glucose and xylose produced the greatest increase in total soluble phosphate, which agrees with other experiments using these carbon sources. Starch produced a steady increase in growth and a corresponding increase in soluble phosphate. There appeared to be a shift in the utilization of starch after the eighth day. This could be due to two degradations of the carbohydrate, first by starch hydrolyzing bacteria, and then by other bacteria capable of using the subunits of starch.

The increased biological activity in the sediment caused the increased levels of soluble phosphate. This is indicated by the effect of the formaldehyde treatment on the release of phosphate from the sediment. In every case where formaldehyde was added and bacterial growth halted, no increase in soluble phosphate was observed (see Figure 13).

The addition, at various periods, of glucose (Figure 14) resulted

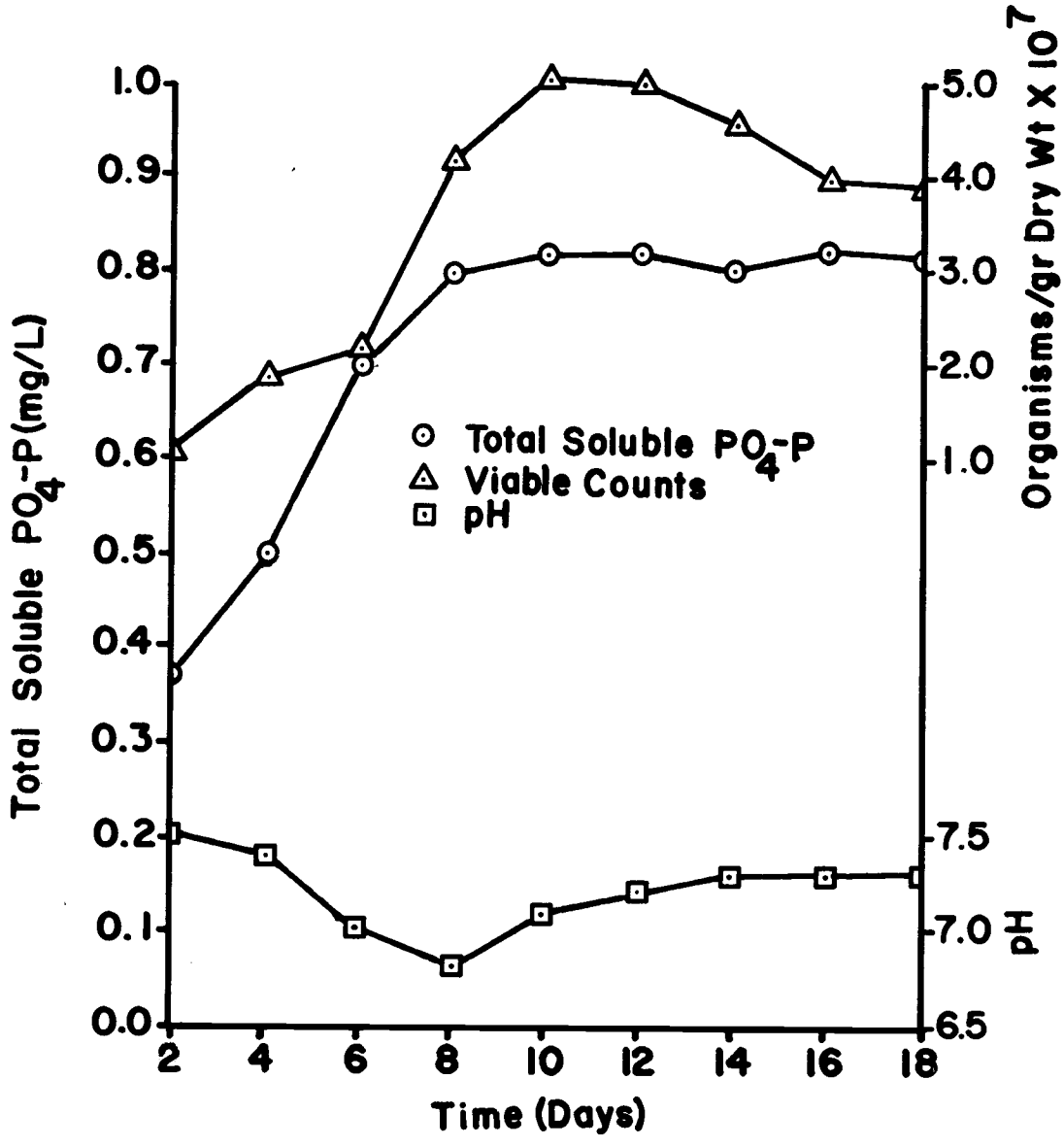

Figure 8. Release of phosphate, changes in viable counts and changes in pH in sediment augmented with one percent glucose and incubated at 15 C.

