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THE DYNAMICS OF PHOSPHORUS IN THE ALGA, CHLORELLA PYRENOIDOSA
AND ITS INTERACTIONS WITH INORGANIC PARTICULATES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Joseph Louis Taraba, B.Ch.E., M.Sc.

* * * * *

The Ohio State University
1978

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Studies in Mass Transfer. Professor Christie J. Geankoplis


Studies in Environmental Pollution. Professors Karlis Svanks and Thomas L. Sweeney
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<table>
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<tr>
<td>AMP - AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP - ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BBM - BBM</td>
<td>Bold's Basal Medium</td>
</tr>
<tr>
<td>CMP - CMP</td>
<td>Cytidine monophosphate</td>
</tr>
<tr>
<td>Co-A - Co-A</td>
<td>Coenzyme-A</td>
</tr>
<tr>
<td>COD - COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>DSTR - DSTR</td>
<td>Continuous Stirred Tank Reactor</td>
</tr>
<tr>
<td>DNA - DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DO - DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>DOC - DOC</td>
<td>Dissolved Organic Carbon</td>
</tr>
<tr>
<td>DOP - DOP</td>
<td>Dissolved Organic Phosphorus</td>
</tr>
<tr>
<td>DT - DT</td>
<td>Doubling Time</td>
</tr>
<tr>
<td>FAD - FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>GMP - GMP</td>
<td>Guanosine Monophosphate</td>
</tr>
<tr>
<td>H₂O(Ext) - H₂O(Ext)</td>
<td>Boiling Water Extraction of Phosphorus Compounds from Cells</td>
</tr>
<tr>
<td>NA - NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>NAD - NAD</td>
<td>Nicotiamide Dinucleotide</td>
</tr>
<tr>
<td>o-PO₄ - o-PO₄</td>
<td>Orthophosphate</td>
</tr>
<tr>
<td>Org-P - Org-P</td>
<td>Organic Phosphorus</td>
</tr>
<tr>
<td>P - P</td>
<td>Phosphorus</td>
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<tr>
<td>Poly-P - Poly-P</td>
<td>Poly Phosphate</td>
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<td>Orthophosphate Determination by Stannous Chloride-Myolybdenum Blue Method</td>
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<td>PO₄(HM) - PO₄(HM)</td>
<td>Orthophosphate Determination by Hydrazine-Molybdenum Blue Method</td>
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<tr>
<td>PP - PP</td>
<td>Particulate Phosphorus</td>
</tr>
<tr>
<td>PPA - PPA</td>
<td>Proteose Peptone Agar</td>
</tr>
<tr>
<td>P-Tot - P-Tot</td>
<td>Total Phosphorus Hydrolysis by Postassium Persulfate Digestion before Determination of Orthophosphate</td>
</tr>
<tr>
<td>RNA - RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SP - SP</td>
<td>Soluble Phosphorus</td>
</tr>
<tr>
<td>SRP - SRP</td>
<td>Soluble Reactive Phosphorus</td>
</tr>
<tr>
<td>SUP - SUP</td>
<td>Soluble Unreactive Phosphorus</td>
</tr>
<tr>
<td>TCA(Ext) - TCA(Ext)</td>
<td>Cold TCA extraction of Phosphorus Compounds from Cells</td>
</tr>
<tr>
<td>TCA - TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TP - TP</td>
<td>Total Phosphorus</td>
</tr>
<tr>
<td>UMP - UMP</td>
<td>Uridine Monophosphate</td>
</tr>
<tr>
<td>UTP - UTP</td>
<td>Uridine Triphosphate</td>
</tr>
</tbody>
</table>
SUMMARY

An experimental study of the dynamics of phosphorus (P) in the alga, *Chlorella pyrenoidosa* (Emerson) was carried out in laboratory axenic batch cultures. As a result of experimental conditions, the formation of inorganic particulate P was found in the medium under basic conditions. The dynamics of the inorganic particulate P was also investigated during the growth. The emphasis of the study was the determination whether a defined fraction of P extracted from the alga correlated with the specific growth rate of the alga when P was stoichiometrically limiting the growth. This experimental approach was concluded from a review of the literature.

Batch cultures of *Chlorella pyrenoidosa* were grown under continuous light in Bold's Basal Medium with the concentration of P at approximately 1.45 mg P/l as orthophosphate (o-PO₄). Cultures were grown at two pH levels (6.5 and 7.5) and two temperatures (25 and 30°C) with CO₂-enriched air (~0.5% CO₂) bubbling through the medium.

Two extraction methods were used to solubilize P from the algal cells, cold TCA (Schmidt-Thannhauser) and boiling water (Fitzgerald-Nelson). The P extracted by each method was divided into the three compartments based on the liberation of o-PO₄ as a result of increasing the intensity of acid hydrolysis. A fourth P compartment was the P not extracted from the algal cell.
It was concluded that the growth rate of *Chlorella pyrenoidosa* during the growth cycle correlated with the most stable P extracted by both cold TCA and boiling water under all the environmental conditions previously mentioned. This compartment contains functional P compounds of which ATP is a major component. The functional P compartment of the algal cells could be determined in the presence of inorganic particulate P that was associated with iron and calcium compounds filtered with the cells. In the experimental work, P was found to coordinate with Fe(OH)$_3$ to form a complex and was found to form hydroxyapatite with calcium.

Several other observations of importance were made in the course of the growth cycle of *Chlorella pyrenoidosa* in batch culture. The specific growth rate during exponential growth based on the cell number concentration, dry weight concentration and total cell volume concentration was in the range of 0.065-0.067 hr$^{-1}$ up to the time of $\sigma$-PO$_4$ depletion from the medium. After $\sigma$-PO$_4$ depletion, the specific growth rate based on cell number concentration increased dramatically while the specific growth rate based on dry weight concentration and total cell volume concentration did not change. At pH = 6.5 and 25°C, the cell number specific growth increased to 0.088 hr$^{-1}$; while at pH = 7.5 and 25°C, the rate increased to 0.111 hr$^{-1}$. All three growth measures indicated the cessation of exponential growth at the same time. Other dramatic changes were observed to occur as the cell number specific growth rate increased. The cell shape of *Chlorella pyrenoidosa* changed from spherical to fusiform as new daughter cells were released. The number of daughter cells per division decreased in the
same time period from 4 or 8 spherical daughter cells per division to 2 fusiform daughter cells per division. The percentage of fusiform cells increased to 100% at the end of the exponential growth phase. Further the cell dry weight density and individual cell volume decreased to a minimum during the increased specific growth rate. With the onset of stationary growth, the percent of fusiform cells decreased significantly while the cell density and individual cell volume increased.

Finally it was observed in the batch growth experiments that as the *Chlorella pyrenoidosa* cells began exponential growth, there was a dramatic increased filterability of the inorganic P, as hydroxyapatite.
CHAPTER I

INTRODUCTION

The study presented in this dissertation deals with the cycling of phosphorus (P) within the aquatic environment between the living (biosphere) and the non-living (abiosphere) world. The research evolved from a proposal that was submitted for funding to study the dynamics of P and clay colloids in natural river systems. To expand on the above division of the aquatic environment, the biosphere encompasses the algae, bacteria and higher organisms while the abiosphere consists of inorganic and organic compounds that are found in the soluble and insoluble states. The insoluble inorganics would include clay particulates, large molecular weight organic polymers and coordinated complexes which form colloids.

The natural environment has a broad spectrum of entities in each category cited above. Limiting the number of entities in laboratory experiments only creates a non-realistic environment, but the limitation simplifies the system so that interactions between a species in each category may be observed. The study, presented in the following chapters, limited the biosphere to a single species, the alga Chlorella pyrenoidosa. The abiosphere colloids included a ferric iron complex and hydroxyapatite which were formed under basic pH conditions in a defined synthetic growth medium. Both inorganic colloids bind orthophosphate (o-PO₄). Each of these inorganic
particulates are found in natural aquatic ecosystems. In laboratory experiments, during which the initial nutrient levels were fixed; the pH, temperature, light intensity and dissolved gases were controlled; P was traced as it was taken from the abiosphere, as $\text{o-PO}_4^\text{-2}$, to the biosphere where the algal intracellular P concentrations were partitioned into four compartments: three levels of acid stable P in the extracted soluble P and the non-soluble P. The P compounds were extracted by hot water and by cold trichloroacetic acid (TCA). This procedure yields results more rapidly than the more detailed sequential extractions of cells found in the literature.

The evaluation of each extracted P compartment of $\text{C. pyr}$. was made to identify the cellular role and correlation with the growth rate. The results determine the applicability of a growth models based on an internal cell P concentration that correlates to the cell growth rate. The cell growth rate as a function of internal cell P has been noted in the literature. Thus a mathematical growth model can be incorporated into P models of the aquatic ecosystems which closely parallels observed phenomena, and an analytical method of obtaining the results for the model from natural aquatic systems. The analytical method must be able to give significant results in an ecosystem which includes inorganic particulates.

In order to orient the reader to the aquatic ecosystem, Chapter II, Introduction to Aquatic Ecosystem, was written. This chapter deals with general observed phenomena. Chapter III, Related Literature, is developed in more detail for the P dynamics in aquatic
ecosystems than the research work covers. Finally, there are a great number of abbreviations used in the writing of this dissertation. A Glossary is included in the preliminaries for the reader's reference.
CHAPTER II
INTRODUCTION TO THE AQUATIC ECOSYSTEM

2.1 General

The thrust of this section is an orientation to the structure of the environment. This structure involves the flow of matter and energy to and from the abiosphere, through a hierarchy of living organisms in the biosphere. The interface of the abiosphere and the biosphere is dominated by simple protist organisms which are primarily algae, bacteria, and fungi. The introduction of matter into the biosphere begins at this point. The cycling of the elements of matter through the interface is important. This section examines the fate of phosphorus (P) in an aquatic environment at the interface. Phosphorus is involved in the transfer of energy in organisms as well as a constituent in the molecule that holds the key to life, DNA.

2.2 General Ecology

The organisms in an environment can be divided into the animal and the plant world based on structural and physiological differences. The foundation of each division is the unicellular organisms, the protists, because of their simple biological organization. Each division has a hierarchy above the protists based on increasing biological organization. In Figure 2.1 the animal division is presented as a pyramid with the foundation of the protists and man at
Figure 2.1 The Relationship Between Organisms in an Animal Ecosystem
the top. The width of each level is the relative number of individuals of that level. The primary interface of the animal world, the biosphere, and the abiosphere is at Level I, the residence of the simple protists, i.e., algae, bacteria and fungi.

The interface between the biosphere and the abiosphere is the major pathway for energy, as sunlight, and matter, as atoms and molecules, to the organisms that make up the animal division. The light energy is converted by photosynthetic related processes to chemical energy, which is in the form of reduced carbon and high energy phosphate esters. These compounds can be stored, utilized immediately for energy or converted to organisms structural material through biosynthetic processes. Wastes from this conversion are returned to the abiosphere principally as oxidized carbon. The chemical energy formed in Level I can be consumed by organisms in Level II to form cell structural material and to satisfy energy requirements. Wastes in this conversion are released to the abiosphere. There is a net loss of energy in this process. The same sequence is continued up the pyramid till man is reached at the pinnacle. Along the upward path there is degradation of the energy taken in at the base as sunlight. Thus a balance is formed when the wastes can be recycled and not any one level consumes all the energy.

In the aquatic environment, the wastes from man's activity generally broadens the population base of Level I, consuming more energy and more of the limited nutrients. This activity results in elimination of higher organisms from the environment. For example, the high organic content of untreated sewage discharged into a body of water is
oxidized by bacteria thus reducing the oxygen concentration to a level at which higher animal organisms will not survive. Therefore the study of the simple protists in Level I in their role of cycling matter and changing light energy to chemical energy at the interface becomes central to the control and understanding of water ecology.

2.3 Chemical Transformation in Level I

A general description of the cycling and transformations of matter and energy in Level I is shown in Figure 2.2(21). The autotrophs can use CO$_2$ as the sole source of carbon and produce organic material by reducing the CO$_2$ and producing oxygen with energy absorbed from light, i.e., photosynthesis. Heterotrophs obtain carbon from organic compounds. Their energy requirement is obtained from respiration, the oxidation of part of the organic compounds. Thus, in algae, energy rich bonds are produced by photosynthesis, distorting the thermodynamic equilibrium. Bacteria and other respiring organisms catalyze the metabolic processes that tend to restore chemical equilibrium (214).

In Figure 2.2 the general term nutrients encompasses, in addition to carbon and oxygen, both nitrogen, phosphorus, sulfur as essential nutrients usually obtained in an inorganic form, other mineral nutrients and organic growth factors (which are specific organic compounds that the cell does not produce) (21). The inorganic nutrients that are utilized by the cell are in proportion to the cell composition.

Table 2.1 gives the elementary composition of a microbial cell (bacterium Escherichia coli (208)). This composition is a typical value since it is subject to change due to the environment, the age of
Figure 2.2 Cycling of Nutrients Between Heterotrophic and Autotrophic Microorganisms (214)
<table>
<thead>
<tr>
<th>Element</th>
<th>Percentage of Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>50</td>
</tr>
<tr>
<td>Oxygen</td>
<td>20</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>14</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>8</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>3</td>
</tr>
<tr>
<td>Sulfur</td>
<td>1</td>
</tr>
<tr>
<td>Potassium</td>
<td>1</td>
</tr>
<tr>
<td>Sodium</td>
<td>1</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.5</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.5</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.5</td>
</tr>
<tr>
<td>Iron</td>
<td>0.2</td>
</tr>
<tr>
<td>All others</td>
<td></td>
</tr>
<tr>
<td>(Cobalt, Manganese, Copper, Zinc, Molybdenum, Boron)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
cell and species of organism. Table 2.2 lists the general physiological functions of the principal elements listed in Table 2.1 (208).

Table 2.3 summarizes some of the typical organic growth factors. There are many organisms that require no growth factors, some require only one, and others required many growth factors. Typically less biologically organized protists require growth factors (208). The incorporation of the elements, in the form of nutrients and organic substrates, into the different metabolic processes of the cell is schematically presented in Figure 2.3 (21).

2.4 Phosphorus Transformations in Level I

Phosphorus has a major role in biological metabolism. It plays a role in energy storage, energy transfer in metabolic processes, in structural components of membranes, and in the storage and transfer of the genetic code. Phosphorus is least abundant in nature of the major nutritional and structural components of organisms (carbon, nitrogen, oxygen, sulfur) (244). In natural waters phosphorus concentrations can limit the net production of organic material. Phosphorus is often derived primarily from the activity of man. The increased loading of the waters with phosphorus both increases the net production of organic material in waters and can result in another element, such as carbon, nitrogen, or trace elements, to limit the productivity (138). These latter elements generally are found in amounts exceeding requirements. Thus a little added phosphorus from man can greatly increase the net production of organic material creating a eutrophic environment. This potential increase in organic matter can be appreciated by noting the generalized molecular formula for algae
Table 2.2 General Physiological Functions of the Principal Elements (208)

<table>
<thead>
<tr>
<th>Element</th>
<th>Physiological Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients:</td>
<td></td>
</tr>
<tr>
<td>Hydrogen</td>
<td>Constituent of cellular water, organic cell material</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Constituent of cellular water, organic cell materials; as $O_2$, electron acceptor in respiration of aerobes</td>
</tr>
<tr>
<td>Carbon</td>
<td>Constituent of organic cell materials</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Constituent of proteins, nucleic acids, coenzymes</td>
</tr>
<tr>
<td>Sulfur</td>
<td>Constituent of proteins, of some coenzymes</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>Constituent of nucleic acids, phospholipids, coenzymes, phosphoproteins, sugar phosphates</td>
</tr>
<tr>
<td>Potassium</td>
<td>One of the principal inorganic cations in cells, cofactor for some enzymes</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Important cellular cation; inorganic cofactor for many enzymatic reactions, including those involving ATP; functions in binding enzymes to substrates; constituent of chlorophylls</td>
</tr>
<tr>
<td>Calcium</td>
<td>Important cellular cation; cofactor for some enzymes</td>
</tr>
<tr>
<td>Silicon</td>
<td>Major requirement for cell wall synthesis in diatoms</td>
</tr>
<tr>
<td>Micronutrients:</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>Inorganic cofactor for some enzymes, sometimes replacing Mg</td>
</tr>
<tr>
<td>Iron</td>
<td>Constituent of cytochromes and other heme or non-heme proteins; cofactor for a number of enzymes</td>
</tr>
<tr>
<td>Cobalt</td>
<td>Constituent of vitamin B$_{12}$ and its coenzyme derivatives</td>
</tr>
<tr>
<td>Copper, Zinc,</td>
<td>Inorganic constituents of special enzymes</td>
</tr>
<tr>
<td>Molybdenum, Boron</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 Typical Organic Growth Factors (21)

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Examples</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamins</td>
<td>Biotin</td>
<td>Fatty acid biosynthesis; CO₂ fixation; carrier of hydrogen atoms</td>
</tr>
<tr>
<td></td>
<td>Cobalamin (B₁₂)</td>
<td>Reduction and transfer of single carbon fragments</td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
<td>Precursor in flavoproteins involved in electron transport</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td>Required for protein synthesis</td>
</tr>
<tr>
<td>Purines and</td>
<td></td>
<td>Building blocks of nucleic acids and coenzymes</td>
</tr>
<tr>
<td>Pyrimidines</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.3 Biochemical Processes in a Cell (21)
There are papers that cite other nutrients that limit the productivity of aquatic systems. Several papers cite carbon as a limiting nutrient \((22,126,134)\). Others have found that the trace element iron was limiting productivity \((153,164)\), and some studies found that nitrogen was limiting \((74,191)\).

The cycling of the element phosphorus in the aquatic system can be represented schematically in Figure 2.4. This representation is adapted from several sources \((75,180,214,242)\). Phosphorus is compartmentalized in the following way. The external sources to the aquatic system are both natural and man initiated. The aquatic system also has an outflow. Within the water environment, the system is divided into a colloidal microenvironment separate from the rest of the system. This compartment is basically the interface between the abiosphere and the biosphere where Level I organisms exist. Level II organisms are represented as zooplankton and higher plants. In the colloidal microenvironment, \(P\) is found in the colloidal state as phytoplankton (autotrophs), bacteria (heterotrophs), and nonviable particulate phosphorus (PP). The remaining \(P\) exists in soluble state as dissolved orthophosphate \((o-PO_4)\), dissolved organophosphates (DOP) and dissolved condensed phosphorus. In an aquatic system the sediments influence the overlying water concentrations of \(P\) in a process that parallels buffering of the acid-base system.

In Figure 2.4 each box, except \(o-PO_4\), contains a broad spectrum of entities in a natural aquatic system. The arrows show the exchange processes between each compartment in the colloidal microenvironment. The influence of each compartment on an exchange process is not
Figure 2.4 Block Diagram of Phosphorus Flow in Aquatic Ecosystem
indicated but will be discussed later. The colloidal compartments exit the microenvironment by precipitation to the bottom sediments. Also there are losses of biological species to the zooplankton by ingestion. The dissolved species also leave the system by being absorbed by the higher plants, the zooplankton, and the sediments. These latter compartments also feed back to the dissolved compartments through excreted P compounds from metabolic activity and dissolution from the sediments. The inflows to the aquatic system contribute dissolved P compounds, particularly the condensed P from sewage; and a significant part of the inorganic colloids, as clay, from erosion.

2.4-1 Biological Activity

The biological compartments of algae and bacteria consist of a large diverse assemblage of nearly all the major taxonomic groups. A number of species tend to dominate the organism population at a particular time. But these species can be succeeded by other species due to changes in the physical and the chemical environment in the aquatic system (21,201). The species that tend to be dominant are the ones whose physiological requirements and tolerance limits to the chemical and physical parameters are met by the environment. The chemical parameters are the concentrations of the nutrients and their chemical form. For example, an algal species may also be able to utilize P in the form of \( \text{O-PO}_4 \) and not as an organophosphate but another species can. Further, one species may not be able to exist at a given concentration or utilize \( \text{O-PO}_4 \) below a given concentration but another species can. The presence or absence of a particular compound that enhances or inhibits the growth of a species is another chemical
parameter. An example of this would be the strict requirement of a vitamin for growth. If it is absent or in extremely limited quantities the species population will not be large. Physical parameters include light intensity, which is a strict requirement for autotrophic growth, and temperature. Thus each species has a niche based on its physiological requirements in relation to all factors in the environment (208,244).

Phosphorus in the biological compartments can be divided into several types of organophosphorus (Org-P) compounds (68,114,145,208). The exact types and their concentrations in the cell are dependent on the species, age, and the environmental conditions. Orthophosphate may remain free in the cell after uptake as a precursor pool. Low molecular weight organophosphates, such as the sugar phosphates, nucleic acids, and fatty acids containing P, are biosynthetic intermediates which derive P from the precursor pool or are taken up from outside the cell. The intermediates are utilized to produce biopolymers and functional groups. The functional groups would be P containing coenzymes, such as ATP, UTP, and Co-A; and the dinucleotides, FAD, NAD. Biopolymers that are functional would be the P containing RNA and DNA. The biopolymers form structural components. The phospholipids derived from the fatty acids are found in cell membranes. The nucleic acids are found in DNA, the genetic code structure, and in RNA. The sugar phosphates are used to produce structural polysaccharides. Finally orthophosphate (Poly-P) structures that are theorized act as P storage and/or energy storage because of the high energy bonds that are formed. A more
detailed discussion of these components is found in a later section.

2.4-2 Dissolved Organic Phosphorus

Organophosphorus compounds exist in the soluble form external to a cell. These compounds make up 15 to 30% by weight of the total P and up to 90% of the total dissolved P during high algal growth (94, 99, 156) in lake waters. Their primary origin is metabolic activity. The broad spectrum of phosphorylated compounds in the cell also can be found outside the cell. These compounds enter natural waters by excretion from live cells, higher organisms, and by release upon death of a cell. Losses from the DOP pool are due to microbial uptake, hydrolysis to $\text{HPO}_4^-$, and adsorption onto nonviable particulates (99, 156). The particulates may also be a source of DOP because of desorption. The chemical nature of the DOP is not well defined in natural aquatic systems. But several studies have tried to characterize DOP. Experimentally in water analysis, the dissolved state is defined as those components that pass through a .45 μm membrane filter (2). Thus many components that are not in true solution will pass through the filter. Minor (156) used gel chromatography to determine the nature of the DOP. He found a large molecular weight component (>50,000), a component of low molecular weight (<2500), and very low concentrations of other DOP between these extremes in lake water samples. The high molecular weight component was approximately 7-13% of the total DOP and approximately half of this was identified as DNA. Golterman (76) showed that DNA was the slowest of the cell organophosphates to hydrolyze in a pure culture of the alga, *Scenedesmus quadricauda*, during autolysis with no bacteria present. Lean (136), also using
gel chromatography to separate DOP in lake water samples, found two components; one a very large molecular weight fraction (>5,000,000), which he identified as colloidal, and a fraction of molecular weight 250. His analysis was based on the distribution of a radiotracer $^{32}\text{P}$ in an isolated unfiltered lake water sample. All the DOP components may not have been labeled and therefore could not be detected. He postulated that the large molecular weight fraction resulted from combining of the low molecular weight fraction. Herbes et al. (94) studied the DOP of lake water samples using enzyme hydrolysis. They found that virtually no phosphatase-hydrolyzable DOP was present. Using gel chromatography to concentrate DOP fractions, they found both a low molecular weight and a high molecular weight fraction that was phytase-hydrolyzable. The phytase hydrolyzable DOP had both a low molecular weight fraction, which was assumed to be inositol Poly-P, and the high molecular weight fraction, which was assumed to be inositol Poly-P in association with proteins or lipids. Another study (99) found nucleic acids and Poly-P in the DOP of lake samples. The above studies only identify approximately half the total DOP. Further some DOP is highly labile which hydrolyses to $\text{o-PO}_4$ and is not detected. Minear (156) found that in the gel chromatography column some of the DOP was hydrolyzed to $\text{o-PO}_4$. In the analysis of natural water, there is an error in the determination of the total DOP in solution. Total DOP is the difference between the total P in solution and the $\text{o-PO}_4$. The molybdenum blue method for the determination of $\text{o-PO}_4$ can hydrolyze the labile DOP (187).
The studies of the growth of pure cultures of organisms and their release and uptake of DOP give insight into the dynamics of the DOP pool. In natural systems the uptake and release of DOP is found in both algae and bacteria during growth and death release (156,187). In axenic (pure culture, no other organism present) algal cultures, the release and the uptake of DOP have been observed during the life cycle of the alga. Lean and Nalewajko (137) observed the release of DOP during the exponential growth phase of the algae, Anabaena flos-aquae, a blue-green alga, Chlorella pyrenoidosa and Scenedesmus quadricauda, green algae, and Navicula pelliculosa, a diatom. When P became limiting, a significant portion of the released DOP was taken up by the algae as 0-PO₄ in the medium was nearing depletion. These same observations were found in marine algae by Kuenzler (124). He also observed algal uptake of DOP excreted by another algal species. Sugar phosphates have been utilized by algae as the sole source of P (125). Kuenzler (124) determined enzymatically that some of the DOP was monophosphate esters that are hydrolyzed to 0-PO₄ by the enzyme alkaline phosphatase. Lean and Nalewajko (137) found a large molecular weight fraction of excreted DOP which persisted after release while lower molecular weight fractions tended to disappear. In addition to direct uptake into the cell of DOP, 0-PO₄ was liberated from DOP through the hydrolytic action of phosphatase enzymes that are released extracellularly from both algal and bacterial cells (59,64). Sorption and desorption of DOP with solid phases have been found. Bentonite and kaolin clays can interact with nucleic acids, nucleotides, sugar phosphates and proteins (5,84,197). Phosphatases
may be sorbed onto the clays and be deactivated or hydrolyze DOP on the clays (197). The activity of zooplankton can contribute to the DOP pool (178,187).

2.4-3 Dissolved Condensed Phosphate

Dissolved condensed phosphates enter the aquatic system through waste waters from man's activity. Condensed phosphates are used as builders in synthetic detergent products (36,45). Dissolved condensed phosphates are tetrapotassium pyrophosphate, sodium tripolyphosphate, and sodium hexametaphosphate. In natural waters, the hydrolysis of condensed phosphate to $\text{O-PO}_4^{3-}$ was primarily due to the microbial activity (36). In lab cultures, green algae can hydrolyze the condensed phosphate, but bacterial activity enhances the hydrolysis to a greater extent (36,45).

2.4-4 Colloidal Phosphorus

The particulate fraction of the aquatic ecosystem is composed of the biologically active fraction (organisms), the organic macromolecules of cell debris, clays and inorganics that have been formed by precipitation from a saturated water solution. The major entities of this latter group are associated with iron and calcium compounds which have low solubilities (75,214,244). The insoluble chemical form in which each is found is dependent on the oxygen potential and the pH of the water. Iron has two ionic states, ferrous (+2) and ferric (+3). If oxygen is depleted in the water, equilibrium is to the ferrous state. If oxygen is present ferric is the equilibrium state. The ferrous state may exist at low pH, but is converted to
the ferric state at a very low rate. As the pH increases, particularly above pH = 7.0, the ferrous state is rapidly converted to the ferric state (204). The pH affects the ionic form of phosphate \((\text{PO}_4^{3-}, \text{HPO}_4^{2-}, \text{H}_2\text{PO}_4^-)\) (204). The ionic state influences the coordination of the ions and thus the form of the insoluble phase.

