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1966

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

DEGRADATION AND ASSIMILATION OF CONDENSED PHOSPHATES BY BLUE-GREEN AND GREEN ALGAE

A DISSERTATION

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degree of

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BY

ERNST MICHAEL DAVIS

Norman, Oklahoma

1966

DEGRADATION AND ASSIMILATION OF

CONDENSED PHOSPHATES BY BLUE-GREEN AND GREEN ALGAE

APPROVED BY

DISSERTATION COMMITTEE

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DEGRADATION AND ASSIMILATION OF CONDENSED PHOSPHATES BY BLUE-GREEN AND GREEN ALGAE

CHAPTER I

Utilization of impoundments for the disposal of waste materials has long been a practice throughout the world. These impoundments are usually called bio-oxidation ponds, photosynthesis ponds, oxidation ponds, sewage lagoons, or waste stabilization lagoons. The name actually describes the function and refers to essentially the same system for treatment of water-borne wastes from either domestic or industrial sources, or, in many instances, a combination of the two with varying degrees of previous treatment, or none. Numerous adaptations of the basic theory are applied to these systems today, all with the object of rendering the waste material innocuous to the surrounding environment. It is interesting to note that the ultimate design of any one of these impoundments is undertaken with the idea in mind of an adaptive biological population establishing itself in

the impoundment, thereby ultimately degrading the waste materials.

The exact date of the beginning of use of ponds for this purpose predates antiquity. Fish cultivation ponds which were fertilized with waste material to encourage algal growth were known to exist in the Orient and in Europe since ancient times (58). Such ponds employing human waste are not used in the United States at the present time for this purpose. In the southwestern part of this country, the earliest recorded application of this type of waste treatment was Mitchell Lake, near San Antonio, Texas (76). It was used in the first decade of this century, and had an average depth of four to five feet and a surface area of 680 acres. Since then numerous ponds have been constructed for municipalities and/or certain industrial sites because of their relatively lower cost and simplicity compared to conventional processes.

There are several classes of bio-oxidation ponds. According to Oswald (56), "anaerobic lagoons" are those in which fermentative processes predominate. These processes are essentially catabolic (biodegradation) processes as opposed to anabolic (synthesis) processes. Fermentative processes and respiratory processes involve basically

differences in the chemical mechanism of biodegradation, namely the oxygen or hydrogen acceptor mechanisms. Facultative lagoons support anaerobic fermentation and photosynthetic reduction; aerobic oxidation processes occur at different rates and times, or consecutively. High rate ponds produce a balance between oxidation and photosynthetic processes yielding complete aerobic stabilization. It is in this last-mentioned class that usually an excessive amount of algae is produced.

The ponds do not necessarily have to be designed for such operational parameters. On the contrary, several factors may dictate whether the pond will operate in an anaerobic, aerobic, or facultative manner. Among these factors are light, depth, volume, loading, C:N:P ratios, and in some cases, season of the year. Fitzgerald (21) maintained that at least three of these factors - weather, light, and pond loading - change the predominant organism in the pond. If this is the case, then it is not difficult to understand why the entire ecosystem would operate as a different type of unit operation. With dense algal blooms and adequate depth, light penetration for activation of the photosynthetic processes may not occur at a depth greater

than one foot (7) since photosynthetic rates are proportional to light intensity up to 1,000 foot-candles (72). Above this value, photosynthetic rate depression occurs. According to Myers (54) the full complement of solar radiation is 10,000 foot-candles. A generally accepted figure for retardation and/or cessation of photosynthesis is 100 foot-candles (7, 77). This would indicate that 99% of the incident light is filtered out at a depth of one foot. Towne (80) reported the same reduction at a depth of six inches, and by comparison to lakes, stated that an average of 23.5 feet of lake water is necessary for 99% reduction in intensity. Very unusual conditions would have to exist in a stabilization lagoon below the one-foot level for photosynthetic processes to occur. Were it not for circulation in the lagoons, anaerobic processes would predominate in any lagoon below the one to two foot level.

The very nature of the organisms which inhabit stabilization ponds indicates that a symbiotic relationship exists between the algae and the bacteria (2, 46). The mechanism, although far from being a simple reaction, involves the oxidation of organic (carbon containing) compounds. This results in a supply of available carbon dioxide, nitrogen, and

phosphorus, all of which are used by the algae in photosynthesis. Therefore, an overall increase in the total volume of cells and perhaps number of cells per unit volume may result. The important point is that the resulting predominant numbers of these bacterial and algal species will be non-pathogenic; pathogenic species of bacteria are at a competitive disadvantage and their numbers tend to be reduced.

It is generally recognized that domestic sewage contains enough macronutrients to support bacteria and at least some genera of algae. The question that often arises is which elements are limiting factors for growth. The answer to this has not been entirely clarified and must be approached by considering the immediate environmental conditions. If stabilization ponds behaved as lakes, then the answer would be obvious. From the work of Hutchinson (40) on lakes, phosphorus is in shortest supply; therefore, it may be a limiting factor. Gerloff (30, 31) on the other hand, doubted whether nitrogen and phosphorus were really limiting elements because of their relative concentrations. Stumm (79) supported the idea that phosphorus was the limiting element but approached the situation from a different viewpoint. He maintained that phosphorus was limiting because the blue-green algae were

able to fix atmospheric nitrogen. Wolterink (87) approached the question of phosphorus being the limiting factor from a far more realistic viewpoint. He maintained that low phosphorus concentrations cause decreases in the rate of glycolysis. The glycolytic cycle is itself an essential part of the metabolism of any cell. Some of the investigators who have supported the theory that nitrogen was the limiting element in the stabilization pond ecosystem were Gates (27) and Allen (2). They did this on the basis of its limitations on culture growth rates. Porges (67) believed both nitrogen and carbon to be limiting in nutrient level quantities while Fitzgerald (21) and Bogan (7) believed organic carbon to be limiting. At least one investigator, Pipes (62), has proposed that light and carbon dioxide were the most critical factors. These authors base their opinions on the relative supply of the elements in question. These considerations support my earlier statement, i.e., other conditions being equal, the consideration of the limiting nutrient is due largely to the immediately defined conditions in the pond in question. Raw sewage influent often has a C:N:P ratio of 10:1:1 while lagoon effluents have been shown (70) to be around 60:6:1. Unless this ratio is lowered by further removal it may well constitute nutritional pollution.

Work carried out on a number of waste stabilization lagoons in central Oklahoma (14, 70) showed interesting trends in the phosphorus content and plankton diversity. On occasions the effluent showed a higher total phosphorus concentration than the influent. During these periods no drastic changes in the bacterial counts or plankton counts appeared. This has also been shown by Porges (67) in Wisconsin ponds. He showed phosphorus concentrations to be lowest in summer and fall and highest in late winter and, at times, no phosphorus reduction at all in winter. Fitzgerald (20) also has shown a higher effluent phosphorus value and attributed it to dissolution of previously precipitated forms. Internal dominant activity is obviously the reason for fluctuations in these lagoons.

In all the lagoons tested in central Oklahoma, a great predominance of blue-green algae occurred in late summer to early fall. In many cases the only algal forms identifiable by standard plankton counts (3) were blue-greens. In relatively few instances did the blue-greens extend their activity into the winter months (14, 70). Palmer (59) believed the blue-greens to be seasonal insofar as Anabaena and Microcystis were concerned. He further stated that

these two genera were supposed to appear in November and December. Porges (67) maintained that there is similarity between algal species in ponds regardless of the geographical location. This is indeed a significant point because then the possibility exists of placing the same parameters on all lagoons regardless of location.

The question then arises whether during times of predominant blue-green planktonic activity, these organisms degrade the complex forms of phosphates or whether the bacterial population accomplishes this. The complex phosphates in question are the two most commonly used commercially as builders in synthetic detergents and the principal phosphate used for water works corrosion control measures. These are tetrapotassium pyrophosphate, sodium tripolyphosphate, and sodium hexametaphosphate, respectively. Neel (55) stated that algae were capable of reducing the phosphate level in a lagoon as shown by comparisons during periods of aerobiasis and anaerobiasis. This daily fluctuation can easily be assessed. The immediate problem is not one of daily fluctuations because lagoons are designed for much longer retention times, but rather over extended periods of time. Abbott (1) claimed that various planktonic forms in lakes can derive their phosphorus nutrition from complex polyphosphates or

organic phosphorus compounds without the aid of the dissolved phosphate equivalent intermediary stage.

My research is, therefore, an attempt to determine the condensed phosphate degradation activity and assimilative capacity of various representative species of blue-green and green algae and, for comparison, the hydrolytic activity of domestic sewage with its usual biological complement. The algal cultures used in this investigation have been limited strictly to single species and, as far as practical under laboratory conditions, are bacteria-free.

CHAPTER II

THE CONDENSED PHOSPHATES

Phosphorus bearing compounds have been known to man since the time of Aristotle (19). Although the elemental form was not discovered until 1669 by a method of distillation, numerous entries in the literature indicate that compounds of phosphorescent nature have been studied and observed. The total role of this element in the metabolism of plants and animals is being investigated continuously. Among the most obvious uses of phosphorus are the following: It is a fundamental plant nutrient, an essential part of nerve and brain substance, and a decisive factor in muscle action and cell growth. Its principal role is in the formation of compounds for the transfer and storage of energy. Actually, elemental phosphorus never occurs free in nature (34). Naturally occurring compounds of phosphorus are apatite and phosphorite, both of which are subject to biological degradation if a sufficient quantity of water is present. When this degradation occurs, especially in the

presence of other organic compounds, a phosphorescent gas may be seen above the area at night. This has long been termed the "ignis fatuus." The cause of this natural phenomenon has been suggested to be phosphine contaminated with other phosphorus compounds such as P_2H_4 igniting spontaneously (87). Gloyna (33) suggested that hydrogen phosphide is lost to the atmosphere from lagoons, accounting for at least some of the reported losses. The hydrosphere holds many forms of phosphorus chemicals. Among them are (1) soluble ionic phosphate phosphorus, (2) dissolved or colloidal organic phosphorus, (3) particulate organic and (4) acid soluble inorganic particulate phosphorus which exists mostly as ferric phosphate. Wolterink (87) claimed that the concentration of ionic phosphate is extremely small, having an order of magnitude of 1×10^{-3} to 2×10^{-3} mg/l. Particulate phosphorus compounds are thought to be those which are retained on a 0.45 micron membrane filter (61, 71).

It is entirely possible for complex forms to be constructed in nature. Grogan (34) suggested the following relationships. Elemental phosphorus burning in air yields phosphorus pentoxide.

$$4P + 5O_2 \longrightarrow 2P_2O_5 \qquad (2.1)$$

This, in turn, combines readily with water to form phosphoric acid.

$$P_2O_5 + 3H_2O \longrightarrow 2H_3PO_4 \qquad (2.2)$$

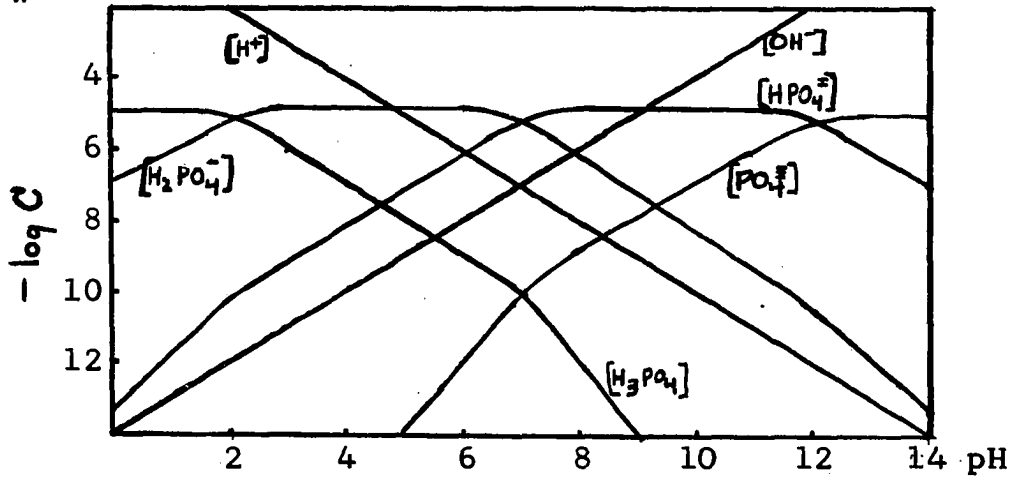
The equilibrium of phosphoric acid is almost entirely pH dependent. Sillen (75) showed the complexity of the existing ionic forms of phosphoric acid in his treatise of graphic presentation of equilibrium data. There are actually three equilibrium constants for phosphoric acid as shown by the following dissociation relationships:

$$H_3PO_4 \longrightarrow H^+ + H_2PO_4^-, \quad pK_1 = 2.12 \qquad (2.3)$$

$$H_2PO_4^- \longrightarrow H^+ + HPO_4^{2-}, \quad pK_2 = 7.21 \qquad (2.4)$$

$$HPO_4^{2-} \longrightarrow H^+ + PO_4^{3-}, \quad pK_3 = 12.3 \qquad (2.5)$$

A graphical presentation of these equilibria in the 10 mg/l concentration range looks similar to the following, assuming $pK_w = 14$, and $C = 10^{-5}$ M.



Partial dehydration of the $H_2PO_4^-$ can occur as indicated by the following reaction which includes the monosodium salt of the anion (34).

$$2NaH_2PO_4 \xrightarrow{-H_2O} Na_2H_2P_2O_7 \qquad (2.6)$$

This is disodium pyrophosphate which, upon further dehydration, produces sodium trimetaphosphate by the following reaction:

$$Na_2H_2P_2O_7 \xrightarrow{-H_2O} (Na_3PO_3)_3 \qquad (2.7)$$

The latter compound can fuse to form sodium hexametaphosphate.

$$n(Na_3PO_3)_3 \xrightarrow{heat} (NaPO_3)_n \qquad (2.8)$$

It is clear then that the two terms, pyro and meta, mean the loss of one molecule of water from two molecules of orthophosphates and the loss of one molecule of water from one molecule of orthophosphate, respectively.

Commercial production of elemental phosphorus, the earth's eleventh most abundant element (34), and that of phosphorus bearing compounds in the United States is increasing in magnitude. Complex phosphates used in detergents and water softening during 1950 amounted to 3.6 lbs/capita (as P_2O_5) and 16 lbs/capita in 1955 (17). In 1962 alone, 451,970 short tons of elemental phosphorus were produced (19).

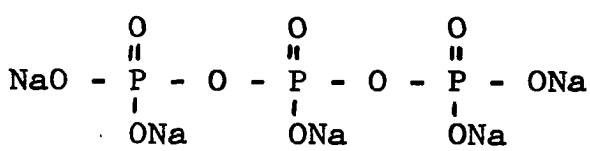
Much of this quantity went into the manufacture of synthetic detergent builders. These complex phosphates are not new to this country. Phosphate cleaners have been in use for many years, and all-purpose synthetic detergents containing phosphates were commercially available. By 1962 they accounted for more than 90% of all the agents used for household cleaning purposes such as dishwashing and laundering (11). By comparison, an adult requires slightly more than a pound of phosphorus per year as a maintenance standard (38).

In today's heavy-duty, non-liquid synthetic detergents there are only two complex phosphates which find use as detergent builders. These are sodium tripolyphosphate (STPP), and to a lesser extent, tetrasodium pyrophosphate (TSPP). In the liquid heavy-duty synthetic detergents, the phosphate which is used is tetrapotassium pyrophosphate (TKPP). In some instances STPP may also be used; however, this is rather rare because of the lower solubility of the sodium salt. Sodium hexametaphosphate (SHMP) has not been used in synthetic detergents but is a widely used corrosion inhibitor (22, 60). Other uses of this chemical will be discussed later in the chapter. They are all sequestering agents, being water soluble after the chelate is formed (48).

Sodium Tripolyphosphate

In addition to its invaluable use as a builder it is also used in the following processes and products (22): water softening, viscosity reduction of oil well drilling muds, clay dispersant for paper coating, clay slips for ceramics, bleach "stripper" in laundries, leveling agent in dyeing, Kier boiling cotton, wool scouring baths, silk degumming, scouring rayon and nylon, water softener in bubble baths, mineral supplement for cattle, pickle for moisture and color retention in curing meats, whipping agent for dried egg whites in pastry, penetrating agent for salting unshelled peanuts, skin softening agent for canned peas, beans, etc., and as an aid in sealing leaky farm ponds, industrial waste lagoons, reservoirs, and irrigation canals.

At 25° C, the solubility of STPP is 18.1 grams per 100 grams water. Determined at an initial pH of 10, 100 grams of STPP will sequester 13.4 grams of calcium, 6.4 grams of magnesium, and 0.18 grams of iron (22). It has a molecular weight of 367.93 and an empirical formula of $Na_5P_3O_{10}$. Structurally the molecule is thought to be the following (48):

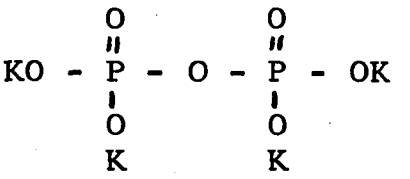


At 30° C the half-life of STPP in 1% solution shows relative stability over a wide pH range (23): at pH = 4, 37.5 days; at pH = 7, 208 days; and at pH = 10, 625 days.

Tetrapotassium Pyrophosphate

This chemical does not have the wide applications that STPP has. Among its uses are the following (22): builder for potash soaps, softener in high-pressure boiler water, ingredient in making low-temperature synthetic rubbers, buffer in electroplating solution, clarifier for liquid soaps and shampoos, rinsability agent in textile processing and in making textile oils and soaps, stabilizer in peroxide baths, processing agent in purifying china clay, and processing agent in latex-based or water-emulsion paints.

Normal tetrapotassium pyrophosphate (TKPP) has an empirical formula, $K_4P_2O_7$, and a molecular weight of 330.34. Its solubility at 25° C is 191 grams per 100 grams of water. The sequestering power of this chemical is not as great as that of STPP. With an initial pH of 10, 100 grams of TKPP sequesters 3.8 grams of calcium, 6.7 grams of magnesium and 0.2 grams of iron (22). The structure of a molecule of this chemical is thought to be the following (48):



At 30° C the half-life of this chemical, in 1% solution, is only reported for a pH of 4 and is 250 days. By comparison, the half-life of this chemical in liquid detergent at a pH of 8.5 is 400 months (21° C) and 8 months (49° C), and at a pH of 11.0 the values are 6,000 months (21° C) and 110 months (49° C) (23).

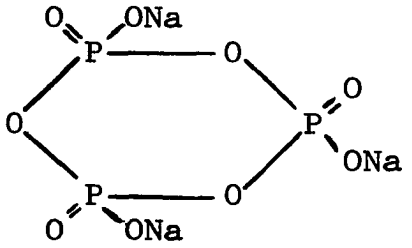
Sodium Hexametaphosphate

This chemical is one of a group called the glassy phosphates. Their uses are wide and varied, among which are the following (22): threshold treatment in corrosion prevention in water systems, pH adjustment and softening boiler water, scale control in water systems, prevention of red water systems, viscosity reduction of oil well drilling muds, formulation of dishwashing compounds, dairy cleaners, laundry mixes, and industrial cleaner formulations, commercial laundry water softener, prevention of lime soap deposits in textile sizing, delustering, dyeing and printing, stabilizing hydrogen peroxide bleach liquors, controlling pitch in making pulp and paper, emulsifier in cheese pro-

cessing, sequestering agent in removing fruit insecticides, washing fruits and vegetables, tanning leather, developing photographic film, mortar and cement additive, metal cleaning before plating or painting, processing eggs, mineral froth flotation, pigment dispersion, additive to curing pickle for precooked meats for color and moisture retention, whipping agent for dried egg whites, and an ingredient in non-dairy coffee "creams."

Attention should be given to the nature of the name sodium "hexametaphosphate." It actually describes a glassy phosphate mixture of various chain lengths with an average length of 13 ($NaPO_3$) units. Nonetheless, the product continues to be known as "hexametaphosphate." The widely used formula $(NaPO_3)_n$ is not entirely correct since it ignores the Na or H atoms on the chain ends (23). A more nearly correct, but less convenient, expression is $(NaPO_3)_n \cdot M_2O$, in which M represents the Na and H atoms on the chain ends. For convenience the brand names "Hexaphos," "Sodaphos," and "Glass H," are applied to the glassy phosphates having the respective empirical formulas $(NaPO_3)_{13}$, $(NaPO_3)_6$, and $(NaPO_3)_{21}$. The abbreviation of the first, (SHMP), corresponding to the formula $(NaPO_3)_{13}$ is used in this work.

The sequestering values of this group are higher than STPP and TKPP for calcium. For 100 grams of SHMP, 19.5 grams of calcium, 2.9 grams of magnesium, and 0.031 grams of iron are sequestered (22). The pH of a 1% solution of SHMP is 6.7 while the pH of 1% solutions of STPP and TKPP are 9.7 and 10.2 respectively. A typical structural composition for the $(NaPO_3)_3$, or trimetaphosphate unit, basic to all glassy phosphates, including SHMP, is thought to be the following (48):



The reversion time to the ortho form is understandably longer as the chain length increases.