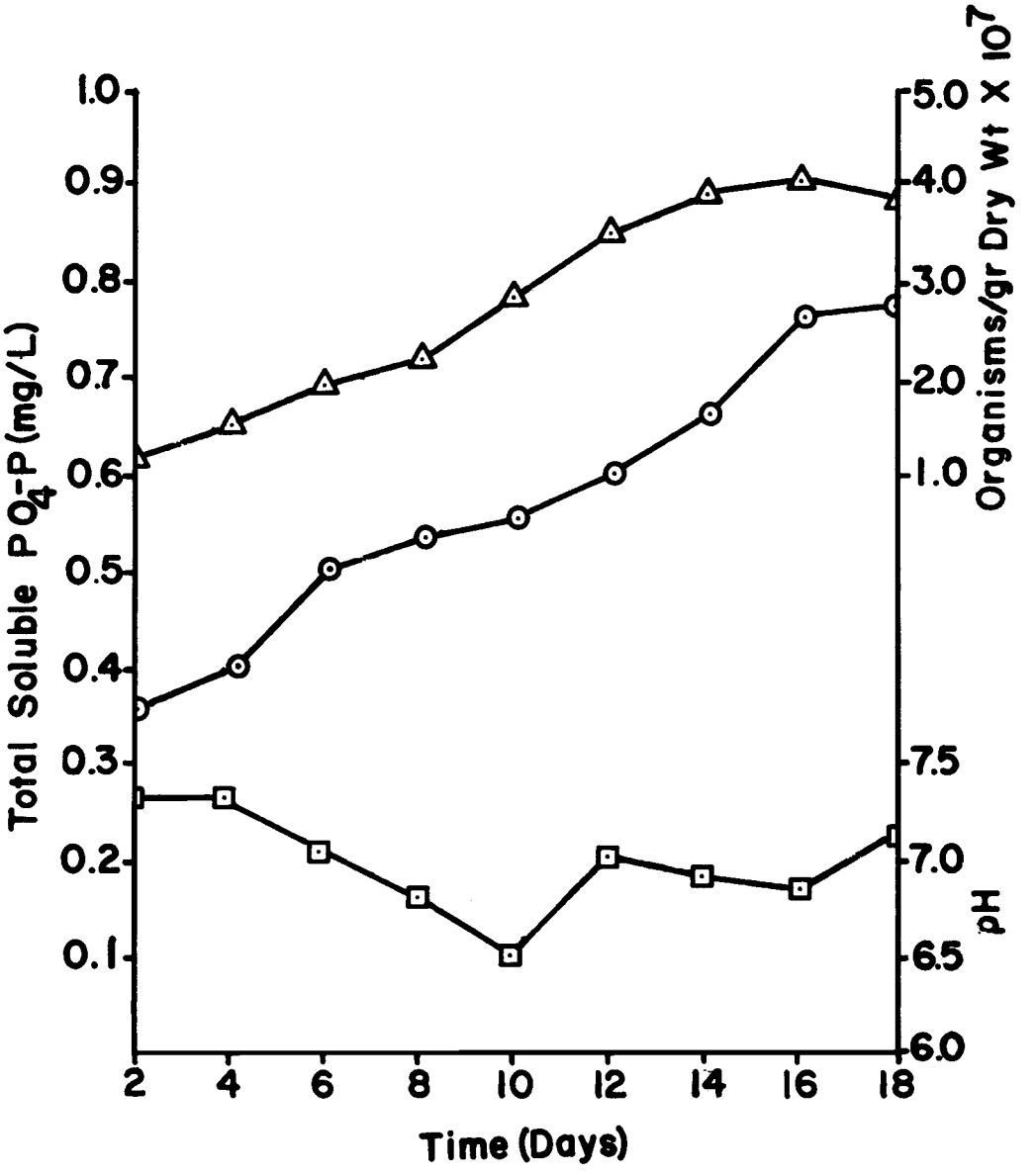

Figure 9. Release of phosphate, changes in viable counts and changes in pH in sediment augmented with one percent starch and incubated at 15 C.

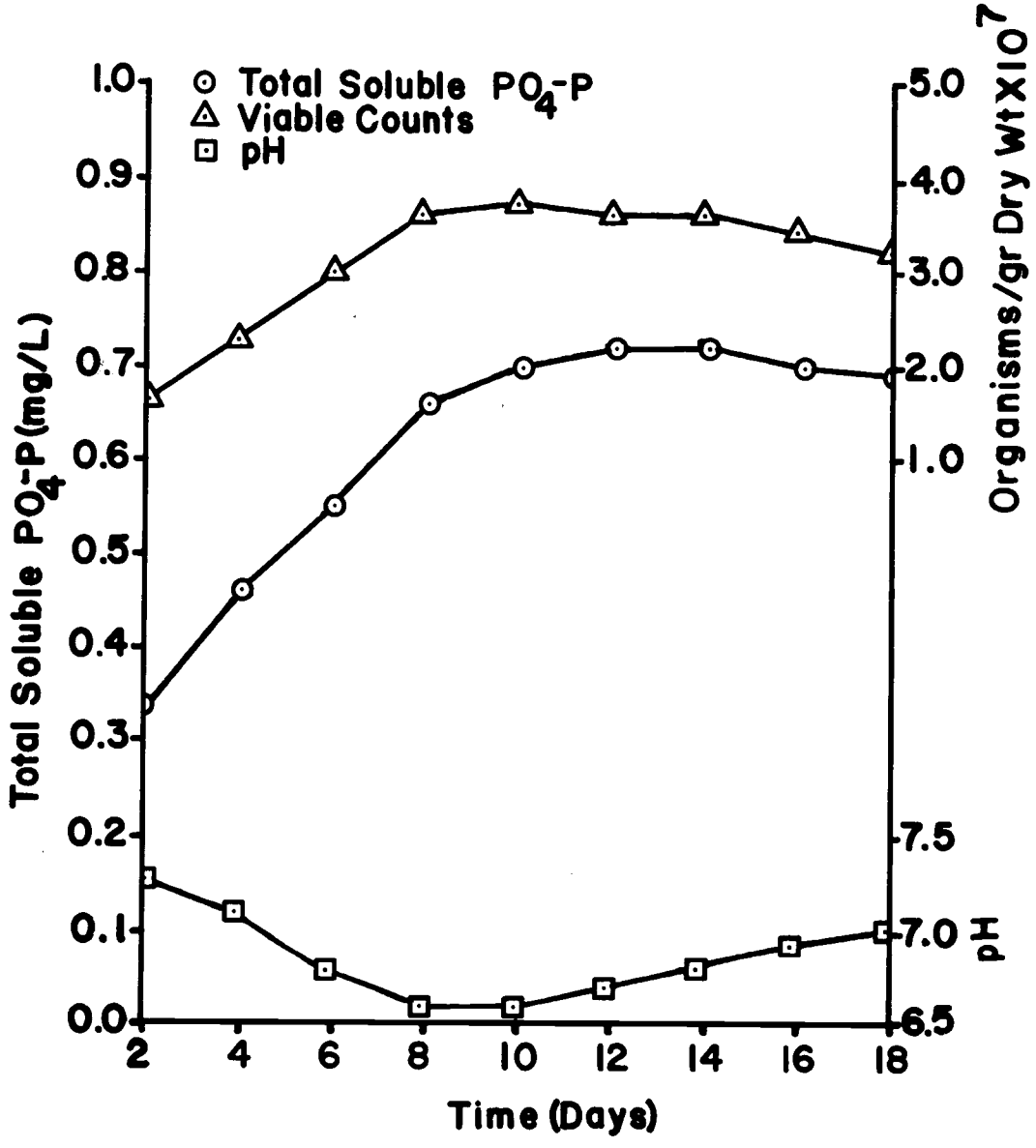

Figure 10. Release of phosphate, changes in viable counts and changes in pH in sediment augmented with one percent xylose and incubated at 15 C.