In natural lake water systems, oxygen generally is present in the water except in the sediments and water near the sediments in eutrophic lakes. The primary insoluble forms that are found in oxygenated natural waters are ferric oxyhydroxides, calcium phosphates, calcium carbonate, and ferric phosphates (29,75,214,244). Both the ferric oxyhydroxides and the calcium carbonate adsorb phosphate which forms complexes or compounds with the iron and calcium. Phosphate adsorbed onto a ferric oxyhydroxide shows a coordination formula of \(\text{Fe}_x\text{OH}_{3(x-y)}\text{(PO}_4)_y\text{z(H}_2\text{O)}\) (209). Calcium phosphates found in natural waters are \(\text{CaHPO}_4\), \(\text{Ca}_3(\text{PO}_4)_2\), \(\text{Ca}(\text{H}_2\text{PO}_4)_2\), \(\text{Ca}_3\text{H}_2(\text{PO}_4)_6\), and hydroxyapatite, \(\text{Ca}_5(\text{PO}_4)_3\text{OH}\) which is the most stable form (27). The compound ferric phosphate, \(\text{FePO}_4\), is found in the insoluble state but its solubility increases with increasing pH (18,204). In natural waters above pH 7.0, an insignificant fraction of the colloidal P is \(\text{FePO}_4\) (18). In lake waters that lack oxygen, iron increases its solubility as it returns to the ferrous state from ferric form (204). This phenomenon releases o-\(\text{PO}_4\) associated with the iron to the soluble state. Clay particles in suspension in natural waters act as a phosphate buffer. Adsorption isotherms for o-\(\text{PO}_4\) have been determined for kaolin, montmorillinite, and illite clays (35,52,92).
2.4-5 Settling of Suspended Particulate Phosphorus

Phosphorus is lost from the colloidal microenvironment through settling of the suspended particulates to the sediments. The inorganic suspended P associated with precipitation of colloidal calcium and iron compounds will settle when the critical particle size is achieved through agglomeration or particle growth (77). Other inorganic P particulates are suspended in the aquatic system through both resuspension of the sediments by water turbulence and external water inputs to the system. Phosphorus associated with clays, the bottom sediment and precipitates will settle when water turbulence subsides (244).

The biological P particulates are cells, both algal and bacterial, and cell remnants of autolysis. Settling rates of viable cells depends on its motile ability, if they are present, the growth stage and other physical or chemical changes (224,244). Some species of algae show an increased settling rate in the stationary growth phase over the exponential phase (224). Since settling of algae takes them out of the light zone, many species have developed methods to decrease their density or change shape to reduce the settling rate (244). By the increase in the surface to volume ratio of the cell, the settling rate is less. The cell can reduce its density relative to water (approximately 1.01 to 1.03) by producing a gelatinous sheath on the external surface of the cell or by the production of excess fats in the cell (244). Finally some cells are able to accumulate gas in an internal cell structure. This structure, gas vacuole, is found in blue-green algae (21).
2.4-6 Sediment Contributions to Phosphorus Dynamics

The sediments are both a source and a sink for P. Phosphorus accumulates in the sediment through settling of both inorganic and organic particulates and through adsorption of $\text{o-PO}_4^-$ from the water (124, 244). Phosphorus is released to the water through desorption processes, dissolution of inorganic particulates, microbial activity, and resuspension of sediment by turbulent water (61, 84, 97).

Orthophosphate is adsorbed and desorbed from sediments under aerobic conditions. The predominant governing factor in the sediments are the metal oxides of iron and numerous other metals (61, 129). Bacteria can solubilize inorganic phosphates, such as FePO$_4$, Ca$_3$(PO$_4$)$_2$, CaHPO$_4$, in the sediments under aerobic conditions because of organic acids and chelating agents released during the metabolism of carbohydrates in the sediments (84). Sediments also release $\text{o-PO}_4^-$ under anaerobic conditions. Under anaerobic conditions, iron has an equilibrium oxidation state of +2, the ferric (+3) is converted to the ferrous (+2) state which is more soluble. The ferric oxide-phosphate complexes and ferric phosphates are solubilized (163).

2.5 Environmental Factors Affecting the System

The aquatic system that has been described is affected by the various environmental factors (light, temperature, pH, nutrients) some of which have been cited. This section summarizes the factors, their levels, and their resultant effects in natural water systems.

Major nutrients (nitrogen, carbon, phosphorus, sulfur, oxygen) vary widely in concentration in freshwaters. Table 2.4 compares typical values of the major nutrients found in natural freshwaters (244).
<table>
<thead>
<tr>
<th>Nutrient(Mg/l)</th>
<th>Typical Values (244)</th>
<th>Lake Erie(30) Epilim.</th>
<th>Hypolim (Anoxic)</th>
<th>Maumee River(236) Above Toledo</th>
<th>Below Toledo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{NO}_3^--\text{N})</td>
<td>0-10</td>
<td>.005-.043</td>
<td>.06 .27-7.2</td>
<td>.59-7.7</td>
<td>(.020-.090)</td>
</tr>
<tr>
<td>(\text{NO}_2^--\text{N})</td>
<td>0-0.01</td>
<td>.004-.011</td>
<td>.009-.024</td>
<td></td>
<td>(0-.004)</td>
</tr>
<tr>
<td>(\text{NH}_3\text{N})</td>
<td>0-5</td>
<td>.026-.035</td>
<td>.060-.139</td>
<td></td>
<td>(.210-.970)</td>
</tr>
<tr>
<td>Particulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Org. N</td>
<td></td>
<td>.050</td>
<td>.050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfur:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{SO}_4^{2-})</td>
<td>5-30</td>
<td>23.</td>
<td>23.</td>
<td>46-133</td>
<td>45-130</td>
</tr>
<tr>
<td>Inorganic Carbon:</td>
<td>pH dependent</td>
<td>73.</td>
<td>88.</td>
<td>74-183</td>
<td>69-190</td>
</tr>
<tr>
<td>Oxygen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{O}_2)</td>
<td></td>
<td>8.8</td>
<td>(0)-4.2</td>
<td>4.9-15</td>
<td>2.8-14</td>
</tr>
<tr>
<td>Phosphorus:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total P (dissolved + particulate)</td>
<td>.010-.050</td>
<td>.012</td>
<td>.012-.027</td>
<td>(.095)</td>
<td></td>
</tr>
<tr>
<td>(\text{PO}_4^{3-}\text{P})</td>
<td></td>
<td>.001-.003</td>
<td>.002-.015</td>
<td>.153-.457</td>
<td>.186-1.37</td>
</tr>
<tr>
<td>Org. P</td>
<td></td>
<td>.002-.004</td>
<td>.004-.006</td>
<td></td>
<td>(.029)</td>
</tr>
<tr>
<td>Particulate P</td>
<td>.007-</td>
<td>.007-.015</td>
<td></td>
<td></td>
<td>(.026)</td>
</tr>
<tr>
<td>Iron:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{Fe})</td>
<td>.05-.20</td>
<td>.008-.027</td>
<td>.023-.200</td>
<td></td>
<td>(.35)</td>
</tr>
<tr>
<td>pH:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature(°C)</td>
<td>20-23</td>
<td>10-12</td>
<td>0-22</td>
<td>0-27</td>
<td></td>
</tr>
</tbody>
</table>
to values determined by the Project Hypo survey of Lake Erie in 1970 (30) and values determined by U.S. Geological Survey (236) in the Maumee River which empties into Lake Erie. The Maumee River values were taken above and below Toledo, Ohio in 1968-1969. Table 2.4 also includes iron concentrations, the pH, and temperature. The Lake Erie values are divided into concentrations found in the epilimnion (warm water near the surface) and the hypolimnion (cold water found near the bottom). The dividing line of these two regions is the thermocline which is the region of highest vertical temperature change. Table 2.5 relates P nutrient concentration to the general biological productivity of a lake.

Table 2.5 General Relationship of Lake Productivity to Average Concentrations of Epilimnetic Phosphorus (244)

<table>
<thead>
<tr>
<th>General Level of Lake Productivity</th>
<th>Total Phosphorus (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-oligotrophic</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Oligo-mesotrophic</td>
<td>Increasing Productivity</td>
</tr>
<tr>
<td>Meso-eutrophic</td>
<td>.005-.010</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>.010-.030</td>
</tr>
<tr>
<td>Hypereutrophic</td>
<td>0.30-.100</td>
</tr>
<tr>
<td></td>
<td>&gt;.100</td>
</tr>
</tbody>
</table>

Light is a factor affecting the aquatic ecosystem. The light intensity, quality, and the diurnal cycle have shown effects on the autotrophic organisms. The chlorophylls or other pigmented structures that absorb light energy, function over a range of light intensities up to a maximum above which no further increase in
photosynthesis rate is found. Too high of a light intensity will inhibit photosynthesis. In the natural water systems organisms near the surface show reduced photosynthesis because of the intense sunlight (237). Light intensity decreases with depth thus reducing photosynthesis rate when the intensity falls below maximum. In general chlorophyll content of an algal cell increases as the light intensity decreases to a maximum as a means to maintain the energy input (206). The level of light intensity affects the excretion of organic compounds from algal cells. Extracellular release of photosynthetic carbon products varies with the light intensity. The release of extracellular carbon is also high at very low light intensities (237). The quality of light or spectral quality also affects the photosynthesis since chlorophylls within organisms and between organisms absorb light at different wave lengths (24). The quality will affect the excretion of the photosynthetic organisms (206). The diurnal variation of the light initiates reproduction in some species of algae (87). The light-dark cycle affects the metabolism of algal cells which must switch energy sources from photosynthesis to internal or external organic carbon compounds (216).

Temperature in aquatic ecosystems range in the majority of cases between 0-30°C. In Project Hypo's study of Lake Erie, temperature during the summer ranged from 10-23°C (30). In year round monitoring of the Maumee River, temperature ranged from 0-27°C (237). The effect of temperature on the growth rate of organisms is viewed from the effect of temperature on chemical reactions and the temperature effects on the enzyme catalysts in the metabolic chemical reactions.
As temperature increases the rate of reaction and growth rate increases up to a maximum or optimal temperature. Any further temperature rise increases the denaturing or deactivation of the enzyme catalyst and the chemical reaction rate decreases, thus the growth rate declines (208).

The hydrogen ion concentration, pH, affects the disassociation rates and the ionic states of both polar inorganic and organic compounds, such as the organic acids (206). The change in the state of these compounds can affect their availability as nutrients for organisms:

(i) Principally, pH affects the ionic state of inorganic carbon, i.e., free CO2, HCO\textsuperscript{3}, CO\textsubscript{3}\textsuperscript{2-}, and that of phosphate, i.e., PO\textsubscript{4}\textsuperscript{3-}, HPO\textsubscript{4}\textsuperscript{2-}, H\textsubscript{2}PO\textsubscript{4}.

(ii) The hydrogen ion concentration affects the solubility of iron and reduces its ability to be taken up by the cell (164).

(iii) The electrical charge on cell surfaces are altered by the pH.

(iv) The change in organism cell wall electrical charge affects membrane potentials and alters the membrane surface enzymes that are utilized for enzyme activated nutrient uptake or which convert nutrients external to the cell (206).

(v) Particulates ability to absorb compounds are affected.
2.6 Measurement of Phosphorus Compartments in Environment-Terminology and Limits

The separation of the phosphorus forms referred to in this chapter is very difficult and requires sophisticated analytical techniques. These techniques are not very useful for making surveys of large bodies of water in order to obtain significant amounts of data for mathematical modeling or water quality assessment of natural systems.

A review of terminology for the phosphorus fractions that are measured in the natural environment and in some laboratory cultures is required. The following definitions are taken from the literature (30,187):

**Soluble reactive phosphorus (SRP)** refers to the value obtained when membrane-filtered water (usually 0.45 µm pore size) is analyzed by one of the variants of the molybdenum blue phosphate technique. This measures the phosphorus that is primarily the \( \text{O}_\text{P}_\text{O}_4 \) form together with some of the easily hydrolyzable forms of Org-P. This term does not imply that the \( \text{O}_\text{P}_\text{O}_4 \) measured was in solution before the addition of reagents. Hydrolyzable P may be associated with inorganic particulates that pass through the 0.45 µm filter. SRP has been referred to as P that is biologically active or easily utilized by organisms for metabolic activity.

**Soluble phosphorus (SP)** refers to the value obtained when membrane-filtered water (0.45 µm) is analyzed after being digested with an oxidizing acid solution. This P would include the biologically active Poly-P which is detected under SP analysis technique (207).
Soluble unreactive phosphorus (SUP) is the difference between SP and SRP. This is also referred to as dissolved organic phosphorus (DOP).

Total phosphorus (TOT-P) is obtained by analyzing whole lake water (no filtration) after acid digestion. It is assumed that the values by this technique are indicative of the true P content of the sample.

Particulate phosphorus (PP) represents the quantity of P in the water that does not pass a 0.45 μm filter. This is the difference between TP and SP. PP is associated with cellular P of both viable and nonviable algae and bacteria as well as the inorganically bound P of the clays and colloidal iron and calcium compounds. A part of the PP associated with the inorganic particulates is considered biologically active.

2.7 Concluding Comments

This chapter was assembled to emphasize the complexity of the aquatic ecosystem. It is a vast, interactive, dynamic system which yields its secrets grudgingly to people who invest vast amounts of time and money for investigations. This chapter emphasized the cycling of the element of phosphorus in the ecosystem and noted many of the primary variables that affect the P and the system dynamics. The comprehensive dynamics of the aquatic ecosystem would include the changes in time of nutrient concentrations and the chemical form; species of organisms, their number, chemical composition, and morphology; the physical parameters such as temperature, pH, light
intensity, weather; and the chemical natural and concentration of nonviable particulates.
CHAPTER III

RELATED LITERATURE

3.1 General

This chapter discusses P-limited growth and the o-PO$_4$ uptake rate of microorganisms that occupy the biological compartments, and the adsorption and complexing dynamics of o-PO$_4$ with inorganic particulates which occupy the biological and colloidal compartments as described in Chapter II. Kinetic expressions of cellular growth are given. A discussion of the adequacy of these biological kinetic expressions in quantitative dynamic models of P in aquatic ecosystems is presented. In light of the observations made, kinetic expressions for cell growth and nutrient uptake based on the internal compartment of the limiting elements are presented. Compartmentalized cellular P compounds are described in terms of functions, concentrations and then dynamics in relation to cellular growth, nutrient uptake and response to environmental parameters, particularly under P limitation. This chapter examines the stability under environmental conditions of the inorganic particulates of iron and calcium and the associated P. Mechanisms of formation and incorporation of o-PO$_4$ into insoluble structures are introduced. Finally, a discussion of microbial availability of Fe and P from the inorganic particulates is presented.
3.2 Cell Growth Dynamics

Biological growth can be defined as an orderly increase of all the chemical constituents of an organism (208). Growth normally results in cellular multiplication. A measure of growth in unicellular organisms is the cell number, cell dry mass or cell volume concentrations (226). A generalized growth curve is shown for a microbial population in a closed system, e.g., a batch of dynamic growth culture. The growth of microbial populations is restricted by the exhaustion of required nutrients from the medium or the buildup of toxic metabolic products in the medium. These changes are brought about by the organisms themselves, thus the cells are self-regulating. After growth has stopped, the cell population decreases as a result of death to individual cells (208).

The generalized growth curve in Figure 3.1 shows four distinct phases of growth (162). During the lag phase, the growth rate is zero, while the cell adapts to the new environment by adjusting its metabolic processes. In the exponential growth phase (or logarithmic phase), the cell growth rate in terms of cell division reaches a constant maximum exponential rate and the increase in cell number (96) follows:

\[
\frac{1}{N} \frac{dN}{dt} = \frac{d(\ln N)}{dt} = \mu_N
\]  

or cell growth rate in terms of dry weight increase may be expressed as

\[
\frac{1}{X} \frac{dX}{dt} = \frac{d(\ln X)}{dt} = \mu_X
\]  

or cell growth rate in terms of cell volume increase is
Figure 3.1 Typical Cell Growth Curve in Batch Culture (162)
\[
\frac{1}{V} \frac{dv}{dt} = \frac{d(\ln \nu)}{dt} = \mu_v
\] (3.3)

where:

X-cell dry weight concentration, \((\frac{mg}{\text{liter}})\),

N-cell number concentration, \((\frac{\#}{\text{liter}})\),

V-cell volume concentration, \((\frac{\mu m^3}{\text{liter}})\),

t-time,

\(\mu_X, \mu_N, \mu_V\)-specific growth rate in terms of dry weight, cell number, and cell volume, \((\text{time}^{-1})\).

The specific growth rates for cell number, dry weight and cell volume concentrations must be identical in balanced growth. During the stationary phase, the growth rate falls to zero when a required nutrient is exhausted from the media or a metabolic product becomes toxic. In the death phase, the growth rate is negative \((l)\).

The environmental conditions are not constant in a dynamic system. The composition of an organism changes at different phases of the growth cycle. Figure 3.2 is an idealized representation of some of the cell changes that are found when stationary phase cells are inoculated into fresh nutrients in a batch system \((l, 96)\). After inoculation and during the lag phase, the individual cell weight and cell dry weight concentration immediately increase exponentially, and there is a rise in the RNA content of the cell for enzyme (protein) synthesis. The individual cell weight and the RNA content increase to a certain level, indicated as \((A)\) in time, then the cell number concentration rises exponentially. During the lag phase the DNA content...
Figure 3.2 Typical Cell Weight, DNA and RNA Concentration in Batch Culture (96)
of the cell decreases to a lower level. In the exponential growth phase, the cell dry weight and cell number concentrations continue to grow exponentially while the individual cell weight and the cell content of RNA and DNA remain constant. At time (B) when a nutrient is exhausted, the individual cell weight and the RNA content of the cell begin to fall. After time (B), the rate of dry weight concentration increase declines since the RNA content decreases. The cell number concentration continues at the same exponential rate to time (C), then the rate falls while the DNA content of the cell begins to rise. At time (D) the cell division ceases and the stationary phase begins. After time (D), the RNA content of the cell and the individual cell weight fall, while the DNA content of the cell has risen to the initial level.

Constant conditions of growth can be maintained indefinitely in a continuous system that is at steady state (chemostat or CSTR) in contrast to the dynamic system discussed above. If the dilution rate (ratio of flow rate to the culture vessel medium volume) is held constant in the continuous system, the organisms continue to grow until the concentration of some essential nutrient is reduced to a level that makes the exponential growth rate equal to the dilution rate. Although the medium of constant concentration is fed to the chemostat, the environment in which the cells are growing and the cell composition are different for each growth rate with respect to the concentration of the limiting nutrient (96). Figure 3.3 presents a generalized example of the cell composition change at different exponential growth rates. The average age of the cells in the chemostat decrease
with increased dilution rate or exponential rate. In a batch system, the validity of constant composition of the cells and constant concentration of the limiting nutrient in the medium can be accomplished if the inoculum cell concentration is small such that several cell divisions can occur before the cell uptake of the limited nutrient has a significant affect on the medium concentration (175).

The phenomena of the cell growth, as expressed in the previous paragraphs, is the expression of a complex autocatalytic system of chemical reactions in which the rate of formation of every component of the system is directly or indirectly dependent on every other component (175). But the exponential growth rate or specific growth rate, \( \mu \), can be expressed as a function of the medium concentration growth limiting substrate or nutrient, \( S \), and an inhibitor.
concentration, \( I_N \), e.g., a toxic metabolic product \((1)\):

\[
\mu_X = \frac{1}{X} \frac{dx}{dt} = f(S, I_N)
\]  

\((3.4)\)

The most commonly used form for the specific growth rate for increase in dry weight proposed by Monod \((162)\) is

\[
\mu_X = \frac{\mu_{X,\text{max}} S}{K_{Sx} + S}
\]  

\((3.5)\)

where: \( \mu_{X,\text{max}} \) - a constant that is the maximum growth rate limit for increasing concentrations of \( S \),

\( K_{Sx} \) - a constant which is equal to the concentration of \( S \) at one-half \( \mu_{X,\text{max}} \).

Equations for \( \mu_Y \) and \( \mu_N \) are of the same form with the constants defined similarly but with the subscripts \( V \) and \( N \) for \( X \). For photosynthetic organisms, growth can be limited by the light energy concentration (light intensity). Equation 3.5 can be modified by substituting the following equation,

\[
S = f(I)
\]  

\((3.6)\)

where: \( I \) - light intensity.

Figure 3.4 is a graphical representation of Equation 3.5 including the determination of the constants, \( \mu_{\text{max}} \) and \( K_S \). It is typically called a saturation curve. The maximum exponential growth of a cell occurs when all nutrients approach saturation values. Equation 3.5 is of the form of the simple model for enzyme-substrate interaction as proposed by Michaelis-Menten \((145)\). Many researchers have applied this equation to the 'master reaction' mechanism for cell growth \((175)\).
The 'master reaction' assumes that the overall rate of a system of linked enzymatic reactions from substrate uptake to the formation of new cell material is controlled by a single slow enzymatic reaction (162). Monod proposed Equation 3.5 as a good empirical fit to data shown in Figure 3.5 (162). In this view the specific growth rate is a function of the medium concentration of the substrate. Dabes et al. (40) extended 'master reaction' by assuming that two enzyme reactions in the reaction sequence were slow. These models have been widely utilized for laboratory and natural organism population dynamics. Other models that predict the specific growth rate with substrate medium concentration are found in Tsuchiya et al. (226). Models (183, 245) have been proposed for the growth cycle in the dynamic system through all four phases. The use of the medium substrate concentration as an independent variable, for predicting specific growth
rate, does not fit all types of data (49,66). In dynamic systems, the utilization of the limiting nutrient from the medium by an organism does not generally correlate with the growth rate of the cell. For steady-state systems, growth of the organism continues with no detectable concentration of the limiting nutrient in the medium. This phenomenon is not confined to laboratory cultures and can be found in natural aquatic systems (174). Monod (162) found that Equation 3.5 did not fit the data for all carbonaceous energy substrates for bacteria systems. Several examples of growth-limiting inorganic nutrients for algae are found in the literature for nitrate (32,223) and phosphate (137,184) and growth factors such as vitamin B$_{12}$ (49) that do not fit Equation 3.5.

New approaches were taken because of the lack of fit of Equation 3.5 to growth data. The following observations have been made. Droop (49) found that the internal or cellular concentration of B$_{12}$ used in a rate model of the form of Equation 3.5 correlated with the cell growth rate. Ferret (175) had suggested the use of an effective limited-nutrient concentration based on the assumption that the medium nutrient concentration exerted an effective concentration within the cell. Herbert (96) proposed the use of the concentration of RNA within the cell to correlate with the cell specific growth rate (see Figure 3.2) since RNA concentration is taken as the cell's ability to synthesize the enzymes which in turn are involved in the cell's ability to grow. There are other distinct compositional changes in cells that depend on the environment and the rate at which they grow (1). Because of these observations, many researchers (1,6,183) have
developed models for the specific growth rate that are "structured," i.e., using various cell components to represent different cell capabilities of growth as opposed to the "unstructured" Monod specific growth rate equation.

Mathematical descriptions based on structure have been proposed. Aiba et al. (1) gives the dry weight specific growth rate based on the cell concentration of RNA:

$$\mu_X = \frac{1}{X} \frac{dX}{dt} = k_2 \left( \ln \frac{N_r}{N_{r0}} \right)$$ (3.7)

where: 
- $k_2$-empirical constant,
- $N_r$-concentration of RNA in cell,
- $N_{r0}$-minimum concentration of RNA in cell.

If the Perret model (175) is expressed mathematically, it is of the Monod form with a different definition for the substrate concentration:

$$\mu_X = \frac{1}{X} \frac{dX}{dt} = \frac{\mu_{X,\text{max}} S_e}{K_s + S_e}$$ (3.8)

where: 
- $S_e$-effective concentration of the substrate in the cell influenced by the external substrate concentration.

The same expression in Equation 3.8 is used when an internal concentration of the limiting nutrient can be determined analytically in a free or combined state. The following equations 3.9 to 3.12, are in the Perret form. Rhee (184) found that, with o-PO$_4$ as the limiting nutrient in the alga Chlorella sp., the cellular P concentration correlated with the cell volume specific growth rate;
\[ \mu_v = \frac{1}{v} \frac{dV}{dt} = \frac{\mu_{v,\text{max}} (q-q_o)}{k_q + (q-q_o)} \]  

(3.9)

where:  
- \( q \): cellular concentration of \( P \),
- \( q_o \): minimum cellular concentration of \( P \) under o-PO\(_4\)

This equation is the same form as Equation 3.8. Cell number specific growth rate, \( \mu_N \), has been related to the Poly-P concentration in Saccharomyces cerevisiae yeast cells, and Corynebacterium diphtheriae bacterial cells (116, 193). In considering the data of Rhee (185) on Scenedesmus sp., the following equation can be suggested:

\[ \mu_N = \frac{1}{N} \frac{dN}{dt} = \frac{\mu_{N,\text{max}} p}{k_p + p} \]  

(3.10)

Where:  
- \( p \): cellular concentration of polyphosphate.

For nitrate-limited growth, Caperon and Meyer (108) found that the cell number specific growth rate correlated with the cellular nitrogen to carbon ratio, N/C, in the following manner:

\[ \mu_N = \frac{1}{N} \frac{dN}{dt} = \frac{\mu_{N,\text{max}} (nc-nc_o)}{k_m + (nc-nc_o)} \]  

(3.11)

Where:  
- \( nc \): cellular N/C,
- \( nc_o \): minimum cellular N/C under nitrate starvation.

Droop (49) found that the amount of vitamin B\(_{12}\) taken up by the alga, Monochrysis lutheri, above the minimum cellular requirement was related to the cell number specific growth rate:

\[ \mu_N = \frac{1}{N} \frac{dN}{dt} = \frac{\mu_{N,\text{max}} (b-b_o)}{b} \]  

(3.12)
where \( b \)-cellular concentration of vitamin \( B_{12} \),

\( b_0 \)-minimum cellular concentration of vitamin \( B_{12} \).