Mechanism of Hydrolysis

The following equations have been suggested for the hydrolysis of some of the condensed phosphates in water (23). Glassy phosphates:

$$(NaPO_3)_n + H_2O \longrightarrow (NaPO_3)_3 + NaH_2PO_4 + (NaPO_3)_{n-4} \quad (2.9)$$

Trimetaphosphates:

$$(NaPO_3)_3 + H_2O \longrightarrow Na_3H_2P_3O_{10} \quad (2.10)$$

$$Na_3H_2P_3O_{10} + 2H_2O \longrightarrow 3NaH_2PO_4, \text{ or} \quad (2.11)$$

$$Na_3H_2P_3O_{10} + H_2O \longrightarrow Na_2H_2P_2O_7 + NaH_2PO_4 \quad (2.12)$$

Tripolyphosphates:

$$Na_5P_3O_{10} + H_2O \longrightarrow Na_3HP_2O_7 + Na_2HPO_4 \quad (2.13)$$

Pyrophosphates:

$$Na_2H_2P_2O_7 + H_2O \longrightarrow 2NaH_2PO_4 \quad (2.14)$$

$$Na_3HP_2O_7 + H_2O \longrightarrow NaH_2PO_4 + Na_2HPO_4 \quad (2.15)$$

$$Na_4P_2O_7 + H_2O \longrightarrow 2Na_2HPO_4 \quad (2.16)$$

There are several interesting points to be considered from the above equations, since the mechanism of hydrolysis, as shown, might well be the same mechanism involved in biological hydrolytic degradation of these compounds. Any polyphosphate formed in the initial reaction, that is, the trimetaphosphate in the SHMP equation, next hydrolyzes according to its own equation. Only the pyrophosphates hydrolyze entirely to orthophosphates in one step; the others can form polyphosphate intermediates in addition to orthophosphates. In the case of SHMP the trimetaphosphate intermediate itself does not have any water-softening

ability but it hydrolyzes to phosphates that do. The useful life of STPP per se is difficult to determine, because the disappearance of the tripolyphosphate ions is accompanied by appearance of pyrophosphate ions. Van Wanzer (83) reported degradation rates of these and other similar phosphates.

The rate of hydrolysis of the condensed phosphates in lagoons is not only affected by biological systems but physiochemical factors as well. Increases in temperature invariably increase hydrolysis rates. The optimum pH varies between the different condensed forms but is in the approximate range of 7-11, that normally encountered in sewage. The presence of calcium ions or excess sodium ions usually tends to increase the rate. Occasionally magnesium ions tend to reduce it. Polyvalent metals generally increase hydrolysis. Comparatively, a concentration increase tends to reduce the rate in the parts per million range while an increase in the per cent range increases the rate. The longer the chain length, the greater the stability. In addition, clay or other suspended matter present increases the rate. The mechanism of this breakdown is unknown. Other conditions being equal, the longer the retention time the greater the amount of reduction to ortho forms. Thus,

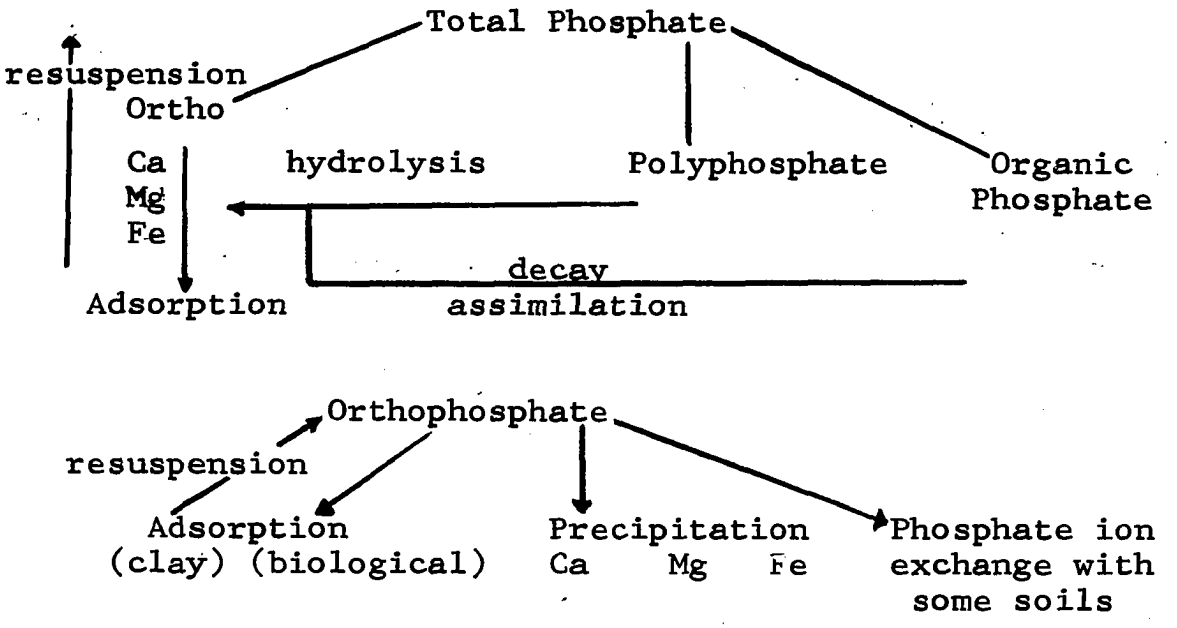
time is a most important factor (11, 23). It has been reported (74) that all three of the aforementioned phosphates exert biochemical oxygen demand values of from 0 to 25 parts per million.

Phosphorus Cycle

The route that phosphorus takes in lakes is a fairly complicated one, yet is known (40). During the summer months, if stratification has occurred, the phosphorus is liberated into the epilimnion from decaying vegetation of the littoral region. At the same time existing littoral vegetation takes up some of this phosphorus. Phytoplankton assimilate additional quantities and release soluble complex forms, less assimilable than ionic phosphate. These forms slowly hydrolyze to the ionic forms. Sedimentation of phytoplankton and other phosphorus-containing seston and fecal pellets from non-protist groups into the hypolimnion constantly occurs. Phosphorus compounds are liberated into the hypolimnetic areas from these sediments or when the sediments reach the mud-water interface. At this point the phosphorus can diffuse from the mud into the waters above if the superficial layer of mud lacks an oxidized microzone. During the time that the body of water stagnates (following

cyclic turnover) equilibrium conditions are attained as far as phosphorus concentrations are concerned. Numerous phosphorus turnover times have been reported (5, 61, 66, 64, 71) varying from values approaching zero to as high as 20 mgP/cu meter/hr, with corresponding times of from a few seconds to up to 15 days. Gest (32) reported that recirculation times for phosphorus are greater in the light periods than in the dark.

In waste stabilization lagoons, the respective depth and the vast quantities of organic and inorganic nutrients, particulate matter, and microorganisms may prevent such a clear cut phosphorus cycle from occurring. Losses from these systems are obviously due to effluent discharge, percolation, and precipitation and not so obviously due to effusion of hydrogen phosphide (33) and aquatic insect emergence (81). Somewhat less than one percent of the total organic matter deposited annually is lost this way. So far as spontaneous gains are concerned, Chalupa (10) found up to 0.16 ppm P_2O_5 added to reservoirs by atmospheric precipitation. Hurwitz (39) outlined a cycle similar to the following one:



The interaction of calcium, magnesium, and iron with orthophosphate occurs at higher pH values. Since algal activity in established stabilization lagoons is more than likely continuous, it is reasonable to assume that a considerable quantity of calcium-, magnesium-, and ferric phosphate precipitates. This is due to the pH change caused by algal activity (41). Resuspension of these forms could be accomplished by a decrease in the pH or by depletion of phosphate content in the surface water. The latter is improbable if the waste is domestic sewage.

Total phosphorus loadings (as P) of lagoons in the central Oklahoma region ranged (70) from 0.4 mg/l to over 9.3 mg/l.

CHAPTER III

THE BLUE-GREEN AND GREEN ALGAE

Division Cyanophyta

The approximately 1,500 species of the Division Cyanophyta, or blue-green algae, comprise a distinctive group sharply delimited from other algae in a number of respects. They are the only algae in which pigments are not located in definite intracellular structures (called chromatophores). Chlorophyll-a is the only type contained. Pigmentation other than chlorophyll-a is due to carotenes, xanthophylls, c-phycocyanin, and c-phycoerythrin. The "central body" of each blue-green algal cell is different from ordinary nuclei in that it lacks a nucleolus and a nuclear membrane. It is sometimes called an incipient nucleus and oftentimes appears less dense than the surrounding protoplasm. Reproduction among the blue-green algae is accomplished by fission, the simplest and most primitive of all types. These organisms lack flagellated reproductive

cells and gametic union. An important distinguishing characteristic of unicellular, colonial, or filamentous forms of this Division is the unusual thickness of the wall surrounding the protoplasm. This is a stratified, or laminated structure consisting of an inner firm portion and an outer gelatinous region. Species identification sometimes depends on the thickness of the gelatinous portion. In filamentous forms this outer wall is termed a sheath.

A person knowledgeable in elementary bacterial physiology would concede the point that, excepting pigmentation, there are relatively few morphological differences between the blue-green algae and bacteria. At least one investigator (25) has found similarity between the blue-greens and gram-negative bacteria. The presence of muramic acid in the cell walls of blue-greens, bacteria, and actinomycetes, but not in other organisms adds weight to the suggestion that these forms are not distantly related. An interesting point to be considered regarding the protoplasm of the filamentous forms was reported by Gusev (35), who claimed that the protoplasm was not in motion. This might be a partial explanation for the comparatively lower metabolic rate and temperature dependence (42) of some of the blue-green forms.

Division Chlorophyta

The green algae number over four times as many species as the blue-greens. The majority of the nearly 7,000 species are freshwater inhabitants; the other species are either marine or terrestrial in nature. Chlorophyll-a and chlorophyll-b both are present, as in the higher plants, along with xanthophylls and carotenes. Individual plants are either unicellular, filamentous, or non-filamentous colonies. The cell walls of green algae are more rigid than those of the blue-greens, consisting chiefly of cellulose and lacking the thick gelatinous sheath. The protoplasm contains a distinct walled nucleus, one or more vacuoles, and one or more chloroplasts. Pyrenoids, or small masses of reserve protein, are found within the chloroplasts, and serve as centers of formation of starch, the principal food stored. Spore formation, fragmentation, and sexual reproduction are the reproductive methods of these organisms. Motile spores and fragment forms are not unknown among the Chlorophyta.

Test Organisms

In order to insure the reproducibility of cultures, all the species used in this investigation were obtained from the culture collection of algae at the University of

Indiana (77). The blue-green algae used for this investigation were representative of genera found in waste stabilization lagoons in the southwest (14). In an extensive coverage, Vinyard (84) found over 640 species and varieties of green algae and 63 species of blue-green algae in the State of Oklahoma, exclusive of waste treatment waters. His work was done on naturally-occurring bodies of water. Since plankton counting is usually reported by genera (3), the corresponding numbers of genera in Vinyard's work were 29 of blue-green algae and 94 of green algae. These numbers of algae all have the potentiality of becoming involved in waste stabilization lagoons. However, relatively few genera are usually seen (14). The blue-greens used for this work were Anabaena cylindrica, Anacystis nidulans, Gloeocapsa alpicola, Oscillatoria borneti, O. chalybia, O. formosa, O. tenuis, and Phormidium faveolarum. Other cultures such as Spirulina sp., Chroococcus sp., and Merismopedia sp. were attempted but could not be used because of persistent gross bacterial contamination or their inability to acclimate themselves to the growth medium.

Members of the Division Chlorophyta used in this work were Chlorella pyrenoidosa, C. vulgaris, Scenedesmus obliquus, S. quadricauda, and Ulothrix fimbriata. Spirogyra sp. was

unable to acclimate to the growth medium used and was therefore not included. Duplication of genera was done purposely to attempt to determine whether or not any metabolic similarity between species existed insofar as phosphate metabolism was concerned.

Table 1 contains research reports dealing with the general topic of metabolic studies and observations of blue-green and green algae and waste treatment. It is clear from the reports summarized in Table 1 that quantitative evaluations of phosphate metabolism are sketchy. A recent report by Maloney (47) showed evidence that the organism Chlorella pyrenoidosa is stimulated by synthetic detergent and that the sodium tripolyphosphate builder is responsible for the stimulation. The limit of syndet stimulus was 3.66 mg/l. Beyond this limit the growth rate was actually inhibited. The minimum amount of the STPP necessary to promote algal growth was shown to be dependent on the intracellular phosphorus concentration, 0.1 mg/l causing exponential growth. Maloney's conclusion was that the organism is capable of utilizing STPP directly or possesses the necessary extracellular enzymes to hydrolyze or accelerate hydrolytic breakdown of STPP to the more usable ortho form. Another

TABLE 1

SOME INFORMATION PERTINENT TO BLUE-GREEN AND GREEN ALGAE IN AQUATIC WASTE TREATMENT SYSTEMS

	Information	References
1.	Phosphate can be removed in secondary sewage treatment by green algae.	21, 55, 86
2.	Phosphorus facilitates the assimilation of nitrogen.	68, 79
3.	Numbers and kinds of algae increase as depth increases. *Anacystis* sp. decreases.	59
4.	Polyphosphates have been demonstrated in various blue-green algae.	35, 43
5.	Excess of medium phosphate causes a "luxury" uptake in blue-greens but cell content of greens remains constant.	30, 43, 44
6.	*Scenedesmus quadricauda* can use condensed phosphate forms only through bacterial intervention.	2, 58
7.	Sewage has macronutrients necessary for algal growth but is not a balanced diet.	7, 57
8.	Extended anaerobiasis permits phosphate accumulation in lagoons.	55
9.	Blue-greens reported in waste-water environments:	
	a) *Oscillatoria* sp.	14, 27, 53, 55
	b) *Phormidium* sp.	14, 25, 55
	c) *Lyngbya* sp.	55
	d) *Aphanizomenon* sp.	55

TABLE 1--Continued

Information	References
e) *Anacystis* sp. (*Microcystis* sp.)	9, 12, 14, 24, 29, 31, 42, 43, 59, 88
f) *Spirulina* sp.	14, 53
g) *Anabaena* sp.	12, 24, 42, 45
10. Green algae reported in waste-water environments:	
a) *Chlorella* sp.	2, 7, 21, 26, 32, 62, 85
b) *Scenedesmus* sp.	2, 7, 21, 32, 85
11. Secretion and/or excretion of large quantities of substances into the surrounding medium is commonplace among blue-greens.	35, 65
12. Tertiary sewage treatment would require 20 hours of processing for complete removal of phosphate expected in secondary sewage plant effluents.	85
13. One milligram of phosphorus in the water is capable of accompanying production of 75 mg of organic material.	68, 79
14. Substrate phosphorylation is intimately involved with the glycolytic cycle and tricarboxylic acid cycle. The Embden-Meyerhof Pathway produces 8 ATP's for a total of 38.	44, 87

TABLE 1--Continued

	Information	References
15.	Blue-greens are known to produce bactericidal substances against Staphylococcus sp. and Clostridium sp.	49, 68
16.	Chlorella sp. does metabolize dissolved organic material in absence of bacteria.	62
17.	Scenedesmus quadricauda utilizes bicarbonate ion as a carbon dioxide source. Chlorella pyrenoidosa cannot.	63
18.	Blue-greens have a higher optimum growth temperature than do greens.	42
19.	Phosphate turnover is much greater in the light than in the dark.	24, 32
20.	Assimilation of 1 mg/l phosphorus accompanies the metabolism of 25-50 mg/l carbon and 2-12 mg/l nitrogen.	6
21.	Blue-greens begin their growth in the nutrient rich bottom and continue in the deficient top regions causing blooms.	30
22.	Some blue-greens begin a "bloom" in surface waters and sink to the bottom to develop large actively growing colonies.	9
23.	Chlorella sp. cell wall enzymes hydrolyze pyrophosphates. These are adaptive enzymes. Polyphosphates up to a chain length of 55 support growth.	26, 47
24.	A critical phosphate concentration for blue-green "bloom" formation is:	
	a) 0.01 mg/l	12
	b) 0.18 mg/l	29

TABLE 1--Continued

	Information	References
25.	The BOD to phosphorus requirement in aerobic systems is 100-300:1.	8
26.	Orthophosphate concentration reduced from:	
	a) 20 mg/l to 5 mg/l in less than 4 hours by green algae.	8
	b) 80% to 90% in 2 hours by green algae.	7
	c) 67% in 5 hours by attached forms.	69
27.	The temperature optimum for several blue-green algae including *Anabaena* sp. and *Anacystis* sp. is above 30° C.	42

conclusion that he reached was that Chlorella pyrenoidosa sorbs phosphorus in excess of its normal metabolic requirements and the amount sorbed is related to the phosphorus content of the surrounding medium. This would indicate an important concept in stabilization pond mechanisms. If an almost unialgal culture exists, as oftentimes is the case, the algal cells may actually sorb quantities of phosphorus from the lagoon showing an overall reduction; but the cells contained in the effluent will be carrying a form of nutritional pollution downstream from the lagoon.

The question of why certain lagoons show almost unialgal cultures during certain times of the year can be at least partially answered by the work done by Hartman (36). Table 2 shows, in part, results of different species of green and blue-green algae being placed together in cultures. The table gives a hint of the multiple chemical limiting factors which probably exist in waters, especially nutrient-rich waste stabilization lagoons, as a result of algal metabolism.

TABLE 2

EFFECTS ON GROWTH AND DEVELOPMENT OF
ALGAE WHEN UNIALGAL CULTURES ARE MIXED

	Test Organism	Interacting Organism	Observed Effects
1.	Chlorella vulgaris	Scenedesmus quadricauda	Inhibition in mixed culture.
2.	Chlorella pyrenoidosa	Scenedesmus quadricauda	Acceleration by filtrate
3.	Scenedesmus quadricauda	Phormidium uncinatum	Stimulation of young culture - inhibition of old culture
4.	Scenedesmus obliquus	Microcystis aeruginosa	Inhibited
5.	Phormidium uncinatum	Scenedesmus quadricauda	Inhibited
6.	Anacystis nidulans	Chlorella vulgaris	Inhibited
7.	Anacystis nidulans	Scenedesmus quadricauda	Inhibited
8.	Microcystis aeruginosa	Scenedesmus obliquus	No effect

CHAPTER IV

EXPERIMENTAL MATERIALS AND METHODS

The original intent of this investigation was to attempt to show if unialgal cultures of blue-green algae would degrade condensed phosphates. The first problem encountered was the selection of a chemically defined growth medium which would support optimum growth of both blue-green and green algae. Numerous nutrient media for algal culturing have been cited in the literature. Starr (77) cited a few for culturing algae. Since the nutrient conditions in a waste stabilization lagoon were under consideration, it was decided that if the inorganic chemical constituents of domestic sewage could be approximated, a desirable medium could then be constructed. Table 3 shows the composition of the medium which was used throughout the study. The inorganic constituents were included in kind and proportion to approximately coincide with the analysis report of domestic sewage secondary treatment effluent cited by Bogan (8). The solution of Hutner's trace elements

TABLE 3

COMPOSITION OF MODIFIED
SYNTHETIC SEWAGE (MSS) MEDIUM

Compound	Amt/l Medium
$Ca(NO_3)_2$	60 mg/l
KNO_3	70 mg/l
NH_4Cl	57 mg/l
KH_2PO_4	20 mg/l
$Na_2SiO_3 \cdot 5H_2O$	20 mg/l
NaCl	70 mg/l
$MgSO_4$	20 mg/l
$NaHCO_3$	125 mg/l
$Na_3C_6H_5O_7 \cdot 2H_2O$	250 mg/l
Hutner's Trace Elements	1 ml
Tap Water	to make 1 liter

was added to supplement the necessary micronutrients necessary for algal growth (82).

Hutner's trace element solution consisted of the following chemicals (in grams per liter);

EDTA - 5.0; $ZnSO_4 \cdot 7H_2O$ - 2.0; H_3BO_3 - 1.0; $CaCl_2 \cdot 2H_2O$ - 0.662; $MnCl_2 \cdot 4H_2O$ - 0.50; $FeSO_4 \cdot 7H_2O$ - 0.50; $CoCl_2 \cdot 6H_2O$ - 0.15; $CuSO_4 \cdot 5H_2O$ - 0.15; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ - 0.10. These salts were added to about 75-100 ml distilled water while stirring. The mixture was brought to a boil, allowed to cool to room temperature, and the pH adjusted to 6.0 with KOH pellets. This was brought up to a volume of one liter with distilled water.

The tap water used in making MSS medium had a pH of 9.0 - 9.5 and showed an average chemical content of the following: chloride - 12.5 mg/l; sulfate - 33 mg/l; iron - 0.0 mg/l; hardness (as $CaCO_3$) - 19.0 mg/l; alkalinity - 66 mg/l carbonate and 245 mg/l bicarbonate. The pH of MSS medium was 8.7--too high for optimum algal acclimation to new cultures; therefore the pH was adjusted to 5.7 - 5.8 with hydrochloric acid. After sterilization in the autoclave for 15 minutes at 17 psig the final pH was 7.5-7.8. This was ideal for algal culture.

There were two sequestering agents present in MSS medium, namely EDTA and sodium citrate. The final concentration of EDTA was 5 mg/l, scarcely enough to sequester the quantities of calcium and other metal ions present. The use of 250 mg/l of sodium citrate had a distinct advantage in that it prevented precipitates from being formed at alkaline pH when the algal metabolism raised the pH of the medium. Kratz and Myers (42) supported the premise that citrate ion was necessary to obtain reproducible results and, in their work with Anacystis nidulans maintained that it was necessary to obtain maximum growth rates.

When mixed as indicated, MSS medium delivered 19.98 mg/l nitrogen (as N) and 4.55 mg/l phosphorus (as P), giving a ratio of N/P of 4.4:1.