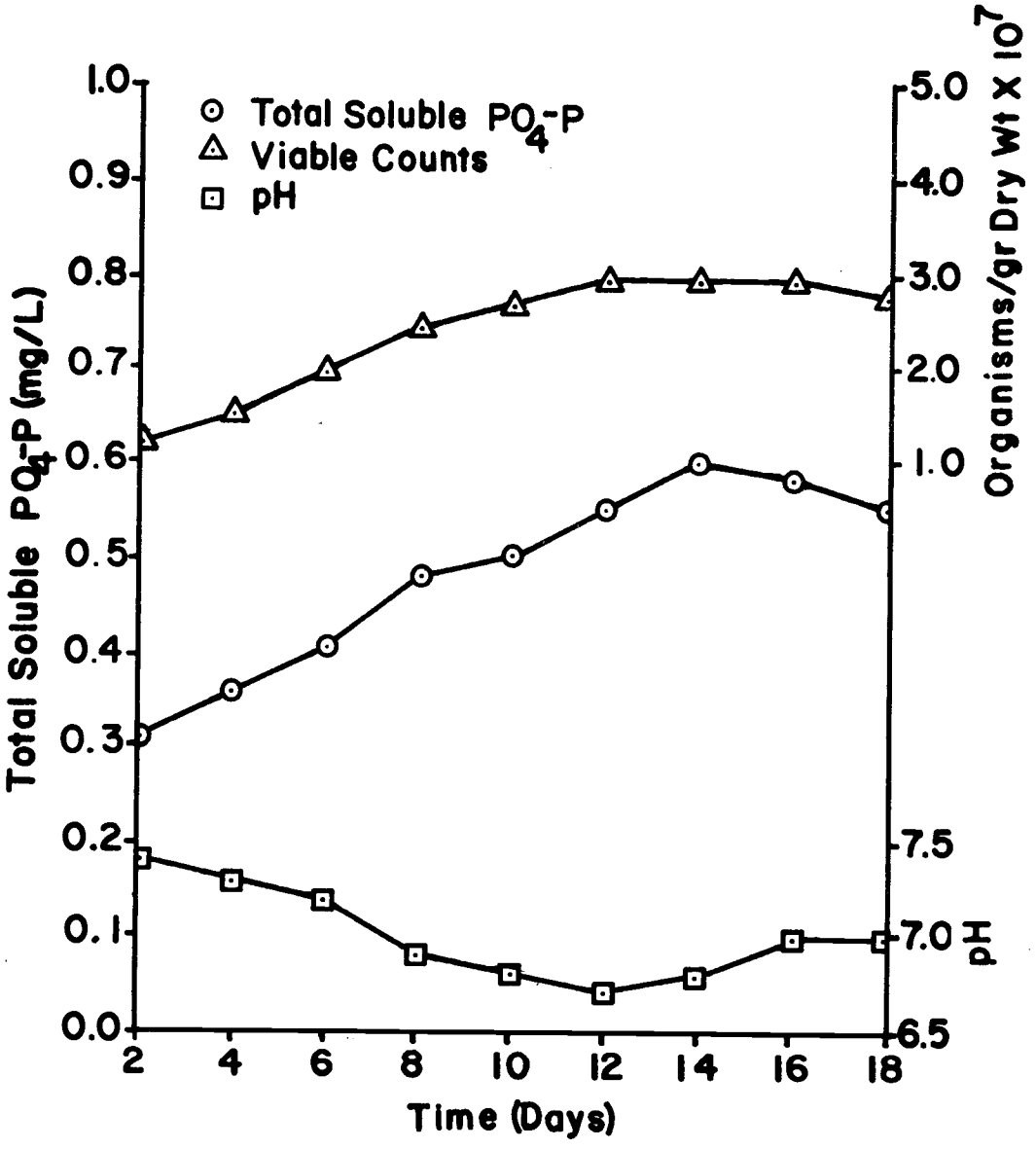

Figure 11. Release of phosphate, changes in viable counts and changes in pH in sediment augmented with one percent mannose and incubated at 15 C.