Equation 3.9 can be reduced to Equation 3.12 if the following relationship of the constants if found to be true, \( K_q = q_o \). Fuhs (68) utilized an equation proposed by Misscherlich which was not based on the 'master reaction' concept. Fuhs correlated the cellular phosphorus concentration with the cell number specific growth rate;

\[
\mu_N = \frac{1}{N} \frac{dN}{dt} = \mu_{N,\text{max}} \left( 1 - 2 \frac{(q-q_o)}{q_o} \right)
\]  

(3.13)

General cell composition properties of the limiting nutrient have been used in Equation 3.9, 3.11, 3.13 to correlate cell growth, i.e., total cell P for \( \alpha\)-PO\(_4\) limiting growth, and the cell N/C ratio for nitrate limited growth. Specific cell chemical species, RNA and \( B_{12} \), or chemical pools, polyphosphate, have been used in Equations 3.7, 3.10, 3.12 to relate to the cell growth rate. One cannot infer from the general cell composition of the limiting element, the specific chemical species or pool chemical that a specific metabolic reaction is controlling the growth. But one can infer that these chemical properties do reflect the trends of the cell growth rate. The cell composition properties of Total P, cell N/C ratio and Poly-P were used because they have relative ease of determination in both laboratory cultures and in field analysis of natural aquatic organisms.

3.3 Phosphorus Models for Aquatic Ecosystems

A quantitative dynamic model of an aquatic ecosystem can become a tool in the prediction of the ecological state of a natural water
environment in response to the stresses of various nutrient loadings and environmental parameters. The development of a mathematical model depends on the qualitative knowledge of the components that make up that system. Chapter II described the aquatic ecosystem qualitatively through compartmentalization of the entities that exist in the system. The mathematical models reviewed in this section were developed by researchers to provide insight into the role and the economy of P. Emphasis of the discussion is on the biological growth rate equations of the models with respect to the information provided in Section 3.2.

The cycling of P between aquatic organisms and the water in which they are suspended maximizes the biological utilization of the element. Three P cycles in natural aquatic systems can be defined (217). One is the annual cycle in which soluble P is incorporated into the biological fraction containing algae and bacteria or adsorbed onto inorganic particulates and sink into deeper waters. In the deeper waters, autolysis on cell death and bacterial activity remineralize the organically bound P to $\text{O}_4^-$PO$_4$. Chemical processes liberate the inorganically bound P as $\text{O}_4^-$PO$_4$. The $\text{O}_4^-$PO$_4$ is returned to the upper water layers seasonally by vertical mixing. The second cycle is the interaction between Level I and Level II organisms, i.e., zooplankton consuming algae and other microorganisms and returning $\text{O}_4^-$PO$_4$ to the water through actions of their intestinal bacteria. This cycle takes place in the epilimnic waters (upper water in lake). The third cycle occurs between the P in the algae, bacteria, suspended particulate inorganic P, and the dissolved P in the epilimnion of a natural water
system. The transients that occur in the last cycle are short term relative to the other cycles. The phenomenon would include the population explosion and crashes of algal blooms and the pseudosteady state of this cycle that takes place otherwise (56,171).

Several mathematical models of P cycling in natural ecosystems are found in the literature. Prober et al. (180) presented a model for the prediction of the dynamics of algal blooms in the time scale of several hours to days. The rate of biomass increase by the photosynthesis under o-PO₄ limitation was found to be the following form:

\[
\frac{1}{X} \frac{dx}{dt} = P(I) F(S')
\]  \hspace{1cm} (3.14)

where: P(I)—the light intensity saturation curve that accounts for the light variation due to water depth and the light variation during the day cycle,

F(S')—the nutrient saturation curve for the limiting nutrient in the ecosystem.

The functions cited above can be defined in the following way:

\[
P(I) = \frac{\mu_{P,\text{max}} I}{K_P + I}
\]  \hspace{1cm} (3.15)

\[
F(S') = \frac{\mu_{n,\text{max}} S'}{K_n + S'}
\]  \hspace{1cm} (3.16)

where: I—light intensity,

S'—sum of the P in solution as o-PO₄ plus the P concentration in the algal cell that is stored as surplus,

Kₚ,Kₙ,μₚ,ₚ,μₙ,ₚ—constants defined as before for the Monod equation.
The P concentrations required for Equation 3.16 could be SRP and PP which are typically obtained on analysis of natural water systems. The PP though is not a direct measure of the stored or surplus P but the total P in the cells.

O'Brien (171) proposed a population growth model for the population explosions and crashes that occurs during algal blooms. The population growth equation was governed by the uptake of the limiting nutrient. Equation 3.5 which is in the Monod form in terms of the specific cell number growth rate would apply. For $\alpha$-PO$_4$ limiting growth, the proposed equation has been shown inadequate as discussed in Section 3.2 because the external $\alpha$-PO$_4$ concentration does not always correlate to the specific growth rate.

Imboden (104) developed a lake model for P cycling that can be applied on a yearly basis. The parameters of the model can be adjusted for the season changes in the lake from summer stratification of the lake water to winter circulation after the overturn of the lake waters. The biochemical reactions are the rate of mineralization of Org-P, through the respiration of bacteria and the algae, and the photosynthesis rate. Imboden simplified these reactions through linearization. The rate of the biological formation of viable PP, basically the formation of biomass, is the difference between these two rates in the following balance:

$$\frac{d(PP)}{dt} = \alpha(SRP) - R(PP) \quad (3.17)$$

where: $R$-respiration rate of algae and bacteria measured in situ,
\( \alpha \)-photosynthesis rate of the algae measured \emph{in situ},

PP-particulate phosphorus concentration,

SRP-soluble reactive phosphorus concentration

Lam and Jaquet (133) developed a lake model for P dynamics that was applied to unstratified lake water. Their formulation of the biochemical reactions is similar to that of Imboden:

\[
\frac{d(\text{PP})}{dt} = \alpha(\text{TP} - \text{PP}) - R(\text{PP}) \tag{3.18}
\]

where: TP-total phosphorus.

The major difference between Equation 3.17 and 3.18 is in the driving force for the photosynthesis where the total phosphorus in solution is the dependent variable for Equation 3.18 as compared to the soluble reactive phosphorus (SRP) in Equation 3.17. The driving force for respiration is particulate phosphorus. Further, the Lam and Jaquet model determined the \( \alpha \) and \( R \) as optimized values by computer fit of all input parameters. The form of the equations used for the biochemical reactions can be compared to Equation 3.14 in which the nutrient variable \( F(S') \) or the Monod growth rate is linearized to

\[
F(S') = (B) (S') \tag{3.19}
\]

when the substrate concentration, \( S' \), is much smaller than \( K_n \), thus

\[
B = \frac{w_{S,\text{max}}}{K_S} \tag{3.20}
\]

In natural water ecosystems the limiting nutrient in many cases shows such behavior as previously mentioned.
The growth model of Tett et al. (222) predicts cell growth rate through the photosynthesis rate as a function of the algal cell chlorophyll to cell carbon ratio, Chl/C, and light intensity. The Chl/C ratio is affected by cell nutrient content. The model was applied to natural aquatic systems and reasonably described growth for 3-4 weeks.

Conceptual models have been developed concerning the pseudo-steady state of the short term P cycle in the epilimnion. Taft et al. (217) developed an expression for the exchange of P between various compartments of the colloidal microenvironment in an estuary ecosystem and is shown in Figure 3.5. The absolute values of each compartment were spectrophotometrically measured using standard methods detailed in Strickland and Parsons (213). The exchange rates, $R_i$, between each compartment were evaluated with the use of radioactive tracer, $^{32}\text{PO}_4^-$. These rates are the fluxes of the biologically active P compounds (SRP, Poly-P, and DOP) in the water that are absorbed or released by the microorganisms in the PP compartment. Taft et al. identified two soluble unreactive phosphorus (SUP) compartments, Poly-P and DOP, that were biologically important which in preceding models of P data were identified as a single compartment, TP - PP. Lean (136) expanded this approach for the short term P dynamics in the epilimnic waters of a lake. The compartments were identified and exchange rates, $R_i$, were evaluated through the use of the radioactive tracer $^{32}\text{PO}_4^-$. Analytically the SP compartments were separated through the use of gel chromatography. The model P dynamics in the colloidal microenvironment that Lean proposed is shown in Figure 3.6. The XP compartment was identified as a fraction that had a molecular weight of approximately 250 and
Figure 3.5 Taft Model of Short Term P Cycle in Epilimnic Waters in an Estuary Ecosystem (217)

Figure 3.6 Lean Model of Short Term P Cycle in Epilimic Waters in A Lake Ecosystem (136)
and was extremely labile. The colloidal phosphorus, which passed through a 0.45 μm membrane filter and was not totally filtered out with a 0.1 μm membrane filter, had a molecular weight of greater than 5,000,000. XP is excreted by the PP compartment through organism activity and can be subsequently both hydrolyzed to PO₄ and complexed to form colloidal P. The colloidal P may become biologically unavailable or hydrolyze to o-PO₄. The percentages in Figure 3.6 refer to the distribution of ^32P after addition as ^32PO₄ to unfiltered lake water.

Extensive data from intensive nutrient surveys of natural water ecosystems are required to formulate and validate models of the nutrient cycles which have been cited above in this section. Lake Erie has been the subject of two intensive studies. The first is Project Hypo conducted by the Canadian Center for Inland Waters in 1970 (30). The P parameters that were measured over the total water column were SP, SRP, PP, and TP. In addition, numerous other nutrients and physical parameters were measured. The nutrient data were determined during seven cruises in the months of July and August covering 27 monitoring stations that were distributed over the central Lake Erie basin. In 1973, the Center for Lake Erie Area Research (CLEAR) at Ohio State University initiated comprehensive nutrient and physical parameter measurements in the western and central basin of Lake Erie. Initially the only P parameters that were obtained were TP and SRP (229). During the years 1974 to 1976, the measured P parameters were TP, SRP, PP, and TP (229). The parameters were determined at 50 stations distributed over the two basins. Numerous cruises per year (approximately seven)
were made during ice-free lake conditions (April to December) covering all the stations and measuring the parameters throughout the water column. Taft et al. (217) conducted an extensive survey during five cruises in the Chesapeake Bay and measured SRP, Poly-P, DOP, and PP at nine stations along the major axis of the bay in the epilimnic water from December 1972 to August 1973.

There is no distinction between P atoms within each compartments used in the models presented in this section. For example, in the soluble compartments, P atoms in organic molecules, such as DNA, are not available for rapid exchange with SRP, Poly-P, or other DOP. Thus rate constants based on the measured DOP concentration will be underestimated (217). Lean (136), Taft et al. (217) and Koenings and Hooper (119) have made improvement in determining the dissolved P, both SRP and SUP. Taft et al. divided the SUP into Poly-P and DOP. Lean found colloidal P, and two SRP components. Koenings and Hooper approached the analysis of the soluble P, defined as the P that passes through a 0.45 μm membrane filter, with an additional filtration step. They further separated the P and Fe by use of a 4.5 nm dialysis membrane. That which passed through the membrane was termed soluble P and Fe and that which did not colloidal P and Fe. The molecular weight cutoff was 12,000 based on protein. The application of these three techniques for comprehensive P analysis of natural water systems seems limited in the case of Lean (136) because of the great amount of time required to apply the technique, but in the case of Taft et al. (217) and Koenings and Hooper (119) there seems to be a possibility of application.
The PP compartment shows the same lack of distinction between P atoms. The only analytical result discussed in this section and generally applied in field analysis is PP. This compartment includes the P associated with the viable organisms of the algae and the bacteria. The PP is associated with cell structure debris from cell lysis after death. But the largest fraction of the nonviable PP is associated with the inorganic particulates (213). These particulates are calcium phosphate and iron phosphate precipitates, and P compounds that are adsorbed onto clays and iron hydroxide floc as previously discussed in Chapter II. To use the PP value from a natural aquatic system as the measure of the cellular concentration of P in the growth rate equations, as suggested in Equations 3.9 or 3.13, is an overestimate of the growth rate driving force under growth rate limitations.

Within the PP fraction P, associated with the iron and calcium phosphate precipitates and absorbed onto iron hydroxide and clay as phosphate or organic phosphate, will go into solution if unfiltered water is analyzed for SRP. Further under these conditions some intracellular phosphorus can be extracted and be detected as SRP (213). Fuhs (68) determined the particulate reactive P by drawing cold 5% perchloric acid slowly through the PP on a 0.5 μm membrane filter. The total amount of P in the acid extract is the particulate reactive P. The inorganic particulate phosphates are soluble in acid and contribute to SRP. Fitzgerald and Nelson (64) extracted P from the PP fraction by boiling the filtered solids for one hour in distilled water that contained the nutrient salts of a typical growth medium (Gorham's) minus the 0-PO₄ with the pH adjusted to 7.0. The amount of SRP in the
water extract was defined as surplus P in cells which Rhee (184) found to correspond to the Poly-P content of a cell in lab cultures. Some of the adsorbed $\text{O}_2\text{PO}_4$ on the inorganic particulates contribute to the extracted SRP. Both of the above techniques were applied to PP in natural aquatic systems. There was a significant drop in the particulate SRP and surplus P extracted when P was limiting the growth.

### 3.4 Multicompartment Models for Biological Growth

The use of radioactive tracers to study the metabolic transformations and flow of an element or compound in a biological system led to the development of compartmental analysis. Previous discussions have utilized compartmentalization to clarify the flow of P in the complex natural aquatic ecosystem. Illustrations of these systems of compartments are shown in Figure 2.4 in Chapter II and Figures 3.5 and 3.6 in this chapter. There is a need for compartmental analysis within the cells for modeling of cell growth as shown in the preceding two sections. It was demonstrated that the specific growth rate in many cases correlates with an internal cellular component or an internal concentration of the limiting element that is incorporated into a cellular biochemical. To investigate the dynamics of the internal P with respect to the specific growth rate of a cell, the internal compartmentalization of P compounds is required. In Section 2.4-1 the P compounds were divided into the following categories: precursor pool—inorganic phosphate; intermediate pool—sugar phosphates, nucleic acids, phosphatidic acids; functional pool—coenzymes and dinucleotides; storage pool—polyphosphates; and biopolymer and
structural pools—phospholipids, DNA, RNA, phosphoproteins, and polysaccharide phosphorus.

Atkins (6) reviews numerous multicompartment models in biological systems for animals, organs tissues and single cells. He defines a compartment as a quantity of a substance which has uniform and distinguishable kinetics of transformation or transport. By this definition, the listed subunits of each pool in the preceding paragraph would constitute a compartment. This approach is in contrast to one compartment model of Monod. The determination of the concentrations and the dynamics of a system of compartments is achieved by direct chemical analysis of a separated compartment or is combined with the use of a radioactive tracer whose activity in each separated compartment is determined. The assumption that makes the combination work to determine the concentration of a compartment is that each compartment is assumed perfectly mixed with respect to the radioactive tracer. Otherwise the technique falls apart and the tracer only gives an indication of the most recent addition of that tracer to the compartment.

Multicomponent analysis is used to develop models for cell growth rate with different limiting nutrients. Anderson and Washington (4) developed a growth rate equation for the bacterium, Pseudomonas fluorescens, with the carbon energy source as the limiting substrate. They developed a multicompartment model for carbon in a batch system that accounted for all carbon compartments inside and outside the cell. Figure 3.7 represents the five-compartment model that was used to analyze the data. The accumulation of carbon in the cell carbon compartment was the rate of cell growth based on biomass.
Williams (245) presented a cell population growth model in which the cell comprised two compartments, a synthetic compartment and a structural/genetic compartment. The synthetic compartment represents the small metabolites in the cell and the structural/genetic compartment represents the cellular macromolecules which were synthesized from the small metabolites. Williams model which includes the external nutrient compartment is in Figure 3.8. Cell division is assumed to occur when the structural/genetic compartment doubles in size over a cell minimum. The model further simulates the cell size by assuming that the cell size is equal to the total of both the cell compartments. The model has two transfer rates: \( R_1 \) is the external nutrient uptake rate, and \( R_2 \) is the rate of synthesis of cell material. The transfer rates, as assumed by Williams (245), are bimolecular; i.e., the rate of transfer between the two cell compartments is proportional to the concentration in each compartment. The uptake rate of nutrient is proportional to the total of both cell compartments and the external compartment. The model exhibited the various universal features of cell culture for both batch and continuous systems. Williams felt this model could deal with the experiments in which nitrogen or phosphorus-starved algal cells demonstrate a rapid uptake of the deficient nutrient from the medium and use it for cell division later. An improvement in the model is to use saturation kinetics of Michaelis-Menten for the transfer rates between compartments.

Grenney et al. (80) extended Williams model approach by the development of a three compartment cell for the nutrient dynamics of algae in a nitrate-limited environment. The model also includes
Figure 3.7 5-Compartment Carbon Model of Bacteria Growth in Batch Culture (4)
Figure 3.8 William's Limiting Nutrient Cell Population Growth Model (245)

Figure 3.9 Grenney's 3-Compartment Nitrogen Dynamic Growth Model (80)
microbial activity in addition to the algae. The Grenney et al. (80) multi-compartment is shown in Figure 3.9. The biomass rate of growth of the algae is proportional to the concentration increase in the protein compartment. Inorganic nitrate is taken up into the cell precursor or storage pool and stored as inorganic nitrogen. The rate of uptake, $R_1$, can be expressed by a Michaelis-Menten enzyme activated equation. The transfer rate, $R_2$, from the precursor pool to the intermediate nitrogenous-organic pool is also an enzyme activated rate. The intermediate pool has losses through the membrane to the environment, $R_5$ (first order rate), and to the structural pool, $R_3$ (Michaelis-Menten enzyme rate equation). The intermediate pool also has additions by uptake, $R_6$ (first order rate), from the environment through the membrane, and a first order breakdown, $R_4$, of the structural proteins. Microbial activity, $R_7$, external to the cell mineralizes the nitrogenous-organics to inorganic nitrate through respiration. This rate is described as a first order rate. Grenney et al. (80) applied the model to both batch and chemostat data. The model demonstrated three phenomena observed in laboratory cultures and in field studies that, as previously has been noted, are not predicted by the Monod growth equation: uptake rate of the limiting-nutrient exceeding growth rate, high growth rates in at very low environmental nitrate concentrations, and change in the ratio of internal cell nitrogen to the population size.

Fuhs (68) used a three compartment cell model for P metabolism in algae in a similar manner as Grenney et al. (218). He divided the cell into structural, functional or synthetic, and storage compartments
for the internal cell P. The size of functional synthetic fraction was considered important to the growth rate since it included the RNA which was used in a previous growth rate equation, Equation 3.7. The storage compartment includes the Poly-P.

Miyachi et al. (158) elucidated the role of the Poly-P in the P metabolism of *Chlorella ellipsoidea*. The Poly-P is not a homogeneous compartment but consists of at least four types. Shown in Figure 3.10 are the probable pathways of P metabolism for the production of the important structural, functional, and genetic P compounds under growth condition where energy is supplied by light during photosynthesis and by organic substrates in the dark (heterotrophic growth). The phosphate precursor pool is shown but the metabolic intermediates and the dinucleotides are not indicated but implied along each pathway. Miyachi et al. (158) used radioactive tracer \(^{32}P\) to obtain the data for the development of the model. They concluded that only part of the Poly-P was for storage of phosphate (Poly-P"B" and "D") and that the remainder (Poly-P"C" and "A") was of a functional nature as an intermediate to the production of DNA and protein-P. The storage Poly-P "B" and "D" supplied phosphate to the precursor pool only under absence of phosphate in the medium under light conditions. It was found, though only under dark conditions, that Poly-P "B" supplied the P for the production of RNA.

Takeba (218) developed a multicomponent model for the P metabolism of cotyledons, specialized leaves that are found in all plant seeds. The model shown in Figure 3.11 is the best fit of data obtained using the radioactive tracer \(^{32}PO_4\). The model is similar in concept
Figure 3.10 Probable Pathways of P Metabolism as Determined by Miyachi (158)
Figure 3.11 Takeba's Multicomponent Model of P Metabolism (218)
to that of Miyachi et al. in Figure 3.10. The model does include breakdown of the macromolecular P polymers which Figure 3.10 does not include. The Takeba model does not include a compartment for DNA since the radioactive P was not incorporated into the DNA-P during the time of the experiments. Further a compartment for Poly-P is not identified. The rate of transfer out of a P compartment is first order in the concentration of the P in the compartment. The use of first order transfer equations are common in multicomponent analysis (6).

Phosphorus metabolism models were determined using laboratory cultures. Miyachi et al. (158) and Takeba (218) have used very sophisticated separation techniques and analysis procedures to obtain data for the model. Fuhs (68) used less sophisticated P identification techniques, but there were advanced in comparison to field data collection. Williams (245) did not deal with the analytical techniques in the discussion of his model. The Grenney et al. (80) study utilized the data of a diverse number of studies for the substantiation of the growth model. These studies have postulated a structure for P metabolism that can be used as a basis to assess techniques of internal cell P analysis which can be applied in the field to obtain significant data of natural aquatic system biochemical reactions.

3.5 Analytical Techniques for Cell Phosphorus Analysis

The sophistication and detail of the cell growth models described in the previous section depends on the analytical techniques that provide the data. Practical application of analytical techniques to field studies and laboratory cultures depend on the ease in terms of
time, manpower and equipment, relevance and accuracy of the results. This section will discuss several analytical approaches to the separation of P compartments in a cell.

Kanai et al. (114) and Miyachi and Tamiya (161) have modified the Schmidt and Thannhauser (199) method of the separation of the P compounds in a cell. The flow sheet of the extraction and separation processes, the Miyachi method, is shown in Figure 3.12. This procedure was used in the development of the P metabolism models of Miyachi et al. (158) in Figure 3.10 and Takeba (218) in Figure 3.11. Each of the above investigations coupled this procedure with the use of the radioactive tracer $^{32}$PO$_4$ as outlined in the previous section in which a quantitative recovery of P from each compartment is not required. The only necessity to determine the true concentration of each compartment in the cell is a recovered sample of that compartment which is analyzed for total P and radioactivity with a scintillation counter. The true cell concentration is calculated with the determined values by the procedure that Atkins (6) outlines. This technique is widely used in the laboratory for P metabolism studies. Tracers are used in aquatic ecosystems but not in the above manner (187). The Miyachi method is used for quantitative determination of the P compartments in the cell (185). The accuracy of the quantitative results is subject to the losses between extractions as well as alterations due to the extraction procedure. This latter factor affects the technique when using tracers. An important observation can be made in the Miyachi method. If the Poly-P's are excluded, the TCA acid extraction divides the cell P into two general categories of cell functions. The
Washed Algal Cells

8X TCA x 2 (for 30 min. and 15 min. at 0°C)

- sup. = [ACID SOLUBLE FR.]
- ppt. = [ACID INSOLUBLE FR.]

Adjust pH to 4.0 with acetate buffer, 0°C
Add Conc. Soln. Ba(NO₃)₂, 0°C

- sup.
  - ppt.
    - Charcoal treat. (30 min., 0°C)
      - Add H₂SO₄ for 1 h, boil 20 min.
        - LABILE NUCLEOTIDIC P
        - INORGANIC P
      - 10X PCA
        - ppt.
          - sup.
            - 0.5N NaOH, boil for 10 min.
              - sup. = [PROTEIN P]
            - 5X PCA, boil for 20 min.
              - Charcoal treat.

- sup.
  - ppt.
    - Charcoal treat.
      - 0.5 NaOH, boil for 10 min.
        - sup. = [PROTEIN P]
      - 10X PCA
        - sup.
          - Charcoal treat.

- sup.
  - ppt.
    - 2N KOH, for 16-20 hr. at 37°C
      - sup.
        - DII. KOH of pH 9.0, x2 (for 1 hr. and 30 min. at 0°C)
          - sup.
            - ppt.
              - Add Conc. Soln. Ba(NO₃)₂, 0°C
                - sup. = [LIPID P]

Figure 3.12 Flow Sheet of Miyachi's Extraction and Separation Processes of Cellular P Compounds (119)
TCA soluble P includes the precursor pool, the intermediate metabolite pool, and the functional compound pool. The TCA insoluble P includes the macromolecules required for structure and genetics. This separation of the cell P into two compartments fulfills the requirements of the Williams (245) cell growth model for P-limitation shown in Figure 3.8. The division of the Poly-P's in the Miyachi method confuses the picture since the Poly-P have shown both a storage of precursor role and a functional intermediate role (83,158) and are divided into both fractions.

Fuhs (68) used a simplified extraction technique for obtaining data for the confirmation of his model. Fuhs extracted the cells with cold perchloric acid (PCA) and determined the total acid soluble P. This was followed by extraction of the cells with EtOH-ether (1:1) and the total P in this extract was the lipd fraction. The residue P from the first two extractions was designated the acid insoluble or residual P. This procedure of extraction follows the initial major separations of the Miyachi method. Fuhs conceptual model divided the cell P into structural, functional/synthetic, and storage P compartments. The structural components, which are required for the maintenance of cell integrity and viability, were DNA (acid insoluble) and membrane phospholipids. The functional/synthetic compartment was the acid soluble P metabolic intermediates and nucleotides, P of the chloroplast lipids, and the acid insoluble RNA. The size of this fraction was assumed to control the growth rate. The storage compartment included the acid soluble and acid insoluble Poly-P. Their presence was postulated as not having an appreciable effect on the
growth rate. At maximum depletion of P in the cell, Fuhs postulated that P would be restricted to the essential components such as DNA and the membrane lipids, leaving no P for functional/synthetic components, nor for the synthesis of additional structural components and no P in the storage compartment. The interpretation of the analytical results of the diatom, Cyclotella nana, during P depletion show that the acid soluble fraction has no structural components while the lipid showed functional/synthetic character. The acid insoluble phosphorus showed the presence of structural components.

Other extraction and separation procedures have been used in P metabolism studies that are similar to the Miyachi method (192, 203).

An alternate procedure for the extraction and separation of the P compounds in a cell is to extract the lipids first followed by the cold TCA extraction. The lipid extraction may include some of the low molecular weight P compounds. The amount and the type of P compounds depends on the lipid extraction procedure. If EtOH-ether (3:1) extractant is used at room temperature, very little of the P metabolites and Poly-P are removed with the P lipids and the cold TCA extraction will give comparable P compounds as in the Miyachi methods (39). An extraction with boiling EtOH-water extractant increases the amount of P compounds removed (76, 79). This method removes the P metabolites and the acid soluble Poly-P (79) in addition to the phospholipids. If the boiling time is one hour or longer, acid insoluble Poly-P will hydrolyze (76). In the latter method, the phospholipids can be recovered by extraction with chloroform (76). The cold TCA extraction following the lipid extraction solubilizes the P
compounds, particularly the nucleotides, that hydrolyze in acid at room temperature (76). Extraction with TCA at 90°C of the residue from the boiling alcohol and cold TCA extraction removes the DNA, RNA, and acid insoluble Poly-P, the latter if not previously removed as noted above. The residue that remains contains P in proteins. Each of the extracted compartments was analyzed for total P.

Fitzgerald and Nelson (64) proposed the extraction of P compounds from cells by boiling them for one hour in water containing the salts of a typical inorganic growth medium (minus the P) that was adjusted to pH 7.0. The method yields three compartments; the water insoluble P in the residue, the o-PO$_4$ and the total P in the water soluble fraction. The determined o-PO$_4$ in the water extract was considered the surplus P in the cell. Rhee (184,185) correlated the o-PO$_4$ in the water extract with the total Poly-P separated with the Miyachi method. In addition, the boiling water extraction is considered to extract the precursor and the intermediate metabolites in a cell (25). Fitzgerald and Nelson (64) do not speculate what P compounds are in the water insoluble fraction or in the non-o-PO$_4$ in the water soluble fraction. The literature gives no further indication of the probable source or the types of P compounds in the compartments.