Acclimation of the stock cultures to this medium took a little more than two months. After that time subculturing was done on MSS agar plates using slightly less than the prescribed 1.5% Bacto-agar to insure against rapid dessication. This aim was also aided by pouring thicker plates than are ordinarily used for bacteriological work. The moisture-laden agar allowed the algal cells, which were streaked on the plate with a flamed inoculating wire loop,

to generate into their typical growth patterns. Filamentous greens and blue-greens grew out, and away from the original streak. In this manner it was possible to pick off cells from the extended filaments in the hopes that relatively bacteria-free cells could be isolated. In the case of the unicellular and colonial forms, such as Anacystis nidulans and Gloeocapsa alpicola, it was not as simple since these forms are not spreaders but grow on agar plates in a fashion similar to that of bacterial colonies. Cells taken from the agar plates were placed in 125 ml Ehrlenmeyer flasks containing 100 ml of MSS medium.

If turbidity appeared within 72 hours after inoculation of the flasks the attempt at isolation of a "pure culture" was a failure and the procedure was attempted again. The turbidity which, more often than not, appeared in the flasks was due to gram-negative bacilli probably originating from the thick gelatinous sheath of the blue-green algae. The same type of contaminant was found among a few of the green algae used but was relatively easy to eliminate by the above-mentioned technique. A full year of transfer and subculturing transpired before a clear supernatant was obtained. Additions of 0.1 - 1.0 mg/l streptomycin, neomycin, and

dihydrostreptomycin aided slightly in ridding the cultures of the bacilli. Overdoses (above 1.0 mg/l) of any one of these antibiotics tended to inhibit algal growth and in some cases had an algaecidal effect. At least one other investigator (78) claimed to have had great difficulty in obtaining bacteria-free blue-green cultures.

No claim is made that the cultures used in this investigation were literally "bacteria-free" as are reported so many times in the literature. The purpose of the isolation and transfer routine was to obtain cultures which were as bacteria-free as possible in order to show as nearly as possible, by laboratory means, the true phosphorus metabolism of these algae.

Phosphate Determination

Several different colorimetric tests for phosphorus were considered before a suitable one was found. The usual test for orthophosphate and the condensed forms, such as poly-, pyro-, and meta-, is based on the formation of a blue complex, molybdophosphoric acid. This is the basis of the stannous chloride test found in Standard Methods (3). This test is more sensitive than the amino naphthol sulfonic acid method given in the same book. Since maximum sensiti-

vity was very desirable the amino naphthol sulfonic acid method was rejected. The stannous chloride method was abandoned for several reasons. A more sensitive modification of the test was found which had fewer interferences from other chemicals in the test solution.

The method for determining orthophosphate was taken from Murphy and Riley (52) and incorporates potassium antimonyl tartrate as a molybdophosphoric acid complex intensifier. The atomic ratio of antimony to phosphorus is 1:1, resulting in a complex which is stable up to 24 hours. The colorimetric determination of phosphate was done with a Bausch and Lomb Spectronic-20 (transistor model) spectrophotometer at 882 millimicrons. Beer's law is obeyed up to 2 mg/l. Murphy and Riley (52) maintained that the stannous chloride method hydrolyzes some organic phosphorus compounds during the color development period, causing error. Since the original method was developed for seawater it was shown that the salt error in this method (52) was less than 1% while that of the stannous chloride method (3) was almost 15%.

The method for total phosphate was chosen from that given by Menzel and Corwin (50) and is simply an extension

of the orthophosphate method by Murphy and Riley (52). Menzel and Corwin (50) claimed that virtually all the phosphorus bonds are broken by oxidation by potassium persulfate ($K_2S_2O_8$). It was found that autoclaving the samples at 250° F and 15-17 psig for thirty minutes hydrolyzed all the phosphate to the ortho form. Tests were run using adenosine triphosphate to determine if all the bonds were broken by hydrolysis. This compound contains 2 P-O-P bonds and one P-O-C bond. Excellent results were obtained. Poor results were obtained in the total phosphate determination when the samples were heated in a boiling water bath for one hour as suggested as an alternate method (50).

A promising method which had to be rejected was the extraction method proposed by the Association of American Soap and Glycerine Producers (4). They separated the analysis into three parts, obtaining what they called orthophosphate, hydrolyzable, and total phosphate. It is assumed that their differentiation classifies PO_4 and simple P_2O_5 as ortho, $(P_2O_5)_n$ and R-(P-O-P) as hydrolyzable, and R-(C-P) and R-(P-O-C) as total organic. Great difficulty was encountered in obtaining reproducible results in the benzene-isobutanol extraction step for orthophosphate. Morgan (51) also had

difficulty and found some condensed forms of phosphate which he claimed were organic groups in the separation procedure. This was reason enough to reject the procedure since emphasis was being placed on a sharp line of demarcation between the ortho form and condensed forms.

In all the analyses during this investigation the samples were filtered through 0.45 micron membrane filters. This allowed colloidal and particulate phosphate compounds smaller than 0.45 to be analyzed in the total phosphate determination. Einsele (16) claimed that colloidal particles did not react with the acidified molybdate reagent. It is not known if he was referring to the ortho test or total test. Menzel and Corwin (50) claimed that they will be broken down by persulfate oxidation in the test for total phosphorus. In a recent article, Edwards, et al, (15) claimed that 99% of the orthophosphate color will be developed in four minutes by the Murphy and Riley method described herein.

One of the most important parts of the analysis for phosphate was not stressed nearly enough by Murphy and Riley or by Menzel and Corwin. Between runs, all the glassware involved in the test was subjected to acid scouring by filling with a solution of 1:10 HCl. There is no doubt that

various forms of phosphorus adsorb onto glass and other types of container walls. This can be redissolved by prolonged exposure to hydrochloric acid, chromic acid cleaning solution, hydrofluoric acid, or to a lesser extent, sulfuric acid. Hassenteufel, et al, (37) showed quantitative measurements of phosphorus on different types of container walls. He showed an optimum pH range of 7.5 - 8.0 for this to occur. Polyethylene and polyvinyl chloride apparently adsorb the phosphate three times faster than glass. Desicote (Beckman Company) was reported to be inefficient in preventing this from occurring.

An excellent quantitative separation technique for industrial phosphates has been shown by Etienne (18). Meta-, pyro-, poly-, and orthophosphates were separated as barium salts at increasing controlled pH.

Environmental Culture Controls

Several types of fluorescent lights and combinations of fluorescent and incandescent lights were tried before optimum lighting conditions were obtained. General Electric Cool White, Warm White, and Daylight Type were tried. Also a special fluorescent bulb for plant growth called Plant-Gro by Westinghouse (similar to Sylvania's Gro-Lux) was tried.

The best results were obtained using standard Daylight Type fluorescent bulbs and incandescent bulbs with a ratio of two watts fluorescent to one watt incandescent (28). The average light intensity on the two-liter Ehrlenmeyer flasks (Figures 1 and 2) was 250 - 300 foot-candles while that on the Chemostat flow-through columns (Figures 3 and 4) was approximately 200 foot-candles. Starr (77) used 500 foot-candles for rapid multiplication of his cultures but lowered the intensity significantly to 50 - 100 foot-candles for optimum growth.

The temperature in and around the culture flasks was maintained as near to 28° C as possible. Usually the temperature ranged from 24° C to 28° C due to the lighting cycle which was held at 16 hours on and 8 hours off for the duration of the tests. It was found that the green algae, and especially the blue-green algae, grew better at this slightly elevated temperature than at room temperature. It is not unusual to see prodigious blooms of blue-green algae in waste stabilization lagoons in the southwest in August when the lagoon temperature approaches, and in some cases maintains a temperature of 33° C.

Apparatus

The two-liter Ehrlenmeyer flasks shown in Figures 1 and 2 each contained 1,500 ml of MSS medium. They were stoppered with a three-hole #9 rubber stopper. One outlet in the stopper was permanently clamped while the other two served important functions. One of these contained an injection cap through which were added the solutions of condensed phosphate by means of a membrane filter Swinney-type hypodermic filter. Sterilization of the phosphate solutions was achieved in these cases by using a 0.1 micron pore size membrane filter. The other outlet in the stopper contained an attached calcium chloride drying tube in which was placed absorbent cotton. This allowed gaseous exchange with the atmosphere permitting aerobic conditions to prevail within the flask and, at the same time, prevented contamination. Sterilization of each complete apparatus (flask) was accomplished by autoclaving at 15 - 17 psig for 15 minutes and 250^{o} F. Note, in Figure 1, the suspension of Anacystis nidulans in the flasks at the right front compared to the recently inoculated clear flasks of Anabaena cylindrica at the left front. Light measurements at peak growth of the organisms will be given in a subsequent chapter. These

measurements were taken with a General Electric light meter, type 213, at the broadest part (approximately six inches) of the flask and show relative decreases in light intensity.

Figure 2 shows the typical growth of Oscillatoria borneti in the foreground and Gloeocapsa alpicola in the background. The filamentous form grows in this manner when there is no rough surface onto which the filaments may attach themselves.

The Chemostat apparatus shown in Figures 3 and 4 is similar to those described earlier (13). The volumetric content of each four-foot column shown in Figure 3 was kept at 2.5 liters. Sterilization of these columns was accomplished by twice evacuating to 15 mm of mercury and filling to 15 psig with Carboxide Fumigant (Linde Corporation Brand of Ethylene Oxide sterilizing gas). This pressure was maintained for 24 to 48 hours, after which time the gas was exhausted and sterile air was passed through the columns to sweep out any remaining gas. The source of air for these columns was the school air supply. This air was passed through sterile absorbent cotton to remove any oil and other impurities and then passed through sterilized bacterial filters of glycerine impregnated glass wool before going through the columns. The

sole purpose of air flow through the columns was to maintain aerobic conditions throughout the duration of the experimentation. Exhausted air was passed into a strong chlorine-water solution contained in 125 ml Ehrlenmeyer flasks.

Filling of columns with MSS medium was accomplished by connecting two-liter Ehrlenmeyer flasks containing 1,500 ml and 1,000 ml of the medium to any of the sample ports at the ends of the column and drawing in the sterile medium with a small vacuum.

The cores of the columns were constructed with three lengths of 22 mm Pyrex tubing tied together with chromel wire. Each of the two cores per column was 22 inches long and was covered with washed and autoclaved unbleached muslin. The purpose of these cores was to give a rough attachable area onto which the filamentous forms could grow.

The minimum surface area, per column, of the cores was 264 square inches. If growth of some forms occurred on the wetted perimeter of the 85 mm diameter column this was an additional minimum of 239 square inches. The approximate wetted perimeter per column was 5.2 inches. The total surface area possible for growth was therefore 503 square inches.

Figure 4 shows the excellent growth obtained by filamentous blue-greens on the cores and one non-filamentous

form (Gloeocapsa alpicola). Note that the filamentous species in the lower column was growing on the wall of the column and on the core. This was Oscillatoria chalybia.

For the flow-through phosphorus extraction studies, 3,600 ml of MSS medium was autoclaved in a four-liter Ehrlenmeyer flask and after cooling, the test phosphate was added through an injection cap in the stopper. This solution was then pumped by a Sigmamotor (Brand) pump at the rate of 100 ml per hour (total 36 hours per run) into the Chemostat (column). The waste fluid was allowed to drain from a sample port at the same height at the opposite end of the Chemostat. This maintained the same wetted perimeter throughout the experimental run.

Tetrasodium pyrophosphate (TSPP) is sometimes used as a detergent builder but since tetrapotassium pyrophosphate (TKPP) is more commonly employed it was felt that use of both TSPP and TKPP would be duplication. Therefore, of the two, TKPP was chosen. Samples of these phosphates were furnished by Stauffer Chemical Company, Victor Division, Hooker Chemical Corporation, Phosphorus Division, and Monsanto Chemical Company, Inorganic Chemicals Division on request. For purposes of calculation during the experimen-

sole purpose of air flow through the columns was to maintain aerobic conditions throughout the duration of the experimentation. Exhausted air was passed into a strong chlorine-water solution contained in 125 ml Ehrlenmeyer flasks.

Filling of columns with MSS medium was accomplished by connecting two-liter Ehrlenmeyer flasks containing 1,500 ml and 1,000 ml of the medium to any of the sample ports at the ends of the column and drawing in the sterile medium with a small vacuum.

The cores of the columns were constructed with three lengths of 22 mm Pyrex tubing tied together with chromel wire. Each of the two cores per column was 22 inches long and was covered with washed and autoclaved unbleached muslin. The purpose of these cores was to give a rough attachable area onto which the filamentous forms could grow.

The minimum surface area, per column, of the cores was 264 square inches. If growth of some forms occurred on the wetted perimeter of the 85 mm diameter column this was an additional minimum of 239 square inches. The approximate wetted perimeter per column was 5.2 inches. The total surface area possible for growth was therefore 503 square inches.

Figure 4 shows the excellent growth obtained by filamentous blue-greens on the cores and one non-filamentous

form (Gloeocapsa alpicola). Note that the filamentous species in the lower column was growing on the wall of the column and on the core. This was Oscillatoria chalybia.

For the flow-through phosphorus extraction studies, 3,600 ml of MSS medium was autoclaved in a four-liter Ehrlenmeyer flask and after cooling, the test phosphate was added through an injection cap in the stopper. This solution was then pumped by a Sigmamotor (Brand) pump at the rate of 100 ml per hour (total 36 hours per run) into the Chemostat (column). The waste fluid was allowed to drain from a sample port at the same height at the opposite end of the Chemostat. This maintained the same wetted perimeter throughout the experimental run.

Tetrasodium pyrophosphate (TSPP) is sometimes used as a detergent builder but since tetrapotassium pyrophosphate (TKPP) is more commonly employed it was felt that use of both TSPP and TKPP would be duplication. Therefore, of the two, TKPP was chosen. Samples of these phosphates were furnished by Stauffer Chemical Company, Victor Division, Hooker Chemical Corporation, Phosphorus Division, and Monsanto Chemical Company, Inorganic Chemicals Division on request. For purposes of calculation during the experimen-

tation and since the results and tests are reported as mg/l phosphorus (as P) the following percentages of the three phosphates used were calculated:

STPP - 25.26% P; TKPP - 18.75% P; and SHMP - 30.38% P.

CHAPTER V

DISCUSSION AND EVALUATION OF DATA

It is the purpose of this chapter to evaluate the data obtained by the two different experimental algal culture methods employed in the investigation. It was presumed at the beginning of this investigation that algal cultures grown in a stationary medium environment behave differently from those grown in an environment in which exposure to a flowing nutrient medium is limited to a predetermined length of time. Preliminary chemical and bacteriological tests on domestic sewage will also be discussed in an attempt to demonstrate the hydrolytic activity of domestic sewage. This is done for comparison purposes in order to demonstrate the relative activities of different environmental situations on condensed phosphate hydrolysis rates. The data should be valuable to any of the disciplines involved with pollution control abatement today, namely members of the sanitary engineering, limnological, aquatic microbiological or algalogical professions.

Hydrolytic Activity by Domestic Sewage

Samples of domestic sewage were taken at the influent to the municipal sewage treatment plant of Norman, Oklahoma. The time of sampling was set at approximately 9:00 a.m. when, it was hypothesized, the industrial or commercial fraction would be at a minimum. The approximate BOD value of this sewage was 245 mg/l. Unfiltered raw sewage was placed in four one-liter Ehrlenmeyer flasks to a volume of 750 ml. For all practical purposes it might be suggested that the contents of the four flasks was uniform because only one grab sample was taken in obtaining the total volume. Analytical discrepancies will be shown later which demonstrate that different fractions of a grab sample are not necessarily uniform. The purpose of this part of the investigation was primarily to gain a partial knowledge of the concentration and fate of condensed phosphates ordinarily found in domestic sewage; secondly, the effect of added quantities of STPP, TKPP, and SHMP on the bacterial population and the rates of hydrolysis of these phosphates in domestic sewage were considered. The data for these runs are shown in Tables 4 through 7. In all cases the contents of the flasks were stirred for ten minutes prior to sampling in an attempt to obtain a relatively homogeneous sample.

TABLE 4

HYDROLYSIS OF SODIUM TRIPOLYPHOSPHATE
IN RAW SEWAGE WITH CORRESPONDING MICROBIAL POPULATIONS

Day	Ortho mg/l-P	Condensed mg/l-P	Coliform #/ml	Total #/ml
0	6.37	6.84	5.18×10^5	1.52×10^6
2	11.61	1.39	1.00×10^5	1.13×10^6
4	10.88	2.22	0.8×10^5	1.01×10^6
5	10.54	2.32	1.6×10^4	5.76×10^5
7	11.34	1.87	1.2×10^4	5.21×10^5
9	11.69	1.21	1.0×10^4	5.00×10^5
11	11.87	1.23	0.2×10^4	4.44×10^5
13	11.88	1.22	$<1 \times 10^3$	3.84×10^5
14	10.21	2.99	$<1 \times 10^3$	3.61×10^5

TABLE 5

HYDROLYSIS OF TETRAPOTASSIUM PYROPHOSPHATE
IN RAW SEWAGE WITH CORRESPONDING MICROBIAL POPULATIONS

Day	Ortho mg/l-P	Condensed mg/l-P	Coliform #/ml	Total #/ml
0	6.40	7.03	3.19×10^5	1.30×10^6
2	12.22	0.98	3.65×10^5	2.02×10^6
4	11.67	1.43	3.20×10^5	1.67×10^6
5	10.60	2.43	3.0×10^3	5.76×10^5
7	11.73	1.66	2.87×10^3	5.70×10^5
9	11.93	1.48	2.01×10^3	5.10×10^5
11	12.03	1.27	1.56×10^3	4.99×10^5
13	11.24	1.81	$< 1.0 \times 10^3$	4.61×10^5
14	11.10	2.30	$< 1.0 \times 10^3$	4.82×10^5

TABLE 6

HYDROLYSIS OF SODIUM HEXAMETAPHOSPHATE IN RAW SEWAGE WITH CORRESPONDING MICROBIAL POPULATIONS

Day	Ortho mg/l-P	Condensed mg/l-P	Coliform #/ml	Total #/ml
0	6.70	6.22	5.76×10^5	1.33×10^6
2	12.22	0.65	0.34×10^5	0.83×10^6
4	11.78	0.93	6.22×10^4	8.24×10^5
5	10.82	2.11	1.91×10^4	5.47×10^5
7	10.75	2.07	1.67×10^4	5.41×10^5
9	10.21	2.69	1.11×10^4	5.96×10^5
11	11.00	1.81	0.45×10^4	3.90×10^5
13	11.21	1.34	$< 3 \times 10^3$	2.78×10^5
14	11.93	0.99	$< 1 \times 10^3$	2.31×10^5

TABLE 7

CONTROL FOR TABLES 4, 5, AND 6
SHOWING NORMAL[a] CONDENSED PHOSPHATE HYDROLYSIS
WITH CORRESPONDING MICROBIAL POPULATIONS

Day	Ortho mg/l-P	Condensed mg/l-P	Coliform #/ml	Total #/ml
0	6.50	2.47	5.99×10^5	1.87×10^6
2	7.33	1.59	5.02×10^5	1.01×10^6
4	7.87	1.08	4.17×10^5	0.99×10^6
5	8.40	0.48	3.00×10^5	8.72×10^5
7	8.55	0.17	2.79×10^4	7.77×10^5
9	8.90	0.03	2.03×10^4	6.35×10^5
11	8.72	0.18	1.16×10^4	6.00×10^5
13	7.72	1.24	0.98×10^4	4.72×10^5
14	8.08	0.87	$< 1 \times 10^3$	3.06×10^5

[a] Means those condensed forms usually found in domestic sewage.

Data shown in Tables 4 through 7 corresponding to day zero represent samples taken at the onset of each run. The apparent wide variation in numbers of organisms per milliliter at the onset of the runs exceeds the experimental error of the membrane filter technique used in the plate count tests. This observation indicates the difficulty of duplicating analyses in a single grab sample of sewage.

The condensed phosphates were hydrolyzed much more rapidly than reported by Sawyer (73). Tables 4, 5, and 6 show hydrolysis of 79%, 86%, and 89.5% of the amount present in the first 48 hours while the forms present in the control were only reduced by 35.6% in the same period of time. The forms in raw domestic sewage were, in all probability, more complex than the poly-, meta-, and pyrophosphate types added. Overall, the addition of known forms had little effect on the reduction of members of the coliform group. The rate of decrease in the total counts appeared to be relatively steady in all flasks; that is to say, the presence of additional phosphorus did not increase the life expectancy of viable bacterial populations in the sewage. Comparisons of the maximum amount of total phosphate taken from the filtrates, presumably used by the microorganisms present in

the sewage were surprisingly similar when expressed in percent of total present at the onset of the run. They were as follows; 2.6% (day 5); 3.0% (day 5); 2.9% (day 13); and 2.8% (day 7). The particular day cited indicates the points in the data at which the maximum amounts of phosphorus were taken from the filtrate. These values are expressed for Tables 4 through 7 respectively.

The Static Experimental Runs

Before studies were undertaken on phosphate hydrolysis and uptake by the different algae, the rates of hydrolysis of the three phosphates in MSS medium were determined. Figure 5 shows these rates based on 10.00 mg/l as 100%. Both vertical axes correspond to one another, that is, the percent of the total hydrolyzed and the actual corresponding concentration. Note the comparatively rapid hydrolysis of SHMP. Seventy percent of the total amount hydrolyzed in 30 days was achieved within the first 7 days. The total amount hydrolyzed in the 30-day period was only 9.9% of the total present. These rates were much slower than the rates of hydrolysis in domestic sewage which were 72.7% (STPP), 76.5% (TKPP), and 66.8% (SHMP) hydrolysis of the total present within the first 7 days.

Figures 6 through 18 present the graphic results of all the static runs involving the test phosphates. The description of this procedure can be found in Chapter IV. Figures 1 and 2 show the equipment used. It should be noted that a point on the curves for condensed forms and a point on the curves for orthophosphate represent one sample taken from the flask containing the particular test phosphate. Interpretation of the meaning of the trend of the ortho and condensed curves is largely left to the individual; however, certain observations can be made concerning their significance. The controls, represented by the growth curves, will be discussed later.