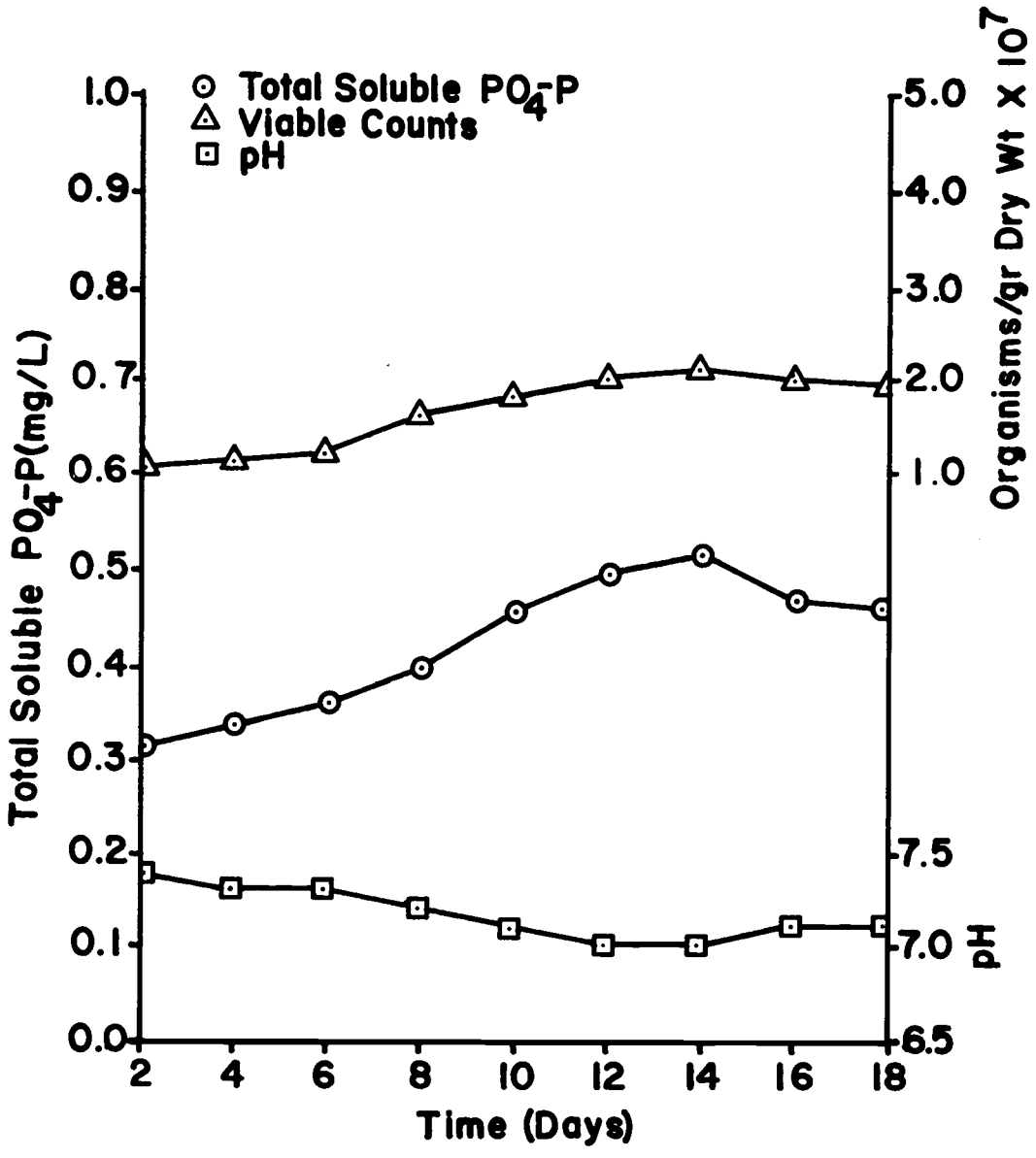

Figure 12. Release of phosphate, changes in viable counts and changes in pH in sediment augmented with 10 percent cell-free algal preparation at 15 C.

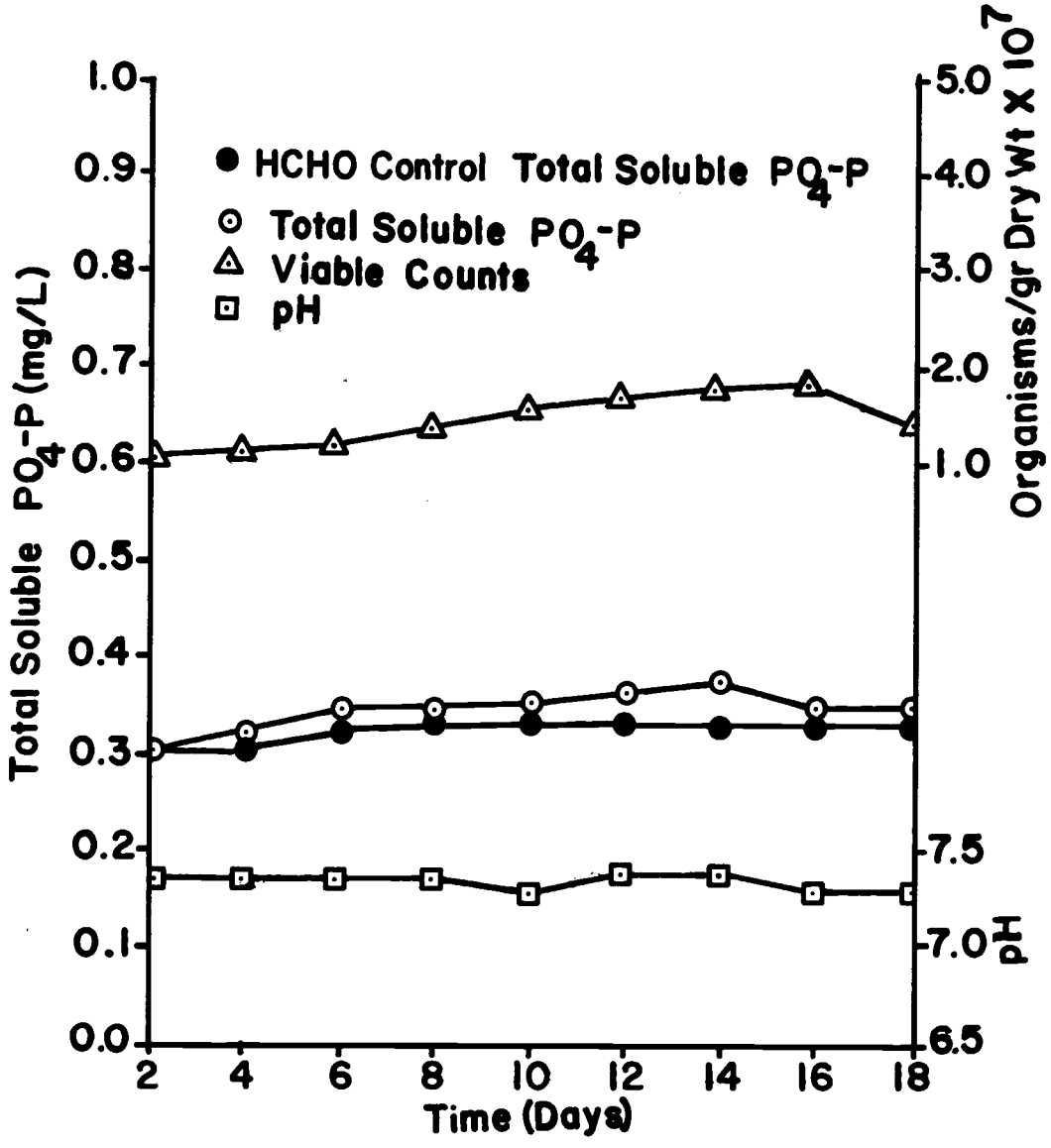

Figure 13. Release of phosphate, changes in viable counts and changes in pH in sediment at 15 C. Release of phosphate in the formaldehyde treated sediment is plotted for comparison.

