Technical Report

Development of An Alternative Phosphorus-Solubilization Procedure for Algal Assay Wastewater Analysis

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EXECUTIVE SUMMARY

A series of laboratory experiments was conducted with the objective of developing a phosphorus-solubilization procedure as an alternative to autoclaving, within the Algal Assay:Bottle Test (AA:BT) protocol for the analysis of wastewater effluents. Autoclaving of wastewater effluent samples is not recommended as an AA:BT pretreatment procedure, due to the potential production of algal toxins from the breakdown of complex organics in effluents under extreme heat and pressure during autoclaving. Pretreatment of effluent samples by only filtering results in the exclusion of the frequently large portion of algal available phosphorus associated with particulates. Consequently, AA:BT analysis of the eutrophication impact of wastewater effluents on receiving waters generally underestimates the bioavailable phosphorus contribution of these discharges, thus limiting the utility of the AA:BT as a decision tool for determining the effectiveness of phosphorus removal operations.

Based on a review of the pertinent scientific literature, which indicated solubilization of particulate P is induced naturally by the action of organic acids produced by many bacteria and some algae, initial experiments were conducted with varying concentrations of glycolic acid, to determine their phosphorus solubilization effectiveness. High turbidity water samples were collected from an eutrophic pond for these experiments. Results of Experiments #1 - #10 indicated that dilute acidification (0.006 M) of a sample with glycolic acid is capable of approximating the autoclaving yield of the sample, but that even a ten (10) percent solution of that sample, after neutralization, greatly inhibits subsequent algal

growth. Therefore, it was decided that glycolic acid treatment was not a viable alternative phosphorus solubilization procedure within the AA:BT protocol.

Subsequent experiments were conducted to determine the phosphorus solubilization effectiveness of acetic acid, hydrochloric acid (HCl) and sodium hydroxide (NaOH) on similar eutrophic pond samples. Results indicated dilute acidification (0.006 M) of a sample with HCl was the most appropriate phosphorus solubilization method. Algal toxicity tests showed that fifty (50) percent dilutions of 0.006 M HCl-treated algal nutrient medium, after neutralization, did not inhibit algal growth, when compared to a control. Four (4) AA:BT experiments were then conducted to compare the algal availability of phosphorus solubilized by 0.006 M HCl treatment to that of autoclaving. These experiments demonstrated that, while slight algal growth inhibition may be encountered at low dilutions (i.e. 50% sample), the incorporations of the HCl-treatment scheme into the AA:BT procedure for wastewater effluent testing allows for the analysis of algal-available particulate phosphorus, which would not be solubilized without the use of autoclaving.

The final experiment conducted during this research applied the 0.006 M HCl-treatment procedure to the AA:BT analysis of a wastewater effluent sample collected of the Marlborough Easterly Wastewater Treatment Facility. Filtered and HCl-treated aliquots were prepared for the AA:BT in dilutions consisting of 1%, 5%, 10% and 20% of the original treated samples. Results of this experiment indicated that 0.006 M HCl treatment can increase the algal growth potential of a wastewater effluent via solubilization of phosphorus from particulates prior to filtration. However, the phosphorus

solubilized during HCl treatment may not all be readily algal available, and/or algal toxins solubilized during the acidification may cause varying degrees of algal growth inhibition.

The research experiments indicated that 0.006 M HCl is the optimal concentration for achieving the dissolved phosphorus yield of autoclaving, while not generally causing gross algal growth inhibition. Although 0.006 M HCl treatment has apparently caused varying degrees of algal growth inhibition in both eutrophic pond samples and a wastewater effluent, it has also been demonstrated to increase the algal growth potential of these waters over their respective untreated algal yield levels. Further study of the 0.006 M HCl treatment procedure is necessary to properly assess its potential as a phosphorus-solubilization technique within the protocol of the AA:BT analysis of wastewater effluents.

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INTRODUCTION

Phosphorus is widely acknowledged to be the most critical nutrient determining the trophic status of freshwater aquatic systems, due to the natural scarcity of its soluble forms in pristine waters. The relative abundance of dissolved phosphorus in wastewater effluents and surface runoff from certain land uses has focused attention on both point and non-point source control measures for attenuating phosphorus loads to water bodies as the most efficient means to combat eutrophication problems. Although diffuse-source loading of phosphorus from agricultural and urban land areas can contribute large amounts of the nutrient to surface waters during rainfall events, complete management of non-point sources is commonly not feasible on a large scale. Control of point-source inputs of phosphorus, primarily wastewater treatment facilities, is generally attainable and only limited by the financial constraints of the community or region served by the plant, and by the local environmental consciousness, as dictated by state and/or federal water pollution regulations.

In the recent past, the eutrophication and effective death of many water bodies were commonly accepted as an unavoidable consequence of human development and progress. The diminution of quality surface water supplies, pollution of groundwater aquifers, and propagation of unbridled growth and expansion of communities have forced many municipalities do seek alternative and/or supplementary sources of drinking water. Water bodies once considered unsuitable as sources of domestic water now often represent one of the few possible solutions to the water shortage problems of towns and cities. Commonly, these waters exhibit some degree of eutrophication, in many instances due primarily to the lack of proper phosphorus loading controls.

These eutrophied water sources are evidence that the consequences of eutrophication can transcend the obvious visual, olfactory, and ecological offenses. The addition of chlorine during disinfection of algal-rich waters can result in the increased formation of trihalomethanes (Hoehn et al., 1980; Jones and Lee, 1982a; Jones and Lee, 1982b). The potential carcinogenic hazards of long-term exposure to trihalomethanes at concentrations commonly found in domestic water supplies are currently a major concern of the public health field. Oliver and Shindler (1980) and Hoehn et al. (1984) have shown that algae and algal byproducts can be potent trihalomethane precursors when disinfection of drinking water is accomplished by chlorination. Therefore, although high levels of dissolved phosphorus forms in aquatic systems are generally not directly harmful to humans, the indirect effects of the overabundance of this nutrient can be of significant magnitude.

As a result, an increased emphasis has been placed on the monitoring of the trophic status of water bodies in recent years, via the measurement of total phosphorus (TP) and orthophosphate, or dissolved reactive phosphorus (DRP), concentrations in the water column. While the TP analysis detects all forms of particulate and dissolved phosphorus present, the DRP analysis is generally used to estimate the amount of the nutrient that is readily bioavailable to algae and other aquatic life. However, use of one or even both of these detection procedures is usually not sufficient for proper monitoring of the eutrophication potential of an aquatic system.

Orthophosphate measurement alone is of little predictive value, since bioavailable P is quickly taken up by organisms in the water that are removed by filtration prior to analysis. DRP concentrations may actually be very low at times under eutrophic conditions, since the P is primarily

incorporated in algae, bacteria, detritus, dissolved organic P, and macrophytes during the growing season (Chiaudani and Vighi, 1974). Conversely, the highest DRP levels in eutrophic lakes are often found during the late winter when biological activity is at a minimum (Levine and Schindler, 1980).

Although TP analysis can be used during any season as a gross estimate of all forms of the element present in a water, it provides no bioavailability information. Despite this shortcoming, TP determinations often constitute the basis for eutrophication control strategies, including the design and operation of wastewater treatment processes for P removal. However, because the percentage of bioavailable P has been shown to vary greatly according to the sample's source and time of collection (Peters, 1981), alternative eutrophication control parameters are being established.

The use of algal bioassays to determine the potential amount of P available to a single algal culture of known characteristics under controlled conditions has gained wide acceptance during the last decade (Greene et al., 1975; Hegemann et al., 1983; Plotkin and Ram, 1983). In accordance with this trend, incorporation of improved P bioavailability data into eutrophication control strategies can result in more cost-effective P-reduction measures, due to a decreased dependence on TP measurements alone. Accurate estimation of potential P availability is critical to the efficient operation of a tertiary wastewater facility, since the cost per unit reduction in the bioavailable P load increases as the bioavailability of the P source decreases (Sonzogni et al. 1982).

Determination of bioavailable P levels in wastewater effluents is also very important for predicting the effectiveness of different levels of P removal at reducing the degree of eutrophication of the receiving water.

Because the amount of algal growth in waters subject to the P-rich discharges from treatment plants is generally limited by the level of inorganic nitrogen present, due to the overabundance of P, removal of inadequate amounts of bioavailable P will cause no change in the trophic status of the aquatic system. Until the levels of P in the receiving water are reduced sufficiently so that algal growth is limited by the bioavailable P concentration, the costs of P removal produce no major environmental benefits. An extensive algal assay study of the Housatonic River, downstream of the outfalls of the Pittsfield, Massachusetts wastewater treatment facility and other P-rich sources, conducted by Ram and Plotkin (1983), corroborated these findings and indicated that the percent P removal necessary to attain P limitation in the river may be beyond the "Best Practicable Technology" level. The potential role of the Algal Assay:Bottle Test (AA:BT) in determining the proper levels of P removal and the economic feasibility of such wastewater treatment operations has thus been demonstrated.

Conventional P removal procedures attempt to minimize the readily bioavailable soluble fraction of TP in effluents via addition of precipitants, causing conversion to particulate forms. The efficiency of P removal is highly dependent on the effective settling of the P solids (Switzenbaum et al., 1981). Consequently, since improper clarifier operation can be a large fraction of the TP in effluents. The bioavailability of particulate P in effluents (Young et al., 1982), fluvial systems (DePinto et al., 1981),lake sediments (Williams et al. 1980), soils (Hegemann et al., 1982),and runoff suspensions (Huettl et al., 1979) has been the subject of considerable research. These studies have indicated

that particulate P can play a critical role in determining the trophic status of a water body.

Although particulate P in effluents is not immediately bioavailable, its long-term impacts can be important. The actual availability of particulate P to algae in natural systems is determined by many factors (Cowen and Lee, 1976; Huettl et al., 1979; Sonzogni, 1982):

1) time of suspension in photic zone,
2) resuspension due to turbulence,
3) temperature (chemical and biological rates),
4) pH,
5) DRP level in receiving water (controls rate of release from particles),
6) chemical and physical characteristics of particles,
7) amount of light,
8) algal species and population,
9) availability of other essential nutrients.

Standard AA:BT procedures for non-wastewater samples involve pretreatment by autoclaving and subsequent 0.45 micron (μm) filtration. Autoclaving causes the release of a portion of the particulate P, and filtration removes indigenous organisms and particles that would interfere with the growth of the test alga and with cell enumeration via electronic particle counting. Because some of the particulate P is solubilized prior to filtration, the AA:BT serves to estimate the potential long-term algal bioavailability of P in a sample. Therefore, since it is conducted under ideal conditions for algal growth, the AA:BT determines an upper limit of

the amount of bioavailable P present (assuming P limitation occurs). The factors listed above may constrain the algal utilization of P in natural systems.

Statement of Problem

Autoclaving of wastewater effluents is not recommended as an AA:BT pretreatment procedure, due to the potential production of toxicants from the breakdown of complex organics in effluents (Miller et al., 1978). Presently, only filtration is utilized to prepare effluent samples for AA:BT analysis. This results in the loss of the frequently large portion of a algal available P associated with particulates (Jadlocki et al., 1976; Saldick and Jadlocki, 1978). Consequently, AA:BT analysis of effluent impacts on receiving waters generally underestimates the bioavailable P contribution from wastewater discharges. This limits the utility of the AA:BT as a decision tool for determining the effectiveness of P removal operations.

Purpose of Investigation

The objectives of this project are:

1) to design an alternative P solubilization procedure for AA:BT analysis of wastewater effluents; and
2) to determine the bioavailability of the particulate P solubilized by the alternative procedure.

Incorporation of an effective P solubilization scheme into the AA:BT effluent analysis technique would greatly enhance the dependability and usefulness of the procedure, by allowing the AA:BT to more accurately assess the P loading impacts of wastewater effluents on the trophic status of receiving waters.

LITERATURE REVIEW

Phosphorus Forms In Aquatic Systems

Phosphorus is essential to the life processes of all organisms, due to its key role in the storage and transfer of cell energy and in genetics. Adenosine triphosphate (ATP) is the universal energy carrier in living cells and phosphate groups are present in nuclei acids. Phosphorus on earth was originally derived from the weathering of igneous rocks containing apatite minerals. Phosphorus is certainly not one of the rarest elements, but it is relatively scarce in comparison to the other major atoms of living organisms (carbon, hydrogen, oxygen, nitrogen, and sulfur), constituting one tenth of one percent by weight of the earth's surface (Cole, 1979). However, most organisms contain approximately two percent phosphorus on a dry weight basis (Snoeyink and Jenkins, 1980). Therefore, when present in available forms, phosphorus is rapidly taken up and concentrated by living organisms.

Phosphate minerals are highly insoluble in water. Rapid uptake via biotic removal and abiotic adsorption on particulates generally assures maintenance of low levels of dissolved inorganic phosphorus in pristine freshwater aquatic systems. Nitrogen, which is generally the limiting nutrient for algal growth in polluted waters with high levels of phosphorus, can be converted from atmospheric nitrogen to available inorganic forms by nitrogen-fixing bacteria and certain algae. Gaseous forms of phosphorus are virtually nonexistent in the earth's atmosphere.

Research has widely indicated that the fraction of phosphorus directly available for uptake by aquatic organisms is dissolved inorganic phosphorus or orthophosphate (PO_4^{-3}). The wet-chemical analytical methods generally utilized to determine the orthophosphate concentration of a water have long

been a subject of much debate and criticism. Rigler (1968) found that the values given by the most commonly used chemical analysis for orthophosphate, the Ascorbic Acid - Molybdenum Blue colorimetric technique (Murphy and Riley, 1962), were generally much higher than the maximum concentrations of dissolved inorganic phosphorus obtained from radiobiological analysis of the same water sample. Rigler qualified his contention by hypothesizing that other forms of phosphorus in aquatic systems could also become bioavailable, due to the rapid transformation rates of the various phosphorus fractions. Recent radiobiological studies of phosphorus bioavailability have shown approximately seventy-five percent of P chemically measured as orthophosphate to be readily available to algae (Jordan and Dinsmore, 1985).

The classification and definition of the different compartments of the aquatic phosphorus cycle also involve a degree of uncertainty. Wetzel (1975) presents a generally accepted categorization of the major fractions of phosphorus found in natural waters:

I. Particulate Phosphorus

A) Phosphorus in living organisms (nuclei acids, enzymes, vitamins, etc.);

B) Phosphorus in mineral phases of rock and soil (apatite) and adsorbed by inorganic complexes (clays);

C) Phosphorus adsorbed by dead particulate organic matter;

II. Dissolved Phosphorus

A) Orthophosphate,
B) Polyphosphates (primarily from detergents),
C) Soluble organic forms (organism excretion and decomposition),
D) Organic colloids.

Figure 1 displays the major compartments of the phosphorus cycle in aquatic systems. It is important to keep in mind that such an analytical classification of the major phosphorus forms does not always involve mutually exclusive categories.