Several chromatographic techniques have been applied to the identification of P compounds in a compartment. The quantitative sensitivity of these techniques were enhanced by labeling the P utilized by the cell during growth with radioactive tracer $^{32}$PO$_4$. Sastry and Kates (196) extracted the lipids from the alga, *Chlorella vulgaris*. The P compounds of the extracted lipids were
separated using paper chromatography. Meyer and Bartlett (154) analyzed the TCA soluble P compounds of the horse blood granulocytes and lymphocytes. The soluble P compounds were separated in an ion exchange resin chromatographic column. Using the same type column, Goodman et al. (79) isolated the P compounds solubilized with 20% boiling EtOH in water from the alga, Scenedesmus.

The difference in the rate of hydrolysis of organophosphates and Poly-P to $\text{o-PO}_4$ have been used to determine the amounts of various extracted P compounds. Chayen et al. (34) found that the hydrolysis to $\text{o-PO}_4$ of Poly-P in hot acid conditions was faster than that of the nucleotidic phosphate. Leloir and Cardini (140) gives hydrolysis rate constants of numerous Org-P compounds. Miyachi method uses this procedure to determine Poly-P in the various soluble P fractions. In Figure 3.12 the $\Delta$15P procedure requires that a 1N $\text{H}_2\text{SO}_4$ solution of the soluble P be boiled for 15 minutes. The released $\text{o-PO}_4$ is the amount of P associated with the Poly-P and the non-hydrolyzed P is a more stable form of P compound. The primary chemical structural difference is that of a $-\text{P-O-P-}$polyphosphate linkage compound to the more stable $-\text{C-O-P-}$ of the nucleotidic linkage (34).

There are many other techniques that are utilized by investigators but the summary given above covers the widely used procedures whose analytical results can be compared to each other.
3.6 Phosphorus Metabolism and Dynamics in Cells

The dynamics of cellular P compartments have been investigated in laboratory cultures using separation techniques cited in the previous section. These studies were conducted on a wide variety of organisms under environmental conditions in which P was either a limiting nutrient or a non-limiting nutrient. The effects of other environmental factors on the cellular P were studied. Extensive discussion of the results and conclusions of the cited literature in this section and following sections are found in the Discussion of Results chapter.

The algae that have been extensively studied are the green algae from the genus *Chlorella*. Miyachi and his colleagues carried out a series of investigations of the P dynamics of *Chlorella ellipsoidea* (157,158,158,161). Using the Miyachi separation and extraction technique shown in Figure 3.12, the changes in the P compartments with time in batch growth cultures were followed when the nutrient $0\text{-PO}_4^-$ was either in excess or absent from the medium under either photosynthetic or heterotrophic growth. In the latter case *Chlorella* previously grown in an excess $0\text{-PO}_4^-$ medium was transferred to medium deficient in $0\text{-PO}_4^-$. With the use of the radioactive tracer, $^{32}\text{PO}_4^-$, as nutrient newly taken up P was followed between compartments during the growth. The conclusions from these studies led to the probable metabolic pathways of P in cells shown in Figure 3.10. Baker and Schmidt (10) and Herman and Schmidt (95) followed TCA soluble P, phospholipids, and the TCA insoluble P compartments during the photosynthetic synchronous growth of *Chlorella pyrenoidosa* in batch culture.
Specific P compartments in *Chlorella* were studied by other investigators. Sastry and Kates (196) studied the incorporation of the $^{32}\text{P}$ from PO$_4$ and glycerophosphate into the phospholipids of *Chlorella vulgaris* in dynamic cultures during photosynthesis. Correll and Tolbert (39) analyzed for the RNA-Poly-P"D" during the synchronous growth of *Chlorella pyr.* with an 18:6 light:dark day cycle in a dynamic culture. Aitchison and Butt (2) studied the Poly-P dynamics in *Chlorella vulgaris* during photosynthetic growth in batch culture. Two dynamic situations were followed: 1) algal cells that were P-starved were transferred to cultures with excess o-PO$_4$ and 2) cells grown in excess of o-PO$_4$ culture were transferred to a medium with no o-PO$_4$. Fitzgerald (62) used the boiling water P extraction to study the dynamic photosynthetic growth of *Chlorella pyr.* Other researchers have incorporated boiling water extraction technique into their studies of the photosynthetic growth under phosphate limitation of the blue-green alga, *Anabaena variabilis* and the green alga, *Scenedesmus specialis* (89,91,184,185,196).

Research of cellular P has also been carried out on other algae. Fuhs and colleagues (68,69) carried studies of the P content in the TCA soluble and insoluble, and lipid compartments of diatoms *Cyclotella nana* and *Thalassiosira fluviatilis* during photosynthetic growth in a chemostat. Sicko-Goad and Jensen (203) investigated the P starvation and P recovery of the blue-green alga *Plectonema boryanum* in batch culture under 12:12 light:dark day cycle growth. They followed the TCA soluble and insoluble P and the lipid P during the experiments. Bieleski (14) studied the changes in lipid P located in the
chloroplasts of the plant *Spirodea* under P limiting conditions.

Extensive cellular P research was carried out by Rhee (184, 185, 186) on the alga *Scenedesmus specialis*. He used the P separation and extraction techniques of the Miyachi method on the alga grown in both batch and continuous cultures that were o-PO₄ limited under both continuous photosynthesis and a 12:12 light:dark day cycle.

Cellular P dynamics of bacterial cells have been studied. *Escherichia coli* was studied by Horiuchi (101), Wade (232) and Maruyama and Mizuno (150). Wade followed the TCA soluble and insoluble P, lipid P, protein P, RNA, and DNA during batch growth in which the energy substrate was limiting. Horiuchi and Maruyama and Mizuno determined the changes of P in the TCA soluble P, the RNA, and the DNA compartments during o-PO₄ limited growth in a batch culture. Hou et al. (102) followed the changes in RNA and DNA in the bacterium *Pseudomonas aeruginosa* in dynamic growth in which o-PO₄ was limited. Sall et al. (192) applied the Miyachi separation method to the P compartment analysis of the bacterium *Corynebacterium diphtheriae*. Visual observations were made on the formation of intracellular volitin granules during the incorporation of extracellular o-PO₄ into Poly-P which has been identified with the granules. The phospho-lipids in the walls of the bacteria *Bacillus subtilis* were studied by Tempest et al. (219) and of *Bacillus licheniformis* by Forsberg et al. (67) in dynamic growth under o-PO₄ limitation. The yeast, *Torulopsis utilis*, was examined for changes in the RNA and the Poly-P "D" in batch culture by Chayen et al. (34).
Investigations of the cellular P dynamics of *Scenedesmus specialis* have been made when nutrients other than P are limiting. Rhee (186) followed the P compartments obtained using the Miyachi method of separation when nitrate was the limiting nutrient. The growth experiments were carried out in continuous light in a steady state culture. Wintermans and colleagues (235,247) studied the effects of the medium concentration of glucose and CO$_2$ on the TCA soluble and insoluble P in *Chlorella vulgaris* in dynamic growth under both photosynthetic and heterotrophic growth. Ullrich (227) studied the TCA soluble and insoluble P fractions in presence and absence of CO$_2$ during photosynthetic growth of *Ankistrodesmus braunii*. Various light quality experiments were also conducted. Golterman (76) followed the hot and cold TCA soluble P and the insoluble residue P compartments of dead *Scenedesmus quadricauda* cells as they autolysed. Tempest and associates (46,220) followed the changes of RNA, DNA, and total cell P of the bacterium, *Aerobacter aerogenes* in continuous culture. The growth of the bacterium was studied when the growth was limited by K$^+$, or Mg$^{2+}$. Hopson and Sack (110) investigated the changes of RNA, hot TCA soluble P and the total P of *Escherichia coli* under carbon energy substrate limitation in dynamic culture.

Reviews by Healey (90), Harold (83) and Hodson (98) discuss other literature concerning cellular P studies.

Many of the investigations cited in this section were directed to the purpose of correlating the RNA content of a cell to the growth rate as suggested by Herbert (96). Different analytical, extraction and separation techniques were studied to enhance the accuracy of RNA
determination. Studies were also made to determine the fate of P in the cell during metabolic conversions in response to different environmental stresses. Much effort was made to identify the role of Poly-P in cell metabolism and its location in subcellular structures.

3.7 Uptake of Phosphorus by the Cell

All cell nutrients and metabolic waste products must pass through the cell wall (if it exists) and membrane. The physical structure and the chemical activity of this cell envelope will affect the passage of chemical entities into and out of the cell. The cell envelope biological activity is affected by both the cell physiological state through the regulatory mechanisms of cell wall and membrane synthesis and the environmental parameters of the medium. The uptake of the P will affect the internal P compartments if the rate of uptake into the primary precursor and storage pools of the cell is greater than or less than the rate of output from the pool due to cell metabolic requirements. This section discusses the kinetics of uptake of P, mechanisms of uptake, influence of cellular physiological state and the effects of the physical parameters of the environment, such as, pH, ionic species concentrations, temperature, P concentration. In cell growth models the uptake of nutrient is a rate that is included in the overall model development since the uptake may be the limiting rate. Uptake rate is considered in the Grenney et al. (80) and Takeba (218) growth models shown in Figures 3.7 and 3.11 respectively. The models of Williams (245) and Miyachi et al. (158) can include the uptake rate into their models as shown in Figures 3.8 and 3.10 respectively.
The uptake of \( \text{o-PO}_4 \) from the medium into the cell is an energy dependent process (189). The transport system of phosphate is activated by enzymes which are located in the cell membrane (16,190). The uptake transport system of algae, yeast, and bacteria are similar and are specific to phosphate (90). Models of energetics of the active uptake of substances into cells and associated metabolic processes have been comprehensively discussed by Blondin and Green (16) and Luria (142).

Dual phosphate uptake mechanisms in *Chlorella pyr.* have been suggested (109,110) and that they reside in the plasmalemma (115), the cell plasma membrane, which, for alga, is found inside the cell wall (20). Dual uptake mechanisms are considered to operate in parallel but one is effective at low concentration of o-\( \text{PO}_4 \) in the medium (less than 1 mM) and the second mechanism that functions at higher concentrations (greater than 1 mM). Dual mechanisms have been observed for other ions, such as rubidium and potassium (198).

The rate of uptake of a cell is affected by the external environment and the physiological state of the cell. The medium concentration of o-\( \text{PO}_4 \) affects the rate of its uptake. Scott (200) found that the rate uptake of P-deficient *Chlorella pyr.* is dependent on the concentration of o-\( \text{PO}_4 \) in the medium during photosynthesis but is independent of the concentration in darkness. Rhee (184) found the uptake rate of o-\( \text{PO}_4 \) P-deficient in *Scenedesmus specialis* was dependent on the medium concentration during photosynthetic growth. Perry (177) correlated the short term o-\( \text{PO}_4 \) uptake of *Thalassiorira pseudonana* with a Michaelis-Menten enzyme kinetics equation;
\[ V_u = \frac{V_{u,max} S}{K_u + S} \quad (3.21) \]

where: \( V_u \) - uptake rate of the substrate,
\( S \) - substrate concentration in the medium,
\( V_{u,max}, K_u \) - Michaelis-Menten constants as previously described.

Rhee (184) observed a decreasing rate of uptake of \( \text{o-PO}_4 \) as the cell accumulated excess \( P \) in the form of \( \text{Poly-P} \). The uptake was expressed as a Michaelis-Menten enzyme equation with an inhibition term for the concentration of the \( \text{Poly-P} \);

\[ V_u = \frac{V_{u,max} S/K_u}{1 + S/K_u + i/K_i} \quad (3.22) \]

where: \( i \) - concentration of the \( \text{Poly-P} \) in the cell,
\( K_i \) - Michaelis-Menten half saturation constant for inhibition.

Other observations of uptake rate dependence on the physiological state have been made. Fuhs et al. (69) observed a decreasing rate of uptake of \( \text{o-PO}_4 \) by the diatom, \textit{Thalassiosira fluviatilis} as the cellular \( P \) increased during photosynthetic growth. Healey and Hendzel (91) and Healey (89) found that the uptake of \( \text{o-PO}_4 \) by \textit{Scenedesmus quadricauda} and \textit{Anabaena variabilis} increased with decreased cell \( P \) at higher cellular \( P \), little change at intermediate cell \( P \) and decreased uptake rate at low cell \( P \). The increased uptake rate of a cell is also related to increased protein production in the cell membrane. The cell is increasing the number of \( \text{o-PO}_4 \) uptake sites by synthesizing more of the transport system enzyme in response to the deficient
cellular P (90,108). Aitchison and Butt (2) present data of the initial rate of o-PO$_4$ uptake as a function of the time that Chlorella vulgaris was in o-PO$_4$ deficient medium. As time increased the rate of uptake increased to a maximum plateau. Uptake studies of other nutrients and organisms are reviewed by Healey (90).

The pH of the medium affects the activity of the enzymes in the cell membrane that are actively engaged in the absorption of ions (190). The ionic states of an ion reaction change with pH and the uptake system may not be as efficient. Jeanjean et al (110) found a broad range of the uptake of o-PO$_4$ by Chlorella pyr. which rose sharply to a maximum rate at pH 6.5 but remained high up to pH 9.0. Uptake of o-PO$_4$ by Anabaena variabilis requires the presence of Mg$^{2+}$ (89). Yeast (198), E. coli (239), and Chlorella pyr. (200) required potassium to enhance the uptake rate of o-PO$_4$, while Ankistrodesmus braunii required both Na$^+$ and K$^+$ for enhanced o-PO$_4$ uptake (228). Kylin (131) studied the effect of light intensity in the presence and absence of CO$_2$ on the uptake of o-PO$_4$ in Scenedesmus. Organic factors that inhibit or stimulate have been studied by Jeanjean (108) in Chlorella pyr.

This section has reviewed the several external medium factors affecting the uptake of P into the cell. Kuhl (127) reviews other research. There is a range of unexplored factors that can affect the cell envelope uptake mechanisms.
3.8 Effect of Phosphorus on the Phosphatase Activity in Cell Envelope

When there is a metabolic deficiency of an inorganic nutrient, a cell has increased ability to use organic forms that nutrient (90, 127). For P deficiency, phosphatase enzyme activity is induced or increased in the cell surface. Further there is found soluble enzyme activity in the medium. The phosphatase is capable of releasing $\mathrm{O-PO}_4$ for organophosphate esters or release $\mathrm{O-PO}_4$ from condensed polyphosphates. Cells can synthesize phosphatase enzyme to operate in an alkaline and/or acid environments which may or may not be repressed by $\mathrm{O-PO}_4$ depending on the cell's genetic code. Phosphatase activity is found in algae (127), bacteria (128) and yeasts (241). The ability of utilizing organic forms of a cell deficient inorganic nutrient is not limited to P.

Phosphatase activity has been detected in laboratory cultures and in natural aquatic systems. Fitzgerald and Nelson (64) have detected alkaline phosphatase activity in a wide range of algal species: green, blue-green algae and diatoms. They found alkaline phosphatase activity in *Chlorella pyrenoidosa*. Rhee (184) found alkaline activity in *Scenedesmus specialis* while Healey (89) and Healey and Hendzel (91) found extracellular alkaline phosphatase activity in the medium in *Anabaena variabilis* and *Scenedesmus quadricauda* cultures. Matagne et al. (151) found in the cell wall of the alga *Chlamydomonas reinhardi* a neutral phosphatase. Ayyakkan and Chandram (8) found bacterial phosphatase activity in sediments of a natural water ecosystem while Berman (13) found alkaline phosphatase activity in lake waters.
Studies on the utilization of organophosphates and condensed phosphates show that the liberation of o-PO₄ occurs in the medium by the soluble phosphatase or on the cell surface by attached extracellular phosphatase before the cell absorbs the o-PO₄ into the cell. Miyachi et al. (160) found the carbon backbone of phospholipids remaining in the medium while *Chlorella ellipsosidea* utilized the o-PO₄. Eppley (55) in multicellular algae and Davis and Wilcomb (45) in *Chlorella pyrenoidosa* and other green algae found that that o-PO₄ was liberated from the condensed phosphate before the o-PO₄ was taken up into the cell. The liberated o-PO₄ was not entirely consumed by the algae. Kuenzler and Perras (125) found that several marine algae utilize the o-PO₄ liberated by cell surface alkaline phosphatase from glucose-6-phosphate, adenosine monophosphate, and α-glycerophosphate. The uptake rate of o-PO₄ by the cells was the same for all three organophosphates. Sastry and Kates (196) determined the α-glycerophosphate can be used as a source of cell P for *Chlorella vulgaris*. They did not indicate whether o-PO₄ was released by enzyme activity before or after entering the cell.

The question of whether algae can absorb organophosphates before hydrolysis of the o-PO₄ is not answered. The ability of algae to liberate o-PO₄ from the low molecular weight organophosphates and condensed polyphosphates have been demonstrated, but there is little evidence of release of o-PO₄ from more stable macromolecular compounds of DNA or RNA.
3.9 The Effect of Phosphorus Nutrient Deficiency on the Cell Composition and Morphology

The response of algal cells to a nutrient deficiency can be found in several general characteristic changes in the cell composition and morphology. The previous three sections have characterized the changes in cell P composition, o-PO$_4$ uptake rate and the phosphatase activity in response to P-nutrient deficiency. This section covers the change in photosynthetic pigments, carbon storage compounds, cell morphology, and the photosynthesis rate.

When cell division stops, the photosynthetic fixation of CO$_2$ into cell material does not cease but declines, thus there is accumulation of C in the cell. As the cell C increases, the cell size increases in the absence of division. This phenomena was noted by Lehman (139) in the alga *Pediastrum duplex* in batch culture. In continuous culture cell size increased with decreased growth rate as observed by Fuhs et al. (69) for *Cyclorella nana* and by Rhee (184) for *Scenedesmus specialis* under o-PO$_4$ limitation, and in the review article by Healey (90) algae under nitrate limitation. Prokop and Ricica (181) found that the cell size decreased at lower growth rates for the alga *Chlorella pyrenoidosa*. This study though was for maximum growth rate conditions in which neither o-PO$_4$ nor NO$_3$ limited the growth but was limited by light in dense continuous cultures. Hase et al. (87) observed that in synchronous growth cultures of *Chlorella*, the cell size at the time of division was smaller in P deficient medium than in complete medium. For algae the form of the excess carbon appears as lipid under N-limitation (90) and as carbohydrate under P-limitation.
(69). Under o-PO$_4$ limitation the bacterium *Aerobacter aerogenes* also accumulates carbohydrate (46,47).

There is a decline in the photosynthetic pigments of nutrient-deficient algae. The chlorophyll content of a cell decreases in response to the decrease in the photosynthesis (90). It was noted in a review article by Bogorad (17) that the chlorophyll content of the algal cell varies during nutrient sufficiency depending on the light intensity. Under equivalent growth conditions the chlorophyll content of a cell generally varies inversely with the light intensity above saturating light conditions. Bogorad (17) summarized the forms of chlorophylls found in the algae. The chlorophyll found in all species of algae is chlorophyll a. The green algae of the *Chlorophyta* division of algae contain chlorophyll a and b with former the predominant in concentration. There are numerous studies of the change in concentration of chlorophyll a with respect to the growth of algae under o-PO$_4$ limitation during both steady-state and dynamic growth. *Anabaena variabilis* (89), *Scenedesmus quadricauda* (91), *Cycotella nana* (69) and *Chlorella pyrenoidosa* (181) show a decrease in the chlorophyll content as growth rate slows or ceases.

Nutrient deficiency can induce changes in the shape of the cell. When the concentration of calcium in the medium was low, the shape of the cell of the diatom, *Phaeodactylum tricornutum* was ovate compared to fusiform for high concentrations (38). Under phosphate depletion conditions, the fatty acids that form the lipids in the cell membrane structure in the gram-positive bacteria change from the P containing teichoic acid to the non-P containing teichuronic acid (67,219). For
Bacillus licheniformis, the loss of the P containing membrane lipids causes changes in the cell shape. With incomplete cell membrane formation in the P-deficient cell, the cell forms irregular spheres but are restored to rod shaped cells when o-PO$_4$ is restored to the medium (67). Algal cell membranes of the green algae contain phospholipids (9). Under phosphorus depletion, the phospholipid level in Scenedesmus specialis was observed (185) to decrease.

Other important phenomena have been found under P-starvation of cells. Lehman (139) found that the extracellular release of organic carbon was large for the P-starved cultures of the green alga, Pediastrum duplex, but the absolute rate of release declined upon the cessation of cell division. Lower o-PO$_4$ concentration increased glycollate release of Chlorella pyrenoidosa (89). Lean and Nalewajko (137) measured DOP in batch lab cultures for Anabaena flos-aquae, Navicula pelliculosa, Chlorella pyrenoidosa, and Scenedesmus quadricauda. During the exponential growth of the culture when o-PO$_4$ was present in the medium, DOP was released to the medium but upon the depletion of the o-PO$_4$, the cells took up the released DOP. Hase et al. (87) found that the number of daughter cells at division of Chlorella ellipsoida decreased from the average of 6.5 in complete medium to 3.5 in P-free medium. For the alga, Prymnesium parvum, toxin production is enhanced under o-PO$_4$ limitation (41).

There are numerous other cell responses under P-deficient growth on the level of individual biochemicals. A detailed literature investigation will reveal these studies. Their application to this investigation are limited.
3.10 Extracellular Carbon Release and Mixotrophic Growth of Algae

During the photosynthetic growth of algae, soluble organic compounds are released into the medium which varied according to the stage of growth. Mixotrophic growth of algae is the parallel incorporation of carbon into the organic carbon pools of the cell by fixation of CO$_2$ through photosynthesis and the uptake of organic carbon from the medium. Further the uptake of extracellular carbon by the algae varies with the stage of growth. The extracellular organic carbon may originate from the cellular release or from the additions of organic carbon in the medium formula.

The sources of dissolved organic carbon in the medium during cell growth can be one of the following processes: 1) release of intracellular organic carbon compounds through the cell membrane by active or passive (diffusion) processes, 2) the dissolution of compounds from the cell wall, 3) the availability of cell remnants from cell division, 4) release of organic compounds from dead cells during the process of autolysis (123). The processes outlined above occur in both algae and bacteria (51). The stage of growth of the culture also affects the rate of excretion and the type of excreted compounds. Cells in the stationary and the lag phases of growth release more organic compounds than cells in exponential growth phase (93). But other research show that the highest release rates are during the exponential growth phase (8,170). The lag phase release can be due to the transfer of unhealthy cells which die or are unable to hold the intracellular compounds (66). Nalewajko and Lean (169) found that
algae release low molecular weight organic compounds during the exponential growth phase but in the older or stationary phase the released compounds were high molecular weight. The low molecular weight were intracellular metabolites which are in high concentration in the cell during the exponential growth phase. The high molecular weight organic carbon compounds are from the polysaccharides produced by the cells for the gelatinous sheaths that are generally formed on the cell surface in the stationary growth phase. Dunstall and Nalewajko (51) observed the same phenomena for the bacterium *Pseudomonas fluorescens*. Fogg (66) suggested that the low molecular weight dissolved organic carbon (DOC) compounds are reutilized by the cells while the high molecular weight DOC is not readily reabsorbed.

Several studies of the extracellular release of DOC of green algae have been made. *Chlorella* has been extensively studied. The release of the low molecular weight gylcollate by *Chlorella pyrenoidosa* was studied by numerous investigators (8, 148, 225, 237). The release of glycollate was dependent on the pH, aerobic conditions, light and the bicarbonate ion during exponential growth. The release of the glycollate increased at high light intensities and low CO₂ concentrations. The amount of DOC released to the medium was proportional to the amount of carbon photosynthetically fixed (237). At low CO₂ or limiting concentrations, the glycollate was taken up by the cell as the cell enters the stationary growth phase and took up glycollate under high light intensities when CO₂ was sufficient (8, 237). Glycollate is the principle extracellular organic product of *C. pyrenoidosa* with a concentration of up to 8 mg./l. (225).
Extracellular organics released by algal cells are not limited to glycollate. Hellebust (93) summarized known DOC for *Chlorella* which included polysaccharides, simple sugars, sugar alcohols, amino acids, and peptides. Maksimova and Pimenova (147) found volatile and other organic acids in cultures of *C. pyrenoidosa*. Nalewko and Lean (169) noted the low molecular weight DOC released early in growth decreased and a high molecular weight DOC which increased as the culture of *C. pyrenoidosa* approached the stationary growth rate. The source of the polysaccharides, the probable high molecular weight DOC, is the capsular sheaths secreted by the algae during their growth (8). One study of *C. pyrenoidosa* found that the total accumulated organic matter in the culture at the end of the exponential growth phase was approximately 100 mg/l. (148).

The quality of the light has an effect on the extracellular release of organic compounds from algae. Brown and Green (24) determined that *Chlamydomonas reinhardtii* released greater DOC in white light over that of blue or red light. They observed that the quality of the released compounds varied with the type of the light. Brown and Green noted that research by others on *C. pyrenoidosa* showed that glycollate release was found in high intensity white and red light but not in blue light. They also analyzed for the identifiable compounds in the metabolic intermediate pool and the released compounds as a function of the light quality. They noted that the cell number growth rate of *C. reinhardtii* was highest for red light and fell in order when exposed to blue, white, and green light.
Organic carbon is released to the environment during the autolysis of algal cells. Jewell and McCarty (112) studied the aerobic decomposition of 
*C. pyrenoidosa* and other green algae. They divided the organic into three categories: 1) a small fraction that which is respired by bacteria in a few hours, 2) a large fraction which decomposes within a year, 3) a large refractory fraction which decomposes only a few percent a year.

Other algal organisms have been studied for the release of DOC and can be reviewed in Fogg (65) and Hellebust (93).

Mixotrophic growth has not been extensively studied in algae. Wintermans (247) observed that the uptake of phosphate by *C. pyrenoidosa* was suppressed by the presence of 0.2% glucose in the medium. They explained this by postulating that the assimilation of glucose and O-PO\(_4\) compete for the energy generated by photosynthesis. Samejima and Myers (194) found that the growth rate of *C. pyrenoidosa* in medium light grew two times faster with glucose than galactose in the medium. But the medium with glucose grew almost four times faster than the control culture with no organic present. Neither fructose nor maltose changed the rate of growth when compared to the control, but sucrose slightly depressed the rate. Though sucrose was not utilized as carbon source by *C. pyrenoidosa* in the Samejima and Myers study, Milner (155) found that sucrose was present as an intracellular component. In the dark sucrose does not support the growth of *C. pyrenoidosa* while glucose and galactose does support growth. Endo and Shirota (54) found that mixotrophic growth with acetic acid as organic carbon source was greater than with glucose for *Chlorella regularis* in the presence of CO\(_2\). The absence of CO\(_2\)
with acetic acid reduced the growth rate to that of autotrophic growth.