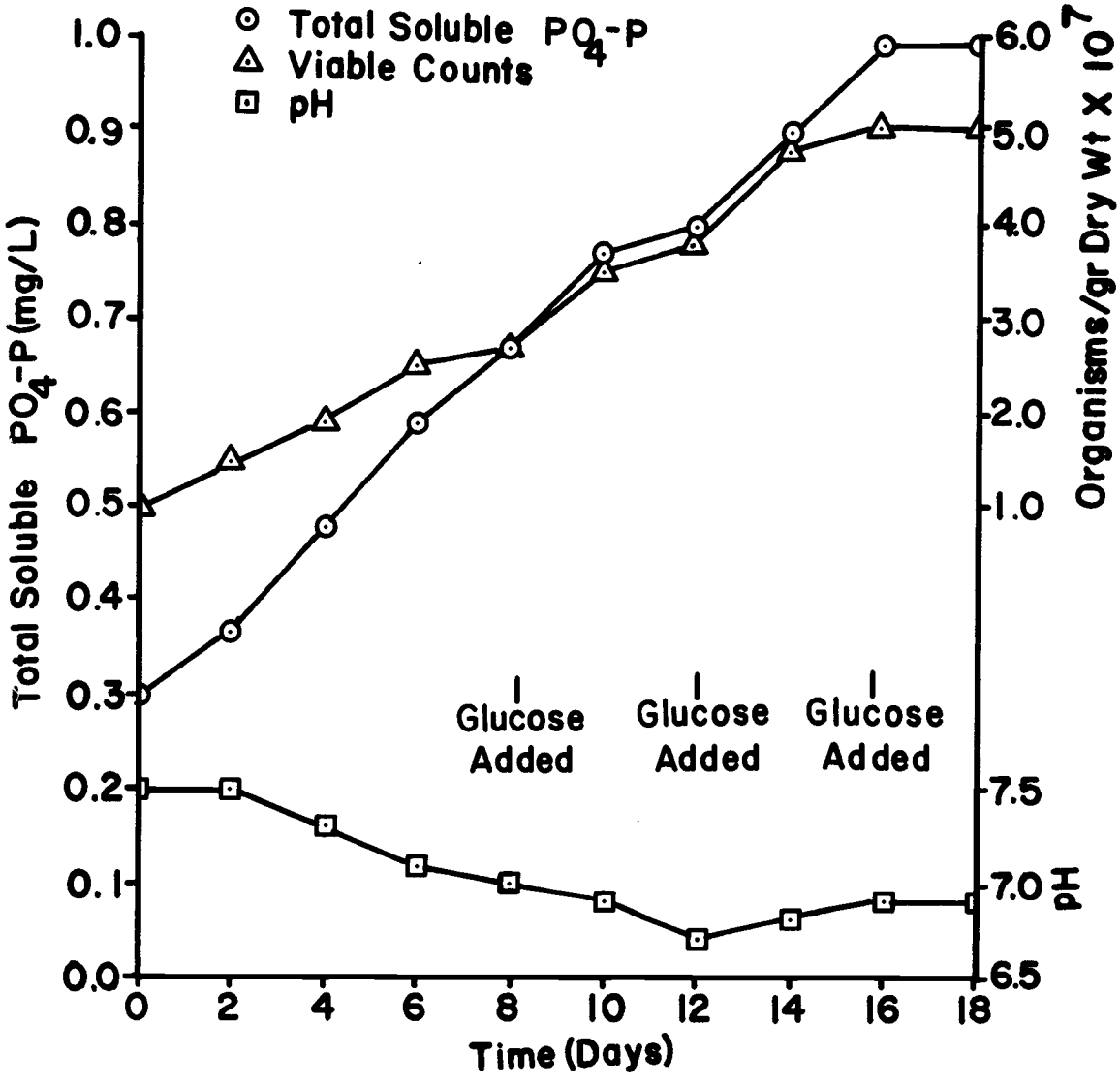

Figure 14. Release of phosphate, changes in viable counts and changes in pH in sediment that has one percent glucose added at Day 0, 8, 12 and 16 with incubation at 15 C.

in a progressive increase of organisms and soluble phosphate. pH fluctuations varied with the carbohydrate, but in each case the decrease in pH was not very significant. Since the organic acids are produced by bacteria, their action on $CaHPO_4$ (or other precipitated phosphates) is probably in microniches (microenvironment) and as a result, the pH electrodes are not capable of recording these pH changes.

This increased biological activity in the presence of carbohydrate must result in the production of organic acids which chelate the metallic phosphates, liberating inorganic phosphate. Some of the phosphate would be taken up by the cells while the remainder is free to support the growth of algae. Release of phosphate from the sediment to the water would result from shifts in chemical equilibrium due to consumption of phosphate from the water due to algae or bacteria. Mixing of the sediment by wind action would stimulate this exchange.

If precipitating ions are added to sediment, there is a decrease in the soluble phosphate. No appreciable changes occurred in viable counts or pH. Ca^{++} and Fe^{+++} were added to the sediment in Figure 15. The soluble phosphate level dropped off rapidly. On the sixth day, one percent glucose was added to stimulate microbial activity in one of the sediment samples. Figure 15 shows the sudden rise in phosphate due to microbial stimulation.