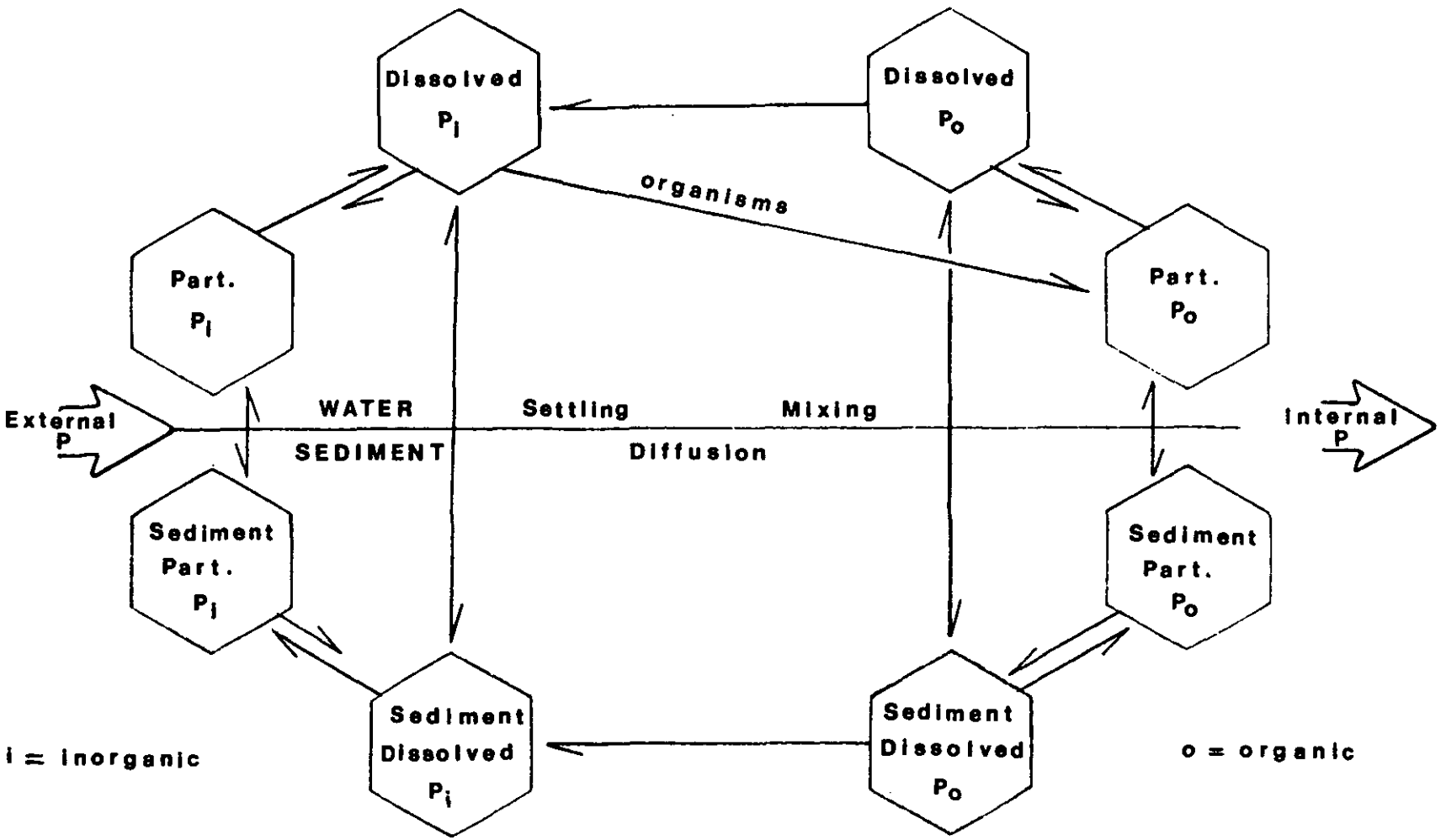


FIGURE 1 Major Compartments of the Phosphorus Cycle in Aquatic Systems. (After: Syers, et al. (1973))

"Dissolved" phosphorus is an operational term, based on the differentiation from the "particulate" fraction by filtration through a 0.45 micron membrane filter (American Public Health Association, 1985). Dissolved phosphorus can be analytically categorized into three fractions by use of the aforementioned Molybdenum Blue method (Meals and Cassell, 1978):

A) "Dissolved (molybdate) reactive phosphorus" (DRP), which includes;
   1) orthophosphate (generally represents the majority of the fraction);
   2) colloidal P solubilized by the acidic molybdate reagent;
   3) hydrolizable soluble organic P;

B) "Total dissolved (molybdate) reactive P" - measured after oxidation and hydrolysis of all soluble P forms;

C) "Dissolved unreactive P" - equal to the difference between A and B and generally consisting of soluble organic P and polyphosphates.

The colloidal P and hydrolizable soluble organic P are believed to account for the discrepancies between DRP measurements and actual P bioavailability pointed out by Rigler (1968).

These classifications demonstrate the importance of properly applied and understood terminology when studying the interactions and effects of various P forms in aquatic systems. This is especially true in the case of the critical orthophosphate fraction, that, if it is to be considered synonymous with the analytical DRP fraction, must be interpreted to include colloidal P and hydrolizable organic P forms that are less than 0.45 microns in diameter.

Phosphorus Bioavailability

A great amount of research has been conducted to study the processes by which phosphorus is exchanged between the different P fractions and to determine the short-term and long-term bioavailabilities of each form. The contributions of particulate and colloidal P and dissolved organic P to the trophic status of aquatic systems have received much attention. The majority of research has involved the study of lakes, as opposed to fluvial systems, due to the greater residence times and resultant eutrophication problems of the former.

Dissolved Organic P

Allen et al. (1977) studied the cycling of dissolved organic P (DOP) in aquatic systems and determined that two major DOP fractions exist: one having a rapid turnover rate to orthophosphate and possibly being hydrolyzable by alkaline phosphatases, P-solubilizing enzymes produced by bacteria and certain algae; and the other fraction being much more stable, with a long residence time. The release of orthophosphate from dissolved complex P compounds in lake water was investigated by Francko and Heath (1979). They found that a low molecular weight (MW) compound common in eutrophic lakes readily released orthophosphate in the presence of alkaline phosphatase, while a high MW compound found in humic bogs resisted enzymatic hydrolysis. Another study indicated high MW DOP in the humic acids of lake tributaries to be resistant to enzymatic breakdown to orthophosphate, but to be subject to degradation by ultraviolet light (Stevens and Stewart, 1982a). Berman and Moses (1972) found high alkaline phosphatase activities in organisms to be induced by low orthophosphate concentrations and to be repressed by high concentrations. Paerl and Downes (1978) demonstrated that in the absence of free orthophosphate in lakewater, soluble organic

compounds can be utilized via alkaline phosphatase activity in P-starved algae. Langowska (1982) determined that phosphatase producing bacteria can increase the availability of synthetic organic P compounds for algal growth. It has also been shown that the hydrolysis of some DOP compounds to orthophosphate by the acid in the Molybdenum Blue reagents can lead to their analytical determination as DRP (Downes and Paerl, 1978). Therefore, the accurate estimation of algal available P levels in an aquatic system via chemical analysis is very difficult, due to the many factors affecting the availability of DOP compounds for algal growth.

Particulate and Colloidal P

Particulate phosphorus can constitute greater than ninety-five percent of the total phosphorus in the water columns of lakes during the summer (Wetzel, 1975). Therefore, particulate P can certainly play a major role in the cycling of orthophosphate. Macromolecular colloidal P forms generally pass through a 0.45 micron membrane filter can be hydrolyzed during the analysis, resulting in their inclusion with the "dissolved" reactive fraction (Twinch and Breen, 1984). However, colloidal P in aquatic systems has been determined to possess orthophosphate cycling characteristics distinct from actually soluble P forms. Lean (1973) has determined that colloidal P is an important intermediary for the exchange of orthophosphate between the water column and the particulate fraction. Peters (1978) found the rate of this exchange to be more rapid in the summer than in the winter, due primarily to the reduced biological activity during colder weather. Peters (1981) pointed out that particulate P consists of an organic fraction (living organisms and their byproducts), which is largely available, and an inorganic fraction (clays and apatites) from which desorption of orthophosphate is less likely. Therefore, both particulate P and colloidal

P forms can become algal available when orthophosphate levels in the water are low and other conditions are conducive to the release of orthophosphate from these fractions.

Algal Availability of Particulate P

A great number of studies have focused on the proportion of particulate P available for algal growth in freshwater. Cowen and Lee (1976a) found particulate P bioavailability to vary widely according to source, with bioassay results indicating an average of twenty percent available for algal growth in tributaries to Lake Ontario. Other studies of particulate P in lake tributaries and drainage waters have also determined that approximately twenty percent is ultimately algal available (Dorich et al., 1980; DePinto et al., 1981; Stevens and Stewart, 1982b). However, Cowen et al. (1978) found the percent available particulate P in such fluvial systems to vary plus or minus twenty percent, depending on sample location and time of collection. Peters (1981) indicated that a much higher proportion of particulate P is algal available in lakes than in their tributaries, because the particulate P in lakes is largely biologically associated and well-removed from sedimentary and riverine sources of insoluble P.

Young and DePinto (1982) and Young et al. (1985) studies the algal availability of particulate P from various sources in the Great Lakes basin and found the availability and release rate of orthophosphate from particulate P to be ranked in order of decreasing magnitude as follows:

1) wastewater solids,

2) lake bottom sediments,

3) tributary suspended sediments,

4) eroding bluff solids.

Young et al. (1982) determined that an average of sixty-three percent of particulate P in wastewaters can ultimately be algal available, with an additional nine percent available over a long-term (greater than two weeks) bioassay; eighty-three percent of TP in wastewaters was indicated to be ultimately available, including virtually all of the dissolved P.

Many studies have concluded that the actual residence time of particles in the water column is a much more important factor affecting overall water quality than the quantity of available P in the particles. For bioavailable P associated with rapidly settling particles, the rate of conversion of particulate P to orthophosphate is more crucial than the fraction which can eventually become available (Verhoff and Heffner, 1979). Therefore, Williams et al. (1980) concluded that the mean residence time for particles in the photic zone of a lake is the most important factor in the assessment of particulate P bioavailability in actual environmental situations; fine clay particles were found to remain in a five meter photic zone for approximately sixteen days under non-turbulent conditions. Klapwijk (1982) indicated algal available P in lake sediments to be positively correlated with the clay content of the sediments. Dong et al. (1983) found P levels to be greater in clay particles than in sand or silt, allowing greater P availability, due to the selective transport in runoff and the much longer suspension times of clays. Stabel and Geiger (1985) determined that such fine particles with a great surface area can also serve as a sink for bioavailable P whenever the equilibrium concentration of orthophosphate is exceeded in the water column. They found a portion of the adsorbed P to be irreversibly bound under normal environmental conditions, while approximately sixty percent was available via desorption.

A study by van Eck (1982) concluded that potentially algal available particulate P is only important when the orthophosphate concentration in the water is low, and that the particulate fraction will become increasingly crucial to the trophic status of waters receiving wastewater effluents, as more dissolved P is removed via tertiary treatment. At lower orthophosphate levels, the rate of release of algal available P from particulates controls the rate of algal uptake, as the particulate P acts as a source of replenishment for the orthophosphate pool, responding to biological uptake of the nutrient (Sagher, 1976).

Processes Affecting Release of Particulate P

The rate and amount of release of bioavailable P to the water column from particulate P are the result of various physio-chemical and biological reactions (DePinto et al., 1981):

1) sorption - desorption,

2) dissolution - precipitation,

3) microbial mineralization of organic P,

4) microbial-induced dissolution of particulate P,

5) coagulation - sedimentation of the particulates.

Cowen and Lee(1976b) found that physical and chemical rather than microbial mineralization processes were most likely the key factors regulating the release of bioavailable P from suspended particles to the solution phase in Lake Ontario tributary waters. Oloya and Logan(1980) indicated that P physically adsorbed to soils and sediments is essentially one hundred percent labile or exchangeable. The conversion of particulate organic matter to inorganic forms via mineralization can also be brought about by changes in the water column due to physio-chemical, as well as bacterial action (Cowen et al., 1977). Therefore, the bioavailability of

particulate P in aquatic systems is greatly dependent on physical and chemical processes, especially sorption/desorption reactions. These reactions are influenced by many factors (Sagher, 1976):

1) cation species associated with solid phase orthophosphate,
2) ionic strength of the environment,
3) presence of competing anionic species,
4) pH,
5) nature of the sorbing surface,
6) time of contact between the solid and solution orthophosphate phases,
7) oxidation-reduction conditions.

However, the algal availability of particulate P in wastewater effluents can be significantly affected by microbial activity, due to the high concentrations of microorganisms. Narasiah and Morasse (1984) indicated that bacteria play an important role in determining seasonal variations of wastewater P species in a temperate climate. Orthophosphate levels were found to be an average of thirty-four percent higher in summer effluents than in winter effluents, with similar influent characteristics. The elevated rates of degradation and hydrolysis of organic P and polyphosphates by microorganisms under higher temperatures were believed to be the cause of this phenomenon.

Methods for Determining Particulate P Algal Availability

There are two major classes of procedures utilized to estimate the amount of particulate P available for uptake by algae. Chemical extractions generally subject solutions containing particulates to mildly basic or acidic conditions, while constantly mixed at a specific temperature for a certain period. Orthophosphate levels in the resultant extraction solutions

are then measured with typical wet-chemical methods. In comparison, bioassay techniques utilize a variety of procedures in an attempt to more directly estimate the amount of algal available particulate P. However, a disadvantage of bioassays is the amount of time required to perform them.

Chemical Extractions - The major acid extraction procedures utilize hydrochloric acid (HCl) in varying concentrations (0.1 to 1.0 N). Such treatments have been found to solubilize calcium phosphates (including apatites) and much of the iron and aluminum phosphates. Base extractions usually use 0.1 N sodium hydroxide (NaOH) solutions and remove non-occluded phosphates bound to iron and aluminum, but not those associated with calcium (Cowen and Lee, 1976a). Anion-exchange resin extractions are also commonly applied to estimate the inorganic P which would be released from particles upon dilution in a P-deficient receiving water. The base and resin extraction methods have generally been found to provide the best estimates of particulate P algal availability when compared to bioassay analyses conducted on the same samples (Cowen and Lee, 1976a; Sagher, 1976). However, Young et al. (1982) found the base extraction procedure to have a low accuracy for predicting particulate P availability in wastewaters. In addition, a study of the algal availability of particulate P from diffuse and point sources in the lower Great Lakes basin concluded that no single chemical extraction procedure can be widely used as a surrogate to time-consuming bioassays (Young and DePinto, 1982).

Bioassays - Bioassay techniques for determining particulate P algal availability generally attempt to simulate natural conditions. The Dual Culture Diffusion Apparatus (DCDA) is a recently developed procedure designed specifically for the analysis of particulates (DePinto, 1982), The DCDA consists of two culture vessels separated only by a thin membrane

filter that allows the diffusion of dissolved substances between an algal culture and a particulate suspension. It has been used to analyze P release from Great Lake tributary suspended sediments (DePinto et al., 1981) and from wastewater particulates (Young et al., 1982). Although the DCDA allows the detailed study of the kinetics of P release from particulates, it is generally more work-intensive than the Algal Assay:Bottle Test (AA:BT) developed by Miller et al. (1978).

As previously described in the Introduction, the AA:BT involves the direct addition of a pure culture (Selenastrum capricornutum) algal inoculum to a pretreated aliquot of a water sample which is then subjected to constant mixing under controlled temperature, humidity, and illumination conditions for a minimum of two weeks, prior to growth quantification. Pretreatment for non-wastewater samples consists of autoclaving to solubilize potentially available particulate nutrients, especially P, followed by 0.45 micron filtration. Due to the toxicant formation problems associated with the autoclaving of wastewater, no particulate P solubilization procedure has been generally applied during the AA:BT analysis of wastewater effluents. In the next sub-section, research regarding autoclaving and alternative particulate P solubilization techniques will be discussed.

Particulate P Solubilization Alternatives

Autoclaving

Autoclaving has generally been shown to increase the amount of particulate P available to algae (Cowen and Lee, 1976a). For example,Cowen et al. (1978) found the algal availability of particulate P in Lake Ontario tributary waters to be increased from approximately six percent to as much as fifty-seven percent by autoclaving. However, autoclaving eliminates

dissolved carbon dioxide from the sample, raising the pH, and sometimes actually causes a loss of available P via precipitation of P salts (Filip and Middlebrooks, 1975). This is usually a problem only with hard (>150 mg/1 as $CaCo_3$ total hardness) waters (Jadlocki et al., 1976; Twinch and Breen, 1982). Saldick and Jadlocki (1978) pointed out that the P species produced by the autoclaving of algal cells which analyze as DRP are not necessarily the same as those found in natural waters which also analyze as DRP. Therefore, the potential of autoclaving to induce chemical transformations that would not occur under natural conditions limits the utility of the procedure as a particulate P solubilization technique within the AA:BT.

Ultraviolet Light

The use of ultraviolet (UV) light in P algal availability studies has not seen wide application. One major reason for this is the oxidation of organic nitrogen compounds to bioavailable forms by UV light (Filip and Middlebrooks, 1975). This creation of algal available nitrogen from fractions generally unavailable to algae, even on a long-term basis, is important when excess P levels allow nitrogen to be the limiting nutrient for algal growth, as with effluents from secondary treatment. UV light has also been found to cause the degradation to orthophosphate of soluble high MW organic P compounds, which are generally resistant to enzymatic hydrolysis by alkaline phosphatases (Downes and Paerl, 1978; Francko and Heath, 1979; Stevens and Stewart, 1982a). With certain waters, this can lead to an overestimation of the maximum levels of algal available P.

P Solubilizing Enzymes

Considerable research has been conducted to study the role of phosphatase (alkaline and acidic) enzymes, produced by many bacteria and

some algae, in the hydrolysis of various dissolved and particulate P forms and subsequent release of orthophosphate. Francko and Heath (1979) found alkaline phosphatases to affect a broad class of P compounds and to be adaptively produced by some organisms when nutritionally stressed. Peters (1981) determined the algal availability of particulate P to be increased due to the enzymatic breakdown of suitable organic P substrates by naturally occurring phosphatases. Alkaline phosphatase activity has been demonstrated in P-starved Chlorella algal cultures (Paerl and Downes, 1978), and the production of both acidic and alkaline phosphatases has been found in the Scenedesmus obliquus alga, allowing direct utilization of synthetic organic phosphates (Langowska, 1982). Keenan and Auer (1974) detected no alkaline phosphatase activity in Selenastrum capricornutum, the most commonly used alga for the AA:BT procedure. However, this was probably due to the neglect of the effect of illumination, as Klotz (1985) found the alkaline phosphatase activity of Selenastrum capricornutum to be inversely related to light intensity. It was hypothesized that decreased phosphate uptake due to greatly reduced light intensities under shaded conditions may stimulate alkaline phosphatase activity, thus increasing the pool of potentially algal available P compounds. The production of phosphatases, which are inactivated during autoclaving (Berman and Moses, 1972), by bacteria can also increase the amount of P available to algae (Langowska, 1982).