All the curves from Figures 6 through 18 have been corrected for hydrolysis rates in order to give a better insight into the actual mechanisms of phosphate utilization by the algae in question.

A comparison of the curves for Anacystis nidulans and Gloeocapsa alpicola (Figures 7 and 8) show dissimilarities between the two unicellular blue-green forms. Gloeocapsa alpicola utilizes comparatively little orthophosphate and TKPP while Anacystis nidulans shows uptake of both during the early stages of growth. It is important to bear in mind

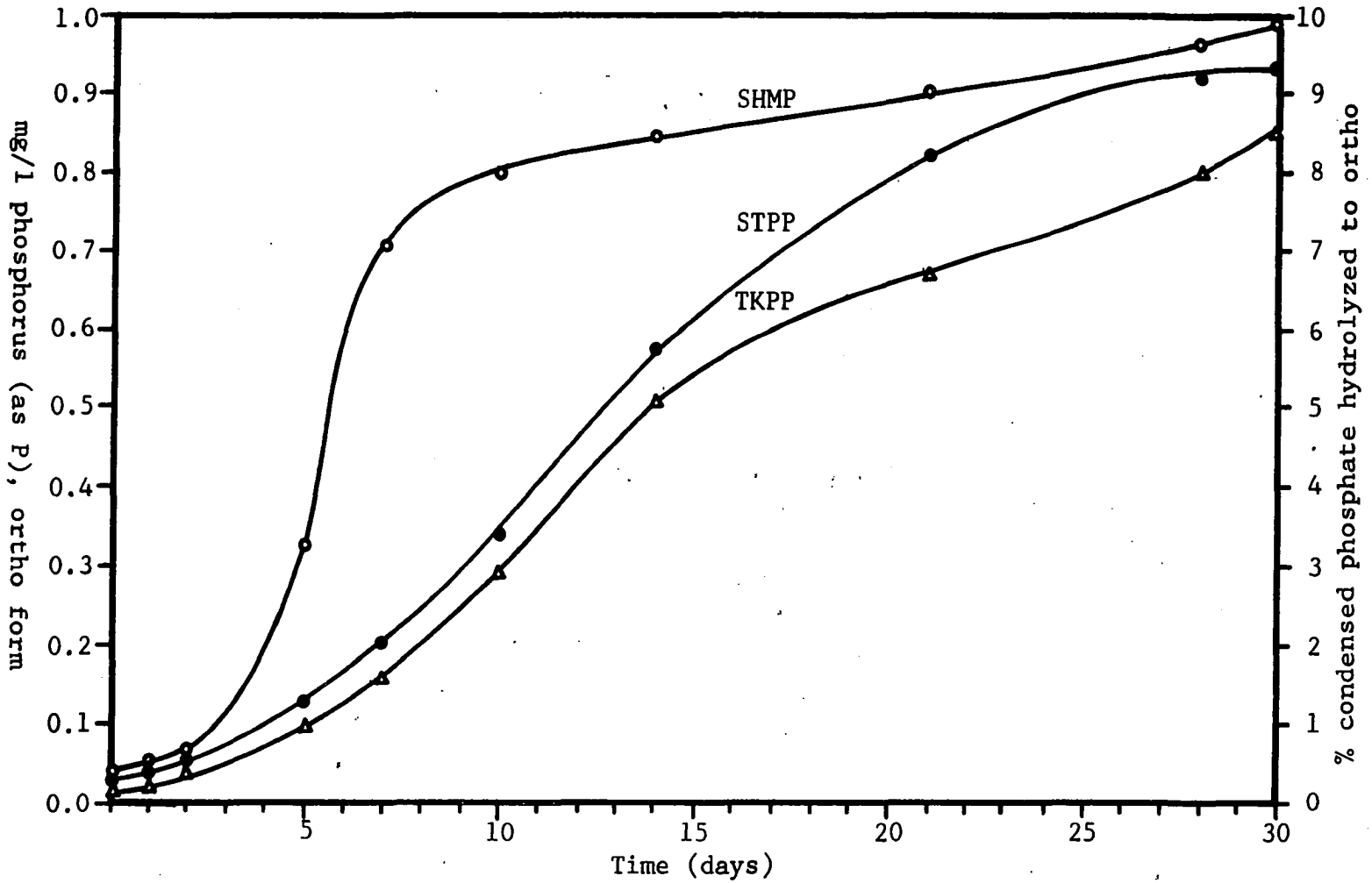


Figure 5. Hydrolysis rates of sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in modified synthetic sewage (MSS) medium based on 10 mg/l as 100%.

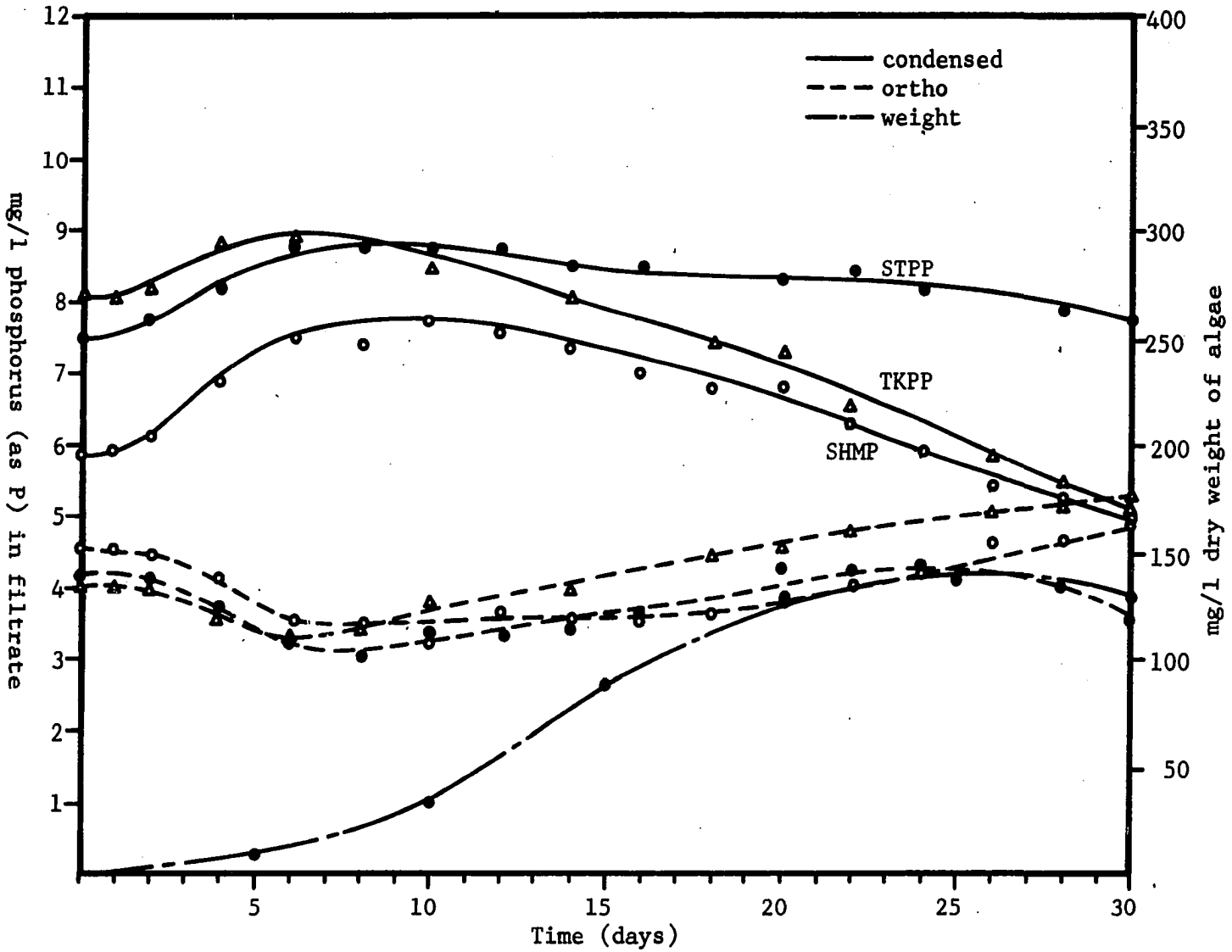


Figure 6. Effect of Anabaena cylindrica on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

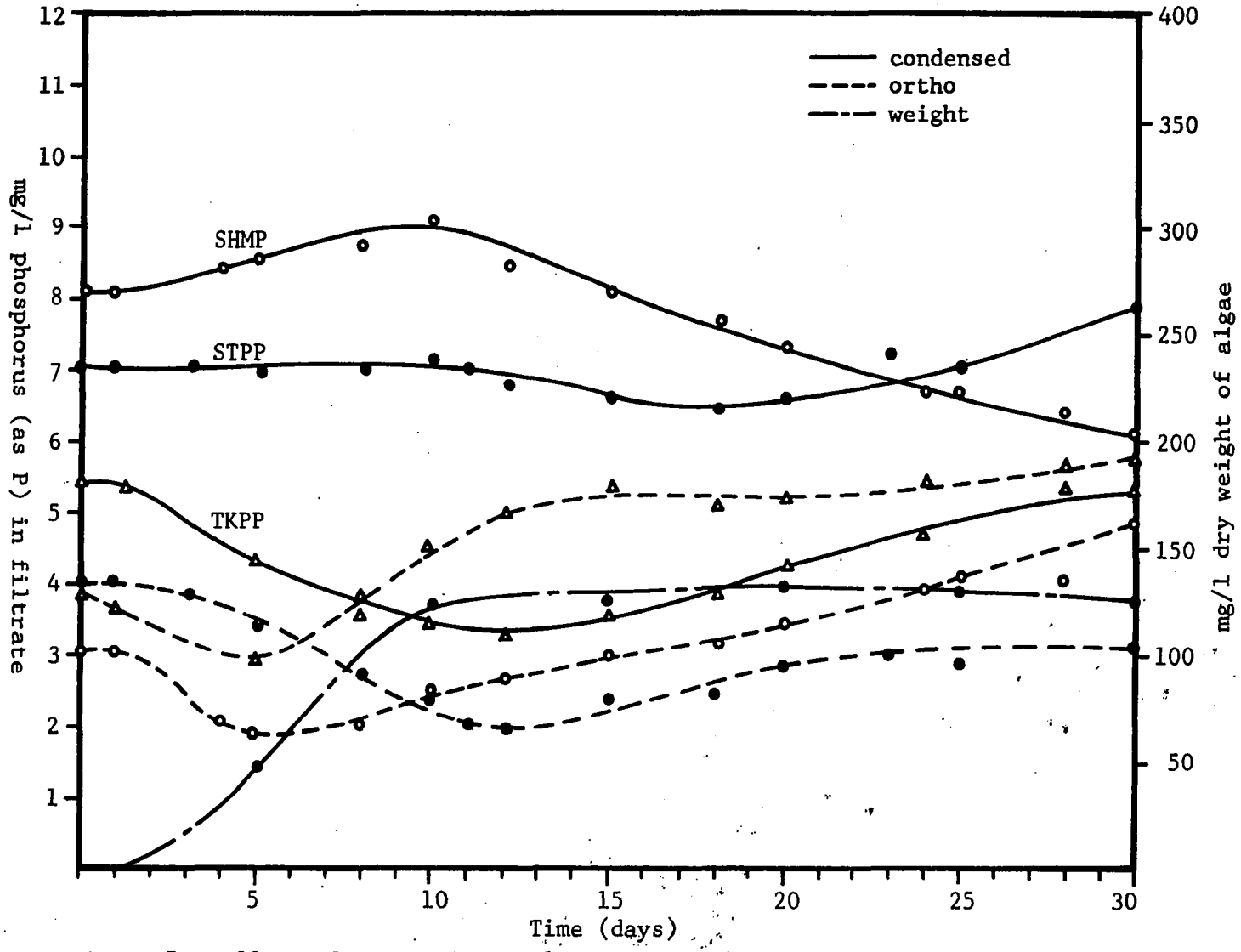


Figure 7. Effect of Anacystis nidulans on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

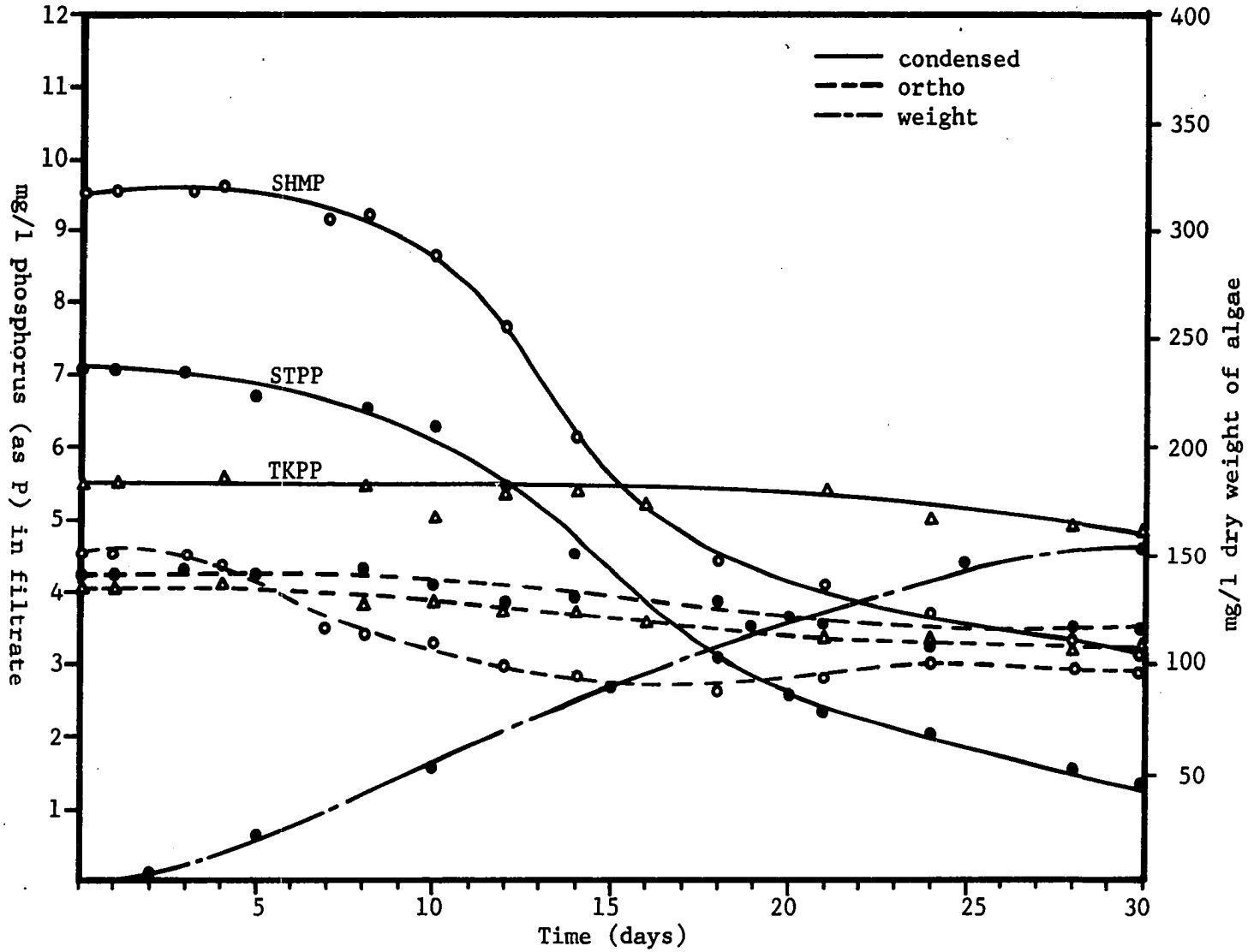


Figure 8. Effect of Gloeocapsa alpicola on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

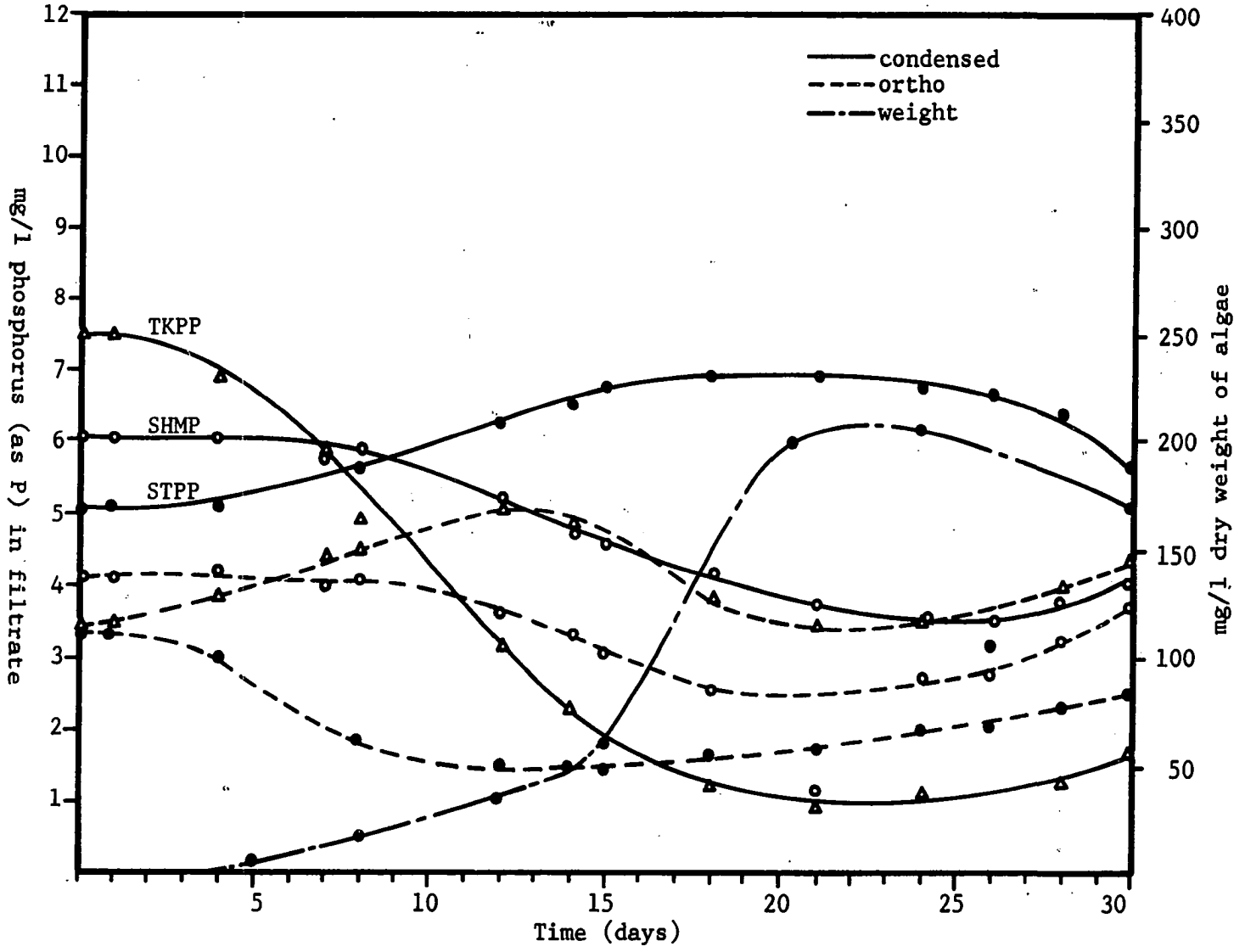


Figure 9. Effect of Oscillatoria borneti on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

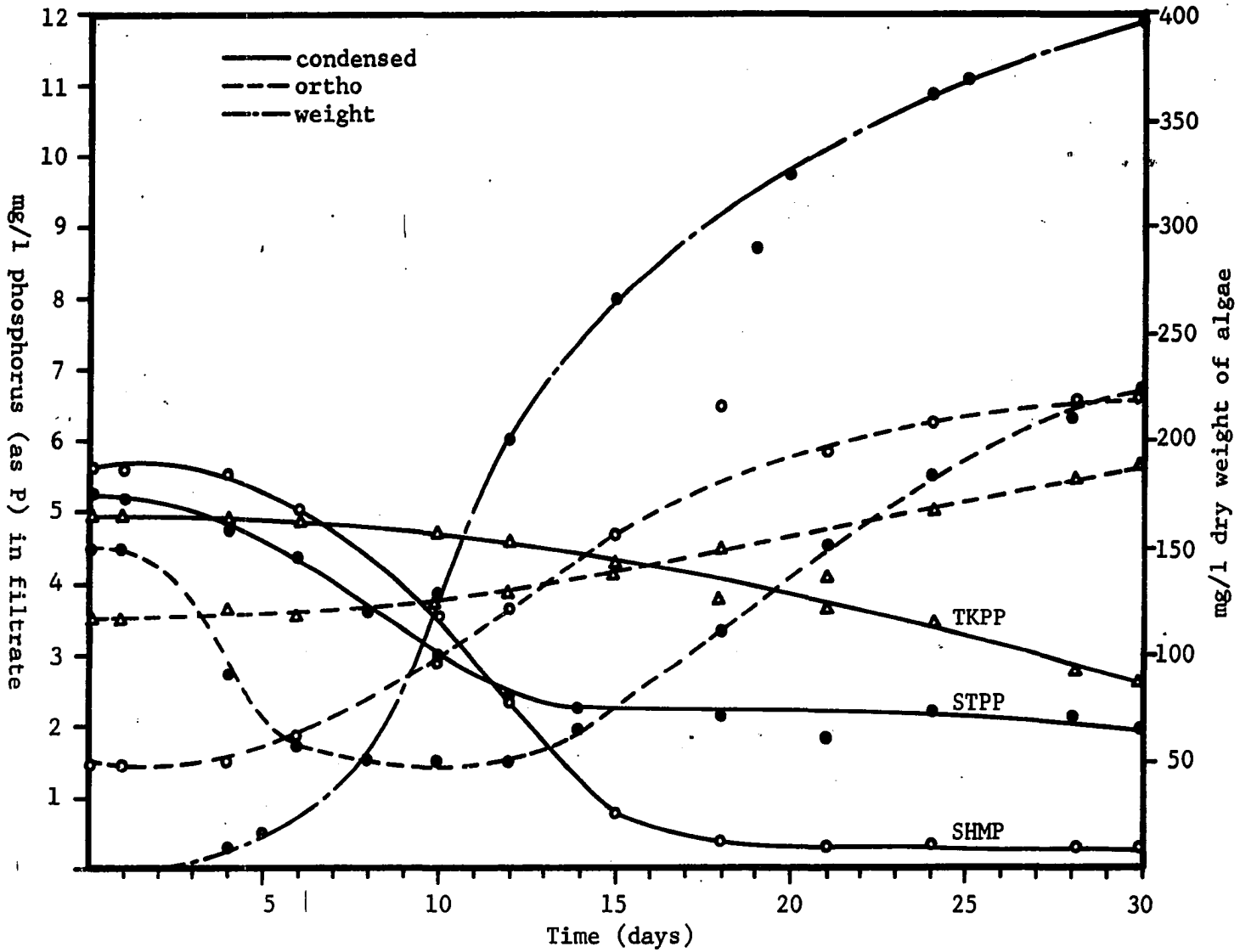


Figure 10. Effect of Oscillatoria chalybia on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