3.11 Inorganic Particulates of Iron, Calcium Phosphates: Their Formation, Stability and Source of Microrbial Nutrients

Inorganic particulates are found in natural aquatic systems and in laboratory culture. These particulates encompass the calcium phosphates-hydroxyapatite \((\text{Ca}_5\text{(OH)}(\text{PO}_4)_3)\), dicalcium phosphate \((\text{CaHPO}_4)\), and tricalcium phosphate \((\text{Ca}_3(\text{PO}_4)_2)\) and iron phosphates—ferric phosphate \((\text{FePO}_4)\) and the oxyhydroxide-orthophosphate complex \((\text{o-PO}_4\) absorbed on \(\text{Fe(OH)}_3\) variously given as \(\text{Fe(OH)}_{3-n}(\text{H}_2\text{PO}_4)_n\) and \(\text{Fe}_x(\text{OH})_{3(x-y)}(\text{PO}_4)_y\cdot z\text{H}_2\text{O}\) \((214,61)\). These particulates exist because of their low solubility products at the environmental conditions that exist in natural aquatic systems and in laboratory culture medium. Other particulates that will not be discussed are clays and the organic particulates on which P compounds are adsorbed. Discussion of these particulates are found in several references \((61,92,119,129,163,246)\).

The discussion in this section covers the ability of the inorganic particulates to supply the nutrients of P and Fe for cells to take up and to use in their metabolism when these nutrients are no longer in the medium in the soluble state. The chemical structure of these particulates depend on the physical environment in which they are formed. The ability of these particulates to release Fe or P depend on the environmental factors as well as the activity of microbial cells.
Iron is an important micronutrient for growth of organisms. In metabolic processes iron is a component of cytochromes and ferredoxin, which are members of the electron transport chains in photosynthesis, respiration and a requirement in several enzymatic reactions (90). Eyster (56) found that the critical concentration of iron in the medium for *C. pyrenoidosa* was $1 \times 10^{-9}$ M for heterotrophic growth and $1 \times 10^{-5}$ for autotrophic growth. Davies (43) reported growth kinetics of iron limitation for marine algae. Wiessner (243) summarizes findings of researchers on iron limited growth in numerous algal species. Knauss and Porter (118) report the cellular iron concentration for various medium concentrations of iron. The difficulty with maintaining the above concentrations of iron in solution is the low solubility of iron. In the range of pH of 5.0 to 11.0 where most aquatic organisms grow, the concentration of Fe(III) iron in aerobic conditions varies from $1 \times 10^{-8}$ M at pH 5 and 11 with a minimum concentration of approximately $4 \times 10^{-11}$ M at pH 8.0 (204). The concentration is controlled by the solubility of ferric hydroxide, Fe(OH)$_3$, and slightly effected by the solubility of ferric phosphate, FePO$_4$, near pH 5. The solubilities of Fe(III) listed above is generally lower than the critical requirement for *C. pyrenoidosa* particularly during photosynthetic growth. The soluble concentration of ferric iron can be higher due to complexing of iron with other inorganic species or organic complexing agents (214). But the organic complexed iron in solution may not be available for cell uptake. Wiessner (243) reviewed literature which stated that Fe(III), complexed with EDTA, was not available to the cell and not broken down
metabolically. Droop (48) observed that growth in laboratory cultures did not proceed unless a chelator was added. Zarnowski (248) found that \textit{C. pyrenoidosa} had higher yields of growth as the ratio of EDTA to Fe(III) increased. Krauss and Specht (121) were able to determine that \textit{Scenedesmus} metabolically broke down EDTA and utilized the iron. Page (172) found that the roots of plants were able to assimilate the iron complexed by EDTA without assimilating the chelator or metabolically breaking it down. Prelog (179) attributed this to the ability of an organism to produce a complexing compound, siderochrome, which has a higher affinity for the iron than EDTA. The source of chelators is not restricted to additions of man-made compounds.

During growth, organisms produce organic extracellular products that are capable of functioning as chelators (103,123). Bacteria have been found in lake sediments that can produce organic acids that solubilize iron particulates (84).

Microorganisms have shown an ability to utilize the hydrous form of iron. Particulates are attracted and adsorbed onto cell surfaces. Clay particles have been observed sorbed on bacteria (149) while Kennedy et al. (117) were able to immobilize microbial cells onto metalhydroxides. Harvey (86) observed Fe(OH)$_3$ particles on the surfaces of the diatoms \textit{Nitzschia closterium} and \textit{Lauderia borealis}. Harvey (86) and Goldberg (72) determined that diatoms utilized iron from the adsorbed ferric hydroxide. Prelog (179) found that cellular siderochromes in alkaline environment were able to combine with the iron from ferric hydroxide and take the iron into the cell. Research has shown that the siderochrome concentration increases with
depletion of iron in a cell. There also is a physical explanation that is possible. The environment at or near the cell surface of algal cells is alkaline due to the depletion of the carbonate ion which is taken up during photosynthesis. As mentioned previously the solubility of ferric hydroxide increases with the pH above 8, thus any iron from ferric hydroxide on the cell surface can go into solution.

The inorganic particulates described in the introductory paragraph of this section can limit the concentration of o-PO₄ in solution. The major culprit is the adsorption of o-PO₄ onto Fe(OH)₃. Chelators are used to keep Fe(III) in solution under aerobic conditions to prevent the precipitation of o-PO₄ with the iron (107). Calcium phosphate compounds also limit the solubility of o-PO₄ (214). Their insolubility becomes important above pH 7.2 (18). The form of the calcium phosphate depends on the time after precipitation (221). The initial precipitated form is an amorphous solid which has been variously described as CaHPO₄ (37) or Ca₃(PO₄)₂ (233), which with time loses phosphate to form a more thermodynamically stable crystal, hydroxyapatite. Ferric hydroxide is also initially precipitated as an amorphous solid with high o-PO₄ absorptive capacity. With time a more thermodynamically stable crystalline form, goethite (α-FEOOH), evolves through loss of water from the hydroxide. This chemical conversion also reduces the amount of o-PO₄ adsorbed relative to the iron (135). Due to reduced coordination ability the time of conversion from the amorphous forms to the crystalline forms is faster in the case of calcium compounds (135,221). Under conditions in
which CaCO₃ precipitates, the formation of initial amorphous calcium phosphate occurs on the surface of the CaCO₃ particles. The conversion to the more stable hydroxyapatite follows (37,60,81,130). In the presence of CaCO₃, the solubility of precipitated calcium phosphate as CaHPO₄ was a minimum at pH 7 (37). The formation of the calcium precipitates are reduced by the increased concentration of Mg²⁺ (60). For ferric hydroxide, complexion with o-PO₄ is reduced by the adsorption of colloidal organic material (119). In the presence of the chelator, EDTA, at pH greater than 8, Mg²⁺ and Ca²⁺ bind actively with EDTA thus displacing Fe(III) and inducing greater precipitation of iron hydroxide. Further during the steam sterilization procedure of synthetic medium, Mg²⁺ and Ca²⁺ combine with EDTA as double salts which are insoluble (182).

The availability of o-PO₄ from the inorganic precipitation for cell growth depend on the chemical form and the chemical parameters of the liquid medium. Golterman et al. (78) determined the availability of o-PO₄ at pH 8 for the growth of Scenedesmus obliquus from the calcium phosphates, hydroxyapatite and Ca₃(PO₄)₂. Hydroxyapatite, 1.0 mg P/l, yielded a growth rate of 43% of the maximum growth rate and a total cell yield which is 34% of the total cell yield of 1.0 mg P/l. as KH₂PO₄, while Ca₃(PO₄)₂ gave a negligible growth rate and only 3.5% yield of the total cell. Griffin and Jurinak (81) determined the release of o-PO₄ absorbed on CaCO₃ surfaces as a relatively slow process in comparison to the growth rate of algae. The availability of o-PO₄ from the calcium phosphates for algal growth is through physical processes of desorption or solution.
There is no evidence of microbial mediated release of o-PO$_4$.

The sorbed o-PO$_4$ on the ferric hydroxide precipitate is complexed with the iron and the hydroxyl groups on the solid surfaces and can be represented with the general formula: Fe(OH)$_3$$_n$(H$_2$PO$_4$)$_n$ or Fe$_x$(OH)$_3$(x-y)(PO$_4$)$_y$.zH$_2$O (214,246). Fitzgerald (63) conducted growth experiments using iron precipitated o-PO$_4$ which constituted 0, 50 and 85% of the total o-PO$_4$ in a chelator-deficient growth medium for the green alga, Selenastrum capricornutum. In each case the maximum growth of the alga was the same at pH 7.0-7.5. Additional experiments were conducted in which the precipitated iron-phosphate complex was separated from the growing cells with either a 0.45 µm membrane filter or a dialysis membrane. The conclusions of these experiments showed no difference when compared to previous results at all levels of insoluble o-PO$_4$. The non-cell mediated release of o-PO$_4$ is substantiated by the work of Stamm and Kohlschütter (209). They conducted phosphate desorption studies on the iron hydroxide-phosphate complex. Their results show the release of o-PO$_4$ at pH 7.0-7.5 but also show a very abrupt increase in the amount released above the pH of 11. The amount and the rate of release of the o-PO$_4$ is dependent on the precipitate content of more crystalline forms of the iron hydroxide. Parfitt et al. (173) found that goethite, α-FeOOH, released o-PO$_4$ more slowly than the amorphous iron hydroxide. Orthophosphate is also made available to the microbial cell during the solution or uptake of iron due to surface cellular activity previously discussed in this section.
The inorganic particulates of calcium phosphate and iron phosphate complexes show an important role in the cycle of phosphorus. Their role, in terms of P, is that of a buffer releasing \( \text{PO}_4^{3-} \) to the medium when the solution \( \text{PO}_4^{3-} \) nears depletion and combining with \( \text{PO}_4^{3-} \) when the concentration increases. The phenomena outlined in this section occur both in the natural aquatic system and in laboratory cultures.

### 3.12 Miscellaneous Phenomena Associated with the Growth of Algae

This section covers phenomena associated with the growth of the algae that are important to the research being presented in this writing. The topics cover the total concentration of the inorganic carbon in the medium, the light intensity during growth, the pre-history of the algal inoculum. The discussion also covers the predominant bacterial contaminant in *Chlorella* cultures.

The inorganic carbon dissolved in the medium is in three forms, \( \text{CO}_3^{2-}, \text{HCO}_3^-, \text{CO}_2 \). The weight fraction of each species is a function of the pH. The total inorganic carbon is a function of the partial pressure of \( \text{CO}_2 \) in the gas over the liquid which is in equilibrium with the aqueous \( \text{CO}_2 \). The data of Goldman et al. (73) on the fixation of \( \text{CO}_2 \) in *Scenedesmus quadricauda* during autotrophic growth show that the total inorganic carbon in the medium should exceed 8 mg/l. in the pH range of 7.0 to 7.5 in order not to limit the growth rate. Myers (167) found that the growth of *C. pyrenoidosa* during photosynthesis was inhibited when the \( \text{CO}_2 \) in the gas phase exceeded 6%.
Light supplies the energy for the growth of algae. Several studies have determined the saturation light intensity for Chlorella. Krauss (120) gave the saturation intensity for C. pyrenoidosa as 500 ft-candles of 25°C while Brown and Richardson (26) found the saturation intensity to be approximately 300 ft-candles and found no inhibition up to 2800 ft-candles. But Myers and Burr (167) determined that light intensities over 12,000 ft-candles inhibit the growth of C. pyrenoidosa.

The prehistory of a cell before it is transferred to a new environment as an inoculum was important in the biological response of the cell in its new environment (177). The growth stage, the limiting nutrient, the light intensity and the temperature all have their effects on the response of the inoculum. It may take one or two generations of cell division to overcome these effects.

Chlorella show bactericidal effects. Enteric bacteria and pathogenic bacterial species did not survive during the photosynthetic growth of C. pyrenoidosa and C. vulgaris (44). The bacteria that survived or became the predominant bacterial species in culture of Chlorella was the genus, Pseudomonas (53, 152, 230, 231, 234). Pseudomonas are common to fresh waters (51).

3.13 Concluding Remarks

This chapter has incorporated material beyond that required to discuss the experimental results of the research work. The purpose was to place the experimental work into an overall perspective from which the research idea evolved. Further the biological field is
vast and complex. The results of diverse biological studies contribute
to the understanding of laboratory microorganism growth experiments
as well as the natural aquatic environment and its resident micro-
organisms.
CHAPTER IV

EXPERIMENTAL PROGRAM

The initial research goal was the modeling of the P dynamics in a laboratory ecosystem in which an alga and a bacterium are grown in a defined medium. The modeling concept was to be based on the Grenney et al. (80) proposal for modeling nitrogen dynamics. In addition, a preliminary study was to be made into the significance of the presence of clay particles on the system P dynamics. The application of extraction techniques to cellular P analysis during the growth of the alga and the bacterium was to be investigated and evaluated in terms of obtaining data for incorporation into the Grenney model. A long term goal, though not included in the research proposal, was the application of developed analytical techniques and mathematical model to a natural aquatic ecosystem. The extraction techniques that are proposed were chosen so that they were simple and would not increase significantly the work load of an extensive nutrient analysis of natural water systems as already applied. The achieved goals were of a more limited nature as related in the remainder of this chapter.

Initial examination of the literature relative to P dynamics in laboratory cultures and in natural ecosystems led to the conclusion that the construction of a batch growth system, over a continuous
system was preferred for the experimental study. This conclusion was not held throughout the experimentation but the batch growth system was locked into the constructed equipment. The choice of the batch growth system was based on three factors. First, initial data found in the literature on algal and bacterial growth and cellular P concentrations in P-limited growth was in batch cultures. These data would be helpful as a good reference to compare with the experimental results. Second, microorganism growth in rivers and first bloom formation in lakes show dynamics that can be simulated in the laboratory with batch cultures. Third, the sample size requirement of necessary analytical techniques was too large to obtain a sample from a continuous culture without significant change of the sample during collection.

The batch growth system was constructed, evaluated and calibrated. The system included environmental controls and monitoring for temperature, pH, light intensity, mixing and dissolved oxygen. The monitoring equipment was chosen to be compatible with interfacing to a data acquisition digital computer.

The alga that was selected for experimentation was *Chlorella pyrenoidosa*, a green alga of the division *Chlorophyta*. This organism has been widely studied. It is a large, spherical, singular, non-motile cell that reproduces asexually and can grow in a simple inorganic medium with continuous light. The requirement of the bacterium was to grow compatibly with the alga in which neither organism inhibited the other except through the reduction of nutrients in the
medium. Thus preliminary work was conducted toward the isolation and identification of a bacterial species.

In the literature it was found that P-limited cell growth rate is a function of an internal concentration of a P compartment in algae. The literature related numerous methods of extracting and separating the P compartments of both algae and bacteria. The most comprehensive method for the separation of P components of the cell was presented by Miyachi et al. (158). This technique plus others noted in Section 3.5 are tedious and lengthy in time. The application of extraction and separation techniques of cellular P that were simple and reliable was required. To achieve this goal there is a sacrifice in the comprehensiveness of the results. Two cellular extraction methods were selected for the research: cold TCA extraction as outlined by Miyachi et al. (158) and the boiling water extraction as presented by Fitzgerald and Nelson (64). Each of these techniques extract a different spectrum of P from fresh cells. The extracted P can be separated into three fractions by determining the amount of o-PO$_4^-$ present in a volume of the extract treated in one of three ways: 1) a volume with no pretreatment is analyzed for o-PO$_4^-$, 2) a volume is subjected to acid hydrolysis at 100°C for fifteen minutes (another volume was also to be hydrolyzed for seven minutes) and the released o-PO$_4^-$ is determined, and 3) a volume is subjected to acid persulfate digestion for total P and the released o-PO$_4^-$ is measured. In addition, the total P of fresh cells is determined. The literature provides information of the P compounds separated in the cold TCA extract during the growth of both algae and bacteria.
There is little information concerning the P compounds separated in the boiling water extraction except that the P determined in the untreated fraction correlates with poly-P in the cell. An assessment of the P compounds will be made from the experimental data obtained during the growth of *C. pyrenoidosa* and compared to data on the cellular P dynamics obtained through other analytical methods cited in the literature.

A series of preliminary experiments were carried out. Growth rate and cell yield was identified for both the *C. pyrenoidosa* under o-PO$_4$$_4$ limitation and the bacterium under the carbon energy source limitation. These experiments were conducted in flasks on a shaker table. A determination was made of the range of operating conditions under which the batch growth of the alga and the bacterium could be operated in the fermentor apparatus. Further these growth runs evaluated the analytical techniques which were proposed to determine inorganic carbon, chemical oxygen demand (COD), nitrate of the growth medium; and the cell counts, cell dry weight, cell volume and cell morphology in addition to the P analyses previously outlined. These runs also determined the feasibility of applying each of the analytical techniques and the time scheduling of analyses in order to obtain experimental data.

The experimental runs of axenic cultures of *C. pyrenoidosa* were made in continuous light, 12,000 ft-candles at the external surface of the culture, at 25°C with CO$_2$ enriched air passing through the medium. An inorganic defined algal medium was used to which adjustments or additions were made in the composition. Experimental growth
runs were conducted at a pH of either 6.5 or 7.5 in which the P was approximately 1.4 mg PO₄-P/l. This concentration of P was limiting the growth of the alga stoichiometrically in terms of the cell yield but did not limit the specific growth rate. The original intention was to reduce the P concentration as experience was gained in the use of the analytical techniques. The composition of the medium was varied. The correlation of algal growth rate with an extracted P fraction was assessed.

During the preceding algal growth experiments P was found to be associated with Fe and Ca precipitates. A series of experiments were conducted to identify the precipitates, the dynamics in a non-growth culture and the error that is contributed to the cell P analysis in the previous experimental runs. Additional experimentation is carried out to determine procedures that avoid the precipitation under the environmental conditions used. The investigation of the inorganic precipitates added a dimension to the original proposal not considered and limited the scope of the research as originally planned.

The research results are discussed in light of the original program goals. Improvements in analytical techniques, medium preparation, and experimental approach are presented.
CHAPTER V

EQUIPMENT, MATERIALS AND PROCEDURES

5.1 General

A controlled environment was required to carry out the study of the phosphorus dynamics during the growth of the alga, Chlorella pyrenoidosa. The fermentor apparatus was constructed to fulfill the requirements outlined in the previous chapter. The discussion in the Equipment section is organized according to subsystems of the fermentor apparatus. Each subsystem section describes the function of the subsystem, the equipment used, and specifications. The subsystem limitations experienced during the research work are discussed in terms of solutions in order to improve the equipment for future research work. Calibration procedures of subsystem equipment and the calibration curves are found in Appendix B.

The analytical techniques and experimental methods used during the research are discussed in general in the Procedures section. The errors and limitations of the methods and procedures experienced during the research work are described. The detailed procedures and methods and the calibration curves are found in Appendix C. The equipment, chemicals, and other material requirements for the analytical analyses or research methods are listed with each detailed procedural outline.
5.2 Equipment

A controlled environment for the growth experiments was provided in a batch fermentor apparatus (see Figure 5.1). The monitoring and control systems were constructed to be compatible for interfacing with the Ohio State University Chemical Engineering Department's Digital Equipment Co. PDP-15 digital computer. Available bench scale fermentor systems and the associated monitoring and control equipment did not meet requirements in terms of suitable DC output from the monitoring instruments for analog-to-digital signal conversion for the computer. Further, any control feedback actions by the computer could not be actuated with relative ease on these systems. Therefore, purchased and built monitoring and control systems were assembled around a purchased fermentor vessel to achieve compatibility. The discussion of the fermentor apparatus is divided into subsections each of which describes a subsystem.

The extent of this research program in terms of equipment was the purchase, assembly and evaluation through experimental use of the monitoring system on the fermentor apparatus. The actual implementation of computer feedback control and required computer software was left for development at a later stage.

5.2-1 Fermentor Vessel

The fermentor vessel consisted of a Pyrex® fermentor jar, head assembly, and baffles. This was a 20 liter VirTis Co. Model 43-120 vessel purchased with external magnetic drive assembly. Dimensions of the vessel and the assembly head arrangement of the vessel probes are
Figure 5.1 Batch Fermentor Apparatus.
shown in Figures 5.2 to 5.5 respectively. Discussion of probes shown in these figures are in the following sections. The insert in Figure 5.2 gives the important dimensional ratios of the fermentor vessel. The ratios used in the construction of the vessel are equivalent to ratios in correlations of experimental mixing vessels (1,11,177). The impellers are 6 bladed flat disk turbines. The internal shaft is isolated from the outside of the vessel. The shaft contains a ceramic magnet which is driven by an external magnet through a thin stainless steel diaphragm. The materials of construction of the fermentor vessel are 316 stainless steel, teflon, and neoprene rubber.

5.2-2 Lighting Subsystem

For photoautotrophic growth of *Chlorella pyrenoidosa*, light was supplied by an array of General Electric Cool White 15-watt fluorescent bulbs arranged in a manifold containing 16 tubes (see Figures 5.6 and 5.7). The spectral radiant output of the cool white bulb is in Figure 5.8 plotted in comparison to sunlight. The bulbs can be turned off or on in pairs and are arranged such that even light distribution can be attained when all, 1/2, or 1/4 of the bulbs are on (see Figure 5.14). The temperature of the air that is stagnant in the space between the manifold and the fermentor vessel can reach a temperature of 50°C. This was a thermal load on the fermentor vessel. Two blower fans (see Figures 5.6 and 5.7) were installed to draw air from the bottom of the manifold in order that cool room air would replace heated air.
Figure 5.2 Fermentor Vessel Dimensions
Figure 5.3 Positions of Probes in Fermentor Vessel
Figure 5.4 Internal Structures and Probes in Fermentor Vessel.
Figure 5.5 Arrangement of Probes and Structures Fermentor Vessel Head.
Figure 5.6 Dimensions of Light Manifold
Figure 5.7 Light Manifold.
Figure 5.8 Spectral Output of Cool White Fluorescent Bulb
This arrangement was able to maintain air temperature in the air space approximately 5°C over the room temperature.

Light intensity was measured on the external surface of the fermentor vessel and internally at a point 2 inches from the external surface. The transducers for the light intensity were two Centralab 52CL Photovoltaic cells with spectral range of 400-1150 nm. The external cell was mounted on the glass surface of the tank at mid-height and circumferentially at a mid point between two adjacent illuminated fluorescent bulbs. The internal cell was sealed in an 8mm Pyrex® tube that extended 8 inches into the fermentor vessel. The calibration procedure and curve for these cells are in Section B.1.

5.2-3 Temperature Control Subsystem

Temperature control of the fermentor vessel consisted of a Benco/Grant Model SB 20 constant temperature bath capable of +0.1°C which had a pumping capacity of 11 liters per minute to the fermentor cooling coil and a heating capacity of 1550 watts. Cooling capacity was provided by the addition of cold tap water and supplemented with a refrigeration unit salvaged from an old Frigidaire refrigerator (see Figure 5.9).

The only difficulty encountered in maintaining the temperature of the bath occurred during the summer months when the temperature of the tap water and room temperature were above the temperature of the fermentor vessel. The refrigeration unit provided the additional cooling capacity for the fermentor vessel and an air conditioning unit cooled the room. Under certain combinations of conditions during
Figure 5.9 Temperature Control System.
summer, when tap water was too hot and room air conditioning was off, the system could not be operated at 25°C but only at 30°C.

The temperature of the fermentor vessel did not control the temperature of the bath. (Adjustments were made to the bath temperature regulator after monitoring the fermentor vessel temperature.) This would be the ideal design of the system and would require the elimination of the bimetal thermostat in the bath and its replacement with a contact thermometer or thermistor that sensed the fermentor vessel temperature and fed back to the temperature bath heater control. Calibrations of the thermocouples used to monitor temperatures of the fermentor vessel, room and the tap water, is found in Section B.2.

5.2-4 Mixing Subsystem

Power was delivered to the impeller in the fermentor vessel through an external ceramic magnet assembly. Power was generated by a B and B Motor and Control Co. DC 1/4 HP motor with 146 in. oz. torque. The power was transmitted through a 3-way Crown bevel gear drive (1:1 ratio) to the external magnet assembly (see Figure 5.10). The variable motor speed was regulated by a B and B Motor and Control Co. (see Figure 5.14) SCR-33 controller. The shaft speed was monitored with a Servo-Tek Products Co. DC generator. See Section B.3 for calibration of the speed transducer.

5.2-5 Dissolved Oxygen Subsystem

The dissolved oxygen concentration in the fermentor vessel was monitored with a New Brunswick Scientific Model M1016-0201 oxygen
Figure 5.10 Impeller Drive Mechanism.
probe. The galvanic current signal from the probe is conditioned with an amplifier designed by Johnson et al (113) (see Figure 5.14). The signal, as output on a microammeter, could actuate either a higher or a low relay about a set point on the meter. These relay signals could initiate a control action to bring the dissolved oxygen concentration back to the set point. The original design of the control system was to effect a partial pressure change in the gas which aerated the fermentor vessel. The relays were to actuate solenoid valves on the oxygen or nitrogen gas lines. Discussion in Section 5.2-6 and Figure 5.11, which shows the relays, expand on the concept. But later evaluation of the fermentor system showed that a change in the speed of the impeller could increase or decrease the rate of oxygen transfer to the liquid phase from the gas phase in the same manner as an increase or decrease in oxygen partial pressure. This seemed to be a more effective means of controlling the dissolved oxygen concentration in a dynamic system. Large quantities of oxygen and nitrogen may be required to effect a partial pressure change of the oxygen. The cost of this approach seemed prohibitive. Moreover, the system was not designed to handle automatic speed control. This type control theory would require either solid state electronic components added to the speed controller or a stepping motor attached to the rheostat on the speed controller each of which could be actuated through computer control.

Two operating difficulties developed during the use of the dissolved oxygen probe. First was the full scale drift of the output of the probe due to the limited internal filling solution which was
consumed with time. The drift was determined prior to the initiation
of a run in the fermentor vessel when a standard oxygen concentra-
tion in the liquid was run in order to calibrate the amplifier (see
Section B.4). The second difficulty occurred during the steriliza-
tion of the fermentor vessel. The New Brunswick Scientific Co. Model
AS00 autoclave cooled too rapidly to prevent flashing or local boiling
in the probe. This caused an over pressure in the probe which
ruptured the diffusion membrane. It was prohibitive to replace the
probe and any further measurements were deleted. Because of this
experience in excessive cooling rate in the autoclave, the vessel
was insulated and a nitrogen cylinder with a pressure regulation was
connected to a port in the pressure vessel in order to maintain the
saturation vapor pressure of water above the bubble point. To insure
sterility of the gas, the nitrogen was passed through a bacterial
filter that was sterilized during the sterilization of the fermentor
vessel, by passing for 2 hours small stream of steam from the auto-
clave through the filter.

Calibration of the dissolved oxygen probe is in Section B.4. The
volumetric oxygen transfer coefficient as a function of gas rate and
impeller speed determined by use of the probe is detailed in Section
B.5.