P Solubilization by Organic Acids

Bacteria can also cause the release of orthophosphate from particulate P by their formation of organic acids. Harrison et al. (1972) isolated bacteria capable of solubilizing inorganic P compounds bound to lake sediments by the production of organic acids that function as chelating agents. The organic acids, which are formed during carbohydrate metabolism,

apparently sequester the metallic ions (calcium, iron, aluminum, and magnesium), allowing the release of bound phosphates to the water column. Craven and Hayasaka (1982) corroborated these findings during their study of P solubilization by bacteria associated with the root systems of sea grass. They concluded that the bacteria solubilized calcium phosphate via production of acetic acid during glucose metabolism and that the acetic acid functioned by chelation. The minimum concentration of acetic acid required to produce detectable increases in orthophosphate levels was 10^{-5}M. In addition to acetic acid, Harrison et al. (1972) identified several other organic acids produced by bacteria: gluconic, glucuronic, glycolic, lactic, and succinic acids. In separate experiments based on the bacterial study, they found glycolic acid (0.056 M) to solubilize the most calcium phosphate.

Glycolic acid or glycolate (MW = 76) has also been determined to be a major algal excretory product (Fogg et al., 1969; Wright, 1975). Sen et al. (1966) found that natural waters may contain glycolate levels of up to 0.5 mg/1 (6.6×10^{-6}M) and that concentrations higher than 20 mg/1 (2.6×10^{-4}M) had an inhibitory effect on algal growth.

Summary

This review of pertinent literature has revealed the following:

1) particulate P can be an important source of the nutrient for algal growth;
2) particulate P in wastewater effluents is readily algal available;
3) the Algal Assay:Bottle Test (AA:BT) offers a reliable and relatively facile means of estimating the algal growth potential of both particulate and dissolved P forms in a water sample:
4) normal AA:BT pretreatment of a wastewater effluent sample does not provide a means for solubilizing particulate P prior to filtration, leading to the underestimation of the algal growth potential of the effluent;
5) an alternative particulate P solubilization method is necessary to accurately diagnose the algal growth potential of wastewater effluents;
6) solubilization of particulate P is induced naturally by the action of phosphatase enzymes and organic acids produced by many bacteria and some algae.

METHODOLOGY

Algal Assay:Bottle Test Procedure

The experimental design, application, and data interpretation protocol for the AA:BT procedure are described by Miller et al. (1978) in a report to the U.S. Environmental Protection Agency (EPA). Plotkin and Ram (1983) provide details regarding the establishment and use of the Algal Assay Laboratory of the Environmental Engineering Program at the University of Massachusetts Department of Civil Engineering.

The AA:BT is based upon a modification of Liebig's Law of the Minimum, which states that "maximum yield is proportional to the amount of a nutrient or combination of nutrients which are present and biologically available in minimal quantity in respect ot the growth requirements of the organisms" (Miller et al., 1978). Therefore, the essential nutrient which is the most scarce relative to algal requirements will limit growth when its concentration is reduced to a level insufficient for uptake by algae. Liebig's Law is applicable to a single nutrient limiting growth at one time only when all other nutrients are present in excess relative to the needs of the algae.

When more than one nutrient is present at a critically limiting concentration, algal growth may be simultaneously constrained by each of these nutrients. This has been demonstrated with the green alga, Selenastrum capricornutum, used in the AA:BT. S. capricornutum takes up inorganic nitrogen (N) and inorganic P approximately in a ratio of 11.3:1 (Plotkin and Ram, 1983). Therefore, when these two nutrients are present in a similar ratio, co-limitation can be expected. Miller et al. (1978) defined a water to be N-limiting when the N:P ratio is less than 10:1, P-limiting when greater than 12:1, and co-limiting when between these two

values. However, exception to these definitions have been described by Weiss (1976), who found co-limitation to occur with N:P ratios between 8:1 and 13:1, and by Chiaudani and Vighi (1976), who determined co-limitation with N:P ratios between 5:1 and 10:1.

Prediction of Algal Maximum Standing Crop

Previous research involving the AA:BT has indicated that the maximum standing crop (MSC) of S. capricornutum can be predicted on the basis of the concentration of total soluble inorganic N (TSIN), consisting of ammonia, nitrate, and nitrite, or the concentration of orthophosphate present in a water, depending on which is growth-limiting (Miller et al., 1978). With an excess of P and all other nutrients and no toxic effects, each mg of TSIN as N/L will produce 38 mg ± 20 percent dry weight of algae/L. With an excess of N and all other nutrients and no toxic effects, each mg of orthophosphate as P/L will support 430 mg ± 20 percent dry weight of algae/L. Therefore, the MSC of S. capricornutum can be predicted, based upon the limiting macronutrient, by using these growth yield factors.

Micronutrient Limitation and Toxicity Inhibitions

The effects on algal growth of a shortage of essential micronutrients and/or the presence of algal toxicants in a water must also be accounted for in the AA:BT procedure. EDTA, which acts as a chelator to both ensure the availability of micronutrients in solution and to reduce the deleterious effects of algal growth inhibitors, especially heavy metals, via complexation, is utilized within the AA:BT protocol in this regard. In order to differentiate between trace element limitation and toxic inhibition, a micronutrient spike is added to a set of water sample aliquots. The components of the micronutrient solution are listed in Table 1.

Table 1

Components of Micronutrient Solution

Compound	Concentration (µg/L)	Element	Concentration (µg/L)
$CaCl_2 \cdot 2H_2O$	4,410	Ca	1,202
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$NaMoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$ZnCl_2$	3.271	Zn	1.570
$CuCl_2$	0.012	Cu	0.004
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$MgSO_4 \cdot 7H_2O$	14,000	S	1,911
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2,904

(After: Miller *et al.*, 1978)

Determination of Nutrient Limitation

The nutrient limitation status of a water is determined by measuring the growth response of S. capricornutum (hereafter referred to as "the algae") to singular and combined additions of N, P, micronutrients, and EDTA to pretreated aliquots of the sample prior to inoculation with the algae. The nutrients are added in amounts which force the solution to become growth-limited by the secondary macronutrient. Table 2 lists the nutrient additions utilized with a typical AA:BT experiment design.

Three replicate 50 ml aliquots of the pre-treated water sample are dispensed into 125 ml Erlenmeyer flasks for each nutrient addition. Three 50 ml aliquots of algal nutrient medium (ANM) are also utilized to monitor algal viability during the experiment. ANM consists of the micronutrients listed in Table 1 and the additional components listed in Table 3. Therefore, the typical AA:BT experiment utilizes thirty-three test flasks. Each flask is inoculated with 1.0 ml of pure S. capricornutum culture containing approximately 51,000 cells and then fitted with a foam plug, which allows gas exchange. The flasks are placed on a shaker table, which continuously oscillates at approximately 100 times per minute, and are maintained under 400 $\pm$ 10% foot-candles of fluorescent lights for 24 hours a day, at a temperature of $24^{\circ}C \pm 2^{\circ}$.

The algal cultures are allowed to grow until the MSC is attained. The MSC is considered to be achieved within 14 days or when the increase in algal dry weight measured on two consecutive days is less than five percent (Miller et al., 1978). Growth monitoring is initiated after fourteen days of incubation. The MSC, in mg dry weight of algae/L, is quantified by use of a Model 5615_{ZBI} Coulter Counter with a Model MHR MCV/Hct/RBC computer. A specific growth coefficient, which has been determined for the algae in

Table 2

Nutrient Additions Used in Determining Algal Assay Nutrient Limitation*

Control
Unautoclaved Control
Control + 0.05 mg P/L
Control + 1.00 mg N/L
Control + 1.00 mg N + 0.05 mg P/L
Control + 1.00 mg $Na_2EDTA \cdot 2H_2O$/L
Control + 1.00 mg $Na_2EDTA \cdot 2H_2O$ + 0.05 mg P/L
Control + 1.00 mg $Na_2EDTA \cdot 2H_2O$ + 1.00 mg N/L
Control + 1.00 mg $Na_2EDTA \cdot 2H_2O$ + 1.00 mg N + 0.05 mg P/L
Control + micronutrients

*Resultant concentration after 1 ml algal inoculum into 50 ml solution.

(After: Plotkin and Ram, 1983)

Table 3

Additional Components in Algal Nutrient Medium

Compound	Concentration (µg/L)	Element	Concentration (µg/L)
$NaNO_3$	25,500	N	4,200
K_2HPO_4	1,044	P	186
$NaHCO_3$	15,000	C	11,001
$Na_2EDTA \cdot 2H_2O$	300.000	-	-

(After: Miller et al., 1978)

previous laboratory work Plotkin and Ram, 1983,is multiplied by the mean cell volume and the cell concentration, as measured by the electronic particle counting, to attain the MSC.

Rehnberg et al. (1982) determined that electronic particle counting cannot differentiate between live and dead algal cells, and concluded that the five percent cut-off rule used in deciding when to terminate AA:BT experiments should not be applied to particle count data. Therefore, MSC values were generally quantified with the Coulter Counter on the fourteenth day following inoculation during this investigation.

Chemical Analyses

Autoclaved and unautoclaved aliquots of a AA:BT water sample are typically analyzed for orthophosphate, total phosphorus, ammonia-N, nitrate-N, nitrite-N, total organic nitrogen, and pH. The methods used for determining these water quality parameters are listed in Table 4.

Table 4

Methods for Determining Water Quality Parameters

Parameter	Method	Reference
Ammonia-N	Scaled down colorimetric determination using indophenol reaction	Ram, 1979
Total Organic N	Micro/Kjeldahl nitrogen digestion of sample followed by indophenol colorimetric determination	Ram, 1979
Nitrate-N	Cadmium Reduction Method	EPA, 1979
Nitrite-N	Cadmium Reduction Method	EPA, 1979
Orthophosphate	Heteropoly Blue-Ascorbic Acid Spectrophotometric Method	Strickland and Parsons, 1972
Total Phosphorus	Potassium persulfate digestion followed by Heteropoly Blue-Ascorbic Acid Spectrophotometric determination	APHA, 1985 EPA, 1979 Strickland and Parsons, 1972
pH	potentiometric method	APHA, 1985

After: Plotkin and Ram, 1983

Development and Design of an Alternative P Solubilization Procedure

The initial step of the research involved the selection and testing of a general P solubilization technique. The use of ultraviolet light was rejected due to its oxidation of organic and inorganic nitrogen compounds. Direct inoculation with P-solubilizing bacteria and the addition of phosphatase enzymes were eliminated as alternatives because of unavoidable detrimental effects on algal growth during subsequent AA:BT analysis. The studies by Harrison et al. (1972) and Craven and Hayasaka (1982), relating the solubilization of orthophosphate by bacteria to their production of organic acids, provided the basis for the selection of acidification as the potential means of P solubilization prior to the AA:BT analysis of a wastewater effluent.

Selection of Source for Water Samples

The results of Harrison et al. (1972) prompted a series of experiments utilizing glycolic acid additions. Due to the greater ease of sampling and handling and the general lack of need for dilutions, it was decided that all preliminary experiments would be performed on samples from an eutrophic lake, instead of actual wastewater effluent samples. Based on the suggestion of the Division of Water Pollution Control (DWPC) of the Massachusetts Department of Environmental Quality Engineering (DEQE), Forge Pond in Granby, Massachusetts (see Figure 2), was selected as the source of the water samples. Forge Pond is highly eutrophied, due largely to the discharge of wastewater effluent from the Belchertown State School secondary treatment plant into a tributary to the pond (Massachusetts DEQE, 1980).

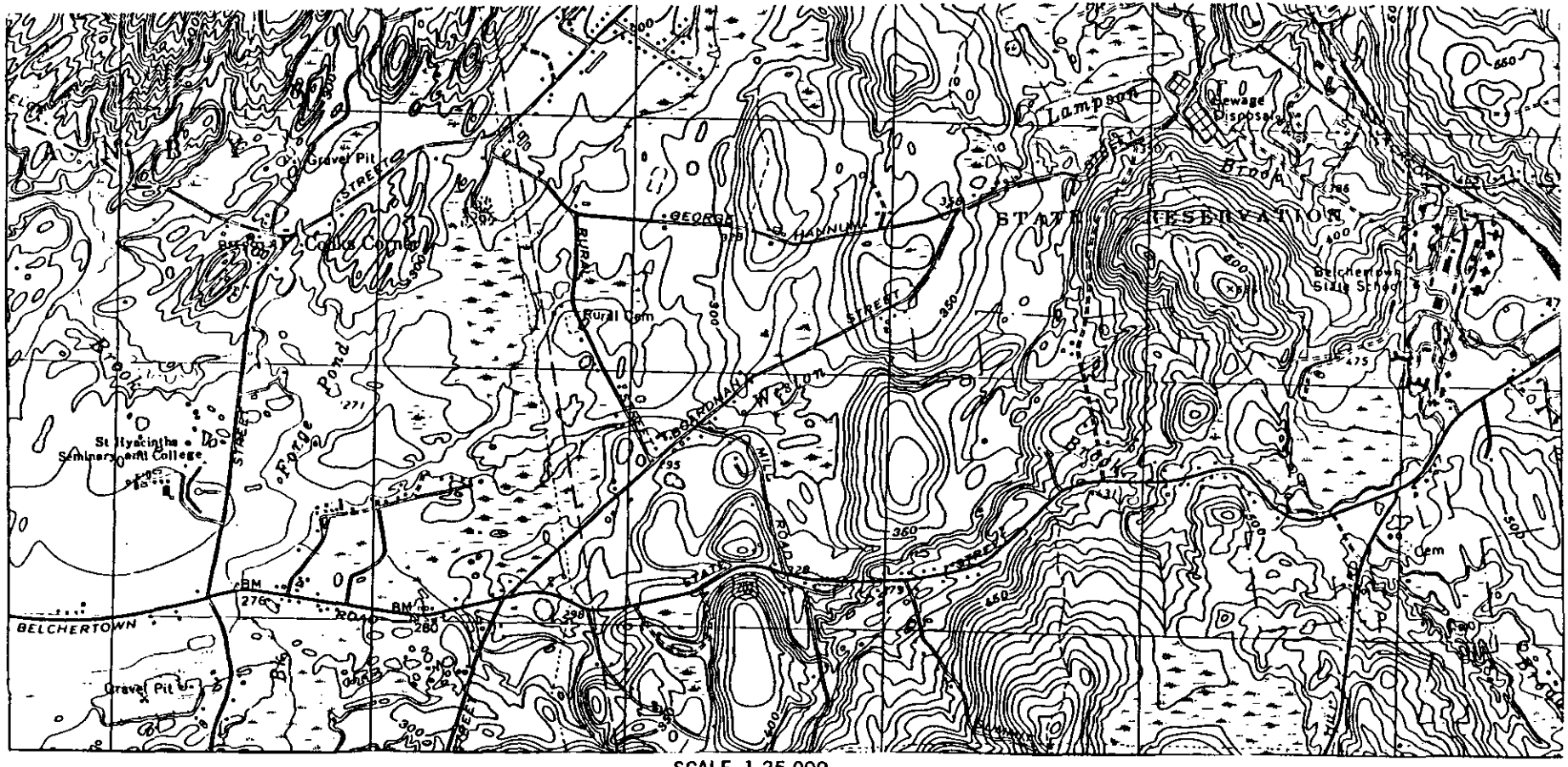


Figure 2 Forge Pond

The high levels of P in Forge Pond were considered optimal for the purposes of the P solubilization experiments.

Sampling Procedures

Water samples were collected near the shoreline of Forge Pond via the use of an extension rod, except during the winter, when samples were taken further from shore through holes drilled with an ice auger. In order to assure a sufficient particulate fraction in the samples, the bottom sediments were generally agitated prior to the collection of samples in acid-washed glass bottles. Samples were stored in a cooler during transport to the laboratory.

AA:BT Analysis of a Forge Pond Sample

An AA:BT analysis of a Forge Pond sample, collected without bottom agitation, was conducted to assess the trophic status of the water. Standard AA:BT protocol, including pretreatment with autoclaving, was followed.