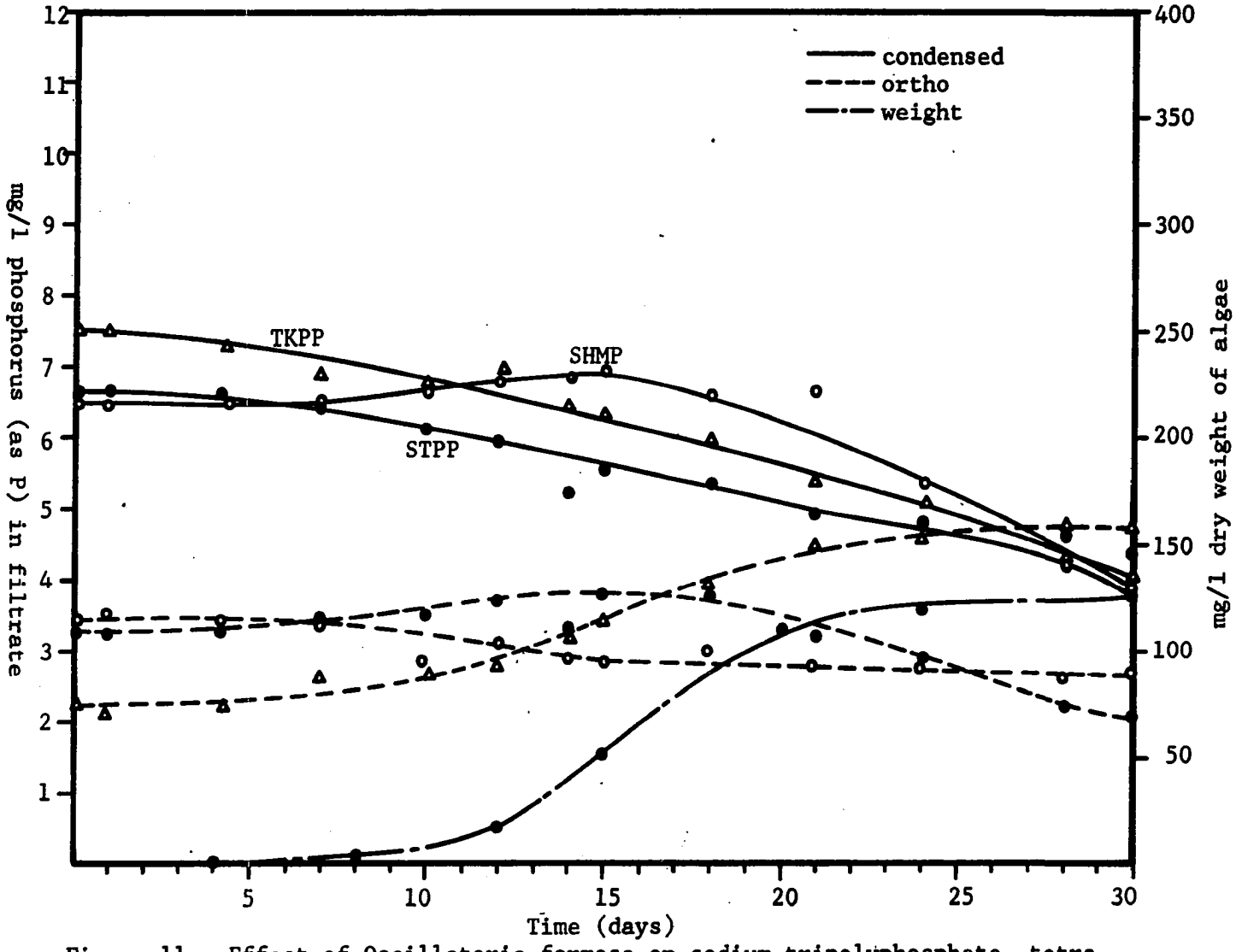


Figure 11. Effect of Oscillatoria formosa on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

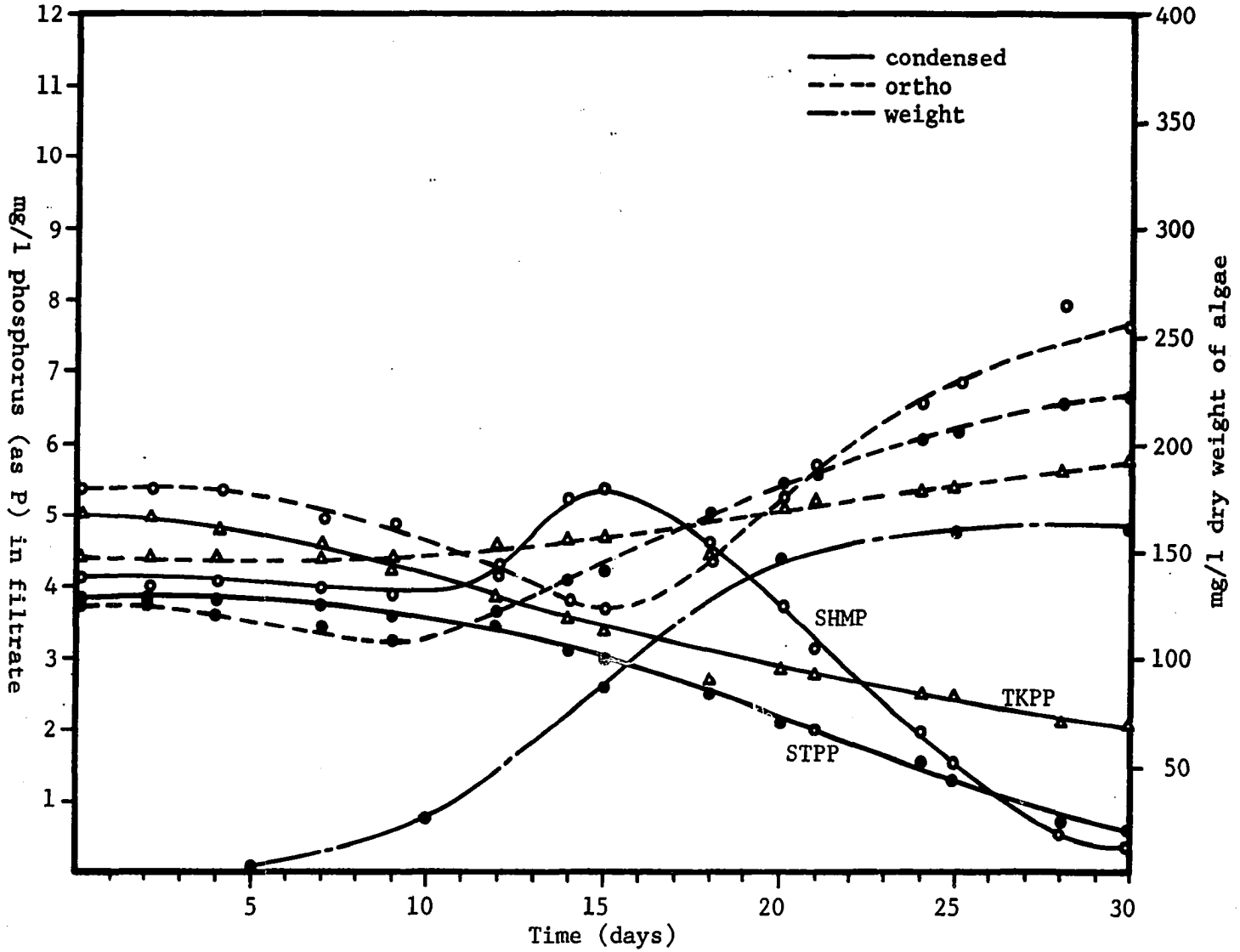


Figure 12. Effect of Oscillatoria tenuis on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

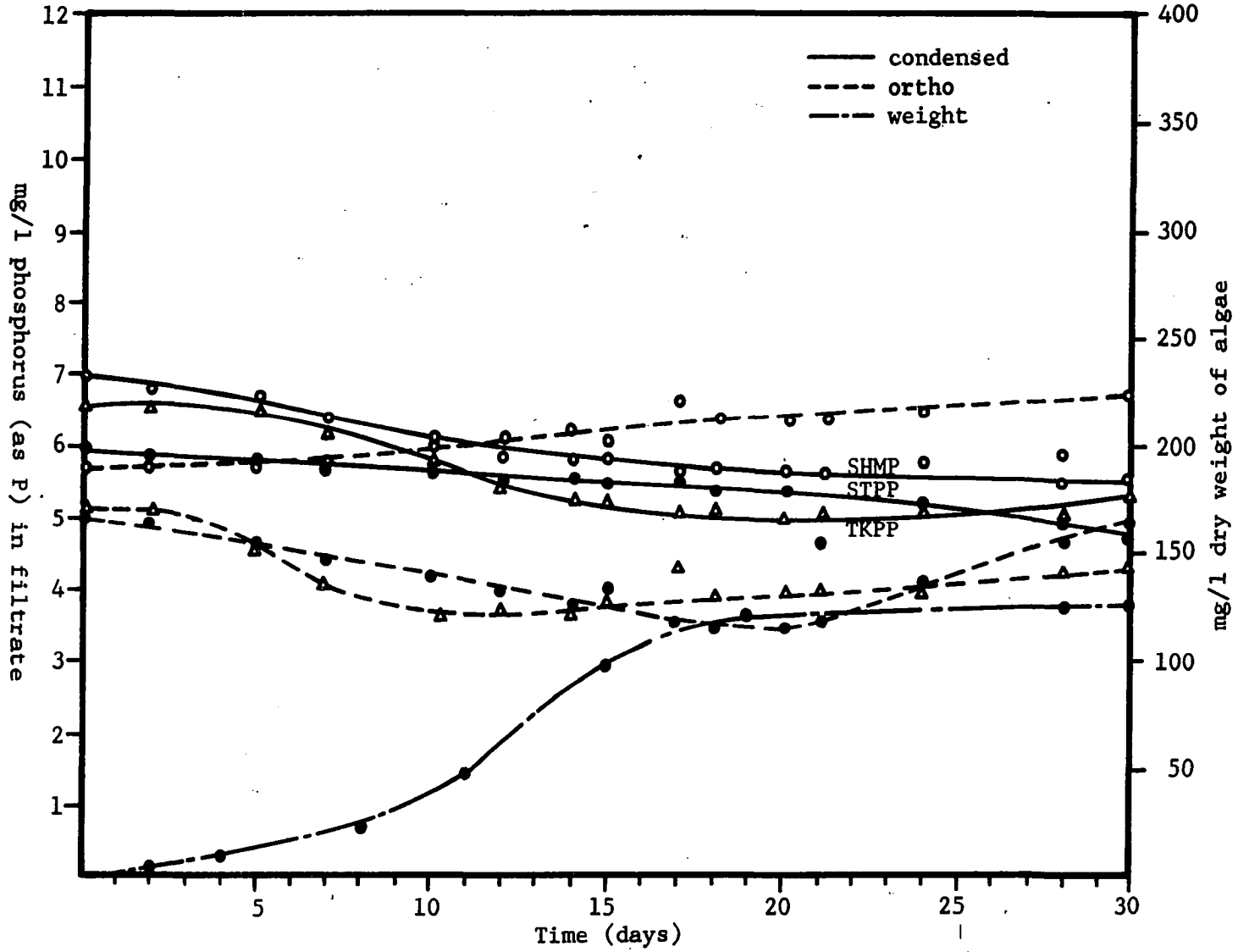


Figure 13. Effect of Phormidium faveolarum on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

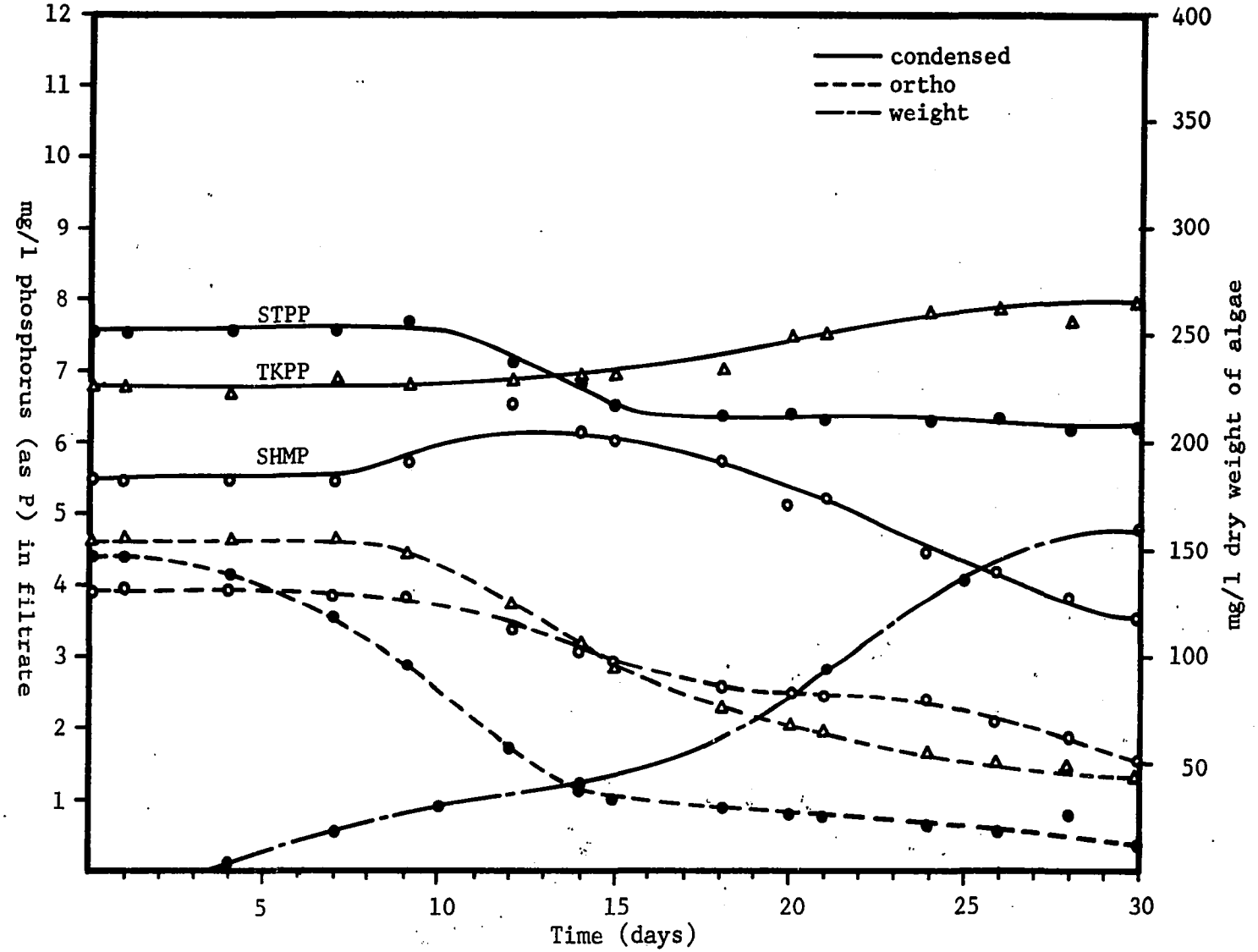


Figure 14. Effect of Chlorella pyrenoidosa on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

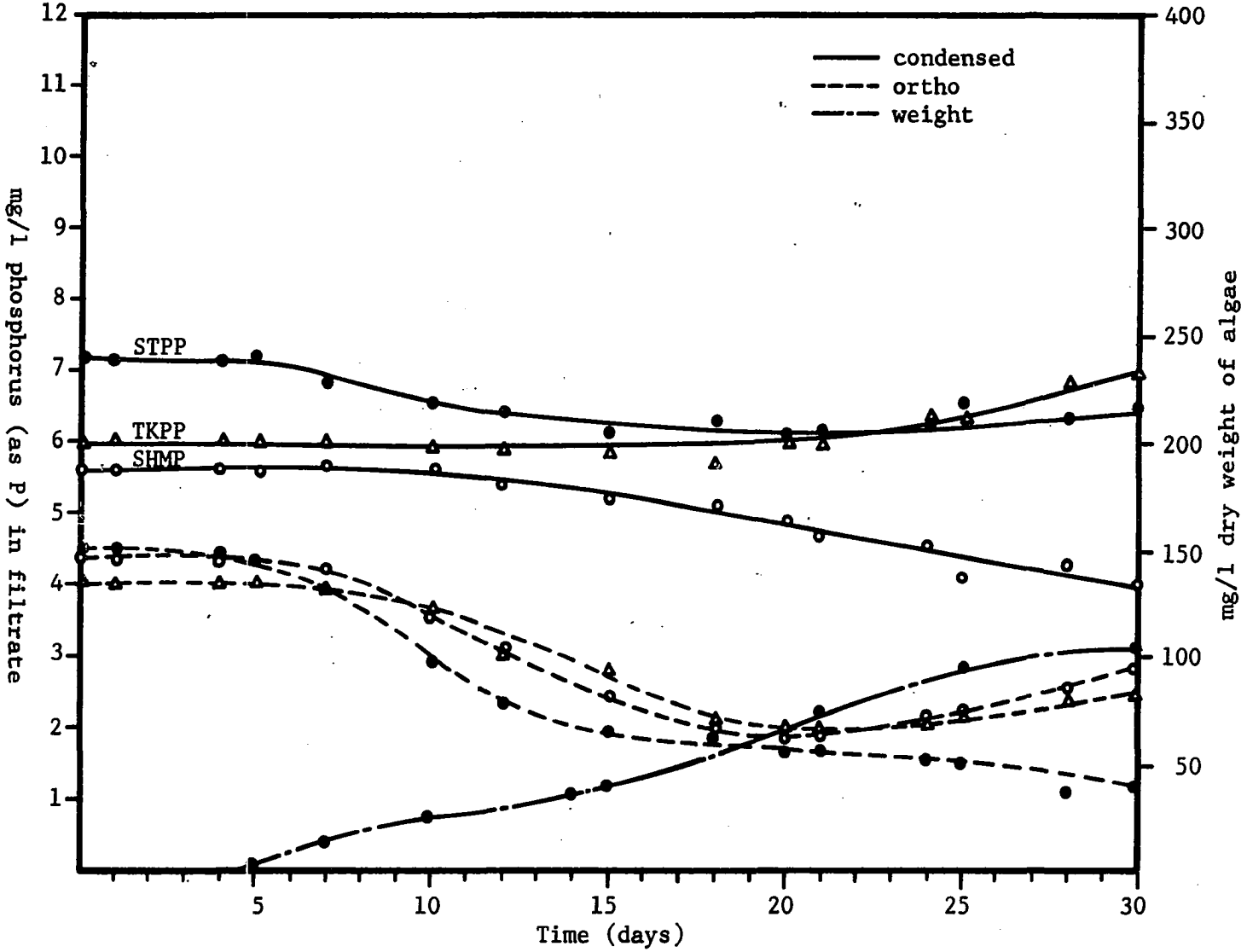


Figure 15. Effect of Chlorella vulgaris on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