5.2-6 Gas Metering Subsystem

The gas metering subsystem was designed for the metering of known
quantities of nitrogen, oxygen, air, and carbon dioxide to the fermente-
tor vessel. The flow diagram is shown in Figures 5.11 and 5.12. The
calibration of the rotameters is discussed in Section B.6. The
Figure 5.11 Gas Metering System Layout
Figure 5.12 Gas Metering System.
pressure of the gas passing through the rotameters was measured after
the rotameter. After mixing the gases, they were humidified to pre-
vent excess evaporation of water in the fermentor vessel, water mist
removed and then passed through a Whatman Gamma-12 in-line filter
with a 0.3 μm filter before the gas entered the fermentor vessel.
The exit gas from fermentor vessel passes through a Nupro 8C check
valve with 1/3 psi opening pressure and through packed glass wool to
filter any backflowing condensed water.

The arrangement is not adequate and has great potential for back
contamination (though none occurred). Further even with inlet air
humified, there was significant water loss from fermentor which con-
densed in the outlet line. This could lead to the problem of back
contamination. This could be solved by placing a small heat
exchanger before check valve to condense water.

The design for oxygen partial pressure control of the gas stream
was to increase the flow of either nitrogen or oxygen in the gas
stream thus decreasing or increasing the molar ratio of oxygen. This
was carried out using solenoid valves (as seen in Figure 5.11) which
could be actuated by relays to increase or turn on the flow of
nitrogen or oxygen. If the intention was to increase the flow of
gas it was necessary to balance the pressure drops in the line with
the solenoid valve and the parallel line using the needle point valves
in each line. This design concept was not deemed as effective as
rpm control as mentioned in Section 5.2-5, but could serve as an
effective supplemental system if rpm control did not achieve the
required change in dissolved oxygen level.
The only operational problem that occurred was the filling of the air rotameter with oil. The level of oil in the air line was so low or dispersed that the demister in front of the rotameter was ineffective. Thus the rotameter became an effective oil separator. If oil did bypass the rotameter, the humidification column should eliminate it during gas contact with the water. With oil in the air rotameter the calibration curves were no longer valid. The air rate was set using the Precision Scientific Wet Test Meter and noting the rotameter level as the guide for the air rate. Total gas rate was also monitored with the Wet Test Meter.

5.2-7 pH Monitoring and Control Subsystem

The pH of the fermentor vessel was monitored with an Ingold combination electrode. The output of the probe was connected to a Leeds and Northrup Model 7415 pH meter through an Orion Research Model 605 Electrode Switch. Another set of pH electrodes, Leeds and Northrup Model 117169 glass electrode and Model 117417 reference electrode, were also connected to the pH meter through the electrode switch for measurement of pH samples external to vessel. Both of these electrode sets were calibrated with standard pH buffer solutions obtained from the Ohio State University Lab Stores.

The recorder output of the Leeds and Northrup pH meter was amplified by a Dana Model 2210 Data Amplifier set at 1000X and recorded on a Beckman Type R Dynograph set at 200 mv/cm. The voltage across the recorder input was read with a Tektronix Model DM601 Digital Multimeter. The output of the pH meter also was amplified
with a Dana Model 2210 Data Amplifier for a pH control circuit (see Figure 5.14). The control circuit consisted of a voltage supression circuit and a microammeter with high and low relays for relays for initiating control actions that would bring the pH back to the set point (see Figure 5.14). In constructing the pH control system the high and low relays were connected to acid and base pumps respectively. The pumps consisted of a 125 ml reservoir, a Masterflex Model 7014 tubing pump, Dupont Viton tubing (for high concentration HCl and NaOH solutions), and Bodine Electric Co. Type NSY gear reduced motor (3 rpm, 1/75 hp) (see Figure 5.13).

The monitoring and control system as described above was ultimately not used due to several important difficulties. The control circuit required an isolation amplifier for which the Dana amplifier should have qualified. No matter which Danna amplifier used, there was feedback from the control circuit to the Leeds and Northrup pH meter causing unreliable readings. The circuit control could be replaced by purchasing a new pH meter with expanded scale and with high and low pH relays built into the meter. For this research project pH was ultimately controlled by acid formed with the absorption of CO₂ forming a carbonate buffering system thus pH control as outlined above was unnecessary.

The second difficulty arose from the Ingold combination pH probe. The combination probe was sensitive to grounding and ground circuits to the pH meter (202). The combination probe could easily be calibrated against standard buffers and the second set of external probes in a glass beaker in which the solution was grounded. But the combination
Figure 5.13 Acid/Base Metering System.
Figure 5.14 Control Panel of Fermentor Apparatus.
when in the electrically grounded fermentor vessel did not respond in the same manner. Initially this could be compensated through zeroing of the pH meter by drawing samples from the fermenter vessel and reading the pH of the solution on the second pH probe set. Ultimately the combination probe could never be zeroed and would shift suddenly to a different pH value. The only explanation for the behavior was spurious electrical ground loops (202) to the pH meter which could not be corrected. Thus the Ingold combination pH probe was no longer used and pH was determined by drawing samples from the fermentor vessel and measuring the pH. The solution for the measurement of the pH in the fermentor vessel would be to use separate glass electrode and reference electrode which are steam sterilizable.

5.3 Materials

The algal organism used in the experimentation was *Chlorella pyrenoidosa* which was obtained from the Culture Collection of Algae at Indiana University (211) and was listed in their inventory as No. 252. The agar used in the preparation of the medium for the maintenance of the alga, as well as agar used in other media formulations, was produced by Difco Laboratories and obtained from the Ohio State University Lab Stores (see Appendix C.1 for medium preparations).

The chemicals used in the experimental runs and in the analyses are listed in the detailed procedures in Appendix C. Unless otherwise noted in the procedures, the chemicals were reagent grade and obtained from the Ohio State University Stores. The term "water" as
used in this dissertation refers only to demineralized double distilled water obtained from the Ohio State University Stores. Water for rinsing glassware was single distilled and supplied to the lab by the building piping system. Chromic acid for cleaning of glassware was prepared by the Ohio State University Lab Stores. Cylinder gases were 99+4 and obtained from the Ohio State University Lab Stores.

5.4 Procedures

This section covers procedures used during the research conducted in the preliminary and the experimental stages. The overall philosophy of the procedures and the accuracy of the analytical procedures are discussed. The detailed step by step procedures, which include the chemical and equipment requirements, are found in Appendix C. The following discussion is divided into four sections: 1) medium preparation, 2) experimental run preparation, 3) integration of the analyses during experimental run, and 4) analyses.

There is one general procedure that applies to all the following subsections. All glassware was cleansed with chromic acid followed by a thorough rinsing with single-distilled water to remove all the chromic acid residuals on the glass surfaces. This procedure removed all organics and the inorganic P, Ca and Fe from the glass surfaces, thus eliminating the contamination of a defined medium or interference with the analytical determinations.

5.4-1 Medium Preparation

Three media formulations were used in the research for the growth of C. pyrenoidosa and bacteria: 1) Bold's Basal Medium (BBM)
(15), 2) Proteose Peptone Agar (PPA) (211) and 3) Nutrient Agar (NA). The formula for each of the media is listed in Section C.1. The medium was prepared in distilled water.

Bold's Basal Medium is a widely used medium for algal growth for which no growth factors are required. This medium was used in a modified form for experimental growth cultures and inoculum culture of C. pyrenoidosa and the isolated bacterium. The modifications were made to meet experimental requirements. For P-limited growth, the amounts of KH$_2$PO$_4$ and K$_2$HPO$_4$ were reduced to obtain levels in the range of 1.5 to 53. mg P/l. The loss of K was replaced, if required, by the addition of KCl. The K concentration was in the range of 2.5 to 84 mg/l. But, if the level of NO$_3$ was increased, both the NO$_3$ and K were added in the form of KNO$_3$. The range of the inorganic nitrogen was 41 to 142 mg/l. A carbon energy source was added to the medium in most experiments. The principle energy source was sucrose, but glucose was also substituted. The energy source concentration range was from 0.2 to 20 gm/l with former being the concentration in all but a couple cases. Though the organic carbon is primarily for bacterial growth, it was present in most of the C. pyrenoidosa growth runs in order to determine the effect of its presence.

Proteose Peptone Agar medium was used to maintain C. pyrenoidosa in stock culture for three months. This formulation was based on BBM plus an organic nitrogen source, proteose peptone. The medium was found by Starr (211) to sustain an active culture for long storage time.
Nutrient Agar was a medium for the cultivation of non-photosynthetic organisms, i.e., yeasts, molds and bacteria. The medium was based on BBM modified with the addition of 200 mg glucose/l. The growth rate of the organisms that require a more complex organic medium are enhanced by the addition of Difco Nutrient Agar which contain beef extract and peptone. This medium was used to maintain a stock culture of the isolated bacterium and for contamination checks of the axenic cultures of C. pyrenoidosa and the pure bacterium cultures.

All the constituents of the media were combined and the agar media boiled to solubilize the agar before sterilization. The sterilization was in a steam autoclave maintained at 15-20 psig for at least twenty minutes.

5.4-2 Run Preparation

This section covers procedures that prepare the fermentor apparatus for an experimental run. The preparation is in two phases; that of the C. pyrenoidosa inoculum and that of the fermentor apparatus. The detailed procedures are found in Section C.2 which also includes the procedure for the preparation of the bacterial inoculum.

The preparation of the C. pyrenoidosa inoculum required that the culture be axenic, that a sufficient number of cells was available for initiation of the experiment and that the inoculum cells were not exposed to an o-PO₄ concentration greater than the experimental run. These conditions were met with a two-stage growth culture process. The first culture provided a dense culture of axenic cells in the stationary growth phase in a nitrate-limited growth medium. These
algal cells stored excess P (186). The second culture was at the same concentration of o-PO\(_4\) as the experimental run, which was P-limited. The cell stored P and the medium P yielded sufficient cells to inoculate the fermentor vessel to give a cell concentration of at least \(1 \times 10^3\) cells/cc.

The function of the preparation of the fermentor vessel for a growth experiment of \textit{C. pyrenoidosa} is to give a sterile environment in which the medium has no chemicals that contaminate the medium. All medium components were added before sterilization. The procedure insured that the monitoring systems were not damaged during sterilization and were functioning at calibrated levels. The control systems for the maintenance of the physical environment were checked to determine if they function properly. These included temperature, pH, impeller speed, light intensity and the gas rates of air and CO\(_2\). When these parameters were at the predetermined values, the algal inoculum was added and the experimental run begun.

\textbf{5.4-3 Integration of the Analyses During the Experimental Run}

The overall integration of the analyses during the experimental run was necessary to conserve both time and minimize the volume of culture required. The step by step procedure of an analysis cycle is listed in Section C.3 and summarized in Figure 5.15.

The procedure evolved during the preliminary experiments in the fermentor apparatus and the initial experimental runs. The complexity of the analysis cycle increased with the addition of the TCA extraction to the boiling water extraction of the algal cells after
Figure 5.15 Flow Sheet of the Overall Analysis Procedure
the first third of the experiments were completed. The goal of developing the scheme was to obtain up to seven consecutive days of data on an experimental culture. The analytical cycle was to be repeated at least once per day with four days, early in the growth run, in which the cycle was repeated twice per day. Since the main goal was to get P data during the growth of C. pyrenoidosa, the analyses for NO₃, COD, Fe and Ca were limited to a few experimental runs.

Several experimental runs were made in which the dynamics of inorganic particulate P was measured in a sterile, non-growth culture. The overall analysis cycle, as discussed above, generally was followed but was not applied daily since time was required for a more intensive analysis of the Fe and Ca in solution and in the precipitate. An experiment, which determined the desorption of o-PO₄ from the separated precipitates, was carried out several times during a run. The procedure is discussed in the following section.

5.4-4 The Analyses

The analyses are divided into four categories: cell observations, cellular P extraction, P determination, and auxiliary culture parameter determinations. Each technique is discussed in terms of purpose, limits and accuracy.

5.4-4A Cell Observations

Cell Count. A measure of the growth is the rate of change of the viable population. The population of C. pyrenoidosa was determined using a counting chamber of known volume under a microscope. The counting chamber was a hemacytometer which is more commonly used for
the counting of blood cells. The hemacytometer was chosen because the size of *C. pyrenoidosa* cells is comparable to that of red blood cells. For ease of enumeration, the cells were stained with iodine solution (82).

A population count of determined statistical significance was obtained by averaging several measurements. For each population determination, two independent collections of a volume of the *C. pyrenoidosa* culture were made and diluted, if necessary, in a graduated centrifuge tube to give an approximate cell count of $2 \times 10^5$ cells/cc. The population of each tube was counted eight times, twenty times if the population is less than $1 \times 10^5$ cells/cc. The average of the counts was recorded as the population.

Additional caution must be taken to obtain significant cell counts. The cell suspensions were well mixed before withdrawal of a sample was made. The cell suspension and the dilution volumes must be accurate. Care was taken when filling the counting chamber with a cell suspension. The proper technique was to allow the chamber to be filled by capillary action, otherwise a uniform distribution of cells in the chamber was not achieved.

Bacterial populations were determined by plate counts. The Petroff-Hausser bacterial counting chamber was not used because of the difficulty in distinguishing bacterial cells from inorganic particulates and algal cell remnants when using either an iodine solution or a methylene blue solution for staining. The former colors carbohydrates and the latter lipid compounds (82). The plate count is a method to enumerate the number of viable cells in a growth culture.
A given volume of a bacterial suspension is successively diluted, $10^2$, $10^4$, $10^6$, etc., times, and a known volume of each dilution was plated onto an agar medium in petri plates. After several days of incubation each bacterial cell will give rise to a macroscopic colony that can be counted. The viable bacterial count of the original sample is the number of colonies on the plate multiplied by the dilution factor and divided by the volume plated. For the bacterial experiments that were performed, 1 ml volume of cell suspension was successively diluted in 100 ml of 1% NaCl solution (the saline solution prevented cell rupture due to osmotic shock). A volume of 0.1 ml of each dilution was plated onto NA plates and incubated for four days at room temperature before the colonies were counted. The plate that gave a colony count of 30-300 was used to calculate the viable bacterial population. The count is only an estimate if the bacteria do not occur singly. Bacteria can exist in pairs, chains, clusters or packets (210). During the plating of a cell suspension, these agglomerations may or may not be separated. The agglomeration characteristics are a function of the stage of growth and environmental parameters.

**Cell Dry Weight.** Another measure of the growth of an organism is the rate of change of the total cell dry weight. Cells from a known volume of a culture are collected on a preweighed filter, dried and reweighed. The increase in the filter weight is the cellular dry weight.

For the research that is being presented in this work, the filters for the cell dry weight determinations were Whatman 4.7 cm diameter glass fiber filters, GF/D (2.7 μm) and GF/F (0.7 μm), which
were prefired at 550°C in a muffle oven for two hours, placed in numbered 50x12 mm plastic petri dishes and stored in a desicator containing anhydrous magnesium perchlorate. The glass filters were originally chosen for two reasons which were not carried through in the experimentation; the dried collected cells can be analyzed to determine the volatile suspended solids by ashing at 500°C and the C, N, O content in a Perkin-Elmer Elemental Analyzer. The reason for the need to prefire the filters at 550°C was to vaporize compounds on the filter in order to prevent error in later weight determinations and analyses when filters go through a similar temperature cycle in the elemental and volatile solids determinations. The filters were weighed on a Perkin-Elmer Autobalance which reads to 0.1 µg. Obtaining an accuracy of ±0.005 mg for a total filter weight of approximately 400 mg, the recorded filter weight was the differential weight between a standard glass filter and the measured filter. The change in the differential weight is the dry weight. During the storage of filters with or without dried cells in a desicator, the drift in weight was less than ±0.005 mg.

Preliminary experiments found that only 1 in 14,000 C. pyrenoidosa passed through the GF/D glass filter and 1 in 3000 bacteria passed through the GF/F filter as determined by plate count. Pressure up to 100 psig, was applied across the filter when the filtration rate decreased and no significant increase was found of cells passing through the filters. Therefore the dry weight in axenic cultures of C. pyrenoidosa was obtained with GF/D filters.
It was found in preliminary investigations that the GF/D filter was able to give a significant separation of bacterium from the *C. pyrenoidosa* when they were grown in the same culture. If a maximum of $1 \times 10^7$ cells of the alga are collected on the GF/D filter from a culture of less than $1 \times 10^5$ algal cells/cc, approximately 50% of the viable bacterial cells, as determined by plate count, passed through the filter. Increasing the number of algal cells collected on the filter reduced the number of bacterial cells that passed through the filter. The additional cells acted as a filter aid. The question not answered is, are the retained bacterial cells on the GF/D filter due to either clumped cells that are too large to pass, bacterial cells that are attached to the algal cells or the passage of the bacterial cells through the filter blocked by the algal cells filling the filter voids? Kroes (122) reported a technique of sweeping the filter surface with a magnetic stirrer placed near the filter surface to prevent significant algal cell buildup. This procedure may increase the efficiency of the separation of the bacteria from the algal cells.

In dense *C. pyrenoidosa* cultures with bacteria, a greater separation efficiency was found if the algal cells were settled out by a short centrifuging and passing the supernatant through the filter first. No further work was conducted into separation since the algal-bacterial cultures were not initiated.

Dry weight was determined by filtering a known volume of a well mixed cell suspension through the appropriate preweighed glass filter. The maximum dry weight collected on the filter should not exceed $2.5 \text{ mg}$ which is approximately $1 \times 10^7$ *C. pyrenoidosa* cells. This
weight is the suggested maximum dry weight sample which the Perkin-
Elmer Elemental Analyzer can take. After the cells were filtered,
20 ml of water was used to wash the walls of the filter holder and to
wash excess medium from the cells and the filter. The dissolved solids
in the medium would increase the dry weight value. The wash was fol-
lowed by an initial drying step with the passage of dry filtered
nitrogen through the filter and the cells for five minutes to remove
excess water. The filter was placed in a covered glass petri dish
whose bottom was filled with 8 mm glass beads. The filter was placed
in an air circulating oven set at 105°C for one hour (146). The
removal of the excess water and the support of the filter on the glass
beads during the oven drying prevented the glass filter sticking to
the petri dish. Loss of some of the fibers when the filter sticks was
an underdetermined weight loss of the filter. The weight loss can be a
significant part of the total dry cell weight on the filter.

It was found during the experimental runs that accurate volatile
suspended solids data could not be obtained. During the ashing
process of cells on the glass filter at 500°C, there was significant
filter weight loss in addition to the cell dry weight volatilization.
This weight loss by the glass filter itself is not a random process
and can be determined in order to correct data. But the presence of
metals from the filtered cells and metals in inorganic suspended
solids increased the rate of glass filter volatilization in an inde-
terminant way. Data that were obtained concerning this effect is
listed in Appendix D.
**Algal Cell Volume.** A third measure of cell growth is the rate of change of the total cell volume. *C. pyrenoidosa* volume was calculated from the cell diameter, if round, or the major and minor diameters, if oblong, measured under a microscope with a calibrated scale. Two or more cells were randomly selected and the diameter(s) measured during a cell count. This process was repeated for each cell count measurement till twenty cell diameters were accumulated. The volume of a round cell was calculated from the formula for the volume of a sphere.

\[ V_c = \frac{\pi D^3}{6} \]  
(5.1)

If the cell was oblong, or fusiform, the formula for the volume of the cell was that of a prolate spheroid.

\[ V_c = \frac{\pi AB^2}{6} \]  
(5.2)

where:
- \( V_c \) - Volume of the cell (cm³),
- \( D \) - Diameter of the round cell (cm),
- \( A \) - Major diameter of an oblong cell (cm),
- \( B \) - Minor diameter of an oblong cell (cm).

After the individual volumes were calculated, the average cell volume was determined and recorded.

**Algal Cell Morphology.** During cell counts, three general morphological changes in the iodine stained *C. pyrenoidosa* cells were followed and noted. First, the iodine staining characteristics gave an indication of the cellular content and localization of carbohydrate. Second, the number of daughter cells formed during the reproduction
process was noted. Finally, there was the determination of the percentage of the total *C. pyrenoidosa* cells that were fusiform.

### 5.4-4B Cellular Phosphorus Extraction

Cellular P extraction techniques were discussed in Chapter IV. The extraction techniques chosen for the research was cold TCA extraction, TCA (Ext), and boiling water extraction, $H_2O$ (Ext). Each extract contained a different spectrum of P compounds.

For each extraction method, *C. pyrenoidosa* cells were collected on GF/D glass filters. The maximum number of cells collected was $1 \times 10^7$. Larger cell numbers give difficulty to spectrometric P determination because the absorption was greater than 0.8. The filter holder walls and the algal cells were washed with 20 mg of the boiling water extractant to remove P associated with the culture medium in the filter and on the cell walls. The water extractant was BBM medium minus $\delta$-PO$_4$ with the total ionic strength maintained with KCl. The pH of the extractant is adjusted to 7.0 with the addition of NaOH as suggested by Fitzgerald and Nelson (64). Excess water was removed by passing dry filtered nitrogen through the filter and the cells for five minutes. The filter was then placed in a petri dish.

For each extraction method the filters and cells were dispersed in the extractant in a teflon pestle homogenizer within thirty minutes of collection. The filter containing the algal cells was placed in the grinding vessel with 10 ml of the extractant and the grinding process proceeded till the glass fiber filter was disintegrated. The homogenizer was thoroughly rinsed with the remainder of the extractant and combined with the first 10 ml.
The TCA extraction, TCA (Ext), was carried out with an 8% (by wt.) TCA solution that was stored at 0°C. At all times the extractant containing the cellular P was kept in an ice bath or in the refrigerator at 0°C. Vessels in which the extract is transferred are precooled to 0°C. After the grinding procedure was completed and the TCA washings of the grinder were combined and mixed with the ground filter (total volume is approximately 45 ml), the extraction time was thirty minutes. The suspension was centrifuged, the supernatant recovered and the residue resuspended in fresh cold TCA for another fifteen minutes (114). Again the suspension was centrifuged and the supernatant recovered. The majority of the glass fibers and the cell remnants are found in the residue, but a significant amount remained in suspension which could interfere in any spectrometric analysis carried out on the extract. The particles were removed by filtration through a 0.45 μm membrane filter. Through the preceding process the extract remaining in the residue, in the membrane filter and on the surfaces of the filter holder accounted for a loss of 1 cc or no more than 2% of the extracted P. The filtrate was made up to 100 ml and stored in the refrigerator until analyzed.

The boiling water extraction, H₂O (Ext), is conducted in a 250 ml erlenmyer flask with 100 ml of extractant. The extraction proceeds for one hour with the evaporated water condensed on a cold finger and returned to the flask. After cooldown, the contents of the flask were transferred to two 50 ml centrifuge tubes, centrifuged to settle the glass fibers and the cell remnants. The supernatant was filtered through a 0.45 μm membrane filter to remove the suspended glass fibers
and cell fragments. The filtrate was collected and saved for analysis. The total loss of extractant during the boiling process was approximately 1 ml which is an error of approximately 1% of the P determination.

The question that must be answered is the importance of the loss of P to or the gain of P from the membrane and the glass filters. Tests were carried out with standard o-PO₄ solutions to answer this question. It was found that MF-Millipore 0.45 μm filter do not contribute to P to P-free solutions if they were soaked in distilled water (50 ml/filter) for 24 hours before use. Membrane filters absorbed an insignificant amount of o-PO₄ when tests were conducted with standard solutions of 0.1 to 1.0 mg PO₄-P/l. To test the absorption of o-PO₄ by glass filters, standard solutions of o-PO₄ in 8% TCA and the water extractant were ground with GF/D and GF/F filters and the extraction procedures were carried out as described in the previous paragraphs. The o-PO₄ absorbed onto the glass fibers was too low to detect.

5.4-4C Phosphorous Determination

The P determination falls into two categories, spectrophotometric determination of o-PO₄ and the liberation of o-PO₄ by hydrolysis from organophosphorus compounds. Organophosphates of biological importance have a range of free energies of hydrolysis (111) and rates of hydrolysis under acid conditions (140). A separation technique was based on the rate of the liberation of o-PO₄ during acid hydrolysis (7,34,140,165). Five hydrolysis procedures were used
on the water and the TCA extracts: 1) the least severe hydrolysis occurs during the stannous chloride-molybdenum blue o-P\textsubscript{4} determination \([\text{H}_2\text{O(Ext)PO}_4\text{(SC)} \text{or TCA(Ext)PO}_4\text{(SC)}]\), 2) hydrolysis at 100°C in 1N HCl for seven minutes \([\text{TCA(Ext)PO}_4\text{(SC)HCl}]\) (184), 3) hydrolysis at 100°C in 1N H\textsubscript{2}SO\textsubscript{4} for fifteen minutes \([\text{H}_2\text{O(Ext)PO}_4\text{(SC)H}_2\text{SO}_4 \text{or TCA(Ext)PO}_4\text{(SC)H}_2\text{SO}_4}]\) (7), 4) hydrazinemolybdenum blue o-P\textsubscript{4} determination (3 and 5) the most severe hydrolysis, acidic persulfate digestion \([\text{H}_2\text{O(Ext)Tot-P} \text{or TCA(Ext)Tot-P}]\). The o-P\textsubscript{4}, released in the number 2, 3 and 5 hydrolyses, is determined by the stannous chloride-molybdenum blue spectrophotometric method (210). Greater details of each procedure are found in Appendix C.

**Liberation of Orthophosphate by Hydrolysis.** The o-P\textsubscript{4} in a sample was determined by the stannous chloride-molybdenum blue method, PO\textsubscript{4}(SC). It is inherent in the procedure that a sample is exposed to acid conditions (pH=1.0) at room temperature for up to fifteen minutes. Chamberlain and Shapiro (33) Rigler (188) and Leloir and Cardini (140) found hydrolysis of labile organophosphates during the application of this technique. This hydrolysis was applied to both the TCA and boiling water extracts in addition to the medium that passes through a 0.45 \(\mu\)m membrane filter.

A more severe hydrolysis was carried out in acidified samples at 100°C for different time periods. Rhee (184) used a seven minute hydrolysis in 1N HCl, PO\textsubscript{4}(SC)HCl, and he and Kanai et al. (114) applied a fifteen minute hydrolysis in 1N H\textsubscript{2}SO\textsubscript{4}, PO\textsubscript{4}(SC)H\textsubscript{2}SO\textsubscript{4}, to release o-P\textsubscript{4} from cellular P samples that contained TCA soluble and TCA insoluble Poly-P. These hydrolysis methods also released o-P\textsubscript{4}
from organophosphates contained in the sample that are less stable than Poly-P. Chayen et al. (34) found that under these conditions the nucleotidic bond with -PO$_4$ was stable. Therefore it was concluded that RNA, DNA and lesser polynucleotides do not release significant o-PO$_4$. Further it was concluded from Kanai et al. (114) that protein-P does not hydrolyze.