Preliminary P Solubilization Experiments with Glycolic Acid

Two preliminary experiments #1 and #2 were conducted to evaluate the P solubilization effectiveness of sample acidification with glycolic acid. Both resuspended particulate solutions and raw samples from Forge Pond were utilized. The basic procedure consisted of the following:

1) filtering of a known volume of raw sample with glass fiber and/or 0.45 micron membrane filters;
2) distilled-water rinsing and/or macerating of filters and resuspension of particulates in one-half of the original volume of distilled water (effectively doubling the particulate P concentration of the original sample);

3) additions of sufficient glycolic acid solution to achieve a concentration of 0.06 M in the aliquots (same concentration used by Harrison et al., 1972);

4) constant stirring of aliquots in the dark for 4 days (based on the results of Craven and Hayasaka, 1982) at 5°C and/or room temperature;

5) filtering of aliquots after 4 days of stirring;

6) neutralization to original pH of raw sample via titration with 1.0 and 0.1 N sodium hydroxide (NaOH) solutions;

7) analysis of filtrates for dissolved reactive P (DRP).

In addition, separate aliquots of the raw samples were autoclaved and acidified with 0.06 M glycolic acid, to compare the effectiveness of the two P solubilization procedures on whole samples. Actinomycin (0.5 mg/L) was added to aliquots alone and in combination with glycolic acid, in an attempt to utilize the antibiotic agent to isolate the effects of the stirring and of the acidification on the release of orthophosphate from particulates. Acidified aliquots of the raw sample and resuspended solution were also stirred under refrigeration during Experiment #1, to minimize microbial activity. Blanks were utilized for both the raw sample and resuspended solution during Experiment #2. Air-driven magnetic stirrers were sued with the refrigerated aliquots and standard electrical vortex stirrers with the non-refrigerated aliquots.

An aliquot of the raw sample was autoclaved (30 minutes at 15 psi and 250°F), filtered, and analyzed for DRP in both experiments. The raw sample was also analyzed for natural (unautoclaved) DRP levels, total P, and total dissolved P, allowing calculation of the particulate P and dissolved organic P concentrations. The "estimated autoclaving yield" for each resuspended

solution was calculated by doubling the difference between the unautoclaved and autoclaved DRP concentrations of the raw sample, since the particulate concentration in the resuspended solutions was twice that of the original samples. Inherent in this estimation was the assumption that the majority of the additional DRP solubilized during autoclaving originated from the particulate fraction, as opposed to the dissolved organic fraction. In both experiments, the total particulate P levels were at lease five times as great as the dissolved organic P levels, justifying this assumption.

Glycolic Acid Multiple Interval Experiments

A series of four experiments (#3 - #6) was conducted to determine the feasibility of utilizing a stirring period of less than four days. These multiple interval tests used the sample glycolic acid solubilization procedure as in the previous experiments, except much larger volumes of the resuspended solutions and the raw sample were acidified, and subsequently sampled and analyzed for DRP at various time intervals ranging from 1 hour to 93 hours. Appropriate blanks and aliquots with additions of actinomycin (0.5 mg/L) and penicillin (0.5 mg/L) were utilized.

Based on the results of Experiments #3 and #4, several changes in methodology were incorporated into the procedure during Experiment #5:

1) thorough rinsing of filters to resuspend the bulk of particulates was substituted for the maceration and inclusion of filters in the resuspended solution;
2) autoclaving, filtration, and analysis for DRP of the resuspended solution were utilized to provide a direct means, as opposed to the "estimated autoclaving yield", to evaluate the effectiveness of the acidification procedure;
3) freshly prepared glycolic acid solution was used.

Bacterial Survival Plate Tests

Because an initially sterile water is required prior to algal inoculation, the antibiotic effects of 0.06 M glycolic acid, 0.5 mg/L actinomycin, 0.5, 5, 50, and 100 mg/L penicillin, and 0.45 micron filtration alone were assessed via plate tests. The standard pour plate method (Brock et al., 1984) was utilized. Agar plates were inoculated with 0.1 and 1.0 ml volumes from each treatment and control aliquot of raw sample and resuspended solution after 24 hours of stirring, and were incubated at 30°C. Colonies were counted after 1 and 3 days.

0.06 M Glycolic Acid AA:BT

Since the 0.06 M glycolic acid procedure had proven generally successful at matching or exceeding the DRP yield of autoclaving a sample, the technique was further evaluated with an AA:BT study. Experiment #7 involved a comparison of the algal growth potential of autoclaved and 0.06 M glycolic acid-treated aliquots of a raw Forge Pond sample. The acidified aliquot was stirred for 24 hours prior to filtration and neutralization. Both sample treatments were subjected to an AA:BT analysis with the following additions and controls, in standard triplicate format:

1) control,

2) + 2 mg/L N,

3) + 2 mg/L N + 1 mg/L EDTA,

4) + 2 mg/L N + micronutrients.

An unautoclaved and an ANM control were also utilized during the experiment. Nitrogen was added to all non-control flasks to force P-limitation and to allow quantification of the algal availability of the solubilized P.

Further P Solubilization and Algal Toxicity Experiments with Glycolic Acid

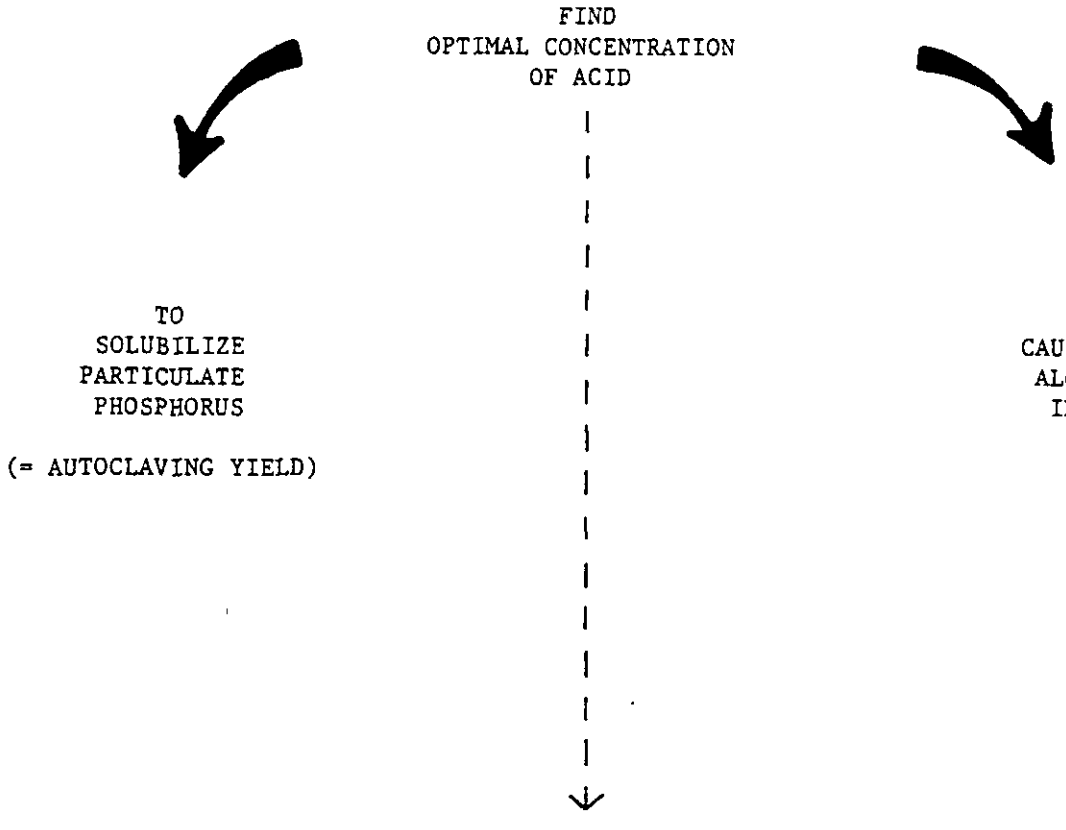
Based on the results of Experiment #7, which indicated that an undiluted 0.06 M glycolic acid-treated sample greatly inhibited algal growth, it was necessary to conduct a series of similar experiments with lower concentrations of the acid. A major factor favoring the success of a general acidification scheme is that wastewater effluents are diluted in distilled water or receiving water (1% - 50% effluent) after pretreatment and prior to AA:BT analysis (Plotkin and Ram, 1983). Figure 3 illustrates the basic goal of the research at this juncture in the study.

P solubilization experiments and algal toxicity experiments were conducted with progressively more dilute glycolic acid concentrations. Figure 4 demonstrates the basic procedures utilized during these tests. The P solubilization studies were conducted using the multiple interval format with glycolic acid concentrations of 0.01, 0.006, and 0.001 M (Experiment #9). Algal toxicity experiments were performed with glycolic acid concentrations of 0.03, 0.01, 0.006, 0.003, 0.001, and 0.0006 M, and also utilized three flasks of 25% ANM as an algal growth control during each test (Experiments #8 and #10).

P Solubilization and Algal Toxicity Experiments Utilizing Other Solubilization Agents

Because of the lack of success at finding a concentration of glycolic acid capable of attaining the P-solubilization yield of autoclaving, while not causing algal growth inhibition after dilutions of fifty percent or greater, similar experiments were conducted with acetic acid, hydrochloric acid (HCl), and sodium hydroxide (NaOH). Acetic acid was selected based on the results of Craven and Hayasaka (1982); HCl on the presumption that the solubilization of P is primarily due to the lowered pH of the solution

BASIC GOAL OF RESEARCH



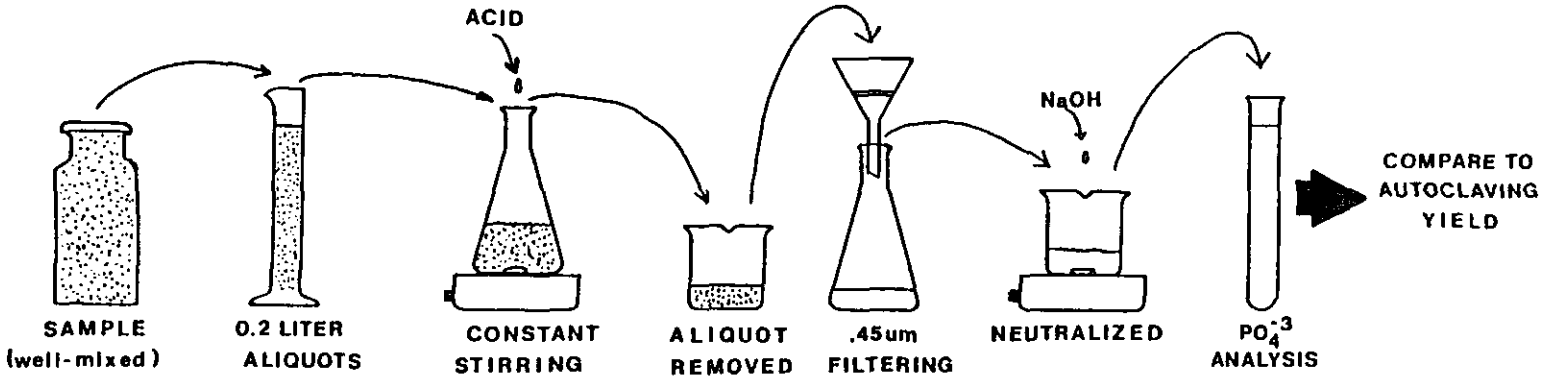
- MAJOR OBSTACLE: ALGAL GROWTH INHIBITORS MAY BE SOLUBILIZED DURING ACID TREATMENT.

- PRO FACTOR: WASTEWATER EFFLUENTS DILUTED BY RECEIVING WATER (50 - 99%) PRIOR TO AA:BT.

Figure 3

BASIC PROCEDURES

P-SOLUBILIZATION EXPERIMENTS



ALGAL TOXICITY EXPERIMENTS

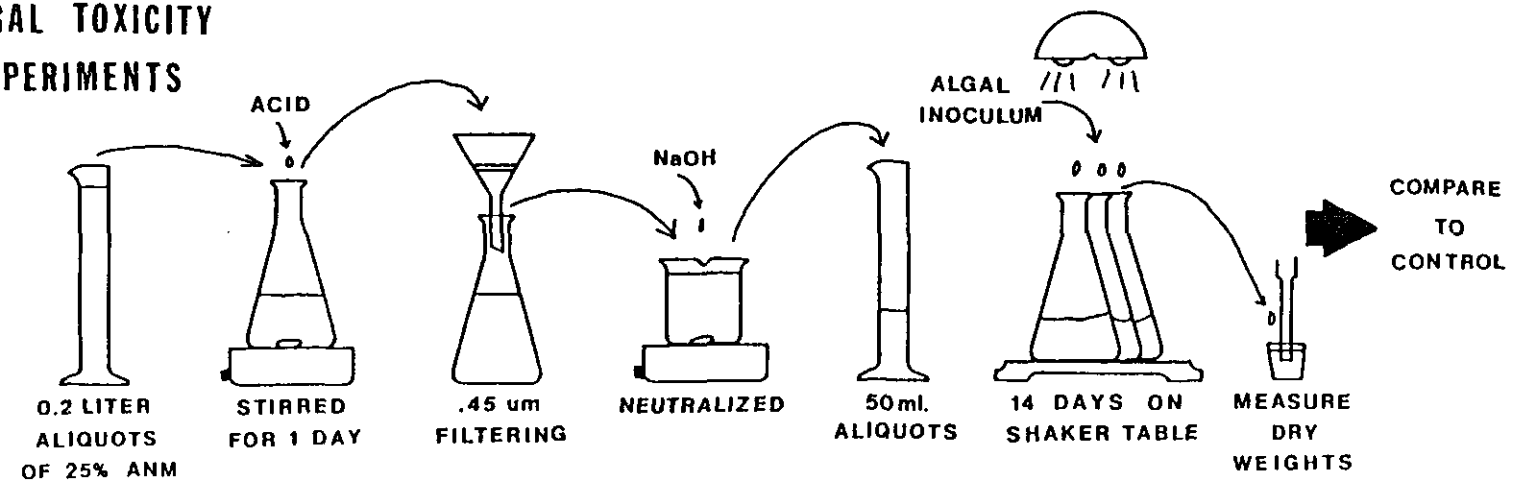


Figure 4

(Diamadopoulos and Benedek, 1984) and the relatively inert nature of the chloride ion residual after neutralization; and the NaOH on its use for the extraction of bioavailable P from soils and sediments (Sagher, 1976).

The P-solubilization effectivenesses of 0.008 M acetic acid; 0.012, 0.006, 0.003, and 0.0006 M HCl; and 0.010 M NaOH were tested (Experiments #11 and #13). Algal growth inhibition tests were performed with 0.010 NaOH; 0.006, 0.003, and 0.0006 M HCl; and 0.003 and 0.006 M NaCl (Experiments #12 and #14). The NaCl aliquots were used to test the algal toxicity of levels of residual chloride similar to those present after neutralization of the HCl solutions.

0.006 M HCl AA:BT Experiments

Based on the demonstrated ability of 0.006 M HCl to achieve the autoclaving DRP yield and to cause no algal growth inhibition at a concentration of 0.003 M in ANM, a series of 0.006 M HCl AA:BT experiments was conducted to compare the algal availability of DRP solubilized by acidification with that released via autoclaving. The procedures utilized were similar to those of Experiment #7, except for the following adaptations:

1) In Experiment #15, all aliquots (autoclaved, 0.006 M HCl-treated, unautoclaved, and ANM) were diluted by 50% in deionized water prior to any nutrient additions;
2) In Experiment #16, all aliquots, except the ANM (undiluted), were diluted by 75%, and 50% dilutions of the autoclaved and HCl-treated aliquots were also subjected to AA:BT analysis, with only 2 mg/L N additions utilized;
3) In experiment #17, all aliquots were diluted by 50% and P limitation was forced by addition of 1 mg/L N instead of the previous 2 mg/L;

4) In Experiment #18, the HCl-treated aliquot was stirred continuously for 12 hours following filtration and neutralization, re-filtered, and then diluted 50%; all other aliquots were also diluted 50%, and 1 mg/L additions were utilized, including with the unautoclaved aliquot.