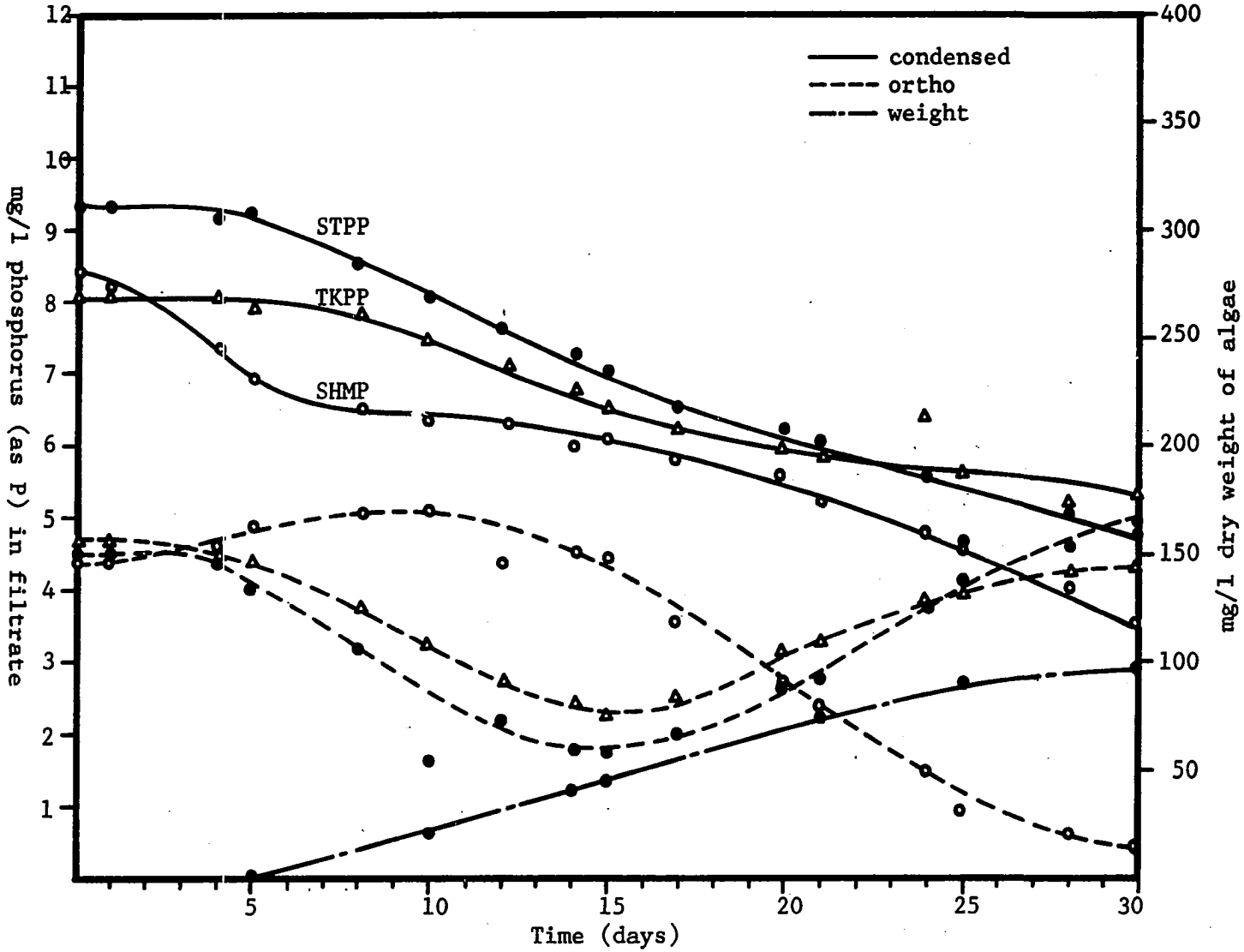


Figure 16. Effect of Scenedesmus obliquus on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

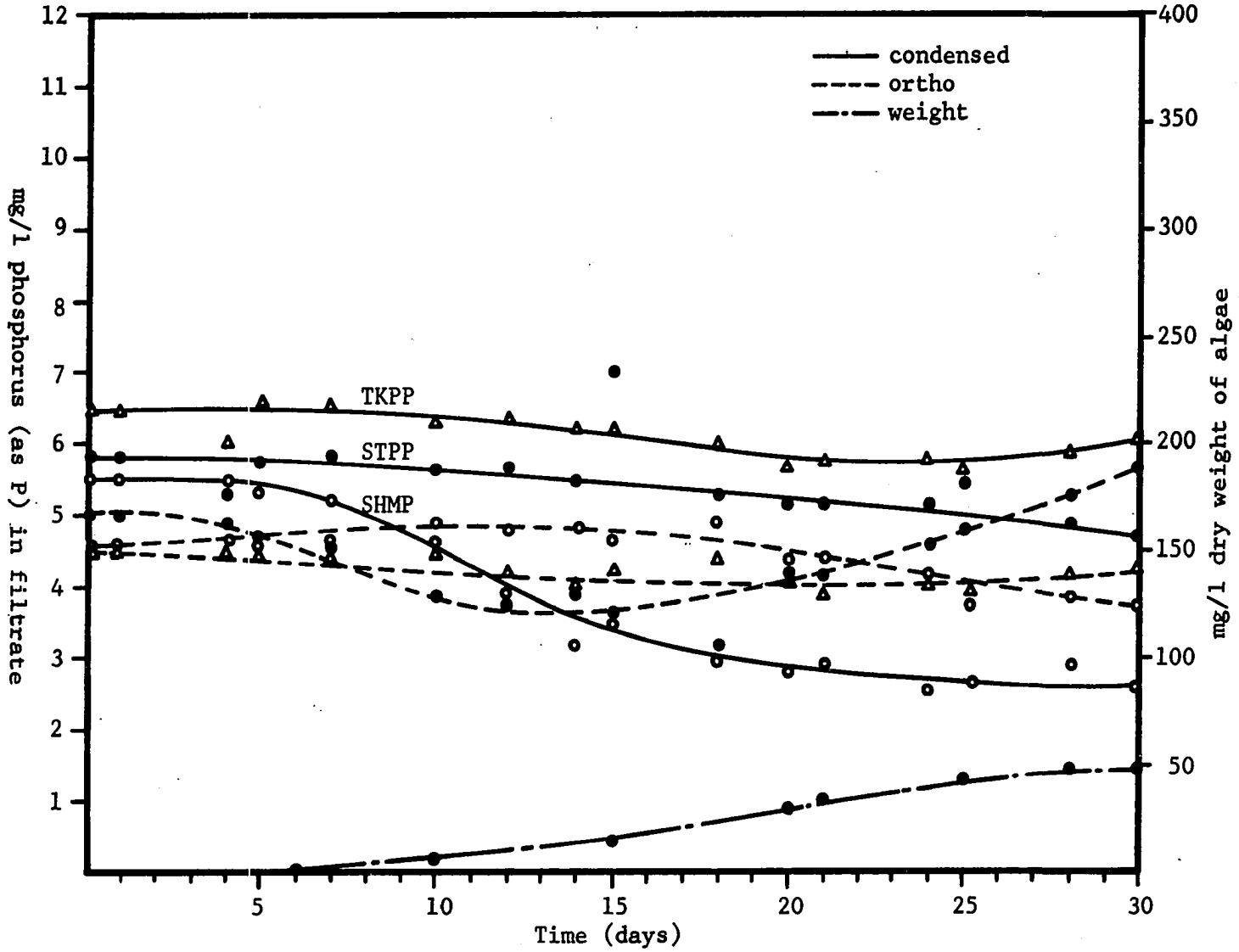


Figure 17. Effect of Scenedesmus quadricauda on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth curve for the organism.

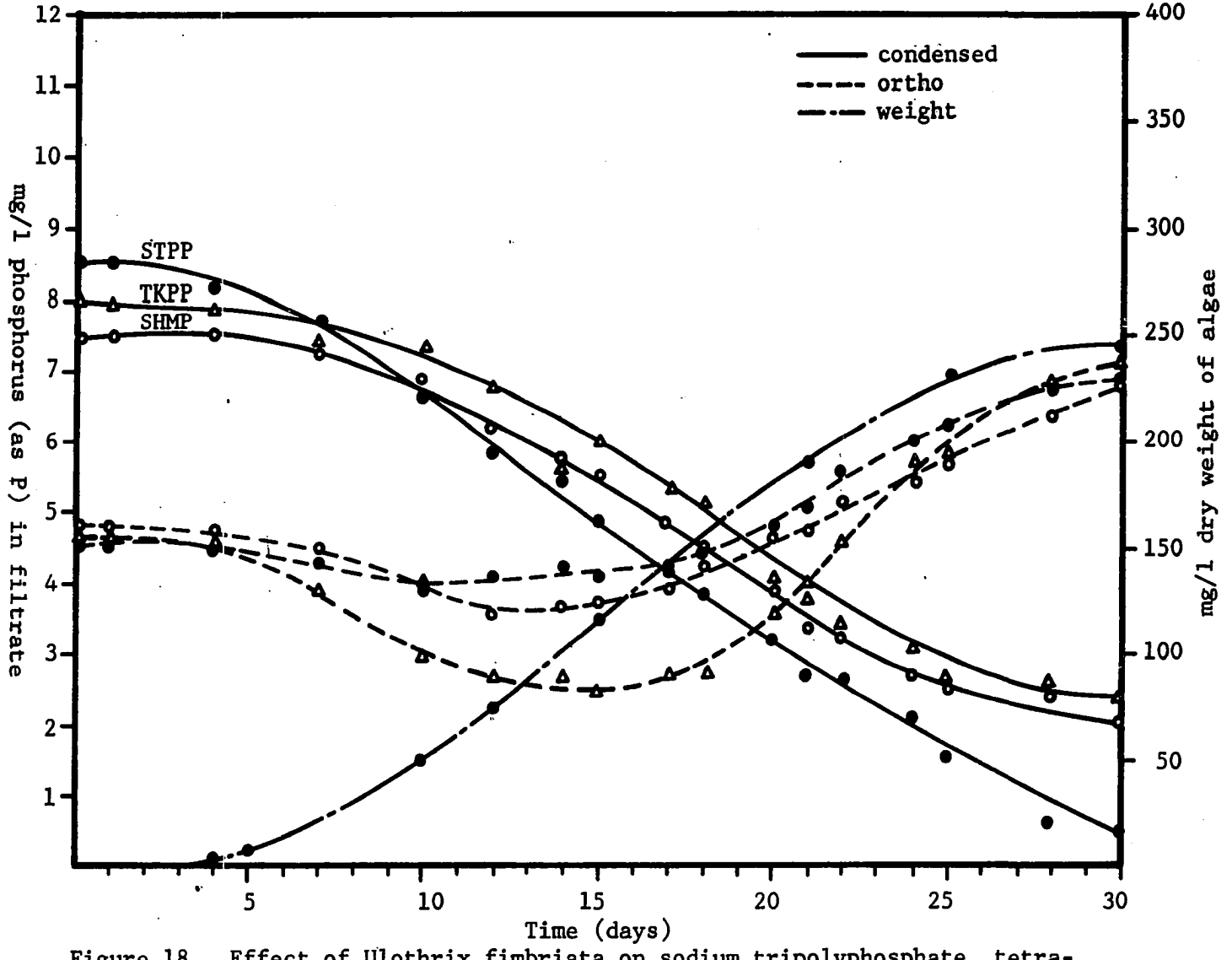


Figure 18. Effect of Ulothrix fimbriata on sodium tripolyphosphate, tetrapotassium pyrophosphate, and sodium hexametaphosphate in MSS medium with a typical growth durve for the organism.

that an increase in any condensed phosphate curve above the concentration indicated at the start of a run is, in all probability, due to effusion or excretion of other inorganic condensed forms or organic forms from the species in question.

Among the filamentous blue-greens tested, Anabaena cylindrica (Figure 6) seems to exhibit incorporation of orthophosphate at about the same rate as it returns condensed phosphate forms to the medium. This might indicate a preference for the ortho form over condensed forms. Slow hydrolysis of all condensed forms is evidently accomplished by this organism as well as by Phormidium faveolarum (Figure 13).

Comparison of different species of the Genus Oscillatoria (Figures 9 through 12) shows more pronounced degradation of the condensed forms. In almost all cases reduction of the condensed phosphate concentrations occurred over the entire period of testing. The only exception was the filamentous blue-green species, Oscillatoria borneti, in the presence of STPP (Figure 9). The orthophosphate curves for the same organisms are not as consistent.

The green algae, on the other hand, show some similarity to the blue-greens in the overall hydrolysis of condensed phosphates. Chlorella pyrenoidosa and C. vulgaris (Figures

14 and 15) show a preference for orthophosphorus during their early stages of growth whereas hydrolysis of the condensed forms is only slight. SHMP was hydrolyzed to a greater extent than STPP or TKPP. Of the other two unicellular species of green algae (Figures 16 and 17), Scenedesmus obliquus showed a greater ability to hydrolyze condensed phosphate forms than did S. quadricauda. Explanation of this difference may well be due to the different growth rates as shown on the respective figures.

The filamentous green algae, Ulothrix fimbriata (Figure 18) exhibits a greater capability of condensed phosphate hydrolytic activity. Obviously some of the condensed is retained in the medium as orthophosphate as shown by the corresponding increase in orthophosphate concentrations. Note that the growth curve is greater for this species than either of the unicellular forms of green algae mentioned above.

Tables 8, 9, and 10 interpret the data of the phosphate curves shown in Figures 6 through 18. In arriving at the percent total phosphorus used, the orthophosphate values were added to the condensed phosphate values at the beginning of every run. This was considered to be the 100% value. The

TABLE 8

RELATIONSHIPS OF TOTAL PHOSPHORUS UPTAKE DURING STATIC RUNS WITH STPP[a]

Organism	% Total Used[b]	Day[c]	k[d]
Anabaena cylindrica	3.25	30	-0.010
Anacystis nidulans	19.00	12	-0.009
Gloeocapsa alpicola	58.90	30	-0.028
Oscillatoria borneti	2.51	30	-0.007
Oscillatoria chalybia	54.55	10	-0.079
Oscillatoria formosa	34.44	30	-0.014
Oscillatoria tenuis	2.68	30	-0.001
Phormidium faveolarum	19.72	20	-0.011
Chlorella pyrenoidosa	45.00	30	-0.020
Chlorella vulgaris	38.30	30	-0.016
Scenedesmus obliquus	36.90	15	-0.032

TABLE 8--Continued

Organism	% Total Used[b]	Day[c]	k[d]
Scenedesmus quadricauda	15.86	15	-0.006
Ulothrix fimbriata	43.30	30	-0.019

[a] Corresponds to data shown in Figures 6 through 18.

[b] Maximum amount of total phosphorus present at t = 0 subsequently taken from medium.

[c] Day observation of (b) made, from t = 0.

[d] Given in $\ln N_o/N = -kt$ units per day from t = 0 to day indicated by (c).

TABLE 9

RELATIONSHIPS OF TOTAL PHOSPHORUS UPTAKE DURING STATIC RUNS WITH TKPP[a]

Organism	% Total Used[b]	Day[c]	k[d]
Anabaena cylindrica	14.38	30	-0.005
Anacystis nidulans	22.85	5	-0.052
Gloeocapsa alpicola	14.88	30	-0.005
Oscillatoria borneti	60.59	21	-0.044
Oscillatoria chalybia	2.08	30	-0.001
Oscillatoria formosa	10.04	30	-0.003
Oscillatoria tenuis	16.24	30	-0.006
Phormidium faveolarum	23.40	21	-0.013
Chlorella pyrenoidosa	19.01	30	-0.007
Chlorella vulgaris	19.60	21	-0.010
Scenedesmus obliquus	31.18	15	-0.025

TABLE 9--Continued

Organism	% Total Used[b]	Day[c]	k[d]
Scenedesmus quadricauda	12.59	24	-0.006
Ulothrix fimbriata	32.80	15	-0.027

[a] Corresponds to data shown in Figures 6 through 18.

[b] Maximum amount of total phosphorus present at t = 0 taken from medium.

[c] Day observation of (b) made, from t = 0.

[d] Given in $\ln N_o/N = -kt$ units per day from t = 0 to day indicated by (c).

TABLE 10

RELATIONSHIPS OF TOTAL PHOSPHORUS UPTAKE DURING STATIC RUNS WITH SHMP[a]

Organism	% Total Used[b]	Day[c]	k[d]
Anabaena cylindrica	5.57	30	-0.019
Anacystis nidulans	6.53	5	-0.013
Gloeocapsa alpicola	52.10	30	-0.028
Oscillatoria borneti	39.40	25	-0.020
Oscillatoria chalybia	2.96	30	-0.001
Oscillatoria formosa	33.83	30	-0.014
Oscillatoria tenuis	14.75	30	-0.005
Phormidium faveolarum	5.51	11	-0.005
Chlorella pyrenoidosa	46.40	30	-0.021
Chlorella vulgaris	33.30	21	-0.019
Scenedesmus obliquus	69.30	30	-0.039

TABLE 10--Continued

Organism	% Total Used[b]	Day[c]	k[d]
Scenedesmus quadricauda	37.42	30	-0.016
Ulothrix fimbriata	28.85	30	-0.011

[a] Corresponds to data shown in Figures 6 through 18.

[b] Maximum amount of total phosphorus present at t = 0 taken from medium.

[c] Day observation of (b) made, from t = 0.

[d] Given in $\ln N_o/N = -kt$ units per day from t = 0 to day indicated by (c).

day indicated in these tables represents the day at which the greatest amount of total phosphate was taken from the medium by the algae. From this the percent of the total used present at day = 0 was calculated. Rate constants were calculated from these figures and are shown in their respective places in the tables. The negative sign simply means that a decrease in the phosphate content occurred.

Examination of the data shown in Tables 8, 9, and 10 reveals wide differences in percentages of phosphorus uptake by the algae. This would appear to be disqualifying insofar as validity is concerned but it must be pointed out that the maximum quantities were taken from the medium at different times during the thirty-day testing. These times are indicated in the second column of figures. Anacystis nidulans reached its maximum uptake earlier than any of the other algal species tested. The importance of this datum lies in the fact that after the times indicated, the algae are returning phosphate compounds to the medium. This is the case of virtually all the species which show a maximum uptake prior to the end of the test run, or thirty days.

The uptake rate constants are shown in Tables 8 through 10 for each species. They were based on the time period

shown. All of the values are relatively low compared to growth rate constants which will be discussed later. Continuous uptake over the entire thirty-day period of all three phosphates tested only occurred in the cases of Anabaena cylindrica, Gloeocapsa alpicola, Oscillatoria formosa, O. tenuis, and Chlorella pyrenoidosa. The rate constants are almost always different in these cases, however, one point of interest is shown. The rate constants are, with one exception, higher for SHMP uptake than TKPP uptake. By comparing the rate constants for SHMP uptake and STPP uptake for the organisms listed above, it can be seen that those for SHMP are higher, or equal, to those of STPP, but never lower. This might indicate that hydrolysis of SHMP by enzymatic action is more easily accomplished than that of STPP or TKPP. This hypothesis is partially substantiated by the relative stability of SHMP in MSS medium (Figure 5).

Controls were run on all species reported using only MSS medium. All other conditions were the same except the container size. In order to obtain accurate weight measurements for the growth curves shown in Figures 6 through 18, 125 milliliter Ehrlenmeyer flasks, filled with 100 ml of

medium were used. The algal contents were weighed on 47 mm membrane filters on the day indicated in the aforementioned Figures. The results of analysis of the filtrate of these flasks for phosphate content is shown in Table 11. Without exception, the condensed phosphate concentration in the filtrate increased during the thirty-day observation period. Concentrations are indicated for each flask on the same day as weight measurements are reported.

Table 12 shows growth rate constants for each algal species used and the day for which the measurements were calculated during control runs. These growth constants are at least tenfold those of phosphorus uptake for all species reported. The constants are not nearly as high as those reported by Kratz et al, (42) and Myers (54) indicating that MSS medium as used in this investigation was not a rapid growth medium but one which permitted optimum growth.

Table 13 shows data calculated from those data shown in Table 11. It relates the maximum amount of phosphorus taken from the medium from zero time to the day indicated and the corresponding percent of the total phosphorus present at the beginning of the control run. From these data the uptake rate constants were calculated. It is apparent that

TABLE 11

PHOSPHATE CONCENTRATIONS IN MSS MEDIUM FILTRATE DURING CONTROL RUNS[a]

Organism	Relative To Figure	Day[b]	Phosphate Form Ortho	Condensed
Anabaena cylindrica	6	0	4.50	0.02
		5	4.01	0.09
		10	3.65	0.15
		15	3.20	0.19
		20	2.99	1.12
		25	2.40	1.40
		30	2.72	1.42
Anacystis nidulans	7	0	4.47	0.01
		5	4.01	0.03
		10	2.13	0.09
		15	3.02	0.62
		20	3.50	0.90
		25	3.77	0.56
		30	3.92	0.21
Gloeocapsa alpicola	8	0	4.54	0.04
		2	3.92	0.04
		5	3.41	0.09
		10	2.62	0.31
		15	2.01	0.46
		19	1.64	0.71
		25	1.42	0.91
		30	1.39	1.61

TABLE 11--Continued

Organism	Relative To Figure	Day[b]	Phosphate Form Ortho	Condensed
Oscillatoria borneti	9	0	4.51	0.01
		5	4.45	0.03
		8	4.02	0.11
		12	3.72	0.15
		15	3.51	0.27
		20	1.62	0.41
		24	1.99	1.12
		30	2.97	1.27
Oscillatoria chalybia	10	0	4.97	0.05
		4	4.77	0.09
		5	4.75	0.11
		10	3.13	0.40
		12	2.68	0.40
		15	2.01	0.31
		19	1.86	0.53
		20	1.35	0.86
		24	1.94	0.99
		25	1.72	1.12
		30	1.88	1.35
Oscillatoria formosa	11	0	3.97	0.02
		4	3.90	0.02
		8	3.65	0.04
		12	3.11	0.09
		15	3.00	0.17
		20	2.08	0.65
		24	2.41	0.71
		30	2.49	0.30
Oscillatoria tenuis	12	0	4.55	0.05
		5	4.50	0.05
		10	4.38	0.04
		15	3.82	0.08
		20	3.51	0.13
		25	3.69	0.15
		30	3.78	0.27

TABLE 11--Continued

Organism	Relative To Figure	Day[b]	Phosphate Form Ortho	Condensed
Phormidium faveolarum	13	0	4.10	0.05
		2	4.03	0.05
		8	3.76	0.08
		11	3.21	0.12
		15	2.91	0.16
		19	3.07	0.29
		28	3.41	0.35
		30	3.50	0.44
Chlorella pyrenoidosa	14	0	4.75	0.02
		4	4.70	0.02
		7	4.26	0.01
		10	4.07	0.05
		14	3.61	0.08
		21	2.19	0.11
		25	2.03	0.14
		30	1.86	0.37
Chlorella vulgaris	15	0	4.75	0.02
		5	4.71	0.03
		7	4.32	0.01
		10	4.10	0.03
		14	3.73	0.09
		15	3.52	0.15
		21	3.10	0.17
		25	3.02	0.44
		30	2.90	0.51
Scenedesmus obliquus	16	0	4.59	0.01
		5	4.57	0.02
		10	4.47	0.09
		14	4.39	0.10
		15	4.35	0.15
		21	4.15	0.29
		25	3.38	0.37
		30	3.27	0.46

TABLE 11--Continued

Organism	Relative To Figure	Day[b]	Phosphate Form Ortho	Condensed
Scenedesmus quadricauda	17	0	4.51	0.05
		6	4.37	0.07
		10	4.30	0.08
		15	4.19	0.13
		20	4.00	0.15
		21	3.50	0.19
		25	3.47	0.27
		28	3.45	0.28
		30	3.43	0.35
Ulothrix fimbriata	18	0	4.61	0.04
		4	4.57	0.04
		5	4.51	0.05
		10	4.13	0.07
		12	3.84	0.11
		15	2.99	0.15
		21	2.36	0.15
		25	2.14	0.19
		30	2.20	0.69

[a] 125 ml Ehrlenmeyer flasks containing 100 ml MSS medium.