The procedure for each hydrolysis method was to add to a 25 ml sample in a 100 ml volumetric flask 5 ml of the appropriate 6N acid to give a 1N acid solution. The sample was brought to 100°C over an asbestos shielded gas flame in 1 1/2 minutes and placed on a hot plate, adjusted to maintain 100°C with no boiling, for the remainder of the hydrolysis period then placed in an ice bath to cool. The seven minute hydrolysis was made on only the TCA extract while both the TCA and water extracts were hydrolyzed for fifteen minutes. The o-PO$_4$ in each sample was determined by the stannous chloride-molybdenum blue method.

Hydrazine-molybdenum blue o-PO$_4$ determination, $P_4O_{10}(HM)$, gives a hydrolysis severity comparable to the fifteen minute hydrolysis above, $P_4O_{10}(SC)H_2SO_4$. This method was not applied to all the experimental runs. The procedure was as follows. A 25 ml sample in a 100 ml volumetric flask was acidified with 1 ml of sulfuric acid followed by the addition of 15 ml of 0.8M Na$_2$SO$_3$ solution. The solution was boiled gently for 30 seconds and immediately 50 ml of freshly prepared coloring solution, ammonium molybdate-hydrazine sulfate, was added. The mixture was heated on a steam bath at 85-90°C for 20 minutes then rapidly cooled to room temperature in an ice bath. The
The sample was diluted to the mark, mixed and read at 830 nm in a spectrophotometer against a blank that was carried through the same procedure. The hydrazine-molybdenum blue method was found to give erroneous results for the determination of o-PO₄ in the TCA extract even though a calibration curve was run with several standards in 8% TCA with the distilled water o-PO₄ standards. Orthophosphate standards were run through the persulfate digestion method and the results fell on the calibration curve determined with distilled water standards. The error of this method was less than 1% with a standard deviation of 0.0004 mg P/l. This method was applied to the water extract, the filtered medium and the persulfate digestion for the determination of the o-PO₄.

The hydrolysis of all the P in a sample to o-PO₄ was by acidic persulfate digestion (5). This method oxidized all the organic compounds releasing the bound P as o-PO₄. Two very stable organophosphates, adenosine monophosphate and uridine diphosphate, were completely hydrolyzed.

The acidic persulfate digestion was as follows. To a 100 ml sample in a 250 ml erlenmyer flask, 1 ml of H₂SO₄ and 8 gm K₂S₂O₈ were added along with a couple glass beads. The mixture was gently boiled for approximately 90 minutes on a hot plate till all the water evaporated and the residual liquid was colorless. If color was present, it was usually dissipated after several minutes as the H₂SO₄ fumes fog the flask. After cooling the flask, the residue was taken up with distilled water. Preliminary experiments showed that splashing, which occurs during the digestion, causes negligible loss of the o-PO₄. The
o-PO₄ was determined by the stannous chloride-molybdenum blue method.

**Orthophosphate Determination.** The o-PO₄ was determined by two methods: hydrazine-molybdenum blue and the stannous chloride molybdenum blue. The former was discussed previously. The stannous chloride-molybdenum blue method is widely used. Each sample to be analyzed must be near neutral pH. For the HCl and H₂SO₄ acid hydrolyses and the acidic persulfate digestion samples, significant amounts of 6N NaOH were added. The procedure is as follows. To a known volume of sample in a 100 ml volumetric flask, one drop of phenolphthalein indicator was added. If sample is colorless, 6N NaOH was added till a pink color developed. The pink color was dissipated with a strong acid solution. The samples were brought to room temperature (20-30°C) in a water bath before the coloration begins. To each sample was added, with thorough mixing after each addition, 4 ml of molybdate reagent, 10 drops of stannous chloride reagent and diluted to the mark with distilled water. The color intensity was read 15 minutes later in a spectrophotometer at 690 nm against a blank that was carried through the same coloration procedure. The temperature of the sample was recorded. The color development is a function of the temperature of the solution, for each 1°C increase there is a 1% increase in the color intensity. The isobutanolbenzene extraction for enhancing the sensitivity was not used. Rhee (184) and others (229) found good sensitivity without the extraction.

Four calibration curves were necessary for the determination of o-PO₄ by the stannous chloride-molybdenum blue method: 1) after the acidic persulfate digestion, 2) after the HCl hydrolysis, 3) after the
H₂SO₄ hydrolysis and 4) for the unhydrolyzed medium filtrate, boiling water and TCA extracts. The errors of the calibration curves were less than 1% with standard deviation of 0.0004, 0.0003, 0.0005 and 0.0005 mg P/l respectively. The standard deviations were determined for equal o-PO₄ concentrations under different hydrolysis conditions. For the TCA extract, the o-PO₄ was determined after hydrolysis procedures 1, 2, 3 and 4 (see above) were applied with a resulting error of less than 3% and a 0.0068 mg P/l standard deviation for a concentration range of 0.08 to 0.29 mg P/l. The water extract was hydrolyzed by procedures 1, 3 and 4 (see above) for a concentration range of 0.05 and 0.16 mg P/l. The error was less than 3% with the standard deviation of 0.0055 mg P/l for the o-PO₄ determined by the stannous chloride-molybdenum blue. An error of less than 2% and a standard deviation for repeated measurements of o-PO₄ in the filtered medium was 0.012 mg P/l when analyzed during a growth experiment in which the concentration of o-PO₄ varied from 1.18 to 0.30 mg P/l. For low concentrations of P (less than 0.30 mg P/l) the error was approximately 5% except for absorption values (<0.005) close to the blank value.

5.4-4D Auxiliary Parameter Determinations

Several culture parameters were determined during the course of a growth run that have not been previously described. These analyses were not applied to every experimental run since there was only limited time available for these additional parameter measurements. The medium, filtered through a 0.45 µm membrane filter, was analyzed for COD, NO₃, total Fe and total Ca. In preliminary non-growth experimental
experiments, the medium was analyzed for total inorganic carbon. The *C. pyrenoidosa* algal cells were analyzed for chlorophyll content as a function of the culture age. In non-growth experimental runs the amount of o-PO$_4$ that can be desorbed from the inorganic suspended solids was measured as a function of age of the solids. The detailed procedures for these determinations are found in Appendix C except for the chlorophyll analysis and the desorption procedure.

**Determination of the Chemical Oxygen Demand.** The COD determination is a measure of the amount of oxygen required to oxidize the portion of the organic matter present in the water sample susceptible to the oxidant used (K$_2$Cr$_2$O$_7$) in the presence of sulfuric acid. The dichromate oxidizes reduced inorganics, such as nitrite to nitrate or ferrous to ferric, and most of the organics. Dichromate does not appreciably oxidize aromatic hydrocarbons and pyridine but straight chain aliphatic compounds are oxidized in the pressure of silver sulfate as catalyst. Ammonia is not oxidized by dichromate.

For this research the COD of the medium filtered through a 0.45 μm membrane filter was determined. The defined BBM medium contained 44.4 mg EDTA/l; but in many of the growth experiments, 200 mg sucrose/l was added to the medium. Sucrose and EDTA were the only oxidizable matter, either organic or inorganic, that was present at the above concentrations, exert 205.6 and 68.1 mg COD/l respectively. Any variation in the COD of the medium was the result of microbial activity, such as cellular uptake of organics, extracellular release of organic matter or the conversion of the initial organics to a form not oxidized by dichromate.
For the COD determination, a known sample volume containing the organic matter was refluxed for two hours in the presence of known amounts of dichromate and sulfuric acid. After refluxing the excess dichromate was titrated with standard ferrous ammonium sulfate to the reddish-brown endpoint of the ferroin indicator. The amount of oxidizable matter, measured as an oxygen equivalent is proportional to the milliequivalents of dichromate consumed during the refluxing. A sample blank was carried through the COD determination to eliminate any contribution of COD from the reagents. The error of the COD determination in the 50-500 mg COD/l was less than 2% with a standard deviation of 11.6 mg COD/l.

**Determination of Iron.** The determination of total Fe of the unfiltered growth medium and the medium filtered through a 0.45 μm membrane filter was made. The amount of Fe that was present on the filters after filtration was also determined. Membrane filters in porcelain crucibles were ashed in a muffle oven and the residue was analyzed for iron. Glass filters were fired in a muffle oven to ash the collected particulates. The residues on the glass filters were analyzed for iron. The detailed ashing procedures are found in Sections C.6-2 and C.6-3.

Iron was detected spectrophotometrically by complexing soluble ferrous iron with phenanthroline. Organically bound Fe was freed by wet ashing (see Section C.6-1) of the organic compounds. This procedure also oxidized reducible organics and inorganics that were present in the sample. To insure the solubilization of particulate iron, the sample was boiled in HCl before the iron was reduced from the ferric state to the ferrous state with hydroxylamine. Elimination of
the reducible organics was necessary before the hydroxylamine was added. After the reduction procedure, the pH of the sample was adjusted to 3.3 with NaOH. The absorbance of the sample at 510 nm was read 30 minutes after the addition of the buffer solution and the 1,10 phenanthroline complexing agent. A sample blank was carried through the coloration procedure for each type ashing process. The error of the calibration curve was found to less than 1% with a standard deviation of 0.0004 mg Fe/l.

**Determination of Calcium.** The concentration of Ca in the medium that passes through a 0.45 μm membrane filter and the Ca concentration of the residues of the suspended solids collected on membrane filters were determined on a Perkin-Elmer Atomic Absorption Spectrometer. The sample preparation before the analysis was important. The dissolution of Ca in the inorganic particulates and the freeing of Ca from organic compounds required a digestion process which maintained low dissolved solids concentration and required no sulfuric acid. Thus, the sample was wet ashed as described in Section C.6-1.

An error of approximately 10% was found in the Ca determination. This error was too high for the detection of Ca by the difference between two samples of similar Ca concentration. Absolute concentrations of Ca of less than 2 mg Ca/l were able to be determined with less than 5% error.

**Determination of Nitrate.** Nitrate concentration was determined in the medium filtered through a 0.45 μm membrane filter. The sample was passed through a column containing granulated cadmium-copper granules which reduces nitrate to nitrite. The nitrite concentration
was determined by combining with sulfanilamide and N-(-napthyl)ethylendiamine dihydrochloride to form a highly colored dye that was detected spectrophotometrically at 540 nm. The absorbance was read 30 minutes after coloration. The efficiency of the column was tested with standard nitrate solution each day. When there was a drop in the efficiency in the column, the granulated cadmium-copper was regenerated. The error of the nitrate determination was less than 1% with a standard deviation of 0.0015 mg N/l.

**Determination of Inorganic Carbon.** The total inorganic carbon and dissolved CO$_2$ was determined only in preliminary non-growth experiments. These measurements were made to find out if Henry's Law predicts the dissolved CO$_2$ concentration. The preliminary analysis determined the total inorganic carbon in the medium as a function of pH and the ionic strength for known partial pressures of CO$_2$ in the gas that aerated the fermentor. Experimental growth run conditions were chosen that gave total inorganic carbon concentrations above the rate limiting values.

The method for soluble inorganic carbon analysis was the Differential Titration Method (3). The principle of the analysis is the titration of a water sample from pH 8.5 to 5.0 with standard alkali and standard acid as appropriate. The sample was then acidified, boiled to remove the inorganic carbon as CO$_2$ and followed by retitration of the sample to the same pH points. The total inorganic carbon was calculated as a function of the change of milliequivalents of base used for the two titrations. The weight fraction of each species, CO$_2$, HCO$_3^-$, CO$_3^{2-}$, is a function of the pH (3).
Determination of the Chlorophyll Concentration of Algal Cells.
The pigment content of the alga was determined. The pigment of *C. pyrenoidosa* are chlorophyll \( \alpha \) and \( \beta \), which convert light energy to chemical energy, and pheophytin, which is unreactive chlorophyll because of the loss of the active metal, Mg, from the chemical structure. The analysis of the alga samples was made by researchers from Project Clear of Ohio State University. The technique used by Project Clear was SCOR/UNESCO which is summarized by Fay (58).

The principle of the method was the extraction of the pigments from the *C. pyrenoidosa* cells with 90% acetone collected on Whatman GF/C glass fiber filters. The absorbance of the extract is measured at four wavelengths on a spectrophotometer and the concentration of each pigment is calculated from the formula presented in the SCOR/UNESCO method.

Desorption of Phosphate from the Inorganic Floc. A measure of the change in the inorganic suspended particulates during non-growth experiments was the desorption of \( \text{o-PO}_4 \) from the floc collected on a membrane filter. One liter of the medium from the fermentor was filtered and the floc was retained on a 0.45 \( \mu \text{m} \) MF-Millipore membrane filter. The filter was removed from the filter holder, which was washed with water, and the filter was replaced in the holder. Three times, 100 ml of a fresh desorbatant was passed through the floc at a rate of 1.5 ml/min. The desorbatant was BBM medium minus \( \text{o-PO}_4 \), Fe and EDTA with the pH adjusted to 7.5. The desorbatant simulated the medium of the fermentor depleted in \( \text{o-PO}_4 \) and determined the ease of desorption of \( \text{o-PO}_4 \) from the floc to the medium. Iron and EDTA was not
included in the desorbant because of possible Fe floc formation and to prevent any free EDTA from solubilizing Fe in the floc on the filter. The desorbant was analyzed for o-PO$_4$ and Fe.

5.5 Concluding Remarks

This chapter has presented the description of both the experimental equipment and procedures. Some conclusions and recommendations were given concerning the experimental apparatus. These comments were made so that improvement in experimental technique can be made if the research program is carried on. The discussion also included results concerning the analytical procedures which were obtained during the development of the procedures.
CHAPTER VI

RESULTS OF THE EXPERIMENTAL WORK

6.1 General

This chapter presents the pertinent data obtained during the experimental stage of the research program. The results are divided into four major sections: 1) preliminary growth experiments of C. pyrenoidosa, 2) identification of the isolated bacterium, 3) the Ca and Fe inorganic particulate dynamics and 4) experimental growth dynamics of C. pyrenoidosa. The preliminary algal growth experiments was an early learning stage during which approximate specific growth rate and ultimate yield of C. pyrenoidosa as a function of the o-PO$_4$ concentration were determined in the erlenmyer flask cultures. The preliminary research also included the initial algal growth experiments in the fermentor apparatus. The next section covers the results of the identification of the isolated bacterium. The bacterium was to be used in the growth experiments with C. pyrenoidosa as outlined in the original proposal of the research program. The experimental growth dynamics of C. pyrenoidosa include all the experiments in the fermentor apparatus during which the extraction of P compounds from the cells were conducted. From the results of these experiments are drawn the information to assess the goals of the research effort as outlined in Chapter IV, Experimental Program. The results and discussion of the inorganic particulate dynamics experiments in the
fermentor apparatus are included in this section. These results were required to evaluate the influence of the particulates on the P assays obtained during *C. pyrenoidosa* growth.

6.2 Preliminary Growth Experiments of *C. pyrenoidosa*

The preliminary growth experiments are divided into two phases: cultures grown in erlenmyer flasks on a shaker table and cultures grown in the fermentor apparatus. The results of each of these phases are limited in scope and will be presented as a summary of major observations.

Experiments were conducted at room temperature on a shaker table (100 rpm) under continuous light (450 ft-cd) in BBM. These growth experiments were carried out with *C. pyrenoidosa* cultures that were contaminated with bacteria. Several general results were determined that had an influence on the acquisition of significant experimental data from P-limited cultures of *C. pyrenoidosa* in the fermentor apparatus. An initial observation was that the concentration of o-P\textsubscript{4}\textsuperscript{-}\textsuperscript{3} initially in the medium used for the inoculum culture must be less than or equal to the concentration of o-P\textsubscript{4}\textsuperscript{-}\textsuperscript{3} in the P-limited experimental culture. Otherwise the cell growth did not reflect the culture medium environment. This phenomenon is greatest when NO\textsubscript{3} was the limiting nutrient in the inoculum. The *C. pyrenoidosa* can store P in excess to its needs under this condition (186).

It was found that the exponential growth phase spanned 3 to 5 days depending on the medium concentration of o-P\textsubscript{4}\textsuperscript{-}\textsuperscript{3} and the initial population of organisms. The exponential growth phase increased in
length with increased o-PO$_4$ concentration or with lower initial cell population of *C. pyrenoidosa*. The exponential phase was observed in a significant number of growth cultures to have a degree of synchronous cell divisions. When only one cell population count was made per day, the specific growth rate was difficult to measure. If two to three cell population counts were made in a 24 hour period, the rate could be determined more reliably. Synchronous growth imposed a sinusoidal function on the specific growth rate. The period of this sinusoidal function was approximately 28 hours.

Other generalized features of the growth curve of *C. pyrenoidosa* growth in a flask were found in the preliminary cultures. The lag phase was observed to have no consistent behavior. The lag phase period varied from 0 to 82 hours. The only influence that may have controlled this behavior was the history of the inoculum. The maximum yield of *C. pyrenoidosa* under P-limitation was 1.12 x 10$^7$ cells/mg P at both 0.026 and 0.133 mg o-PO$_4$-P/l. The competition of bacteria for o-PO$_4$ in these growth cultures was minimized by not providing an organic energy source to the cultures.

The maximum specific growth rate of *C. pyrenoidosa* was observed to be a function of the glucose concentration under P-limitation in the cultures contaminated with bacteria. Under N-limitation the maximum specific growth rate of *C. pyrenoidosa* cultures contaminated with bacteria increased 15 percent in the presence of 200 mg glucose/l. Glucose has been shown to increase the specific growth rate for axenic cultures of *C. pyrenoidosa* (194). The affect of the presence of glucose was illustrated in another experiment. The cells
of an inoculum of *C. pyrenoidosa*, which were grown under P-limitation, were washed in saline solution to remove any traces of P on the surfaces of the cells and transferred to cultures in which no P was present. The cultures either contained 200 mg glucose/l as an energy source or no energy source. The culture containing the glucose was found to have a maximum specific growth rate of 0.002 hr⁻¹ while the culture containing no glucose did not increase in cell population.

Several factors limit the application of the results of the growth experiments of *C. pyrenoidosa* in the erlenmyer flasks. The temperature of the batch cultures was not controlled to ±0.5°C. Carbon dioxide limitation will be encountered resulting in the increase of pH when high concentrations of the alga are in the exponential phase. The agitation of the flasks by a shaker table does not provide a high rate of CO₂ mass transfer from the air to the medium that will meet the demand.

With the information gathered from the growth of *C. pyrenoidosa* in the erlenmyer flasks, a series of four preliminary growth experiments were carried out in the fermentor apparatus. The purpose of these preliminary experiments was to both assess the maximum specific growth rate and to determine operating conditions which will minimize the shortcomings of the flask cultures as expressed in the previous paragraph. Further the cultures of the alga in the fermentor apparatus provided sufficient cell masses and medium volumes to evaluate the analytical techniques that were proposed to obtain data during the dynamic growth of *C. pyrenoidosa*. The importance of this work was both to discover the limitations in the techniques in order both to
improve or to eliminate a technique and to gain information for the integration of all the techniques for efficient data acquisition.

The major results obtained from the preliminary growth experiments of \textit{C. pyrenoidosa} in the fermentor apparatus will only be summarized. The maximum specific growth rates of the alga fell in the range of the growth rates obtained in the flask experiments (0.07-0.09 hr\textsuperscript{-1}). The growth of the alga was found to be limited by CO\textsubscript{2} near the end of the exponential growth phase. A CO\textsubscript{2} volume fraction of 0.005 in the air fed to the fermentor apparatus was sufficient to overcome this limitation. A minimum air rate was determined which could maintain a constant DO concentration of the medium during maximum bacterial utilization. This was required if later bacterial experiments were carried out in the fermentor apparatus. This condition was to be used during the growth of the axenic cultures of \textit{C. pyrenoidosa} to obtain data under equivalent DO conditions that would be used when both a bacterium and the alga were grown in the same culture. A minimum air rate of 5.0 l(STP)/min. was required for an organic energy source concentration of 200 mg sucrose/l when P did not limit the bacterial growth.

6.3 The Isolated Bacterium

In the course of conducting the preliminary growth experiments of \textit{C. pyrenoidosa}, which were microbially contaminated, it was found that one microbial type would predominate in the experiments conducted in the flasks as well as the fermentor apparatus. The nature of the colony formed by the predominant contaminating microbe on NA plates was
similar in all cases. The colony characteristics were as follows.
The colony edge can be described as entire (circular and smooth) with
a profile that was umbonate (protruding nipple in the center surface).
The surface of the colony was smooth and shiny. The colony was
opaque to the transfer of light.

The contaminating microbe described above was isolated from a
contaminated culture of C. pyrenoidosa by the successive dilution
technique which was used in microbe population counts and is described
in Section 5.4-4A. A microbial colony of the predominant contaminant
was chosen from an NA plate on which a colony was found to be segre-
gated from other colonies as indicated by microscopic inspection. A
portion of the colony was transferred to sterile BBM with 200 mg
sucrose/l and incubated for a week at room temperature. This culture
was submitted to the same isolation treatment twice in succession to
ensure that the isolated microbe was pure. This procedure was required
before the identification of the microbe could proceed. Initial
microscopic inspection of the microbe showed a pure culture that was a
rod-shaped bacterium, approximately 1.0 μm long. The Gram stain was
negative.

The bacterium was submitted to a microbiological procedure for
identification. The procedure tests the ability of the bacterium to
grow on different medium preparations. The series of media include
differential media and a series of media which are complete but differ
in the type of the organic energy source. The identification proced-
ure was made possible by Dr. P. Dugan of the Microbiology Department
at The Ohio State University. With the knowledge of the reaction of
the bacterium to the different media and the guidance of Bergey's Manual of Determinative Bacteriology (28), an identification of the bacterium could be made. The reaction of the isolated bacterium to the media is listed in Appendix D with the list of the media used. The bacterium was identified as the genus Pseudomonas. Identification of the species of Pseudomonas would have become an extensive project and was not carried out. Pseudomonas are ubiquitous soil organisms found also in the air and water (21). Pseudomonas are found to be a predominant bacterial contaminant of Chlorella in numerous studies (53, 152, 230, 231, 234).

No detailed growth dynamics of the Pseudomonas were carried out since the experimental work of this research concluded with only axenic cultures of C. pyrenoidosa.

6.4 C. Pyrenoidosa Growth Experiments and Inorganic Particulate Dynamics

This section presents the principle results of the experimental research. The goal of the research effort was to fractionate the P components of the alga C. pyrenoidosa into groups to identify the components in general terms and to relate them to the growth dynamics of the alga. Therefore, growth data of C. pyrenoidosa is presented along with the dynamics of the P compartments. In addition to this primary data, information concerning the morphological changes in the cell and changes in important uncontrolled parameters of the medium during dynamic growth are shown.

An alteration in the steady state value of a chemical parameter of the medium, pH, from a slightly acidic, 6.4-6.8, to a slightly
basic, 7.5, environment for the experimental growth runs introduced a significant change. A pH of 7.5 could not be maintained at a 0.5% CO₂ in air. In fact, air without CO₂ enrichment gave pH values below 7.5. It was found that the addition of the base NaOH to the medium formed a buffering system of CO₃²⁻, HCO₃⁻ and CO₂ which was able to maintain the required pH under an enriched CO₂ concentration in air. The unanticipated side effect was the formation of a precipitate that bound or adsorbed o-PO₄ giving a significant error in the amount of P components extracted from C. pyrenoidosa. Further, the precipitate introduced a less significant error in the cell dry weight determinations. Dynamics of these particulates in terms of concentration, composition and ability to contribute error to P extractions are presented in this section. These results were required to evaluate the particulate effects on the P extraction data and the dry weight data.

6.4-1 General

There were 18 experimental runs made in the fermentor apparatus; 13 were C. pyrenoidosa dynamic growth runs and 5 were inorganic particulate dynamic runs. Table 6.1 summarizes the run parameter data.

The initial nutrient concentration of P, N and K in the fermentor were different than the nutrients found in the BBM (see Table C.1). In addition an organic energy source, sucrose, was added. The concentration of P, the most important nutrient of this study, was 1.39 ± .01 mg PO₄-P/l (excluding run F-6) at the beginning of an
## TABLE 6.1 Run Conditions

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Conc. PO₄ as P (mg/l)</th>
<th>Conc. NO₃⁻ as N (mg/l)</th>
<th>Conc. K⁺ (mg/l)</th>
<th>Light Intensity</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>Stirring Rate (rpm)</th>
<th>Sucrose Conc. (mg/l)</th>
<th>Hours before Inoc.</th>
<th>5N NaOH Added (ml)</th>
<th>Lag (Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-6</td>
<td>1.96</td>
<td>41.2</td>
<td>3.1</td>
<td>1/2</td>
<td>6.4 ± .1</td>
<td>25</td>
<td>450</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+2</td>
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<tr>
<td>F-7</td>
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<td>41.2</td>
<td>2.4</td>
<td>1/2</td>
<td>6.8 ± .1</td>
<td>25</td>
<td>450</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>-10</td>
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<tr>
<td>F-8</td>
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<td>*</td>
<td>1/2</td>
<td>6.75 ± .1</td>
<td>24.5</td>
<td>450</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-13</td>
</tr>
<tr>
<td>F-9</td>
<td>1.43</td>
<td>*</td>
<td>1/2</td>
<td>6.70 ± .1</td>
<td>24.5</td>
<td>450</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-16</td>
</tr>
<tr>
<td>F-10</td>
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<td>*</td>
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<td>6.80 ± .1</td>
<td>25</td>
<td>450</td>
<td>0</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>+7</td>
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<td>F-11</td>
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<td>41.2</td>
<td>86</td>
<td>1/2</td>
<td>7.5 ± .1</td>
<td>25</td>
<td>450</td>
<td>200</td>
<td>75</td>
<td>10</td>
<td>-22</td>
</tr>
<tr>
<td>F-12</td>
<td>1.25</td>
<td>41.2</td>
<td>*</td>
<td>full</td>
<td>7.5 ± .1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>5</td>
<td>13</td>
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</tr>
<tr>
<td>F-13</td>
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<td>*</td>
<td>*</td>
<td>7.5 ± .1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>48</td>
<td>15</td>
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</tr>
<tr>
<td>F-14</td>
<td>1.33</td>
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<td>*</td>
<td>*</td>
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<td>*</td>
<td>*</td>
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<td>*</td>
<td>*</td>
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<td>*</td>
<td>-5</td>
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<td>*</td>
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<td>*</td>
<td>26.5</td>
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<td>*</td>
<td>*</td>
<td>72</td>
<td>*</td>
<td>+12</td>
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### Non-Growth Experiments

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<th>Run No.</th>
<th>Conc. PO₄ as P (mg/l)</th>
<th>Conc. NO₃⁻ as N (mg/l)</th>
<th>Conc. K⁺ (mg/l)</th>
<th>Light Intensity</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>Stirring Rate (rpm)</th>
<th>Sucrose Conc. (mg/l)</th>
<th>Hours before Inoc.</th>
<th>5N NaOH Added (ml)</th>
<th>Lag (Hrs)</th>
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<td>*</td>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>F-19</td>
<td>1.40</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>F-20</td>
<td>1.46</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>F-21</td>
<td>1.45</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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</table>
experimental run. The variation of the P concentration was primarily due to the sterilization procedure during which evaporation of water from the medium was found to be variable and dependent on operating procedure and cool down procedure. A consistent procedure was not always possible due to other demands for autoclave use as well as time constraints on the initiation of an experiment. Since P was the only nutrient concentration measures consistently at the initiation of an experimental run, the other nutrient concentrations should also reflect the same relative variation.