0.006 M HCl-Treatment of a Wastewater Effluent

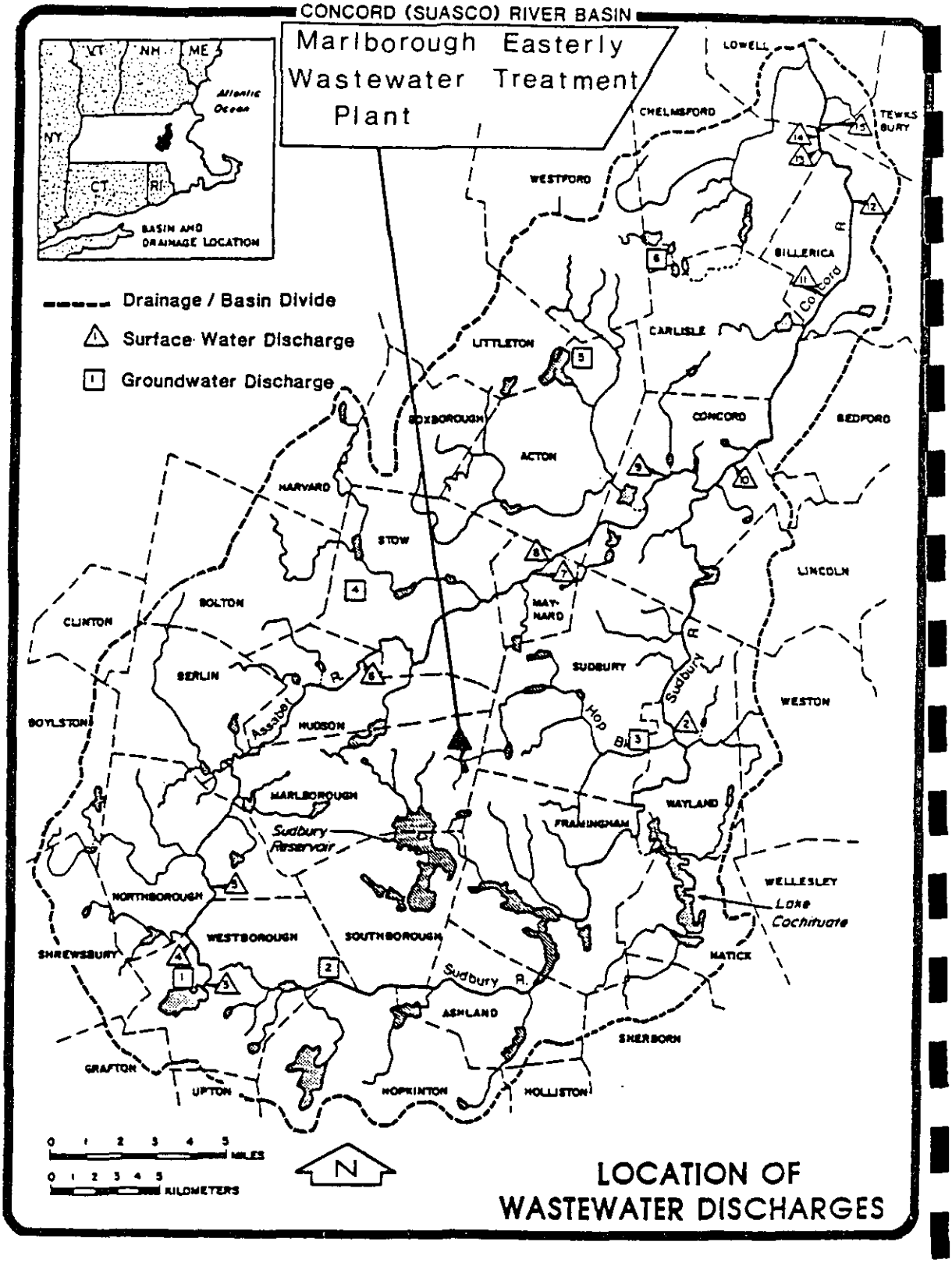
It was hypothesized that the greater dilutions (1-20% sample) generally utilized during the AA:BT analysis of wastewater effluents would minimize the varying algal growth inhibition problems encountered during Experiments #15 - #18. An AA:BT experiment was conducted to test this hypothesis and to evaluate the overall feasibility of applying the 0.006 M HCl-treatment procedure to the AA:BT analysis of a wastewater effluent.

The Marlborough Easterly Wastewater Treatment Plant (Figure 5) was selected by the Massachusetts DWPC as the source of the effluent for the experiment. This facility utilizes advanced treatment to convert ammonia-N to nitrate-N and to remove P. Since a P-limited effluent is required to properly assess the algal availability of the DRP in the sample aliquots, a treatment plant that removes P was the most desirable case for this study.

A 24-hour composite sample of the effluent was collected prior to chlorination by DWPC personnel on August 15-16, 1985. The sample was picked up on August 16, 1985, at the Westborough offices of the DWPC and transported on ice to the University of Massachusetts.

Upon arrival at the Environmental Engineering Laboratory, an aliquot of the effluent was immediately 0.45-micron filtered, another aliquot was subjected to 0.006 M HCl-treatment, and the pH of the raw sample was

Figure 5



After: Mass. DEQE, 1983

measured. After 28 hours of stirring in darkness, the HCl-treated aliquot was 0.45-micron filtered and neutralized to the initial pH of the sample with 0.1 N NaOH.

An AA:BT experiment was set up to evaluate the ability of the HCl-treatment procedure to solubilize algal available P in the effluent. A series of dilutions (1%, 5%, 10%, and 20%) of the filtered aliquot and the HCl-treated aliquot was prepared utilizing deionized dilution water. Each dilution set consisted of three replicate flasks with no additions and three replicate flasks with 1 mg/L EDTA added, to monitor potential algal growth inhibition. The flasks were then inoculated with algae and placed on a shaker table under constant fluorescent light for two weeks, before growth quantification. The DRP, total P, nitrate-nitrite N, and the ammonia-N of the untreated and the HCl-treated aliquots and the total P of the raw sample were measured within a week of collection.

RESULTS AND DISCUSSION

AA:BT Analysis of a Forge Pond Sample

The results of the chemical analyses conducted on the Forge Pond water column sample, collected without bottom agitation, are shown in Table 5. The low N/P ratio of the water indicates that Forge Pond is N-limited, due to an excess of P. On this basis, Forge Pond was initially judged favorable as the source of water samples for subsequent research.

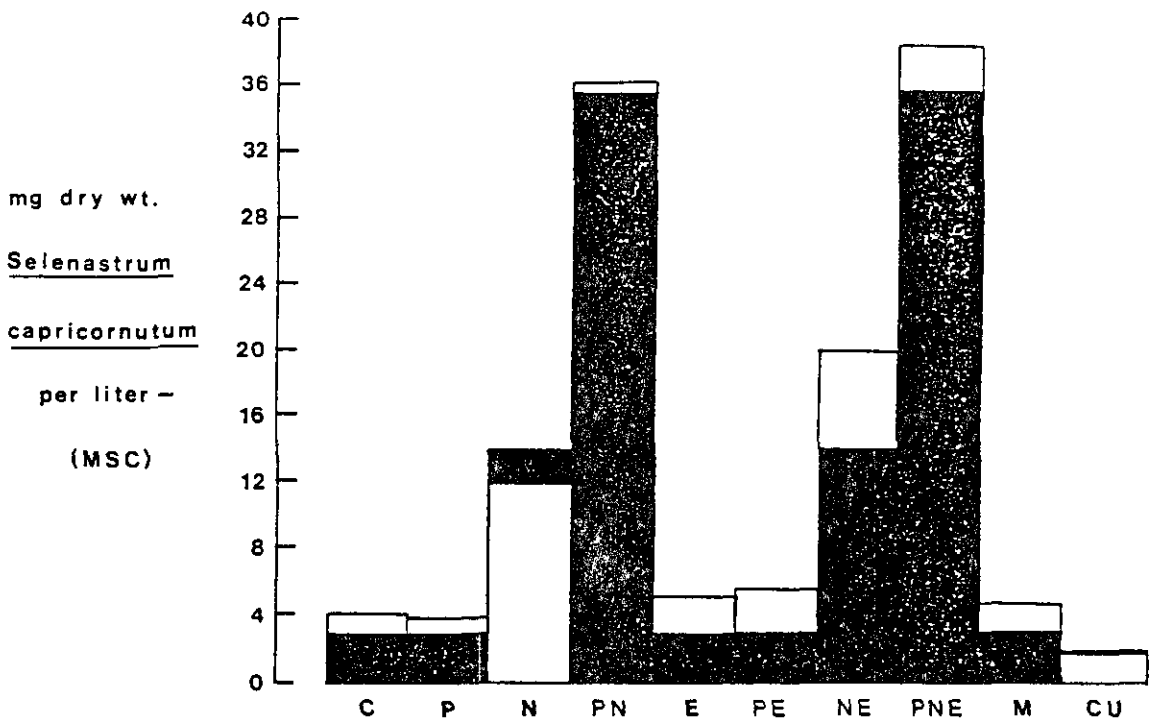
Figure 6 reveals the results of the AA:BT analysis. The predicted yields (shaded) were calculated by using the appropriate growth yield factor, depending on the N/P ratio in each treatment aliquot. The actual yields (unshaded) were measured via the Coulter Counter. The N-limitation of Forge Pond is confirmed by these results, as the N additions produced much more growth than the autoclaved control, while the P additions produced approximately the same amount of growth as the control. However, once sufficient N was added, any excess P resulted in a large augmentation in growth. All EDTA aliquots showed slightly greater growth than the corresponding non-EDTA aliquots, while the micronutrient addition caused no growth increase over the control, indicating a small degree of algal growth inhibition in the sample, possibly due to the presence of low levels of toxic substances. The growth yield of the autoclaved control was more than twice that of the unautoclaved control, demonstrating the important effect of autoclaving; although in this case, the extra growth was due to the additional N solubilized, as both aliquots were N-limited. Nonetheless, the results of the AA:BT showed minor algal growth inhibition, N-limitation due to excess P, and excellent growth response to P additions in the presence of

Table 5 Results of Chemical Analyses of Forge Pond Sample

(mg/L)

CHEMICAL PARAMETER	AUTOCLAVED	UNAUTOCLAVED	RAW SAMPLE
PO_4-P	0.032	0.018	-
NO_3-N + NO_2-N	0.052	0.043	-
NH_3-N	0.028	0.004	-
TSIN	0.080	0.047	-
N/P	2.5/1	2.6/1	-
Total P	0.063	0.040	0.068
Particulate P	-	-	0.028
Total N	-	-	0.438
Total Organic N	-	-	0.391
pH	6.9	6.85	6.85

ALGAL ASSAY OF FORGE POND (11/1/83)



Predicted Yields - Shaded

Actual Yields - Outlined

C = control

P = + phosphorus

N = + nitrogen

E = + EDTA

M = + micronutrients

CU = control, unautoclaved

Figure 6

excess N. Based on these results, Forge Pond was selected as the source of water samples for future experiments.

Preliminary P Solubilization Experiments with Glycolic Acid

Experiment #1

The P fractions of the raw sample from Forge Pond (bottom-agitated) and the autoclaved aliquot used in Experiment #1 are listed in Table 6. Autoclaving solubilized 19 micrograms per liter (ug/L) DRP, as determined by (7) minus (4). Therefore, the estimated autoclaving yield of the resuspended solution was 38 ug/L.

The results of Experiment #1 are shown in Figure 7. The 0.06 M glycolic acid treatment (at $5^{o}C$) was found to closely approximate the autoclaving yield of the raw sample. Similar treatment of the resuspended solution did not prove as successful. However, the acidified aliquot of the resuspended solution with 0.5 mg/L actinomycin, at room temperature, exceeded the estimated autoclaving yield. The control resuspended solution aliquot, which has only 0.5 mg/L actinomycin added and was stirred at room temperature, showed relatively little solubilization of DRP.

The results of Experiment #1 indicated the following:

1) 0.06 M glycolic acid treatment can match or exceed the autoclaving yield of a sample;

2) DRP solubilization via acidification is partially inhibited by lower temperatures during stirring;

3) the effects of stirring alone cannot account for the solubilization of the majority of DRP during acid treatment.

Experiment #2

The P fractions of the raw sample from Forge Pond and the autoclaved aliquot used in Experiment #2 are listed in Table 6. Autoclaving

Table 6 P Analyses for Experiments #1 and #2

P Fraction	Concentration (µg/L) Experiment #1	Experiment #2
• Raw Sample	pH = 7.05	pH = 6.85
(1) TP	119	120
(2) TDP	60	59
(3) TPP	59	61
(4) DRP	53	48
(5) DOP	7	11
• Autoclaved Aliquot		
(6) TDP	86	114
(7) DRP	72	92
(8) DOP	14	22

TP = Total P

TDP = Total Dissolved P

TPP = Total Particulate P

DRP = Dissolved Reactive P

DOP = Dissolved Organic P

EXPERIMENT 1

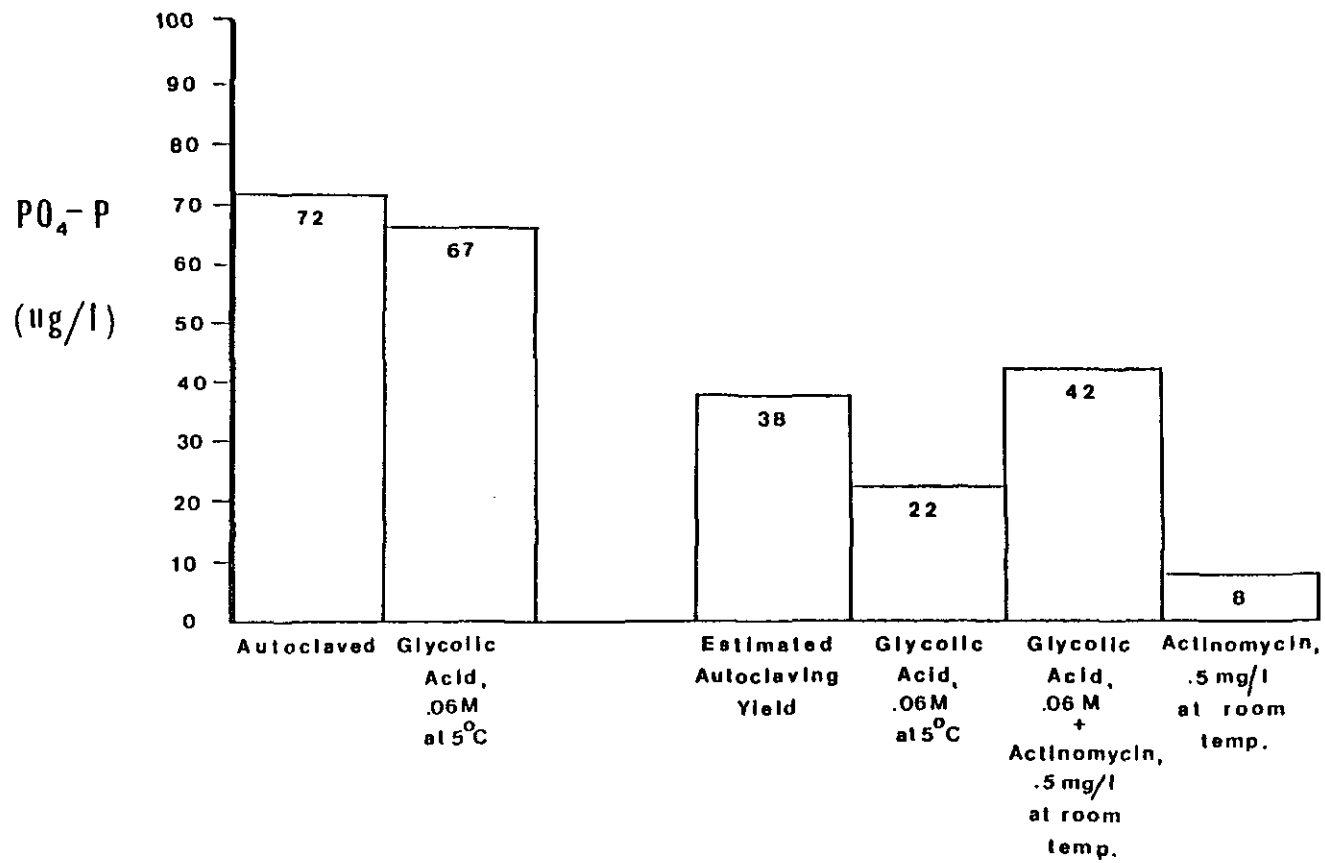


Figure 7

solubilized 44 ug/L DRP, and the estimated autoclaving yield of the resuspended solution was 88 ug/L.

Figure 8 illustrates the results of Experiment #2. The actual temperature of each aliquot was measured immediately prior to DRP analysis, to determine any variations in heat generation by the different vortex stirrers used. The 0.06 M glycolic acid treatment (23^{o}C) exceeded the autoclaving yield of the raw sample by 30%. Stirring alone at a higher temperature (37^{o}C) solubilized relatively little DRP. Acidification of the resuspended solution (23^{o}C) matched the estimated autoclaving yield, while the control aliquots with 0.5 mg/L actinomycin (29^{o}C) and no additions (29^{o}C) showed very low levels of solubilized DRP. However, the DRP level of the latter aliquot exceeded that of the former aliquot by 50%, evidencing P solubilization due to bacterial activity.

The results of Experiment #2 indicated the following:

1) 0.06 M glycolic acid treatment should be conducted at or slightly above room temperature;
2) the effects of stirring and bacterial activity cannot account for the solubilization of the majority of DRP during acid treatment.