Microbial action on glucose was also capable of releasing phosphate from sediment containing 100 mg of $CaHPO_4$ or 100 mg of

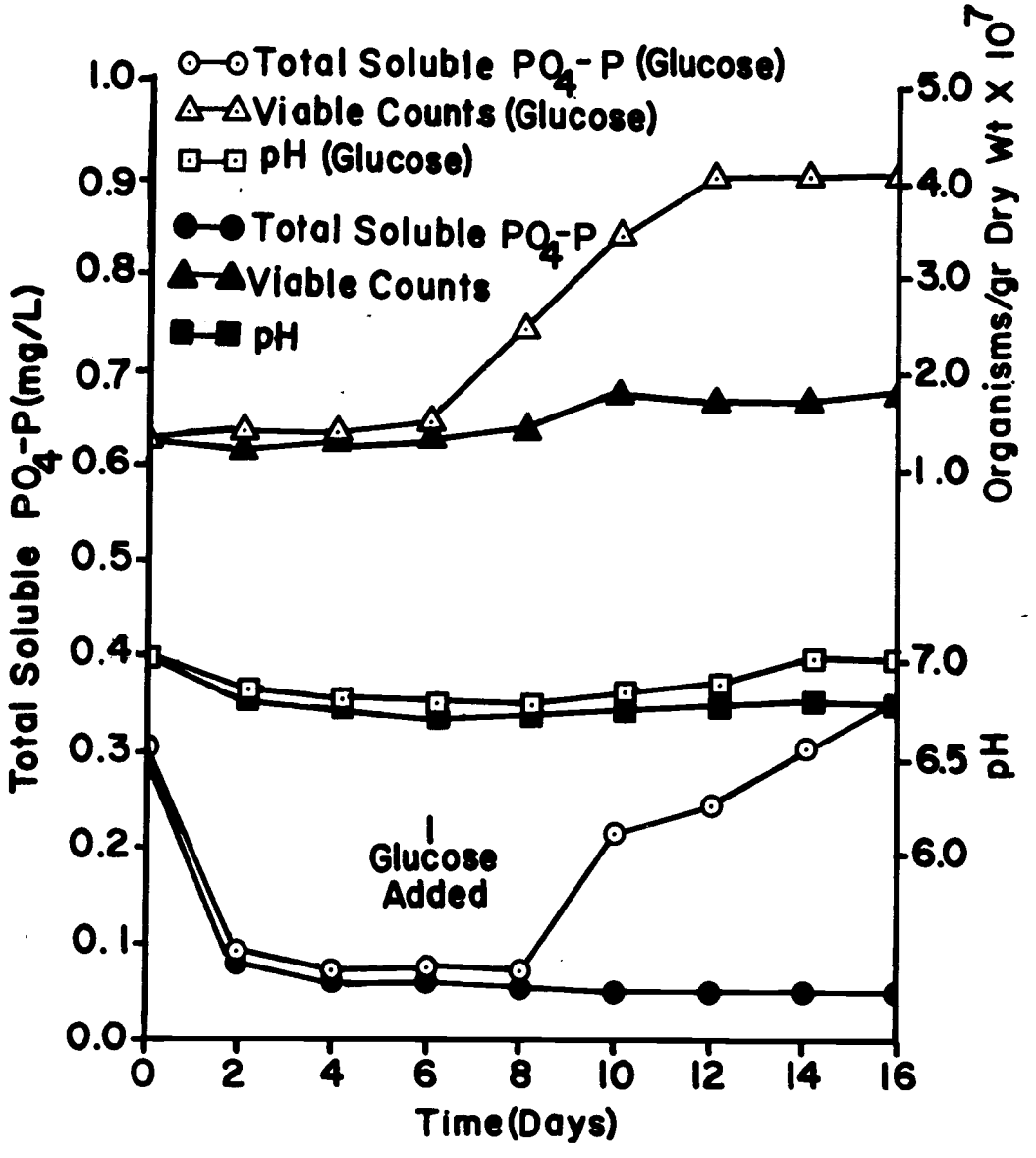

Figure 15. The effect of precipitating ions (Ca^{++} and Fe^{+++}) on levels of phosphate, changes in viable counts and changes in pH. On the sixth day, one percent glucose was added to one set of the sediment samples.

$FePO_4$ added to three liters of sediment. Levels of phosphate released were well above that normally released in the absence of added phosphate. These data are given in Figure 16.