[b] Indicates same points as shown on growth curves on Figures 6 through 18.

TABLE 12

GROWTH CONSTANTS OF ALGAE
GROWN IN MSS MEDIUM

Organism	Relative to Growth Curve On Figure	k[a]	Day
Anabaena cylindrica	6	0.19	25
Anacystis nidulans	7	0.24	20
Gloeocapsa alpicola	8	0.07	30
Oscillatoria borneti	9	0.23	23
Oscillatoria chalybia	10	0.20	30
Oscillatoria formosa	11	0.16	30
Oscillatoria tenuis	12	0.17	30
Phormidium faveolarum	13	0.16	30
Chlorella pyrenoidosa	14	0.17	30
Chlorella vulgaris	15	0.16	30
Scenedesmus obliquus	16	0.15	30
Scenedesmus quadricauda	17	0.13	30
Ulothrix fimbriata	18	0.20	30

[a] In ln N/N_o = kt units per day on dry weight basis.

phosphorus uptake among algae differs among genera and species and can only be interpreted by examination of the environment at one particular time.

Comparison of the phosphorus uptake rate constants of Tables 8, 9, 10, and 13 shows a rather striking fact. Excepting those constants for the green algae species, Scenedesmus obliquus, and with few other individual exceptions, the rates of phosphorus uptake were greater when only orthophosphate was present than when condensed forms were present. This may indicate a slight inhibition of the phosphorylation mechanism among the algae when a predominance of condensed phosphate is present.

Table 14 shows data on the reduction in light intensity by approximately six inches of algal growth in MSS medium at the day peak growth was attained. The species showing the greatest light reducing ability were two unicellular forms, Gloeocapsa alpicola and Chlorella pyrenoidosa. The reason for the incident light readings varying from 275 foot-candles to 285 foot-candles as shown in the table was the relative position of the culture flask to the light source. These data are shown only as supplementary data, but they do show the relative reduction as may occur in the first six inches of a lagoon.

TABLE 13

MAXIMUM PHOSPHATE REMOVED FROM MSS MEDIUM DURING CONTROL RUNS[a]

Organism	(Maximum) mg/l-P Removed	Day	% of Total Present at Onset of Run	k[b]
Anabaena cylindrica	1.13	15	25.0	-0.023
Anacystis nidulans	2.26	10	50.5	-0.070
Gloeocapsa alpicola	2.25	25	49.2	-0.027
Oscillatoria borneti	2.49	20	55.2	-0.040
Oscillatoria chalybia	2.94	12	59.6	-0.073
Oscillatoria formosa	1.26	20	31.6	-0.019
Oscillatoria tenuis	0.96	20	20.8	-0.012
Phormidium faveolarum	1.08	15	26.0	-0.020
Chlorella pyrenoidosa	2.60	25	54.5	-0.032
Chlorella vulgaris	1.50	21	31.5	-0.018
Scenedesmus obliquus	0.77	30	17.1	-0.006
Scenedesmus quadricauda	0.87	21	19.1	-0.010
Ulothrix fimbriata	2.32	25	49.9	-0.028

[a] Related to data shown in Table 9.

[b] $\ln N_o/N = -kt$ units per day.

TABLE 14

MAXIMUM REDUCTION OF LIGHT INTENSITY BY ALGAL CULTURES IN STATIC TWO-LITER FLASK RUNS

Day	Relative To Figure	Reduced From (ft.-c.)[a]	to (ft.-c.)[b]	% Reduction
25	6	280	200	28.5
20	7	280	110	60.8
30	9	280	90	67.9
24	0	285	190	33.4
30	10	275	150	45.4
30	11	275	120	56.4
25	12	280	100	64.3
30	13	285	100	64.9
30	14	275	90	67.3
30	15	275	100	63.6
30	16	280	150	46.4
30	17	275	185	32.7
30	18	275	200	27.2

[a] Incident light.

[b] Measured through approximately 6 inches base of two-liter flask.

The Chemostat Runs

The apparatus used for these phosphorus extraction studies is shown in Figures 3 and 4 and has been described in Chapter IV. The purpose of these runs is stated at the beginning of this chapter. The same algal species used in the static runs were used for this phase of the investigation with the exception of Oscillatoria formosa and O. tenuis. Gross bacterial contamination of the cultures prevented these strains from being used.

In all instances sterile conditions were maintained throughout the experimentation. After each 36-hour run involving a Chemostat, the culture was allowed to stabilize itself for a period of at least 24 hours before another run was started.

Tables 15 and 16 show the data taken during the Chemostat runs. The meaning of the expected value of the phosphorus concentration of the Chemostat contents shown in Table 16 is based on the assumption that complete mixing does occur in the Chemostat during a run. This was calculated mathematically by the following relationship. Since the pumping rate of stock condensed phosphate MSS medium is constant, $(0.1\ 1/hr)(x_s/1\ mg/l)(dt\ hrs)$ enters the column

and (0.1 l/hr)(x_c/1 mg/l)(dt hrs) flows out of the column. The notation x_s = the concentration of ortho- or condensed phosphate in the stock solution being added and the notation x_c = the concentration of ortho- or condensed phosphate content of the Chemostat at time = t. Then the amount of either ortho- or condensed present in the column at time = t is shown by the difference betweer that portion entering and that portion flowing out. This reduces to the differential equation,

$$dx = 0.1(x_s)dt-0.1(x_c)dt \qquad (5.1)$$

which reduces to,

$$\frac{dx}{x_c-x_s} = -0.1dt \qquad (5.2)$$

and upon integration, the relationship

$$\ln (x_c-x_s) = -0.1\ t + \ln C \qquad (5.3)$$

is resolved. Substitution of concentrations at t = 0 solves for the constant. Then the expected (theoretical) value at t = 36 hrs can be obtained. These are the values shown in Table 16. Partial substantiation of the proof that complete mixing did occur throughout the 36-hour period of pumping was shown on a control column by pumping a dye. The dye used was crystal violet and observations were made as the pumping progressed. At two hours after the onset of pumping

TABLE 15

ANALYSES OF CONDENSED PHOSPHATES ADDED TO CHEMOSTAT AND CHEMOSTAT CONTENTS AT T=0

Chemostat With:	Form Added	Stock Analysis, mg/l-P: Ortho	Stock Analysis, mg/l-P: Condensed	Contents in mg/l-P: Ortho	Contents in mg/l-P: Condensed
Anabaena cylindrica	STPP	0.09	3.50	3.14	0.00
	TKPP	2.02	4.18	3.26	1.37
	SHMP	0.39	4.74	1.76	1.83
Anacystis nidulans	STPP	0.06	6.01	0.01	0.24
	TKPP	0.46	4.37	1.14	0.74
	SHMP	0.13	5.34	1.66	0.01
Gloeocapsa alpicola	STPP	0.00	4.63	0.07	0.13
	TKPP	0.07	4.77	0.59	0.02
	SHMP	0.65	7.85	0.62	1.25
Oscillatoria borneti	STPP	<0.01	6.16	0.36	2.13
	TKPP	0.05	4.20	0.63	1.46
	SHMP	0.48	3.97	0.26	0.17
Oscillatoria chalybia	STPP	0.05	6.05	0.01	0.24
	TKPP	0.06	5.94	0.88	0.78
	SHMP	0.03	5.55	0.50	0.55
Phormidium faveolarum	STPP	0.01	5.66	0.01	1.42
	TKPP	0.03	4.30	0.01	0.09
	SHMP	0.09	4.86	0.17	0.26
Chlorella pyrenoidosa	STPP	0.00	6.20	1.70	2.02
	TKPP	0.08	4.75	1.95	0.63
	SHMP	0.41	5.79	2.22	1.70
Chlorella vulgaris	STPP	0.05	4.90	3.00	0.01
	TKPP	0.39	4.31	1.53	1.08
	SHMP	0.13	5.87	0.85	0.98

TABLE 15--Continued

Chemostat With:	Form Added	Stock Analysis, mg/l-P: Ortho	Stock Analysis, mg/l-P: Condensed	Contents in mg/l-P: Ortho	Contents in mg/l-P: Condensed
Scenedesmus obliquus	STPP	0.13	2.87	2.61	3.74
	TKPP	0.13	3.00	3.13	0.13
	SHMP	0.00	4.17	3.00	0.00
Scenedesmus quadricauda	STPP	0.03	6.56	1.70	1.04
	TKPP	0.13	4.35	2.48	0.26
	SHMP	0.06	3.73	1.37	0.00
Ulothrix fimbriata	STPP	0.13	3.59	5.49	0.19
	TKPP	0.08	4.79	2.12	1.29
	SHMP	0.13	6.07	3.85	1.73
Control	STPP	0.06	6.14	6.00	0.38
	TKPP	0.04	4.83	4.25	0.36
	SHMP	0.03	4.28	4.49	0.10
	STPP	0.02	5.04	4.20	0.09
	TKPP	0.03	4.83	4.52	0.10
	SHMP	0.03	5.11	4.55	0.39

TABLE 16

ANALYSES OF CHEMOSTAT CONTENTS AT 36 HOURS AND EXPECTED VALUES OF CONTENTS

Chemostat With:	Form Added	Content Analysis, mg/l-P[a]: Ortho	Content Analysis, mg/l-P[a]: Condensed	Expected Values, mg/l-P[b]: Ortho	Expected Values, mg/l-P[b]: Condensed
Anabaena cylindrica	STPP	2.74	0.72	0.17	3.41
	TKPP	2.35	1.50	2.05	4.11
	SHMP	3.26	1.37	0.40	4.66
Anacystis nidulans	STPP	0.72	0.46	0.06	5.85
	TKPP	3.07	0.13	0.48	4.27
	SHMP	1.96	0.68	0.17	5.20
Gloeocapsa alpicola	STPP	1.89	0.14	0.00	4.55
	TKPP	2.38	0.16	0.08	4.64
	SHMP	3.46	0.38	0.65	7.67
Oscillatoria borneti	STPP	0.60	4.66	0.02	6.05
	TKPP	0.38	2.34	0.07	4.13
	SHMP	1.04	1.08	0.47	3.87
Oscillatoria chalybia	STPP	0.46	1.76	0.05	5.89
	TKPP	0.14	2.08	0.08	5.80
	SHMP	0.47	1.96	0.04	5.41
Phormidium faveolarum	STPP	0.07	0.07	0.01	5.55
	TKPP	0.80	0.24	0.03	4.19
	SHMP	0.89	0.93	0.09	4.74
Chlorella pyrenoidosa	STPP	0.59	2.42	0.05	6.09
	TKPP	1.67	0.97	0.13	4.64
	SHMP	1.51	1.75	0.46	5.68
Chlorella vulgaris	STPP	0.78	2.22	0.13	4.77
	TKPP	1.63	2.86	0.42	4.22
	SHMP	0.46	1.50	0.15	5.74

TABLE 16--Continued

Chemostat With:	Form Added	Content Analysis, mg/l-P[a]: Ortho	Condensed	Expected Values, mg/l-P[b]: Ortho	Condensed
Scenedesmus obliquus	STPP	1.51	1.36	0.20	2.89
	TKPP	2.45	0.60	0.21	2.92
	SHMP	1.37	1.76	0.08	4.06
Scenedesmus quadricauda	STPP	0.78	1.90	0.08	6.41
	TKPP	1.24	1.63	0.19	4.24
	SHMP	1.37	1.50	0.09	3.63
Ulothrix fimbriata	STPP	2.22	0.83	0.27	3.50
	TKPP	1.87	1.67	0.13	4.70
	SHMP	1.39	1.48	0.23	5.95
Control	STPP	0.18	5.72	0.22	5.98
	TKPP	0.09	4.57	0.15	4.71
	SHMP	0.12	3.95	0.15	4.17
	STPP	0.11	4.80	0.13	4.91
	TKPP	0.05	4.49	0.11	4.65
	SHMP	0.09	4.61	0.14	4.83

[a] Related to data shown in Table 15.

[b] Computed by equation 5.1.

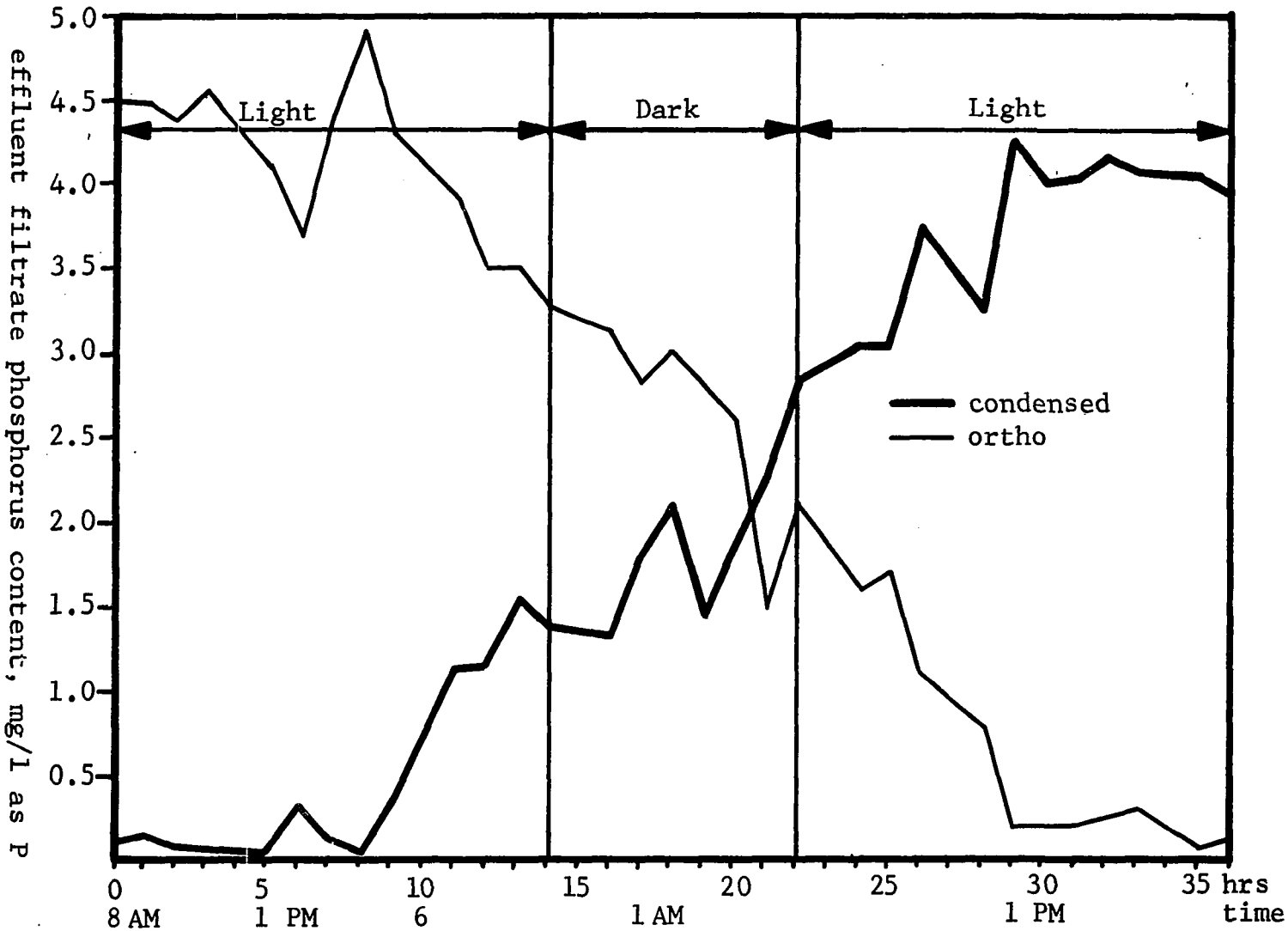


Figure 19. Phosphorus content of Chemostat effluent. Control run with SHMP added.

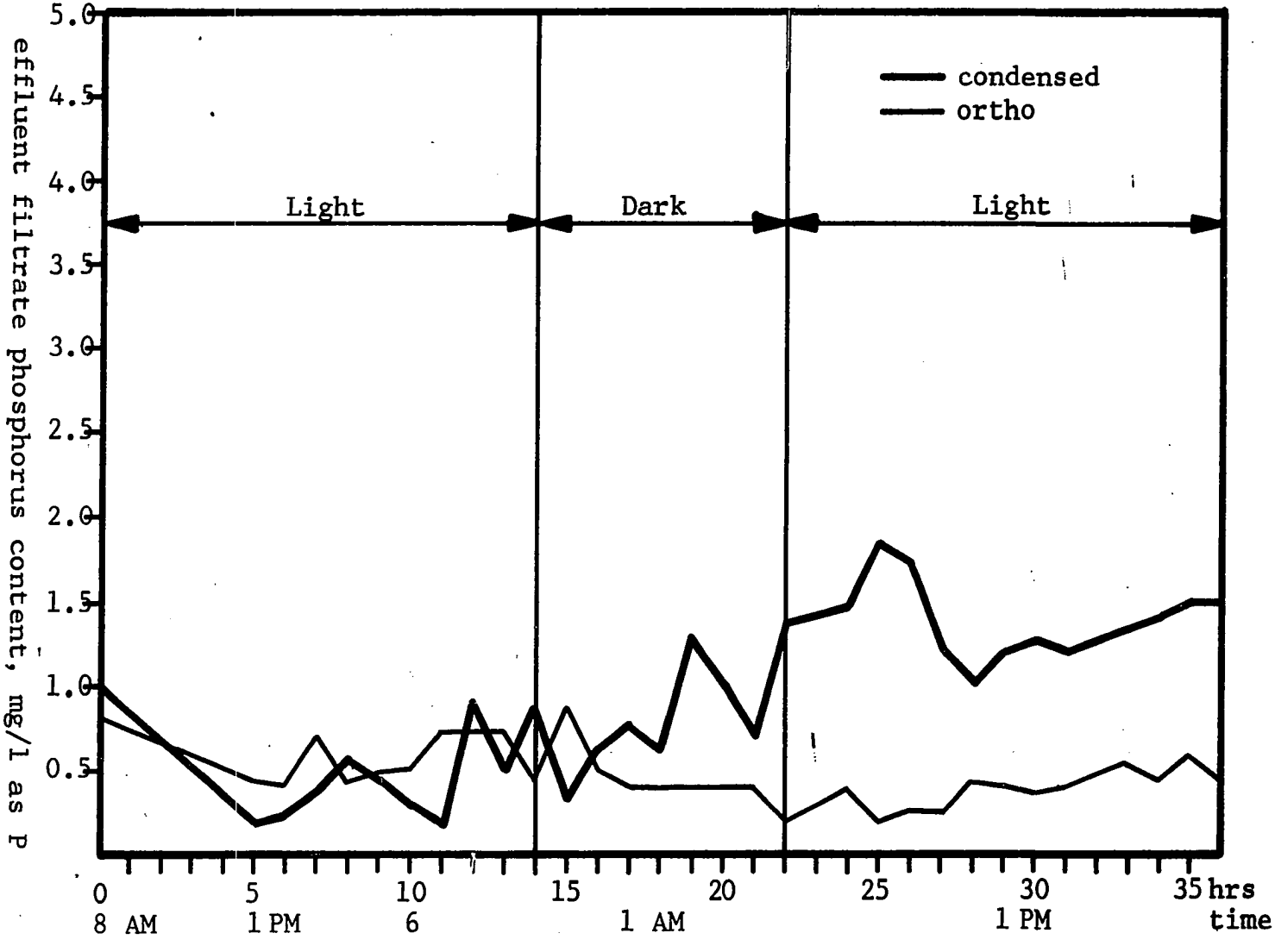


Figure 20. Phosphorus content of Chemostat effluent with Chlorella vulgaris. SHMP added.