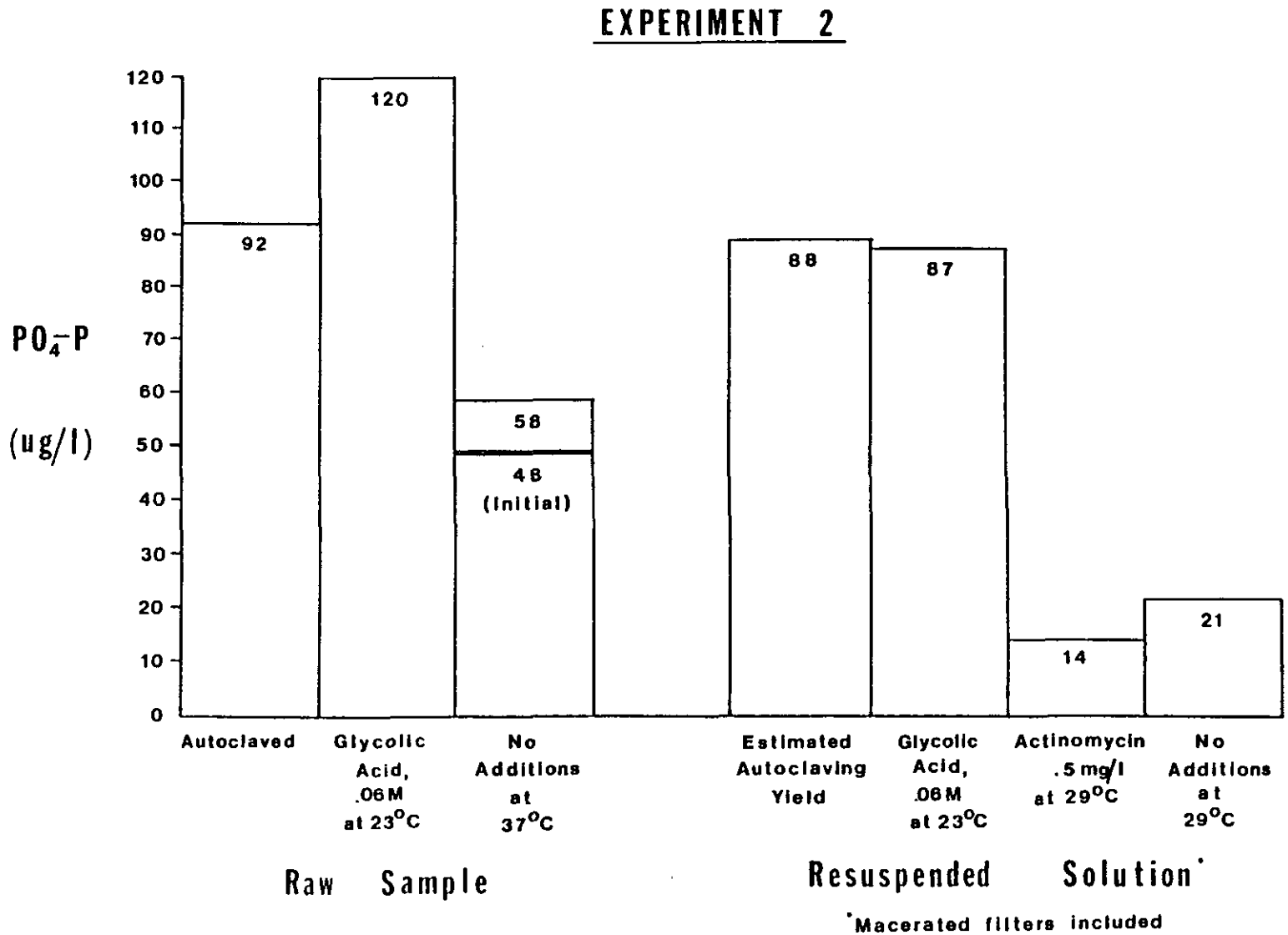

EXPERIMENT 2
PO_4^-P
(ug/l)
120
110
100
90
80
70
60
50
40
30
20
10
0
92
120
58
48
(Initial)
88
87
14
21
Autoclaved
Glycolic Acid, .06M at 23°C
No Additions at 37°C
Estimated Autoclaving Yield
Glycolic Acid, .06M at 23°C
Actinomycin .5 mg/l at 29°C
No Additions at 29°C
Raw Sample
Resuspended Solution*
*Macerated filters included

Figure 8

Glycolic Acid Multiple Interval Experiments

Experiment #3

The Forge Pond water sample used in this experiment was collected at a time when a thin ice layer was present that was not strong enough to support the investigator's weight. Therefore, it was extremely difficult to obtain an optimally bottom-agitated sample. Because the sample had to be taken through small openings in the ice close to the shoreline, the water contained a considerable amount of sediments, duckweed, and organisms, including many large aquatic insects. This accounted for the extremely high total P and particulate P concentrations (see Table 7). The estimated autoclaving yield of the resuspended solution, which was brought up to original volume due to the high particulate levels, was 411 ug/L.

The results of Experiment #3 are shown in Table 8. The maximum amount of DRP was achieved via glycolic acid treatment after approximately 3 days of stirring, although only 68% of the estimated autoclaving yield was attained. This was probably due to the extremely high levels of particulates in the sample, which apparently reduced the P-solubilization effectiveness of the acidification procedure.

The results of Experiment #3 indicated the following:

1) stirring periods of less than four days may be feasible, while maintaining the effectiveness of DRP recovery via glycolic acid treatment;
2) samples containing significant levels of particulates may not be amenable to DRP solubilization via acidification.

Table 7 P Analyses for Multiple Interval Experiments

P Fraction	Concentration (µg/L) Exp. #3	Exp. #4	Exp. #5	Exp. #6
• Raw Sample				
(1) TP	1,020	178	109	600
(2) TDP	82	35	51	56
(3) TPP	938	143	58	544
(4) DRP	15	22	32	32
(5) DOP	67	13	19	24
• Autoclaved				
(6) TDP	840	135	80	354
(7) DRP	426	76	56	181
(8) DOP	414	59	24	173

TP = Total P

TDP = Total Dissolved P

TPP = Total Particulate P

DRP = Dissolved Reactive P

DOP = Dissolved Organic P

Table 3 Results of Experiment #3

0.06 M Glycolic Acid Solubilization Test

Time (hours)	0.06 M Glycolic Acid DRP (µg/L)*	Percent of Estimated Autoclaving Yield
1	171	42%
2	184	45%
3	190	46%
24	244	59%
45	251	61%
73	279	68%
93	278	68%

* NOTE: A blank containing a similar proportion of macerated glass-fiber filters and 0.45 micron membrane filters as the resuspended aliquot was acidified and analyzed for DRP after 4 days of continuous stirring. The blank DRP level of 15 µg/L (apparently originating primarily from the glass-fiber filters) was subtracted from all glycolic acid DRP concentrations.

Experiment #4

The Forge Pond sample utilized during Experiment #4 had much lower total P and particulate P levels than the previous sample (see Table 7). As a result, glass-fiber filters did not have to be used to pre-filter the sample, greatly reducing the DRP level of the blank solution, which contained only macerated 0.45 micron membrane filters, to 2 ug/L. The estimated autoclaving yield was 108 ug/L. As a more reliable quantification of the autoclaving yield of the resuspended solution, an aliquot was autoclaved and its DRP concentration measured to be 130 ug/L.

The results of Experiment #4 revealed that even after 97 hours of stirring, 0.06 M glycolic acid treatment was only able to solubilize 51% of the actual autoclaving yield of the resuspended solution. The percent DRP recovery levels of Experiment #3, which was conducted on a sample with a much higher particulate P level, were not even attained. Therefore, it was hypothesized that the stock glycolic acid solution utilized, which had been kept under refrigeration between experiments, had lost effectiveness during storage.

The results of Experiment #4 indicated the following:

1) the estimated autoclaving yield of a resuspended solution is not always a reliable surrogate measure for the actual autoclaving yield;
2) a fresh stock solution of glycolic acid should be prepared for each experiment.

Experiment #5

The P fractions of the sample used for Experiment #5 are shown in Table 7. The autoclaving yield of the resuspended solution was 29 ug/L. Styrofoam insulation was placed between each aliquot flask and vortex

stirrer, to assure more uniform solution temperatures. Filters were just rinsed free of particulates, not macerated and included in the resuspended solution,due to previous problems with incomplete maceration and maintenance of a uniform solution.

Table 9 reveals the results of Experiment #5. The autoclaving yields of both the resuspended solution and the raw sample were achieved in significantly less time than 4 days. Therefore, the results of Experiment #5 indicated that acid treatment with freshly prepared 0.06 M glycolic acid can attain the autoclaving yield of a sample after 1 day of stirring.

Experiment #6

Table 7 shows the concentrations of the P fractions in the Forge Pond sample used during Experiment #6. High concentrations of total P and particulate P were measured. The autoclaving yield of the resuspended solution was 118 ug/L.

The results of Experiment #6 are displayed in Table 10. Two aliquots of resuspended solution were subjected to 0.06 M glycolic acid treatment. The DRP levels of both aliquots exceeded the autoclaving yield of the solution after 6 hours of stirring, and each showed increasing DRP levels over at least the next 40 hours. The DRP concentration of the acidified raw sample exceeded the autoclaving yield after just two hours of stirring, indicating possible conversion of a portion of the dissolved organic P, and it continued to increase over the next 68 hours.

The results of Experiment #6 indicated the following:

1) a stirring period of 1 day during 0.06 M glycolic acid treatment is sufficient to achieve the autoclaving yield of a sample (even one with high particulate levels);

Table 9 Results of Experiment #5

0.06 M Glycolic Acid Solubilization Test

DRP Concentration (µg/L)

	RESUSPENDED SOLUTION				RAW SAMPLE	
	0.06 M Glycolic Acid		0.5 mg/L Penicillin		0.06 M Glycolic Acid	
Stirring Time (hours)	DRP*	%Auto.	DRP	%Auto.	DRP*	%Auto.
2	27	93	-	-	-	-
22	30	103	-	-	55	98
44	34	117	-	-	52	93
73	33	114	-	-	56	100
92	24	83	10	35	52	93

*NOTE: A blank containing 0.06 M glycolic acid in distilled water was determined to have a DRP level of 3 µg/L. This value was subtracted from all glycolic acid DRP concentrations. The blank with 0.5 mg/L penicillin showed no measurable DRP.

Table 10 Results of Experiment #6

0.06 M Glycolic Acid Solubilization Test

DRP CONCENTRATION (μg/L)

	Stirring Times (hours)					Autoclaving Yield
	2	6	22	46	70	
• Resuspended Solution						
0.06 M Glycolic Acid #1	100	120	143	148	158	118
0.06 M Glycolic Acid #2	107	132	145	161	161	118
5 mg/L Penicillin	-	-	25	29	-	118
• Raw Sample						
0.06 M Glycolic Acid	201	238	276	283	293	181

2) acidification of raw samples may cause the hydrolysis of dissolved organic P to DRP, in addition to the solubilization of particulate P.

Bacterial Survival Plate Tests

The results of several plate test experiments involving both resuspended and raw samples, treated with 0.06 M glycolic acid and/or various levels of antibiotics, or subjected to 0.45 micron membrane filtration, indicated the following:

1) 0.5 mg/L actinomycin and concentrations of penicillin of up to 100 mg/L are ineffective for sample sterilization (after stirring periods of 24 hours or more):
2) 0.06 M glycolic acid alone and in combination with antibiotics is also ineffective for sample sterilization, but less so than any of the above referenced antibiotic concentrations singly;
3) 0.45 micron membrane filtration alone is effective at removing the majority of microorganisms from samples, eliminating the need for any supplementary sterilization procedures following acidification and prior to algal inoculation.

0.06 M Glycolic Acid AA:BT

The results of the chemical analyses conducted on the bottom-agitated Forge Pond sample are shown in Table 11. The DRP yield of 0.06 M glycolic acid treatment exceeded that of autoclaving, while the total soluble inorganic N (TSIN) yields of each treatment were nearly equivalent.

Table 11 Results of Chemical Analyses for Experiment #7

(µg/L)

Chemical Parameter	Autoclaved	Unautoclaved	0.06 M Glycolic Acid-Treated	Raw Sample
DRP	123	73	141	-
$NO_3 + NO_2$-N	72	69	58	-
NH_3-N	46	41	61	-
TSIN	118	110	119	-
N/P	0.96/1	1.5/1	0.84/1	-
TP	176	102	-	217
PP	-	-	-	115
TN	-	-	-	1100
TON	-	-	-	1000
pH	6.75*	-	6.75*	6.75

TSIN = Total Soluble Inorganic N

TN = Total N

TON = Total Organic N

*NOTE: The autoclaved and 0.06 M glycolic acid-treated aliquots were neutralized to the original pH of the raw sample prior to analysis.

Table 12 reveals the results of the AA:BT conducted during Experiment #7. It was evident that the neutralized, undiluted 0.06 M glycolic acid-treated aliquot inhibited algal growth. It was hypothesized that the inhibition may be due to the levels of glycolate remaining after neutralization to the initial pH of the raw sample.

The results of Experiment #7 indicated the following:

1) 0.06 M glycolic acid inhibits algal growth (after neutralization);
2) 0.06 M glycolic acid-treated aliquots must be diluted prior to inoculation.

Further P Solubilization and Algal Toxicity Experiments with Glycolic Acid

Experiment #8

Table 13 shows the results of the algal toxicity test conducted during Experiment #8. Concentrations of glycolic acid down to 0.006 M were indicated to cause significant algal growth inhibition. All aliquots were neutralized to a pH of 7.5 prior to inoculation.

Experiment #9

The results of the multiple-interval P solubilization test performed during Experiment #9 are revealed in Table 14. The minimum concentration of glycolic acid capable of approaching the autoclaving yield of the bottom-agitated Forge Pond sample after approximately 1 day of stirring was 0.006 M.

Experiment #10

The results of the algal toxicity experiment conducted with glycolic acid concentrations of 0.003, 0.001, and 0.0006 M in 25% algal nutrient medium (ANM) were similar to those of Experiment #8. All concentrations were found to greatly inhibit algal growth.

Table 12 Results of Experiment #7

AA:BT After 0.06 M Glycolic Acid Treatment

(mg/L)

Solution	Mean Algal Dry Wt.	Predicted Algal Dry Wt.
• Autoclaved		
Control	5.17	4.48
+ 2 mg/L N	63.0	52.9
+ 1 mg/L EDTA + 2 mg/L N	60.1	52.9
+ Micronutrients + 2 mg/L N	60.9	52.9
• 0.06 M Glycolic Acid		
Control	0.25	4.52
+ 2 mg/L N	0.53	60.6
+ 1 mg/L EDTA + 2 mg/L N	0.78	60.6
+ Micronutrients + 2 mg/L N	0.54	60.6
Unautoclaved Control	6.21	4.18
Algal Nutrient Medium	108.4	80.0

Table 13 Results of Experiment #8

Glycolic Acid Algal Inhibition Tests

Solution	Mean Algal Dry Wt. (mg/L)	Predicted Algal Dry Wt.
Control (25% ANM)	18.2	20.0
0.03 M Glycolic Acid in 25% ANM	0.20	20.0
0.01 M Glycolic Acid in 25% ANM	0.34	20.0
0.006 M Glycolic Acid in 25% ANM	0.62	20.0

NOTE: Glycolic acid aliquots were adjusted to pH = 7.5 $\pm$ 0.1 prior to inoculation.

ANM = Algal Nutrient Medium

Table 14 Results of Experiment #9

Glycolic Acid Solubilization Tests

DRP CONCENTRATION (µg/L)

Glycolic Acid Concentration in Raw Sample	Stirring Times (hours) 22	44	70	Autoclaving Yield
0.010 M	131	139	137	148
0.006 M	130	133	133	148
0.001 M	94	86	73	148

Initial [DRP] = 56 µg/L

[DRP] solubilized by autoclaving = 92 µg/L

[TP] = 370 µg/L

Summary of Experiments #8 - #10

The results of Experiments #8 - #10 indicated the following:

1) the minimum concentration of glycolic acid capable of approaching the autoclaving yield of a sample is 0.006 M;
2) a 10% solution of 0.006 M glycolic acid (0.0006 M) greatly inhibits algal growth.

Based on these results, it was decided that glycolic acid treatment is not a viable alternate P-solubilization procedure within the AA:BT protocol for wastewater effluent analysis.

P Solubilization and Algal Toxicity Experiments Utilizing Other Solubilization Agents

Experiment #11

Table 15 shows the results of the P solubilization tests conducted during Experiment #11. The most important findings were the demonstration of the ability of 0.006 M HCl treatment to attain the autoclaving yield of the raw sample after 1 day of stirring, and the failure of higher concentrations of acetic acid and NaOH to accomplish this.