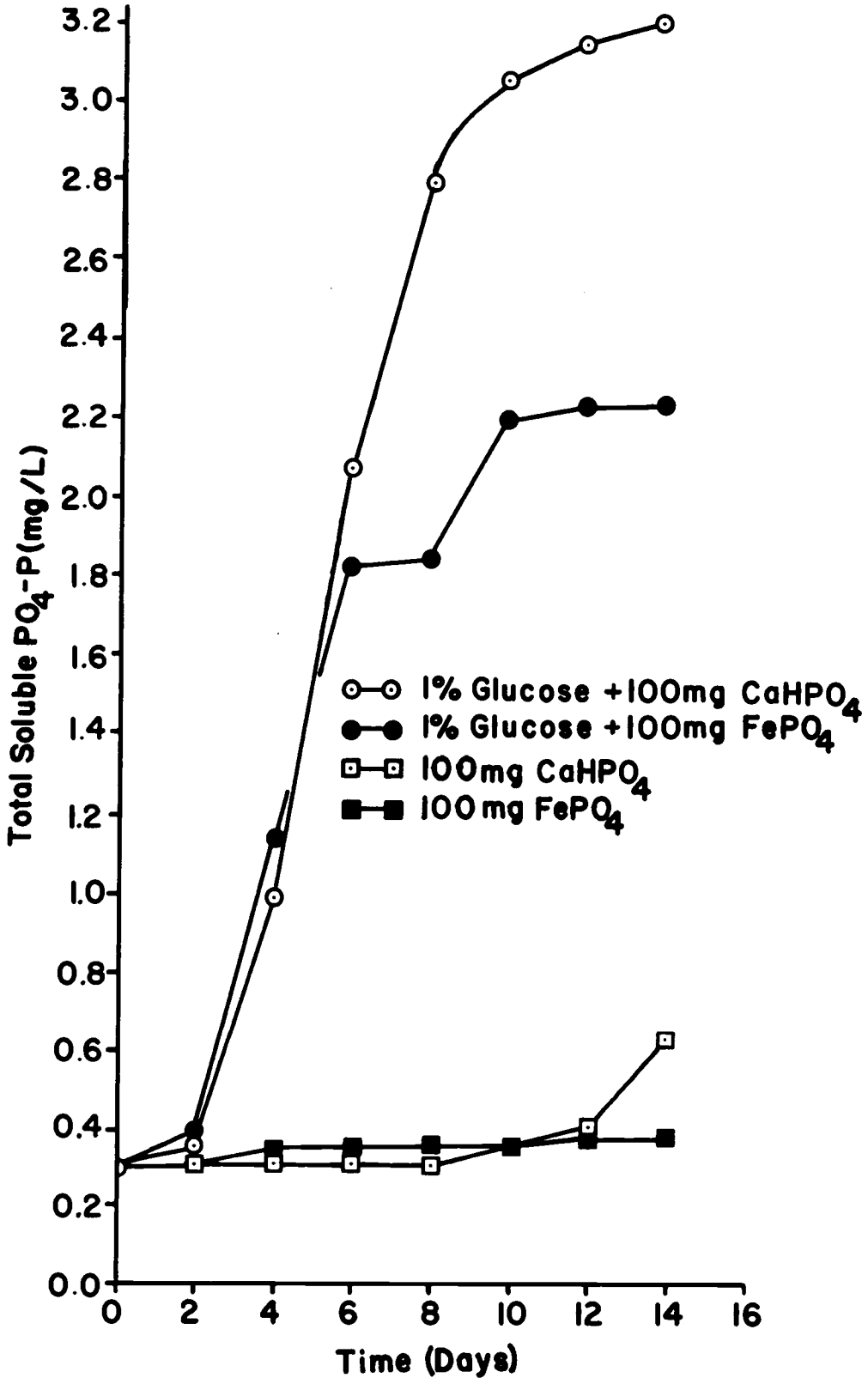

Figure 16. The effect of one percent glucose on the release of phosphate augmented with 100 mg of $CaHPO_4$ or 100 mg of $FePO_4$. Sediment was incubated at 15 C.

SUMMARY

In examining the role of bacteria in phosphate exchange in the sediment, the effects of bacteria, zooplankton and algae in the water must be considered. Of equal importance is the direct physico-chemical exchange of phosphates that occurs. Pomeroy et al. (30) studied the physico-chemical exchange of phosphates in the sediment.

When bacteria in the water are inhibited by antibiotics, there is a rapid loss of phosphate to the sediment (10). However, if bacteria in the sediment are inhibited, there is no apparent change in the levels of phosphate. If bacteria in the sediment are stimulated by increasing the levels of carbon, phosphates are released from their interaction with the sediment particles. The phosphates are then either consumed by the bacteria or released to the water.

The release of phosphate from the sediment appears to be due to the production of organic acids. These organic acids, especially the ones that are carboxyl or hydroxyl group substituted, sequester the phosphates by forming five and six member rings with the cations by coordinate and covalent bonding (22). This reaction is probably analogous to the action of EDTA.

Once in solution, the phosphates can be adsorbed by the bacteria in excess of their own physiological needs. Under anoxic conditions in the sediment, this phosphate could be released to the water. The aquatic bacteria are also capable of rapid uptake of phosphates as well

as the conversion of organic phosphate forms to the inorganic form which is utilizable by zooplankton and phytoplankton.

Johannes (13) claims that it is the zooplankton that are primarily responsible for nutrient regeneration of phosphate, due to grazing of algae and bacteria. Other workers (2, 8, 26, 29) have found that planktonic excretions of total phosphate would be sufficient to support blooms of phytoplankton. The role of zooplankton may be secondary to bacteria since zooplankton cannot adsorb phosphate but must get it from grazing. In bacteria-free preparations of zooplankton, the organisms were unable to concentrate phosphates and could not use organic phosphates (8). Pomeroy, Mathews and Min (29) conducted calculations on phosphate turnover and found that in shallow water (as in Upper Klamath Lake), zooplankton could only supply one-third to one-tenth of the phytoplankton requirements for phosphate.

While biological exchange of phosphates in the sediment appears to exist, it is difficult to illustrate conclusively. The extent of its effect on the total exchange is equally difficult to measure since the exchange occurs below the surface of the sediment. However, based on the work of other researchers and this research, several conclusions are warranted.

1. Bacteria are partially responsible for the exchange of phosphates (organic and inorganic) from the water to the sediment.

2. Bacteria in the water and in the sediment are capable of

storing and concentrating phosphates under aerobic conditions.

3. Anoxic conditions in the sediment and the water would cause the bacteria to liberate their stored inorganic phosphates for use by zooplankton and phytoplankton.

4. Bacteria are capable of removing phosphates bound to the sediment due to the production of organic acids which sequester metallic cations.

5. Organic phosphates are degraded to inorganic phosphates and either stored by bacteria or released upon death or under anoxic conditions.

6. Cryptic growth may be of importance in maintaining a level of inorganic phosphate during periods of stress.

7. With inorganic phosphate in equilibrium between the water and the sediment, removal of phosphates by precipitating ions or by consumption by aquatic life will result in the release of phosphates from the sediment to maintain this equilibrium. Bacteria in the sediment appear to be directly responsible for this release.

BIBLIOGRAPHY

1. Allen, Mary Mennes and R. Y. Stanier. Selective isolation of blue-green algae from water and soil. Journal of General Microbiology 51:203-209. 1968.

2. Barlow, John F. and John W. Bishop. Phosphate regeneration by zooplankton in Cayuga Lake. Limnology and Oceanography, Supplement 10:R15-R24. 1965.

3. Batelle-Northwest. An investigation of the release of phosphorus from lake sediments. Monthly Progress Report Nov., 1969.

4. Buch, M. L., Rex Montgomery and W. L. Porter. Identification of organic acids on paper chromatograms. Analytical Chemistry 24(3):489-491. 1952.

5. Buck, John D. and Robert C. Cleverdon. The spread plate as a method for the enumeration of marine bacteria. Limnology and Oceanography 5(1):78-80. 1960.

6. Duff, R. B., D. M. Webley and R. O. Scott. Solubilization of minerals and related materials by 2-keto-gluconic acid-producing bacteria. Soil Science 95:105-114. 1963.

7. Gerretson, F. C. The influence of microorganisms on the phosphate intake by the plant. Plant and Soil 1(1):51-81. 1948.

8. Harris, Eugene. Radiophosphorus metabolism in zooplankton and microorganisms. Canadian Journal of Zoology 35:769-782. 1957.

9. Harris, R. F. and D. R. Keeney. Towards standardization of soil extracts for microbial media. Canadian Journal of Microbiology 14:653-659. 1968.

10. Hayes, F. R. and J. E. Phillips. Lake water and sediment. IV. Radiophosphorus equilibrium with mud, plants, and bacteria under oxidized and reduced conditions. Limnology and Oceanography 3:459-475. 1958.