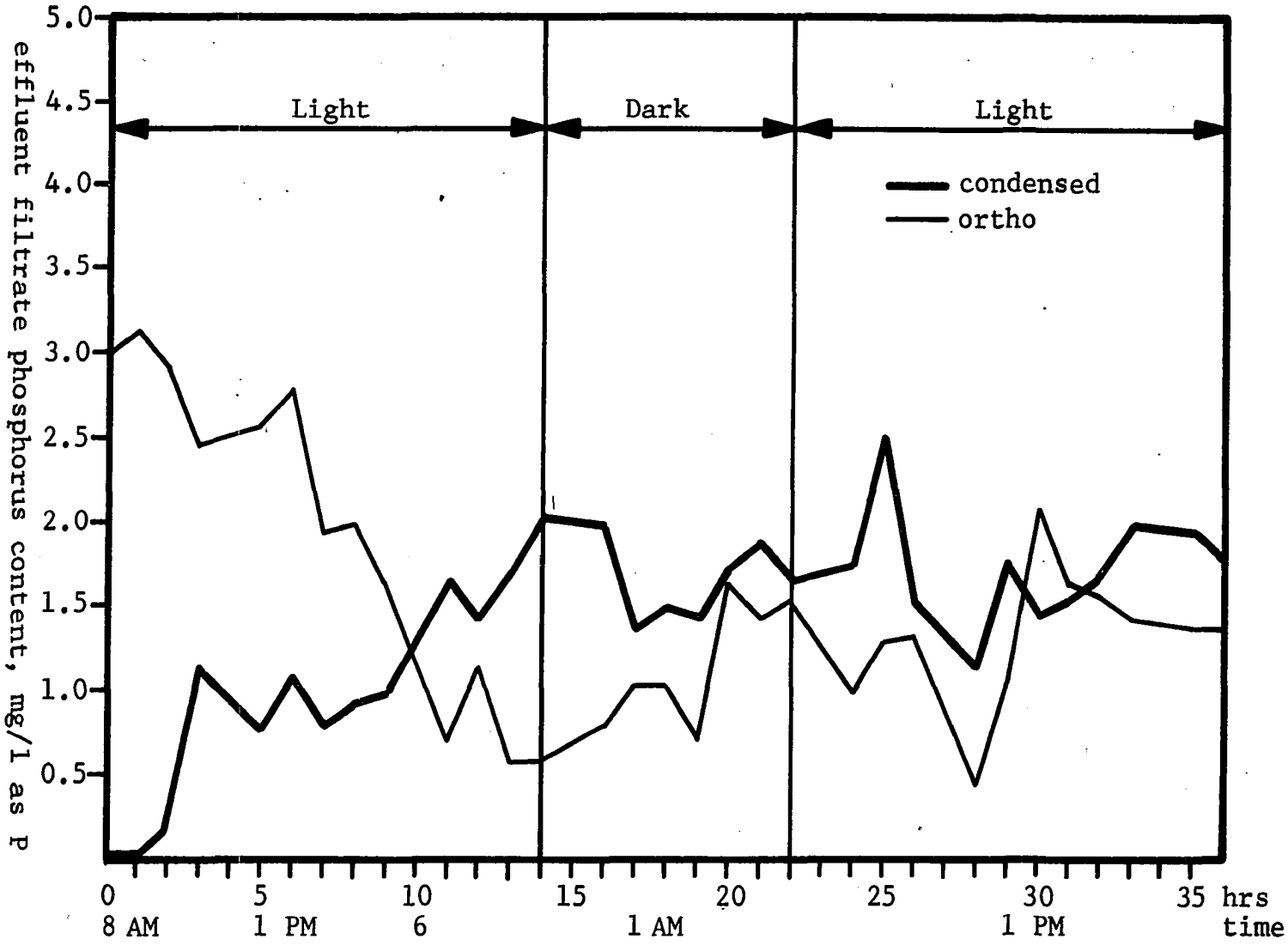


Figure 21. Phosphorus content of Chemostat effluent with Scenedesmus obliquus. SHMP added.

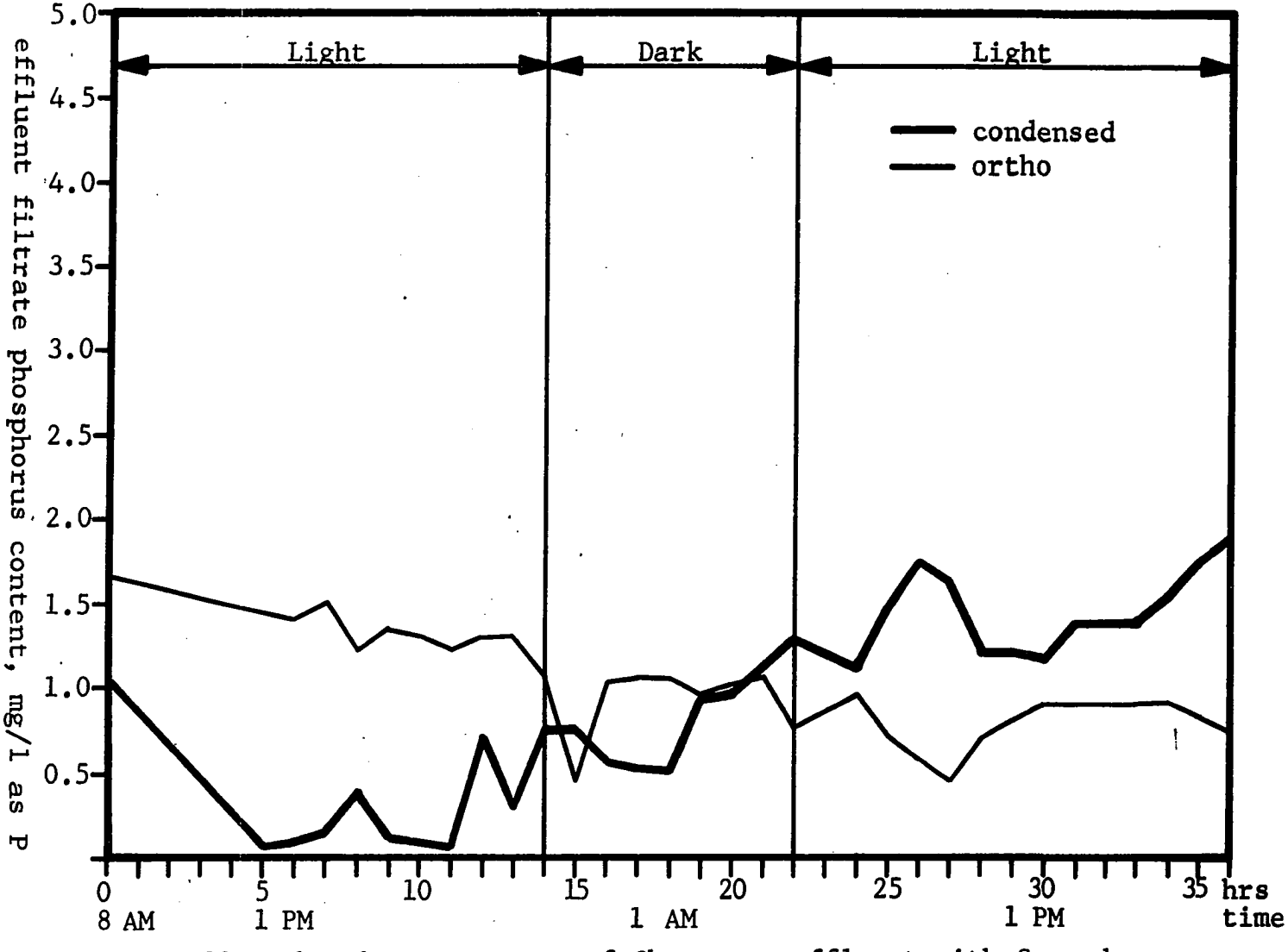


Figure 22. Phosphorus content of Chemostat effluent with Scenedesmus quadricauda. STPP added.

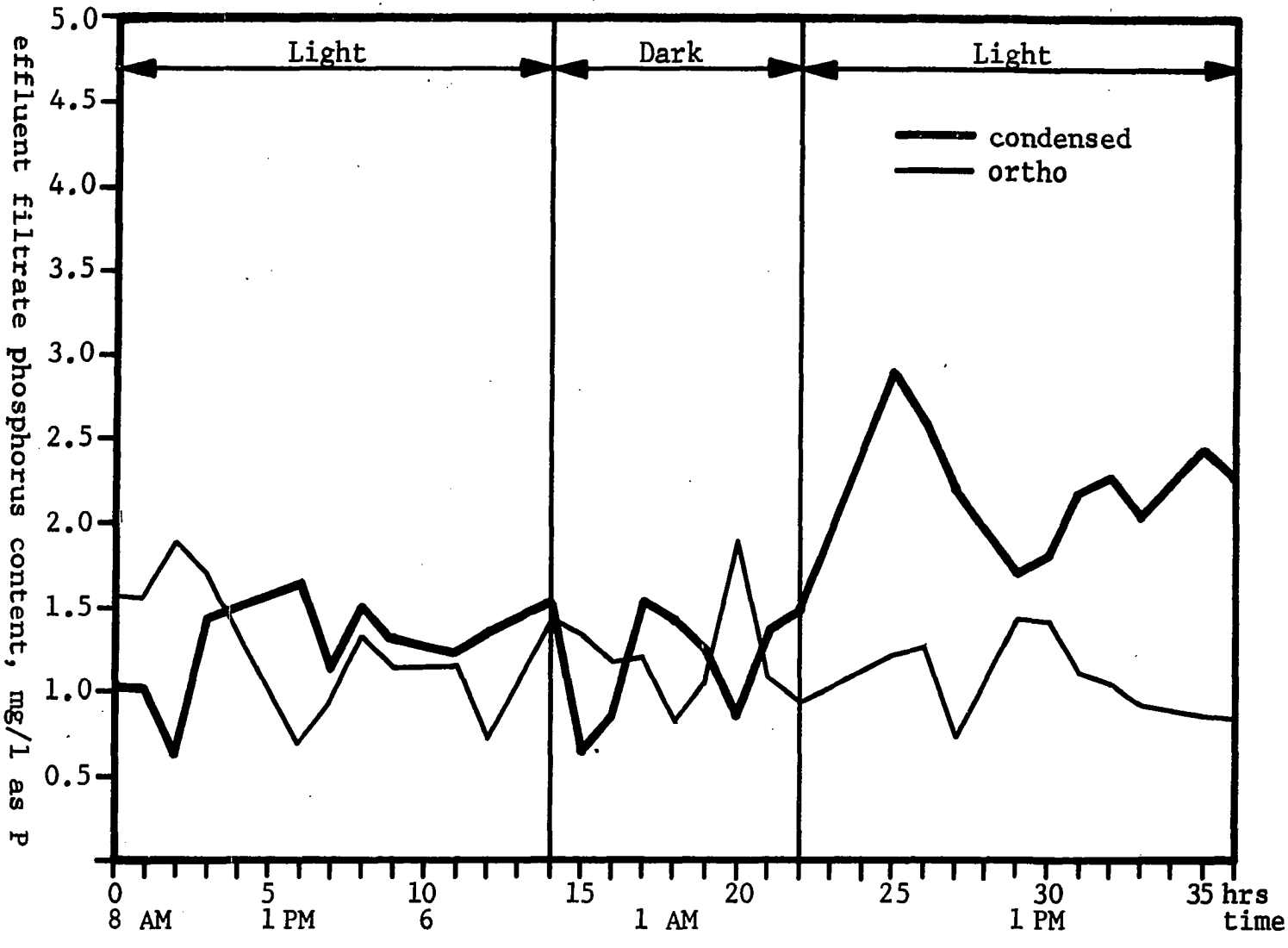


Figure 23. Phosphorus content of Chemostat effluent with Scenedesmus quadricauda. STPP added. Repeated run.

the dye had mixed throughout one half of the Chemostat contents. At four hours, very little difference in color intensity was observed throughout the Chemostat. Spectrophotometric observations of the dye during the run would have been meaningless since the muslin, rubber stoppers, and, to some extent, the glass absorbed some of the dye. These observations, however, validate the usage of the equations (5.1 through 5.3) insofar as mixing is one of the assumptions of the equation. Better proof is shown by the theoretical expected values and the analysis values of the controls shown in Table 16.

Further validation of the use of the Chemostat apparatus was accomplished by hourly sampling of the contents during control and actual experimental runs. These data are shown in Figures 19 through 23. Duplication of the run on Scenedesmus quadricauda and STPP as the test phosphate is included to show the high degree of duplicability obtained by these techniques. The data for these runs are included in Tables 15 and 16.

Interpretation of the data in Figures 19 through 23 was done by analysis of the area under each curve on a weight basis and these figures were compared to the weighed control

TABLE 17

COMPARISON OF PHOSPHORUS DIFFERENCES BY HOURLY SAMPLING AND SINGLE SAMPLE METHODS

Run	Weight Basis (mg/l)[a]: Ortho	Condensed	Total	Single Sampling (mg/l)[b]: Total-P
Scenedesmus obliquus + SHMP	1.02	0.51	1.53	1.01
Chlorella vulgaris + SHMP	1.97	1.07	3.04	3.93
Scenedesmus quadricauda + STPP. Run 1.	1.41	1.09	2.50	3.81
Scenedesmus quadricauda + STPP. Run 2.	1.36	0.36	1.72	2.89

[a] Differences between control and run, on weight of curve basis, from data shown in Figures 19 through 23.

[b] Differences between expected total and single value total-P at the 36 hour point. Corresponds to data shown in Figures 15 and 16.

curves. The results of these calculations are shown in Table 17. The values for the weighed basis extraction are, with one exception, lower than those for the actual point value estimation method. This will allow any reporting of reduction in phosphorus content by the single sample (at 36 hr) method to be the maximum value. The difference in the expected total values and actual total values, corrected for control values will, therefore, represent the maximum quantity of phosphorus taken from the medium in t = 1.5 days. These actual control values are shown at the end of Table 16. Duplicate control runs with the three forms of condensed phosphates were run to obtain average figures for reduction from theoretical expected values to actual assay values. Comparison of the expected values with those obtained at 36 hours shows surprisingly little phosphorus taken from the medium by sorption mechanisms. It is suspected that the glass, rubber, and muslin surfaces were already saturated. The amounts of phosphorus sorbed during the control runs are the following:

STPP = 5.5%; TKPP = 4.5%; SHMP = 5.5%.

Table 18 is a compilation of the data from Tables 15 and 16. The rate constants for phosphorus uptake shown

TABLE 18

RATE CONSTANTS FOR TOTAL PHOSPHORUS TAKEN FROM MEDIUM DURING CHEMOSTAT RUNS[a]

Chemostat With:	Form Added	k[b]	% Total Used[c]
Anabaena cylindrica	STPP	-0.019	2.95
	TKPP	-0.310	37.48
	SHMP	-0.069	8.50
Anacystis nidulans	STPP	-1.075	80.20
	TKPP	-0.267	32.60
	SHMP	-0.472	50.00
Gloeocapsa alpicola	STPP	-0.537	55.40
	TKPP	-0.413	46.20
	SHMP	-0.513	53.70
Oscillatoria borneti	STPP	-0.093	13.25
	TKPP	-0.288	35.20
	SHMP	-0.475	51.20
Oscillatoria chalybia	STPP	-0.655	62.60
	TKPP	-0.650	62.30
	SHMP	-0.537	55.40
Phormidium faveolarum	STPP	-0.916	97.49
	TKPP	-0.932	75.40
	SHMP	-0.650	62.30
Chlorella pyrenoidosa	STPP	-0.450	50.90
	TKPP	-0.392	44.60
	SHMP	-0.421	46.80
Chlorella vulgaris	STPP	-0.325	38.70
	TKPP	-0.006	1.00
	SHMP	-0.732	66.70

TABLE 18--Continued

Chemostat With:	Form Added	k[b]	% Total Used[c]
Scenedesmus obliquus	STPP	-0.004	7.0
	TKPP	-0.013	2.5
	SHMP	-0.185	26.3
Scenedesmus quadricauda	STPP	-0.588	58.6
	TKPP	-0.288	35.2
	SHMP	-0.169	22.7
Ulothrix fimbriata	STPP	-0.138	19.1
	TKPP	-0.204	26.8
	SHMP	-0.509	53.6

[a] Corrected for sorption losses in 36 hours.

[b] $\ln N_o/N = -kt$ units per day.

[c] % total phosphorus taken from medium in 36 hours.

indicate the differences of the 36-hour content analyses and the expected values at 36 hours corrected for sorption rates. The negative sign indicates a decrease in phosphorus content. Without exception, the uptake rate constants for all the blue-green algae in the Chemostats are much higher than those for the static runs (Tables 8, 9, and 10). The green algae indicate a similar rate increase with the exception of Chlorella vulgaris with TKPP and Scenedesmus obliquus with STPP and TKPP.

These data clearly indicate that a nutrient medium passing over actively growing mats of algae has a much greater potential for being reduced in phosphorus concentration than in a static situation. Also, examination of the orthophosphate content analysis column in Table 16 shows (excepting the controls) that, in virtually all instances, the orthophosphate values were higher than the expected values. This can only mean a higher rate of hydrolysis from the condensed forms to the orthophosphate form in comparison to a static situation.

CHAPTER VI

SUMMARY AND CONCLUSIONS

There is no doubt that the aquatic environment in waste stabilization lagoons is composed of a heterogeneous mixture of organisms acclimated to the high nutrient conditions existing therein. Despite man's attempts to control this environment by design parameters such as retention time, depth to surface area optimization, and pretreatment devices, the desired results of nutrient removal are sometimes not attained. There are many reasons for this. In some cases short circuiting may have occurred in the lagoon if it is a single stage operation. Changes in the microbiological population may occur in which case it should be obvious that different organisms, having different growth and assimilation rates, reduce the available nutrients accordingly.

For the last reason it was necessary to observe at least part of the microbiological population in pure culture,

while maintaining strict control measures in the laboratory. The data obtained from such a study should furnish information in part about the activities of the organisms when they are predominant in the lagoon.

The present investigation concerned itself with the question of whether species of blue-green and green algae were capable of hydrolyzing condensed phosphates to the form of orthophosphate. Of equal importance was the behavior of these species with regard to overall phosphorus metabolism during predetermined continuous culture time periods. The results have been compared in two different ways corresponding to the discussion given in detail in the preceding chapter, and a summary of the conclusions is given below. In general, the results indicate a high degree of correlation within each type of experiment, namely static culture methods and flowing systems, but little correlation between the two types.

1. Experimental evidence has shown that it is absolutely essential to maintain cultures in a chemically defined medium if any reproducibility is desired.
2. The condensed phosphates used as syndet builders and in the water softening industry are hydrolyzed by the enzymatic action of raw domestic sewage up to 89.5% within 48 hours.

3. Condensed phosphates alone have little, if any, effect on the rate of decline of numbers of coliform organisms in raw domestic sewage.
4. At least one blue-green algal species tested shows preference for orthophosphate over condensed phosphate forms for its metabolism.
5. Dissimilarities of phosphate degradation and assimilation between genera of blue-green and green algae and even between species of the same genus are apparent.
6. Condensed inorganic and/or organic forms of phosphate may be returned to the aqueous environment during certain growth phases of both blue-green and green algal species.
7. In static conditions, filamentous blue-green and green algae are not the only types capable of forming a bottom mat of actively metabolizing cells. Unicellular forms also have this characteristic behavior.
8. Continuous uptake of sodium hexametaphosphate, sodium tripolyphosphate, and tetrapotassium pyrophosphate over the entire thirty day testing period was shown to occur in five species; Anabaena cylindrica, Gloeocapsa alpicola, Oscillatoria formosa, O. tenuis and Chlorella pyrenoidosa.

9. The average rate constants for total phosphate uptake of all species tested in static conditions were -0.019, -0.016, and -0.017 when in the presence of STPP, TKPP, and SHMP respectively. This indicates overall similarity in phosphate assimilation on the part of blue-green and green algae.
10. Enzymatic hydrolysis may be more easily accomplished in SHMP than in STPP or TKPP.
11. All algal species tested were shown to return some amounts of condensed phosphates to the medium during the latter stages of their growth cycles. This and earlier observations may account for occasional lagoon effluents being higher in total phosphate than the respective influents.
12. Phosphorus uptake rates are higher when only ortho-phosphate is present as opposed to situations where both ortho- and condensed forms are present.
13. Under less than maximum laboratory growth rate conditions, a six inch depth of various species of algae, during peak growth periods, can filter out up to 70% of the incident light of the environment.

14. The average growth rate (k value) of all algal species tested was +0.17, almost exactly ten times the rates of phosphate uptake exhibited by the same algae.
15. Nutrient media have a much greater potential for phosphorus reduction when passing over actively growing mats of algae than when in a static situation.
16. The average total phosphorus uptake rate constants for all algal species tested in the Chemostat units for 1.5 days are -0.436, -0.342, and -0.430 for STPP, TKPP, and SHMP respectively. These correspond to 22.9, 21.4, and 25.3 times those values cited in summary paragraph 9, bearing out the statement in paragraph 15.

It must be kept in mind that the results obtained by carefully controlled laboratory experimentation, such as those presented herein, may not necessarily represent the exact mechanisms which occur in waste stabilization lagoons. Nevertheless, if any true picture of the nutrient reduction is to be obtained, the work must be done first on pure cultures of the organisms involved. After all the factors related to waste stabilization lagoons have been investigated on pure cultures <u>in vitro</u>, the same restrictions and controls

must be applied to heterogeneous cultures. The current scarcity of detailed information of the physiological characteristics of lagoon organisms obviously demands more investigation. The work on heterogeneous cultures should not be limited to mixtures of algae and bacteria alone. It should be obvious to anyone who has even casually observed a lagoon, or any other body of water, that a definite ecological balance is attained in that hydrosphere with time. This balance between algae, bacteria, fungi, invertebrates, and other organisms should be investigated further, at least *in vitro*. The ultimate type of investigation will have to be done by means of yet undetermined methods of the hydrosphere ecosystem *in situ*.

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