The results of Experiment #11 indicated the following:

1) 0.006 M HCl treatment can produce sufficient DRP after a stirring period of 1 day;
2) sample pretreatment with acetic acid or NaOH does not appear to be capable of attaining the autoclaving yield of a sample.

Experiment #12

The algal toxicity experiment conducted with 0.006 M HCl and 0.010 M NaOH in 10% ANM demonstrated that both solutions caused a significant algal growth inhibition, possibly due to the levels of residual sodium and/or chloride after neutralization. The results of Experiment #12 indicated that further dilutions of a 0.006 M HCl solution are necessary to reduce algal growth inhibition.

Experiment #13

Table 16 reveals the results of the P solubilization tests conducted during Experiment #13. Pretreatment of the raw, bottom-agitated Forge Pond sample with 0.003 and 0.0006 M HCl both failed to approach the autoclaving yield, even after 102 hours of stirring. The results of Experiment #13 indicated that HCl concentrations greater than 0.003 M are necessary to attain the autoclaving yield of a sample.

Table 15 Results of Experiment #11

HCl, Acetic Acid, And NaOH Solubilization Tests

DRP CONCENTRATION (µg/L)

Raw Sample Treatment	Stirring Times (hours) 23	47	Autoclaving Yield
No additions	38	42	166
0.012 M HCl	201	219	166
0.006 M HCl	161	182	166
0.008 M Acetic Acid	114	78	166
0.010 M NaOH	61	68	166

Initial [DRP] = 38 µg/L

[DRP] solubilized by autoclaving = 128 µg/L

[TP] = 520 µg/L

Table 16 Results of Experiment #13

HCl Solubilization Tests

DRP CONCENTRATION (µg/L)

Raw Sample Treatment	Stirring Times (hours) 25	47	79	102	Autoclaving Yield
No additions	11	17	27	26	129
0.0030 M HCl	81	90	89	88	129
0.0006 M HCl	25	22	24	36	129

Initial [DRP] = 26 µg/L

[DRP] solubilized by autoclaving = 103 µg/L

Experiment #14

Table 17 shows the results of the algal toxicity tests conducted during Experiment #14. None of the treatments were demonstrated to inhibit algal growth in 25% ANM, including the 0.003 and 0.0006 M sodium chloride (NaCl) solutions.

The results of Experiment #14 indicated the following:

1) 50% dilutions of neutralized 0.006 M HCl solutions, the minimum concentration for sufficient DRP recovery, may not cause algal growth inhibition;
2) NaCl concentrations equivalent to residual levels after neutralization of the HCl solutions do not inhibit algal growth.

Table 17 Results of Experiment #14

HCl and NaCl Algal Inhibition Tests

(mg/L)

Solution	Mean Algal Dry Wt.	Predicted Algal Dry Wt.
Control (25% ANM)	28.9	20.0
0.0030 M HCl in 25% ANM	30.8	20.0
0.0006 M HCl in 25% ANM	37.2	20.0
0.0030 M NaCl in 25% ANM	28.5	20.0
0.0006 M NaCl in 25% ANM	29.4	20.0

NOTE: 1) HCl solutions were stirred for 24 hours, filtered, and neutralized to pH = 7.5 ± 0.1 prior to inoculation;

2) Control and NaCl solutions were filtered prior to inoculation.

0.006M HCl AA:BT Experiments

Experiment #15

Table 18 reveals the results of the 0.006 M HCl AA:BT study performed during Experiment #15. The 0.006 M HCL-treated aliquot was stirred for 24 hours, filtered, neutralized to the initial sample pH, and diluted by 50%, prior to inoculation. Although the maximum standing crops (MSC's) in the 50% dilutions of the 0.006 M HCl-treated Forge Pond aliquots were less than predicted, significant growth inhibition was not in evidence. Algal toxicity was not clearly demonstrated, since the EDTA aliquot of the HCL-treated sample did not show a significant MSC increase over the other treatments.

The results of Experiment #15 indicated the following:

1) The algal availability of the DRP solubilized by 0.006 M HCl treatment may be less than that of the DRP produced via autoclaving;
2) 0.006 M HCl treatment may represent a viable alternate P solubilization procedure within the AA:BT protocol for wastewater effluent analysis.

Experiment #16

Table 19 shows the results of the P analyses for the different aliquots used during Experiment #16. Note that the DRP concentration produced by 0.006 M HCl treatment was much lower than that of the autoclaved aliquot. It is theorized that this was due to the re-adsorption of DRP on particulates during the glass-fiber filtration step, which was necessitated by the high particulate levels of the sample. During the filtration, an unusually thick cake was formed.

Table 18 Results of Experiment #15

AA:BT After 0.006 M HCl Treatment

(mg/L)

Solution	Mean Algal Dry Wt.	Predicted Algal Dry Wt.
• 50% Autoclaved		
Control	4.0	-
+ 2 mg/L N	29.0	16.3
+ 1 mg/L EDTA + 2 mg/L N	27.5	16.3
+ Micronutrients + 2 mg/L N	29.2	16.3
• 50% 0.006 M HCl-Treated		
Control	3.4	-
+ 2 mg/L N	10.7	16.8
+ 1 mg/L EDTA + 2 mg/L N	11.9	16.8
+ Micronutrients + 2 mg/L N	10.9	16.8
50% Unautoclaved Control	2.5	-
50% Algal Nutrient Medium	64.6	40.0

50% autoclaved [DRP] = 38 µg/L

50% 0.006 M HCl-treated [DRP] = 39 µg/L

50% unautoclaved [DRP] = 10 µg/L

[TP] of raw sample = 200 µg/L

Table 19 P Analyses for Experiments #16 - #18

Parameter	Concentration (µg/L) Exp. #16	Exp. #17	Exp. #18
25% Autoclaved [DRP]	46	-	-
50% Autoclaved [DRP]	92	44	58
25% 0.006 M HCl-Treated [DRP]	20	-	-
50% 0.006 M HCl-Treated [DRP]	40	42	58
50% Re-Stirred, HCl-Treated [DRP]	-	-	57
25% Unautoclaved [DRP]	9	-	-
50% Unautoclaved [DRP]	18	28	43
50% Autoclaved [TP]	-	56	68
50% 0.006 M HCl-Treated [TP]	-	48	62
50% Re-Stirred, HCl-Treated [TP]	-	-	61
50% Unautoclaved [TP]	-	37	48
Raw Sample [TP]	390	130	160

Table 20 reveals the results of the AA:BT study conducted during Experiment #16. The algal growth achieved by the 25% HCl-treated aliquots was much less than predicted, except in the case of the EDTA aliquot. These differences in MSC between the aliquots were of sufficient magnitude to indicate that algal toxicants were possibly solubilized during acidification. However, the MSC achieved by the 50% HCl-treated aliquot, with only N added, nearly attained its predicted growth. This occurrence is very hard to rationalize, since the 50% aliquot would be expected to have twice the levels of any toxicants possibly present in the 25% HCl-treated aliquot. Perhaps, the higher concentrations of P and other nutrients in the 50% aliquot helped the algae to overcome the inhibitory effects of any toxicants present.

The results of Experiment #16 indicated the following:

1) Care should be taken to avoid the formation of thick cakes during filtration of HCl-treated aliquots, so as to minimize the loss of DRP via re-adsorption;
2) 0.006 M HCl treatment may result in the solubilization of algal toxicants;
3) The growth inhibition effects of these toxicants may be partially negated by the presence of excess nutrients through minimal dilution of 0.006 M HCl-treated aliquots.

Experiment #17

Table 19 shows the results of P analyses conducted on the aliquots utilized during Experiment #17. The close DRP levels of the HCl-treated and autoclaved aliquots indicate that the loss of DRP during filtration of the HCl-treated aliquot was minimal, due to the lower particulate levels in the Forge Pond sample than encountered in Experiment #16.

Table 20 Results of Experiment #16

AA:BT After 0.006 M HCl Treatment

Solution	Mean Algal Dry Wt. (mg/L)	Predicted Algal Dry Wt. (mg/L)
• 25% Autoclaved		
Control	5.75	-
+ 2 mg/L N	47.5	19.8
+ 1 mg/L EDTA + 2 mg/L N	49.6	19.8
+ Micronutrients + 2 mg/L N	48.8	19.8
• 50% Autoclaved		
+ 2 mg/L N	75.9	39.6
• 25% 0.006 M HCl-Treated		
Control	1.53	-
+ 2 mg/L N	2.34	8.6
+ 1 mg/L EDTA + 2 mg/L N	7.03	8.6
+ Micronutrients + 2 mg/L N	3.27	8.6
• 50% 0.006 M HCl-Treated		
+ 2 mg/L N	14.3	17.2
25% Unautoclaved Control	3.88	-
100% Algal Nutrient Medium	111.2	80.0

Figure 9 displays the results of the AA:BT study performed during Experiment #17. Although the MSC values of the autoclaved aliquots were greater than those of the 0.006 M HCl-treated aliquots for each respective addition, the predicted dry weights of the latter aliquots (18.1 mg/L) were exceeded in all cases. The magnitude of the differences between the MSC values for the EDTA aliquot and the other HCl-treated aliquots, and the lack of a similar trend with the autoclaved aliquots, evidenced that slight algal growth inhibition may have occurred due to the acidification.

The results of Experiment #17 indicated that 0.006 M HCl treatment may represent a viable alternative P solubilization procedure, but that its potential for also solubilizing toxicants, which is dependent on the sample characteristics, may limit its application within the AA:BT protocol for wastewater effluent analysis.

Experiment #18

Table 19 shows the results of the P analyses conducted on the aliquots of the bottom-agitated Forge Pond sample utilized during Experiment #18. The DRP yields of autoclaving and the 0.006 M HCl treatment were equivalent. As described in the Methodology section, the re-stirring procedure, which was tested for its potential as a toxicant-reduction measure, did not significantly lower the DRP concentration of the HCl-treated aliquot.

Figure 10 displays the results of the AA:BT study performed during Experiment #18. The 0.006 M HCl-treated aliquots failed to attain the MSC values of the autoclaved aliquots, although the levels of algal growth in the EDTA aliquots were significantly closer than the other additions, once again demonstrating the presence of toxicants due to acidification. The MSC values of the re-stirred, HCl-treated aliquots were slightly greater than all of the respective HCl-treated aliquots, perhaps due to a slight

Experiment 17

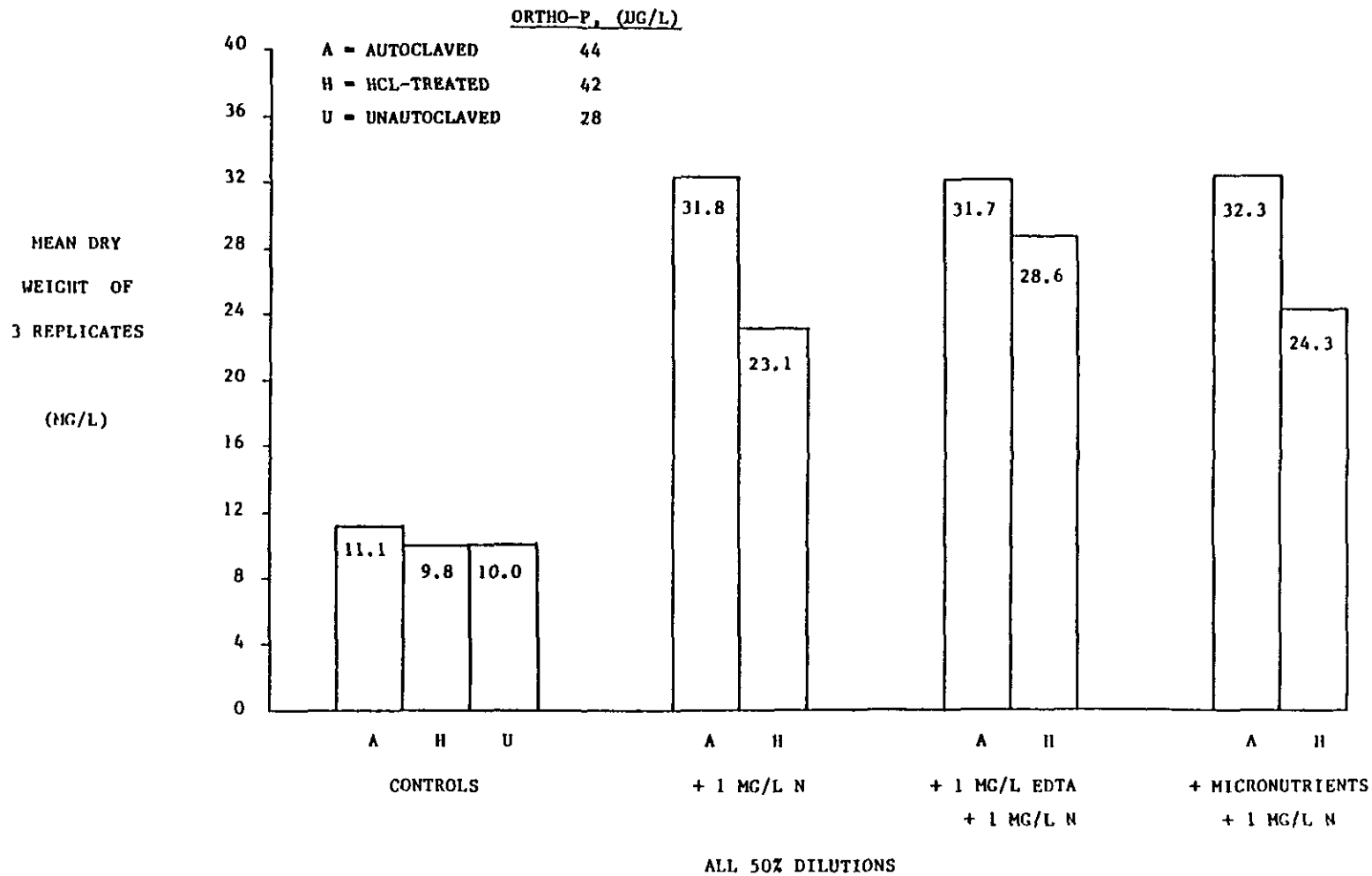


Figure 9

Experiment 18

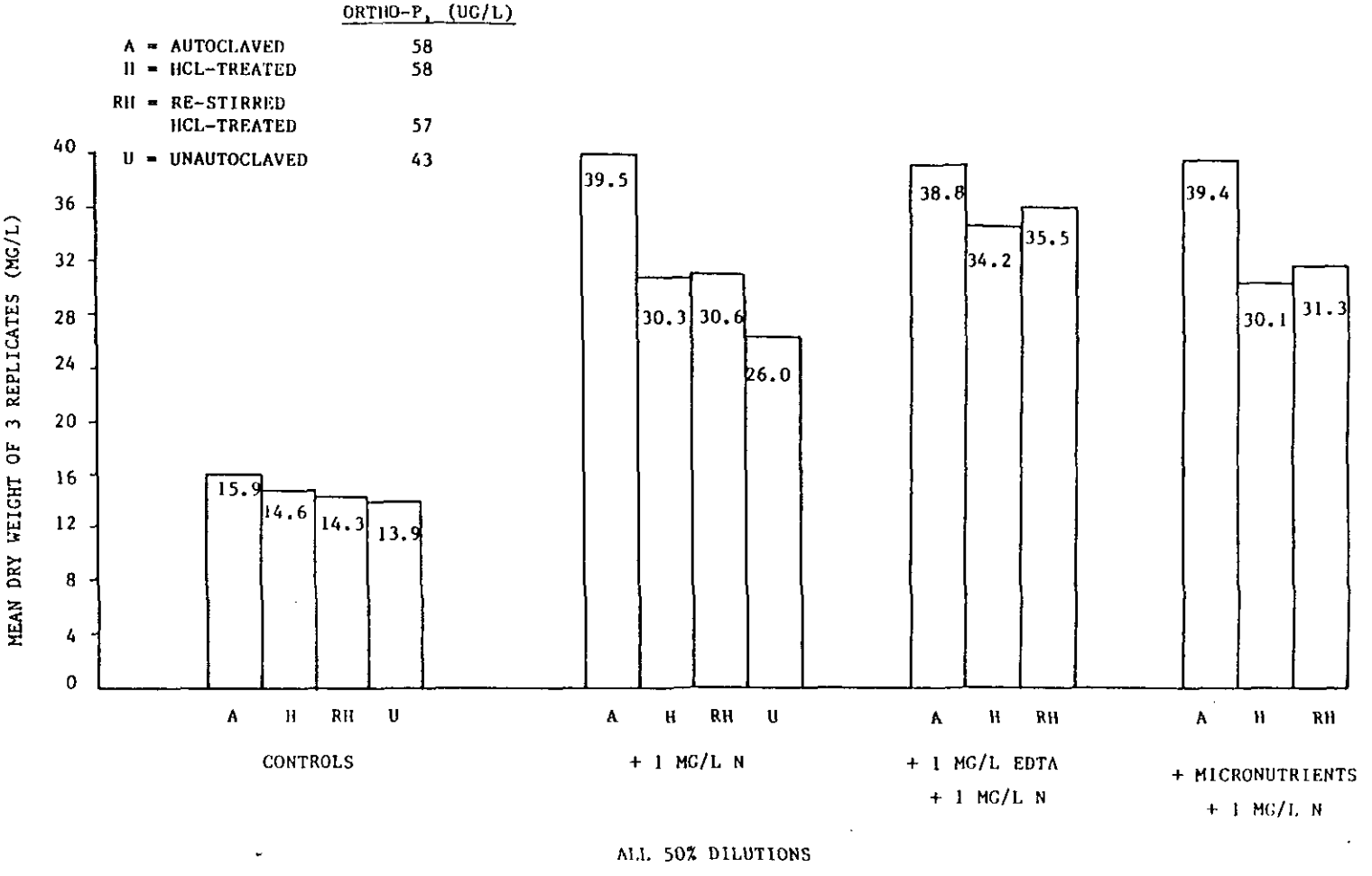


Figure 10

reduction in toxicant levels as a result of the re-stirring procedure. The MSC values of both the HCl-treated aliquots with 1 mg/L N added were significantly higher than the MSC value of the respective unautoclaved aliquot, demonstrating the additional algal growth potential attained due to the acidification procedure.