11. Hough, Leslie and J. K. N. Jones. Chromatography on paper. In: Methods in carbohydrate chemistry, ed. by Roy L. Whistler and M. L. Wolfrom. Vol. 1, New York, Academic. 1962. 589 p.

12. Hugh, Rudolph and Einer Leifson. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. Journal of Bacteriology 66(1): 26-28. 1953.

13. Johannes, R. E. Nturient regeneration in lakes and oceans. Advances in Microbiology of the Sea 1:203-213. 1968.

14. Johnston, H. W. The solubilization of "insoluble" phosphates. II. A quantitative and comparative study of the action of selected aliphatic acids on tricalcium phosphate. New Zealand Journal of Science and Technology, sec. B 36:49-55. 1954.

15. _____ The solubilization of "insoluble" phosphates. V. The Action of some organic acids on iron and aluminum phosphates. New Zealand Journal of Science 2:215-218. 1959.

16. Katznelson, H. and B. Bose. Metabolic activity of phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere and non-rhizosphere soil. Canadian Journal of Microbiology 5:79-85. 1959.

17. Katznelson, H., E. A. Peterson and J. W. Rouatt. Phosphate dissolving microorganisms on seed and in the root zone of plants. Canadian Journal of Botany 40:1181-1186. 1962.

18. Kovacs, Nicholas. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature 178:703. 1956.

19. Leifson, Einer. Staining, shape and arrangement of bacterial flagella. Journal of Bacteriology 62(2):377-389. 1951.

20. Levin, Gilbert V. and Joseph Shapiro. Metabolic uptake of phosphorus by wastewater organisms. Journal Water Pollution Control Federation 37(6):800-821. 1965.

21. Louw, H. A. and D. M. Webley. A study of soil bacteria dissolving certain mineral phosphate fertilizers and related compounds. Journal of Applied Bacteriology 22(2):227-233. 1959.

22. Mehltretter, C. L., B. H. Alexander and C. E. Rist. Sequestration by sugar acids. Industrial and Engineering Chemistry 45(12):2782-2784. 1953.

23. Miller, William E. and Jerry C. Tash. Interim report Upper

Klamath Lake studies, Oregon. Washington, D. C., 1967. 37 p. (U. S. Federal Water Pollution Control Administration. Research Series no. WP-20-8)

24. Pelczar, M. J. (ed.). Manual of microbial methods. New York, McGraw-Hill, 1957. 315 p.

25. Perotti, R. Über de biochemischen Kreislauf der Phosphorsäure in Ackerboden. Zentralblatt für Bakteriologie 2(25):409-419. 1910.

26. Phillips, John E. The ecological role of phosphorus in waters with special reference to microorganisms. In: Principles and applications in aquatic microbiology, ed. by H. Heukelekian and Norman C. Dondero, New York, John Wiley and Sons, 1964. p. 61-81.

27. Phinney, H. K. and Peek, C. A. Klamath Lake, an instance of natural enrichment. In: Algae and metropolitan wastes, U. S. Public Health Service, p. 22-27. (SEC TR W61-3)

28. Phinney, H. K., C. A. Peek and M. C. McLachlan. A study of the phytoplankton problems in Klamath Lake. Corvallis, Oregon State University. 1959. 52 p. (Mimeo)

29. Pomeroy, Lawrence R., Hbney M. Mathews and Hong Shik Min. Excretion of phosphate and soluble organic phosphorus compounds by zooplankton. Limnology and Oceanography 8(1):50-55. 1963.

30. Pomeroy, Lawrence R., E. E. Smith and Carol M. Grant. The exchange of phosphate between estuarine water and sediments. Limnology and Oceanography 10(2):167-173. 1965.

31. Postgate, John R. Viability measurements and the survival of microbes under minimum stress. Advances in Microbial Physiology 1:1-26. 1967.

32. Raghu, K. and I. C. MacRae. Occurrence of phosphate-dissolving microorganisms in the rhizosphere of rice plants in submerged soil. Journal of Applied Bacteriology 29(3):582-586. 1966.

33. Rose, R. E. Techniques for determining the effect of micro-organisms on insoluble inorganic phosphates. New Zealand Journal of Science and Technology, July, 1957. p. 773-780.

34. Sackett, W. G., A. J. Patten and C. W. Brown. The solvent action of soil bacteria upon the insoluble phosphates of raw bone meal and natural raw rock phosphate. Zentralblatt für Bakteriologie 2, 28:688-703. 1908.

35. Shapiro, Joseph. Induced rapid release and uptake of phosphate by microorganisms. Science 155:1269-1271. 1967.

36. Shewan, J. M., G. Hobbs and W. Hodgkiss. The Pseudomonas and Achromobacter groups of bacteria in the spoilage of marine white fish. Journal of Applied Bacteriology 23(3):463-371. 1960.

37. Skerman, V. B. D. A guide to the identification of the genera of bacteria. 2d ed. Baltimore, Williams and Wilkins. 1967. 303 p.

38. Sperber, Joan I. The incidence of apatite-solubilizing organisms in the rhizosphere and soil. Australian Journal of Agricultural Research 9:778-781. 1958.

39. _____ Solution of apatite by soil microorganisms producing organic acids. Australian Journal of Agricultural Research 9:782-787. 1958.

40. Stewart, Denton M. and Gerald A. Rohlich. Eutrophication -- a review. State Water Quality Board, Resources Agency, State of California, Publication no. 34. 1967.

41. Strickland, J. D. H. and T. R. Parsons. A manual of sea water analysis. 2d ed., rev. Fisheries Research Board of Canada, Bulletin 125:47. 1965.

42. Waksman, Selman A. Principles of soil microbiology. Baltimore, Williams and Wilkins. 1927. 897 p.

43. Wang, Thomas S. C., San-Yao Cheng and Helen Tung. Extraction and analysis of soil organic acids. Soil Science 103(5):360-366. 1967.

44. Weiss, Charles M. Relation of phosphates to eutrophication. Journal of the American Water Works Association 61:387-391. 1969.

45. U. S. Coast and Geodetic Survey. Klamath Falls section map. Washington, D. C. 1956. 1 sheet.