The results of Experiment #18 indicated the following:

1) 0.006 M HCl treatment can increase the algal growth potential of an unautoclaved sample, due to additional solubilization of particulate P;
2) 0.006 M HCl treatment may also solubilize toxicants which can inhibit algal growth;
3) Re-stirring and re-filtration of the HCl-treated aliquot, subsequent to initial filtration and neutralization, may be able to reduce the levels of toxicants produced during acidification, without significantly decreasing DRP levels.

Summary of 0.006 M HCl AA:BT Experiments

The 0.006 M HCl AA:BT's have demonstrated that, while slight algal growth inhibition may be a problem at low dilutions (i.e., 50% sample),the incorporation of the HCl-treatment scheme into the AA:BT procedure for wastewater effluent testing allows for the analysis of algal available particulate P that would not be solubilized without the use of autoclaving. However, since greater dilutions (e.g., 1% - 15% sample) of wastewater effluents are generally used during algal assays, the relative effects of growth inhibitors solubilized during HCl treatment will be reduced.

0.006 M HCl Treatment of a Wastewater Effluent

The results of the nutrient analyses conducted on the effluent sample from the Marlborough Easterly Wastewater Treatment Facility are shown in

Table 21. Some interference was evident in the ammonia analysis, reducing the reliability of the concentrations obtained. The 0.006 M HCl treatment of the effluent increased the DRP concentration by 55% over the aliquot that was only filtered. The combined levels of nitrate and nitrite in both aliquots were equivalent.

Figure 11 displays the results of the AA:BT study performed during this experiment. The 1% effluent samples for both aliquots showed insignificant growth, and thus are not represented. A two-tailed Student t test (Bethea et al., 1985) was utilized to calculate the 90% confidence intervals for the algal dry weight values of each treatment. It should be noted that because triplicate flasks are typically used during an AA:BT study, the degrees of freedom for each case are very low, allowing the confidence intervals to be very sensitive to any variations in results.

For the samples with no EDTA addition, the MSC of the HCl-treated aliquot was significantly greater than that of the untreated aliquot only in the case of the 10% effluent. With 1 mg/L EDTA added, the algal yield of tne HCl-treated aliquots were significantly increased over the untreated aliquots with both the 5% and 20% effluent samples. In general, all EDTA aliquots possessed greater MSC values than the corresponding non-EDTA aliquots, both for the HCl treatment and for no treatment. Therefore, algal growth inhibition was evident in the raw, filtered effluent prior to HCl treatment.

The results of this experiment indicated the following:

1) 0.006 M HCl treatment can increase the algal growth potential of a wastewater effluent via solubilization of DRP from particulates prior to filtration;

Table 21 Nutrient Analyses for the Marlborough Easterly Wastewater Treatment Facility Effluent

(mg/L)

Parameter	Whole Sample	Filtered	0.006 M HCl-Treated
DRP	-	0.360	0.560
TP	0.680	0.410	0.610
Nitrate-Nitrite	-	19.8	19.8
Ammonia	-	1.1	0.8

pH of raw sample = 6.85

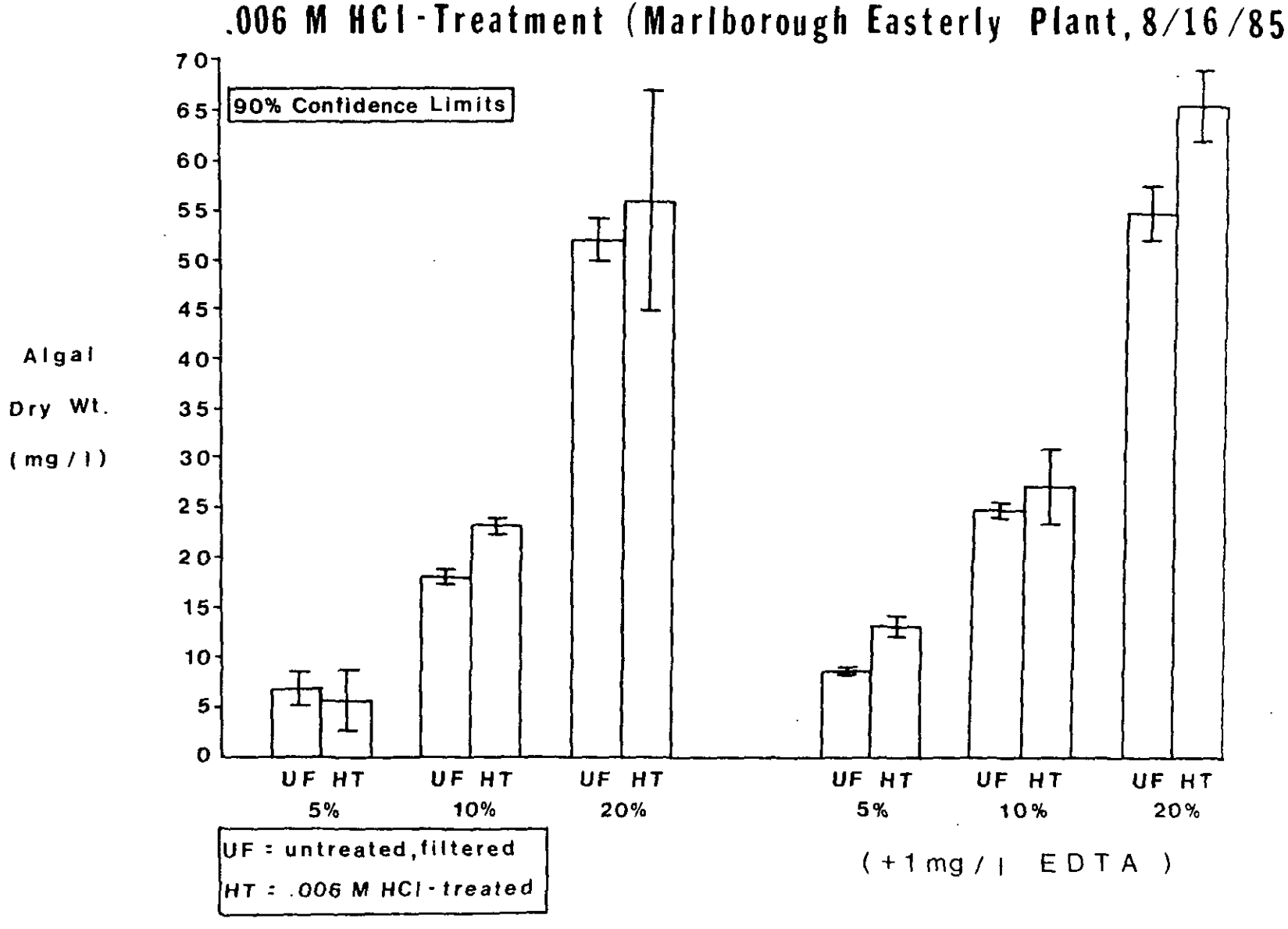

.006 M HCl-Treatment (Marlborough Easterly Plant, 8/16/85)
90% Confidence Limits
Algal
Dry Wt.
(mg/l)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
UF HT
5%
UF HT
10%
UF HT
20%
UF HT
5%
UF HT
10%
UF HT
20%
(+1 mg/l EDTA)
UF = untreated, filtered
HT = .006 M HCl-treated

Figure 11

2) The DRP solubilize during 0.006 M HCl treatment may not all be readily available, and/or algal toxicants solubilized during the acidification may cause varying degrees of algal growth inhibition.

CONCLUSIONS AND RECOMMENDATIONS

A series of experiments was conducted with the objective of developing a P-solubilization procedure as an alternative to autoclaving, within the AA:BT protocol for the analysis of wastewater effluents. The following conclusions are made, based on the results of these experiments:

1) Sample pretreatment with dilute glycolic acid, although effective at achieving the DRP yield of autoclaving, is not a viable means of P solubilization within the scope of the AA:BT procedure, due to the magnitude of its algal growth inhibition effects;
2) 0.006 M HCl is the optimal concentration of the acid for the purposes of this research, in terms of being capable of attaining the autoclaving DRP yield of a sample, while not generally causing gross algal growth inhibition;
3) Although 0.006 M HCl treatment has been indicated to cause varying degrees of algal growth inhibition in both eutrophic pond samples and a wastewater effluent, it has also been demonstrated to increase the algal growth potential of these waters over their respective untreated MSC levels;
4) Further study of the 0.006 M HCl treatment procedure is necessary to properly assess its potential as a P-solubilization technique within the protocol of the AA:BT analysis of wastewater effluents;
5) This additional research should involve the application of the 0.006 M HCl treatment technique to a number of wastewater effluents from varied sources; only in this way can the value of the procedure as a widely applicable eutrophication management tool be properly evaluated.

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APPENDIX A

Raw Algal Count Statistics

ALGAL ASSAY OF FORGE POND
(mg/L)

Treatment	Number	Mean	Standard Deviation
Control	3	4.05	0.065
P	3	3.83	0.393
N	3	11.7	0.69
EDTA	3	5.00	0.280
N + P	3	35.8	0.87
EDTA + P	3	5.30	0.803
EDTA + N + P	3	38.4	1.28
Micronutrients	3	4.74	0.261
Unautoclaved Control	3	1.76	0.042
Algal Nutrient Medium	3	125.7	1.60

EXPERIMENT #7
(mg/L)

Treatment	Number	Mean	Standard Deviation
Autoclaved Control	3	5.17	0.493
Autoclaved + N	3	63.0	1.92
Autoclaved + N + EDTA	3	60.1	0.40
Autoclaved + N + Micronutrients	3	60.9	1.32
0.06 M Glycolic Acid Control	3	0.25	0.025
0.06 M Glycolic Acid + N	3	0.53	0.093
0.06 M Glycolic Acid + N + EDTA	3	0.78	0.062
0.06 M Glycolic Acid + N + micro-nutrients	3	0.54	0.023
Unautoclaved Control	3	6.21	0.231
Algal Nutrient Medium	3	108.4	1.06

EXPERIMENT #8
(mg/L)

Treatment	Number	Mean	Standard Deviation
25% ANM	3	18.2	1.33
0.03 M Glycolic Acid in 25% ANM	3	0.20	0.092
0.01 M Glycolic Acid in 25% ANM	3	0.34	0.092
0.006 M Glycolic Acid in 25% ANM	3	0.62	0.083

EXPERIMENT #10
(mg/L)

Treatment	Number	Mean	Standard Deviation
25% ANM	3	27.2	0.50
0.003 M Glycolic Acid in 25% ANM	3	1.02	0.636
0.001 M Glycolic Acid in 25% ANM	3	0.46	0.042
0.0006 M Glycolic Acid in 25% ANM	3	0.96	0.392

EXPERIMENT #12
(mg/L)

Treatment	Number	Mean	Standard Deviation
10% ANM	3	12.04	0.220
0.006 M HCl in 10% ANM	3	0.122	0.020
0.010 M NaOH in 10% ANM	3	1.96	1.128

EXPERIMENT #14
(mg/L)

Treatment	Number	Mean	Standard Deviation
25% ANM	3	28.9	0.26
0.003 M HCl in 25% ANM	3	30.8	2.10
0.006 M HCl in 25% ANM	3	37.4	1.41
0.003 M NaCl in 25% ANM	3	28.5	1.30
0.0006 M NaCl in 25% ANM	3	29.4	1.89

EXPERIMENT #15
(mg/L)

Treatment	Number	Mean	Standard Deviation
50% Autoclaved Control	2	4.0	0.14
50% Autoclaved + N	3	29.0	0.36
50% Autoclaved + EDTA + N	3	27.5	0.64
50% Autoclaved + Micronutrients + N	3	29.2	0.60
50% 0.006 M HCl-Treated Control	3	3.4	0.23
50% HCl-Treated + N	3	10.7	1.14
50% HCl-Treated + EDTA + N	3	11.9	2.04
50% HCl-Treated + Micronutrients + N	3	10.9	0.03
50% Unautoclaved Control	3	2.5	0.40
50% ANM	3	64.6	0.51

EXPERIMENT #16
(mg/L)

Treatment	Number	Mean	Standard Deviation
25% Autoclaved Control	3	5.75	0.384
25% Autoclaved + N	3	47.5	0.45
25% Autoclaved + N + EDTA	3	49.6	0.85
25% Autoclaved + N + Micronutrients	3	48.8	1.81
50% Autoclaved + N	3	75.9	2.08
25% 0.006 M HCl-Treated Control	3	1.53	0.341
25% HCl-Treated + N	3	2.34	0.203
25% HCl-Treated + N + EDTA	3	7.03	0.225
25% HCl-Treated + N + Micronutrients	3	3.27	0.545
50% HCl-Treated + N	3	14.3	1.58
25% Nonautoclaved Control	3	3.87	0.031
100% ANM	3	111.2	2.37

EXPERIMENT #17
(mg/L)

Treatment	Number	Mean	Standard Deviation
50% Autoclaved Control	3	11.1	0.62
50% Autoclaved + N	3	31.8	0.59
50% Autoclaved + N + EDTA	3	31.7	0.60
50% Autoclaved + N + Micronutrients	3	32.3	0.70
50% 0.006 M HCl-Treated Control	3	9.82	0.67
50% 0.006 M HCl-Treated + N	3	23.1	1.32
50% 0.006 M HCl-Treated + N + EDTA	3	28.6	0.60
50% 0.006 M HCl-Treated + N + Micronutrients	3	24.3	1.18
50% Nonautoclaved Control	3	9.97	0.387
50% ANM	3	57.1	1.95

EXPERIMENT #18
(mg/L)

Treatment	Number	Mean	Standard Deviation
50% Autoclaved Control	3	15.9	0.61
50% Autoclaved + N	3	39.5	0.21
50% Autoclaved + N + EDTA	3	38.8	0.53
50% Autoclaved + N + Micronutrients	3	39.4	0.60
50% 0.006 M HCl-Treated Control	3	14.6	0.51
50% 0.0006 M HCl-Treated + N	3	30.3	0.49
50% 0.006 M HCl-Treated + N + EDTA	3	34.2	0.21
50% 0.006 M HCl-Treated + N + Micronutrients	3	30.1	0.17
50% Re-stirred 0.006 M HCl-Treated Control	3	14.3	0.32
50% Re-stirred HCl-Treated + N	3	30.6	0.55
50% Re-stirred HCl-Treated + N + EDTA	3	35.5	0.85
50% Re-stirred HCl-Treated + N + Micronutrients	3	31.3	0.31
50% Nonautoclaved Control	3	13.9	0.47
50% Nonautoclaved + N	3	26.0	0.40
50% ANM	3	59.5	2.47

EXPERIMENT #19
(mg/L)

Treatment	Number	Mean	Standard Deviation
5% Unautoclaved Control	3	6.62	0.945
5% Unautoclaved + EDTA	3	8.62	0.245
10% Unautoclaved Control	3	18.9	0.38
10% Unautoclaved + EDTA	3	24.7	0.40
20% Unautoclaved Control	3	51.7	1.33
20% Unautoclaved + EDTA	3	54.7	1.59
5% 0.006 M HCl-Treated Control	3	5.45	1.82
5% 0.006 M HCl-Treated + EDTA	3	13.0	0.60
10% 0.006 M HCl-Treated Control	3	23.2	0.45
10% 0.006 M HCl-Treated + EDTA	3	27.1	2.31
20% 0.006 M HCl-Treated Control	3	56.0	6.49
20% 0.006 M HCl-Treated + EDTA	3	65.4	2.12
50% ANM	3	58.1	0.75