The concentration of NO$_3$-N must be 30 times greater than the concentration of P in order that N will not be the growth limiting stochiometric nutrient of the growth of *C. pyrenoidosa* (186). An analysis for NO$_3^-$ in solution during the stationary growth phase of run F-14 indicated no detectable level. Thus any variation in P or N during the formulation of the medium should shift the nutrient limitation from P to N as the nutrients are exhausted. Therefore in runs after F-14, the concentration of N was increased to 145 mg NO$_3$-N/l.

The concentration of K$^+$ was lower in the medium in the first 5 runs, F-6 to F-10, since the only source of K$^+$ was the phosphate. With the reduction of o-PO$_4$ in the BBM, there was also a reduction in K$^+$. In runs F-11 to F-14 the K$^+$ level was restored to the BBM level with KCl thus restoring the ionic strength of the medium. It was later found that the concentration of K$^+$ was important to the uptake of o-PO$_4$ into cells and that a concentration of 0.01M should be maintained (200). Therefore in runs after F-14, the K level was increased to 292 mg K$^+/l$. The increased levels of both K and NO$_3$
occurred at the same time and both requirements were met with the addition of KNO$_3$. The reduction of the uptake of o-PO$_4$ should not be greatly effected by the low K$^+$ concentration. The positively charged K$^+$ ion is primarily required to maintain charge balance in the cell during the uptake of the negatively charged o-PO$_4$ ion. The K$^+$ requirement has been shown to be met by the positively charged Na$^+$ ion in the absence of K$^+$ (200).

An organic energy source that was found to be utilized by the bacterium, Pseudomonas, but could not be respired by C. pyrenoidosa was sucrose. Samejima and Myers (194) indicated that sucrose could not support the growth of C. pyrenoidosa. Growth runs F-6 and F-10 did not have sucrose in the medium but all remaining runs listed in Table 6.1 had an initial concentration of 200 mg sucrose/l. Some preliminary growth runs for bacteria indicated that 200 mg sucrose/l gave significant bacterial activity and utilized no more than 0.35 mg PO$_4$-P/l after 200 hours of growth in a flask. The growth experiments of C. pyrenoidosa in the fermentor apparatus would not have made use of an organic energy source if no bacterial growth runs in the presence of the alga had been anticipated at this stage of the experimental work.

The pH is a chemical parameter of the medium. The availability of nutrients to the cell is affected by the pH. The growth runs F-7 to F-10 were maintained at a pH of approximately 6.75 and run F-6 at 6.4. The slightly acidic medium was the equilibrium value that was attained when CO$_2$ enriched air (\(^0.5\%\)) was bubbled through the medium. In runs F-11 to F-25 were controlled at a pH of 7.5. In the basic
medium, 15 cc of 5N NaOH was added in most cases to the medium after sterilization. The problems that were brought about with the addition of the base were previously discussed. Later discussion in this section will detail the phenomenon.

The justification for the shift to a basic medium was enhancement of the experimental goals. The literature revealed that the maximum uptake of o-PO$_4$ occurred in *C. pyrenoidosa* at a pH of 7.5 (110). Further, at this stage of the experimental work, a growth system of *C. pyrenoidosa* and *Pseudomonas* was still anticipated. Preliminary P analysis of the growth culture medium of *Pseudomonas* revealed the presence of Org-P compounds in solution. The ability of *C. pyrenoidosa* to use Org-P as a source of o-PO$_4$ was to be followed. Fitzgerald and Nelson (64) determined that *C. pyrenoidosa* had an alkaline phosphatase activity in the pH range of 7.3 to 7.6. Alkaline phosphatase is an enzyme that is able to release o-PO$_4$ from some Org-PO$_4$ compounds. Therefore growth experiments after F-10 were controlled at a pH of 7.5. Similarly the inorganic particulate dynamic experiments were carried out at the same pH. There was no evidence of precipitation in the weak acidic medium.

The variables of the physical environment in the fermentor apparatus that are controlled during a growth experiment are temperature, light intensity and mixing intensity (see Table 6.1). Temperature was 24.5 or 25°C throughout most growth experiments and particulate dynamics experiments. A majority of the growth runs in the literature were at 25°C. If data comparisons were to be made, it was felt that this temperature be used. Runs F-24 and F-25 were at
26 and 30.5°C respectively. Both runs were made in August, 1976 when the cooling for the experiment water temperature was never below 26°C and the temperature of the room in which the fermentor apparatus was placed was never below 27°C and was usually at 33°C. The light intensity was at two levels. In runs F-6 to F-10, the light intensity was 1/2 or 8 of the 16 fluorescent bulbs around the fermentor vessel were turned. For the remainder of the runs, all the fluorescent bulbs were on. Finally the mixing intensity was maintained at a constant 450 rpm. Preliminary experiments revealed that a higher rpm level did not increase the maximum growth rate of C. pyrenoidosa.

The prehistory of inoculum cells affects the initial growth response when transferred to a new environment (e.g., fresh medium). Table 6.2 summarizes the conditions of the C. pyrenoidosa conditioning and inoculum cultures. The preparation of the algal cells for the experimental growth run was a two stage procedure; generally an N-limited conditioning culture was followed by a P-limited inoculum culture. The o-PO₄ concentration of the conditioning cultures for runs F-6 to F-10 was 53.3 mg PO₄-P/l. The P concentration was reduced to approximately 5 mg PO₄-P/l for the conditioning cultures of runs F-11 to F-17. The conditioning culture for run F-14 was contaminated and a P-limited inoculum culture was used to initiate the in

The P concentration was reduced because there was a desire not to expose the cells of C. pyrenoidosa to a high o-PO₄ concentration. The medium of the inoculum cultures did not contain P in runs F-6 to F-13. The P concentration was increased but the N/P ratio was greater than

30 in runs F-14 to F-17. For the growth runs F-14, F-24 and F-25,
<table>
<thead>
<tr>
<th>Run No.</th>
<th>Inoc. Culture No.</th>
<th>Mg P/l (inoc)</th>
<th>Age (hours)</th>
<th>Cond. Culture No.</th>
<th>Mg P/l (cond.)</th>
<th>Age (hours)</th>
<th>KCl Addn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-6</td>
<td>P-5</td>
<td>0</td>
<td>159</td>
<td>C-3</td>
<td>53.3</td>
<td>269</td>
<td>X</td>
</tr>
<tr>
<td>F-7</td>
<td>P-7</td>
<td>0</td>
<td>159</td>
<td>C-4</td>
<td>53.3</td>
<td>437</td>
<td></td>
</tr>
<tr>
<td>F-8</td>
<td>P-10</td>
<td>0</td>
<td>140</td>
<td>C-3</td>
<td>53.3</td>
<td>625</td>
<td></td>
</tr>
<tr>
<td>F-9</td>
<td>?</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>F-10</td>
<td>P-14</td>
<td>0</td>
<td>186</td>
<td>C-4</td>
<td>53.3</td>
<td>412</td>
<td></td>
</tr>
<tr>
<td>F-11</td>
<td>P-19</td>
<td>0</td>
<td>273</td>
<td>C-14</td>
<td>5.1</td>
<td>412</td>
<td>X</td>
</tr>
<tr>
<td>F-12</td>
<td>P-22</td>
<td>0</td>
<td>268</td>
<td>C-15</td>
<td>5.0</td>
<td>145</td>
<td>X</td>
</tr>
<tr>
<td>F-13</td>
<td>P-24</td>
<td>0</td>
<td>240</td>
<td>C-18</td>
<td>5.2</td>
<td>117</td>
<td>X</td>
</tr>
<tr>
<td>F-14</td>
<td>C-19</td>
<td>.07</td>
<td>288</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>F-15</td>
<td>P-27</td>
<td>.25</td>
<td>274</td>
<td>P-25</td>
<td>.25</td>
<td>144</td>
<td>X</td>
</tr>
<tr>
<td>F-17</td>
<td>P-36</td>
<td>.5</td>
<td>132</td>
<td>C-26</td>
<td>5.0</td>
<td>98</td>
<td>X</td>
</tr>
<tr>
<td>F-24</td>
<td>C-37</td>
<td>2.5</td>
<td>410</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>X</td>
</tr>
<tr>
<td>F-25</td>
<td>C-42</td>
<td>2.5</td>
<td>519</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 6.2 Inoculum Culture History

(Concentration of NO₃ in all cultures = 41.2 mg NO₃-N/l)

Contamination was found in the inoculum cultures and conditioning cultures were required to initiate a fermentor run. The latter two runs were inoculated with algal cells that were grown under N-limitation since the N/P ratio was less than 30. There was not a sufficient excess of P in these cultures for the algal cells to accumulate a large amount of stored P under N-limitation to cause significant transition difficulties upon transfer of the algal cells to fresh medium in the growth experiments. It should be noted that KCl was added to the medium in the inoculum and the conditioning cultures after run F-10. The reasoning for the addition is the same as previously discussed for the fermentor medium.

The *C. pyrenoidosa* culture ages are listed in Table 6.2 for the inoculum and conditioning cultures. The age is the time since the
culture was initiated to the time when a transfer from the culture for
the initiation of another culture was made.

6.4-2 C. pyrenoidosa Growth Dynamics

Three measures of the growth of C. pyrenoidosa were taken during
the experimental work; the increase in the cell number, cell dry
weight and the total cell volume with time. Before the presentation
of the growth data, the time parameter of each run was adjusted by the
addition or the subtraction of hours in order to compare equivalent
runs. Further, corrections of the dry weight data were required.

Correction of the time parameter was required in order to make a
comparison of run data at the same growth stages of the growth curve.
The time correction is applicable to all runs and all data taken at a
point in time. The adjustment was kept for all further manipulations
of growth experiment data. The correction was required because of the
differences in the length of time of the lag growth phase between runs.
Further, the variability of the initial cell population of a run would
give a time shift in the data. For example, if a low cell concentra-
tion of C. pyrenoidosa was inoculated into a run, it will require a
period of time for the cell population to increase to a level that is
equivalent to a run inoculated with a higher cell concentration.

A standard must be set in order to make the time corrections.
Run F-15 was chosen as the standard run with an "initial" concentra-
tion of algal cells of 0.55x10^4 cells/cc. The "initial" cell con-
centration of run F-15 was set by extrapolation of the maximum growth
rate slope line back to time zero. The maximum specific growth rate
slope line of each run was extrapolated to the cell concentration of 0.55x10^4 cells/cc. The time at which this concentration was found was added to that run if the extrapolated time was negative and subtracted if the time was positive.

The dry weight data that was obtained on the GF/D glass fiber filter required correction. If the medium pH was basic, there was an additional adjustment required. After the sterilization of the medium in the fermentor apparatus, there were present particulates of dust from the distilled water and the nutrient solutions, and there were cell fragments that were not entirely removed during cleaning. This dry weight error must be subtracted from any dry weight determination of C. pyrenoidosa. In Table 6.3 there are listed the dry weight corrections that were determined for four runs in the fermentor apparatus before any addition of NaOH was made. All other runs were corrected using the average of the listed data (1.099 mg/l).

Table 6.3  Dry Weight of Medium before Addition of NaOH and/or Inoculum (on GF/D Filter)

<table>
<thead>
<tr>
<th>Run</th>
<th>Dry Wt. (Mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-20</td>
<td>1.620</td>
</tr>
<tr>
<td>F-21</td>
<td>0.992</td>
</tr>
<tr>
<td>F-22</td>
<td>0.713</td>
</tr>
<tr>
<td>F-25</td>
<td>1.070</td>
</tr>
<tr>
<td>Ave.</td>
<td>1.099</td>
</tr>
</tbody>
</table>

The second dry weight correction only applies to the runs to which the base NaOH was added, algal growth runs F-11 through F-25. Run F-24 is not included in this correction and will be discussed separately. As previously mentioned, the addition of the base to the
medium and the control of the medium pH as slightly basic, permitted
the formation of an inorganic precipitate that can be filtered from
the medium with the GF/D glass fiber filter. Without getting into
the details of the composition and formation of the precipitate, it
was found that from the time of the formation of the precipitate there
is a decay or solution of the solid phase with time till an apparent
steady state value is obtained. Figure 6.14 presents the data ob-
tained from experimental runs in which all conditions of the basic
growth runs of _C. pyrenoidosa_ were repeated (see Table 6.1) except
that the medium was not inoculated with the algal cells. Figure 6.14
indicates that the steady state value of 1.25 mg/l begins about 50
hours after the addition of NaOH. There is a large variation in the
data of Figure 6.14, but the data does reveal an obvious trend which
can be used to correct the dry weight data of the _C. pyrenoidosa_
growth runs, F-11 through F-25 (except F-24). Table 6.1 lists the
time before the inoculation of a growth run at which NaOH was added
to the medium. The time that is listed is added to the uncorrected
time of that run and gives the total hours of the abscissa in Figure
6.14. The dry weight of the inorganic particulates that is read off
the ordinate is subtracted from the experimental dry weight which has
been corrected as previously mentioned. Growth run F-24 was not cor-
rected in the above manner but was dealt with in the same way as runs
F-6 to F-10. The addition of NaOH to this culture medium was carried
out over a three hour period. This procedure did not show an increase
in dry weight as determined by filtration through a GF/D glass filter
before the growth run was inoculated with _C. pyrenoidosa_. But complete
absence of formation of inorganic particulate P is not certain. In the other growth runs NaOH was added in a ten second period.

The corrected cell concentration and dry weight data of \textit{C. pyrenoidosa} were compared to each other and four distinct groupings based on the growth dynamics were revealed. Table 6.4 enumerates the groupings. Noting Table 6.1, Group 1 runs are acidic pH and no sucrose, Group 2 runs are acidic pH and 200 mg sucrose/1, Group 3 runs are basic pH with fast addition of NaOH, Group 4 run was basic pH with the slow addition of NaOH.

Table 6.4 Separation of \textit{C. pyrenoidosa} Growth Runs Based on Growth Dynamics

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Growth Runs</th>
<th>Run Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F-6, F-10</td>
<td>Acidic, no sucrose</td>
</tr>
<tr>
<td>2</td>
<td>F-7, F-8, F-9</td>
<td>Acidic, sucrose</td>
</tr>
<tr>
<td>3</td>
<td>F-11, F-12, F-13, F-14, F-15, F-17, F-25</td>
<td>Basic, sucrose, fast NaOH addition</td>
</tr>
<tr>
<td>4</td>
<td>F-24</td>
<td>Basic, sucrose, slow NaOH addition</td>
</tr>
</tbody>
</table>

Figures 6.1 to 6.5 present the growth data of \textit{C. pyrenoidosa} based on cell concentration, dry weight and total cell volume. The cell concentration growth curves of Groups 1 and 2 (Figure 6.1), Group 3 (Figure 6.3), and Group 4 (Figure 6.5) each reveal four growth phases. Below approximately 20 hours, there is a transition phase during which the inoculum cells are adapting to the new environment. The initial transition phase is followed by the exponential growth phase which in all four groups show an initial specific growth rate which for cell concentration growth is followed by a period with a
Figure 6.1 Growth of *C. pyrenoidosa* in Batch Culture
Based on Cell Number and Dry Weight - Groups 1 and 2
Figure 6.2 Growth of *C. pyrenoidosa* in Batch Culture Based on Total Cell Volume - Groups 1 and 2
Figure 6.3 Growth of *C. pyrenoidosa* in Batch Culture Based on Cell Number and Dry Weight - Group 3
Figure 6.4 Growth of *C. pyrenoidosa* in Batch Culture Based on Total Cell Volume - Run F-25 (Group 3)
Figure 6.5 Growth of *C. pyrenoidosa* in Batch Culture Based on Cell Number and Dry Weight - Run F-24 (Group 4)
higher specific growth rate. The initial part of the exponential growth period begins at approximately 20 hours and lasts till 61 hours for Group 1, 65 hours for Groups 2 and 3, and 70 hours for Group 4. The second part of the exponential growth phase extends to approximately 88 hours for Group 1 and 94 hours for groups 2, 3 and 4. It should be noted that the increase in the specific growth rate of the exponential growth phase occurs at the same time (≈65 hours) as the depletion of the $\ce{PO}_4$ from the medium as seen in Figure 6.11. The exponential growth phase is followed by a transition phase to the stationary phase. Several of the growth experiments of Group 3 were carried into the stationary phase as seen in Figure 6.3.

*C. pyrenoidosa* growth based on dry weight concentration is shown in Figure 6.1 (Group 2), Figure 6.3 (Group 3) and Figure 6.5 (Group 4). Group 1 data was too scattered to draw a meaningful curve. Growth of the alga based on total cell volume is found in Figure 6.2 for Groups 1 and 2 and Figure 6.4 for run F-25 in Group 3. Other growth data based on the cell volume was not taken. One can see that the initial transition period, transition phase from the exponential growth to the stationary growth and stationary growth (Group 3 data) are found in the growth curves. The exponential phase in all the curves show only a single constant specific growth rate and not the two rates found in the cell concentration growth curves. The exponential phase of the dry weight and the total cell volume growth curves span the same time periods as their respective group cell concentration growth phase.
Table 6.5 summarizes the specific growth rates and the corresponding doubling time (DT) of the exponential growth phases of the data presented in Figures 6.1 to 6.5. The specific growth rate during the exponential phase of the cell number (first part), dry weight and total cell volume are equal or nearly equal within a group except for Group 1 where the cell volume specific growth rate is higher. Group 2 has the highest specific growth rate followed by Group 1, based on total cell volume, and then followed by Groups 3 and 4.

Table 6.5  Specific Growth Rate and Doubling Time during Exponential Growth

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Concentration Growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ( \mu_N ) (hr(^{-1})) ( DT^* ) (hr)</td>
<td>0.053 [20-60]**</td>
<td>0.069 [20-65]</td>
<td>0.067 [20-65]</td>
<td>0.065 [20-70]</td>
</tr>
<tr>
<td>2 ( \mu_N ) (hr(^{-1})) ( DT^* ) (hr)</td>
<td>0.075 [60-88]</td>
<td>0.088 [65-90]</td>
<td>0.111 [65-95]</td>
<td>0.101 [70-95]</td>
</tr>
<tr>
<td>Dry Weight Growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \mu_X ) (hr(^{-1})) ( DT^* ) (hr)</td>
<td>---</td>
<td>0.069 [10-80]</td>
<td>0.067 [20-95]</td>
<td>0.065 [25-90]</td>
</tr>
<tr>
<td>Cell Volume Growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \mu_V ) (hr(^{-1})) ( DT^* ) (hr)</td>
<td>0.069 [20-95]</td>
<td>0.069 [15-80]</td>
<td>0.062 [20-95]</td>
<td>---</td>
</tr>
</tbody>
</table>
| * \( DT \) - doubling time.  
** [ ] - Growth time period-hr. |
The stationary growth phase in Figure 6.3 does show a slow rise in both the cell number and cell dry weight with time giving a specific growth rate of .0020 and .00028 hr\(^{-1}\) respectively. The rise could be attributed to dead cells but the cell count eliminated any cell that did not show a stain or, if there was a stain, it did not count the cell if no internal structures were stained. Some cell division was noted during cell counts.

The growth data presented does distribute about the curves that were drawn in Figures 6.1 to 6.5. The variation in data during the stationary growth phase and the transition to the stationary growth phase is the result of both the stoichiometric limitation of *C. pyrenoidosa* by \(\alpha\text{-PO}_4\) and variable accumulations of algal cells on the vessel walls. The wall accumulation of cells becomes significant only during and after the transition to the stationary growth phase. The cells are deposited on the vessel walls from splashing from the liquid surface due to the agitation and the gas bubbles breaking the surface. The variation of the growth data during the exponential growth phase is a function of both the enumeration technique and the possibility of some synchronous growth.

6.4-3 Dynamics of the Observer Physical Characteristics of the *C. pyrenoidosa* Cell

Several physical characteristics of the *C. pyrenoidosa* cell were observed or measured during the course of its dynamic growth. The dynamics of an individual algal cell dry weight and volume are presented. During the cell count procedures, the algal cell shape was observed. Further the number of daughter cells produced during the
division of *C. pyrenoidosa* was noted. Finally the cell concentrations of chlorophyll *a* and *b* and pheophytin were determined analytically during exponential growth.

The *C. pyrenoidosa* growth curves of cell number, total cell volume and dry weight of all four Groups from Figures 6.1 to 6.5 provided the averages of the measured data for the determination of the dynamic change in the dry weight of an average algal cell which was determined from the ratio of the average dry weight concentration and the cell number concentration. Figure 6.6 presents the result of this ratio for Groups 2, 3 and 4. The results cover the exponential growth phase, the transition to stationary growth phase and the stationary growth phase. For the first part of the exponential growth phase (from 20 to 65 hours) the dry weight per cell is constant for Groups 2 and 3 while Group 4 shows a slight negative slope. Group 2 has a steady state value of $1.5 \times 10^{-7}$ mg/cell, Group 3 has a value of $1.87 \times 10^{-7}$ mg/cell and Group 4 decreases from $2.55 \times 10^{-7}$ to $2.5 \times 10^{-7}$ mg/cell during this phase. At the cessation of the first part of the exponential growth when the $\alpha$-PO$_4$ in the medium is depleted, there is a precipitous fall in the cell dry weight which stops at 96 hours for Groups 3 and 4 which is the point of the end of the exponential growth phase. Group 1 data continues to decrease for 10 more hours at which point no further data was taken. Groups 2 and 3 fall upon the same curve while Group 4 data lags approximately 7 hours. After the exponential growth phase, the data of Groups 3 and 4 fall upon each other. The dry weight per cell values increased to a point where the dry weight increases very slowly around the value of
Figure 6.6 Cell Weight Dynamics in Batch Culture
1.12 \times 10^{-7} \text{mg/cell}. A near steady state begins at about 195 hours. This is the point of the beginning of the stationary growth phase (see Figure 6.3).

Figure 6.7 illustrates the dynamic behavior of the average volume of a \textit{C. pyrenoidosa} cell for Groups 1, 2 and 3. The data presented is the ratio of the average total cell volume and the average cell number from Figures 6.1 to 6.4. Group 3 is represented by the data taken for run F-25. Each group shows a different dynamic behavior. During the first part of the exponential growth phase, Group 1 data constantly increases from $2.0 \times 10^{-10}$ cc/cell at 10 hours to $4.25 \times 10^{-10}$ cc/cell at 64 hours. Group 2 cell volume rises slowly from $4.1 \times 10^{-10}$ to $4.2 \times 10^{-10}$ cc/cell. After 30 hours the cell volume for Group 3 falls slowly from $4.3 \times 10^{-10}$ cc/cell to $4.0 \times 10^{-10}$ cc/cell at 65 hours. The cell volumes of all three Groups are approximately equal before the volume decreases. During the second part of the exponential growth phase the cell volume is reduced rapidly for all three Groups. Group 3 data reaches a minimum value of $1.05 \times 10^{-10}$ cc/cell at 95 hours. Group 2 data continues to become smaller after the 88 hour point is passed but the decrease is at a lower rate. The Group 2 data seems to be approaching a steady state value of $1.7 \times 10^{-10}$ cc/cell. This later phase occurs in the transition to the stationary growth phase. Groups 1 and 3 increase in cell volume after the exponential growth phase stops. Group 3 data increases to a steady state value of $3.9 \times 10^{-10}$ cc/cell at approximately 195 hours, the point of the start of the stationary growth phase. Group 1 data was not obtained beyond 125 hours and no steady state was observed.
Figure 6.7 Cell Volume Dynamics in Batch Culture
The iodine staining characteristics of *C. pyrenoidosa* were observed under a microscope during the cell counts. Table 6.6 summarizes the observations for all groups in average terms at 24, 48, 72, 96 and 216 hours. It can be generalized that the algal cells

Table 6.6 *C. pyrenoidosa* Stain and Division Characteristics

<table>
<thead>
<tr>
<th>Time Hours</th>
<th>Growth Phase</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>Inoculum - Dark circular</td>
</tr>
<tr>
<td>25</td>
<td>1st stage exponential growth phase</td>
<td>Circular dark (some medium) stain, some darker internal cell structure, 4 to 8 daughter cells</td>
</tr>
<tr>
<td>98</td>
<td></td>
<td>Circular medium (some dark, light), more prominent dark stained internal cell structure, 4 to 8 daughter cells</td>
</tr>
<tr>
<td>72</td>
<td>2nd stage exponential</td>
<td>Cell lightly stained, prominent dark stained internal cell structure, Circular cells from 4 to 8 daughter cell division, Fusiform cells from 2 daughter cell division</td>
</tr>
<tr>
<td>96</td>
<td>Transition to stationary growth phase</td>
<td>Cell lightly stained, prominent dark stained internal Cell structure (smaller than before), Fusiform cells from 2 daughter cell division, Round cells from 4 daughter cell division</td>
</tr>
<tr>
<td>216</td>
<td>Stationary growth phase</td>
<td>Dark stain, circular</td>
</tr>
</tbody>
</table>

Note: Daughter cells lighter than mother cell - would give range of cell stain intensity - lorca after daughter cells separate retains stain.

decrease in stain intensity as the exponential growth phase continues till o-PO₄ is depleted from the medium. The inoculum cells are dark and the cells at the end of the exponential growth phase are light
(96 hr.). Dark cells are again found at 216 hr. which is during the stationary growth phase. It was noted that the daughter cells were lighter than the mother cells. The lorca, the parental cell wall, also was observed to retain a stain which may give rise to the observed stain characteristics.

Table 6.6 also notes the number of daughter cells that are formed during reproduction. For the first 48 hours, 4 to 8 daughter cells were formed from a single mother cell. At 72 hours when the o-PO$_4$ in the medium is depleted, there are 4 to 8 daughter cells per mother cells but there are also mother cells that give rise to only 2 daughter cells. It was found that the cells from the latter type division were fusiform or prolate spheroids in shape as compared to round cells from the other type divisions. The incidence of a mother cell giving rise to two fusiform cells increases at 96 hr. and a mother cell having 8 daughter cells was no longer found. The percentage of fusiform cells were determined during the cell counts. These data are presented in Figure 6.8 for all Groups. The percent of fusiform cells in the first 60 hours of the growth culture varies from 0 to 10%, with the majority of observations having no fusiform cells. After the 60 hour point the percentage of the fusiform cells increase with time up to a maximum of 100% at 120 hours. The increase in the percentage of the fusiform cells occurred during the second part of the exponential growth phase when the o-PO$_4$ was depleted. The maximum percent of fusiform occurs during the transition to stationary growth phase. The maximum value of fusiform cells does not persist. Observations made
Figure 6.8 Percentage Non-spherical Cells During Growth of *C. pyrenoidosa* in Batch Culture
during the stationary growth phase show that 65 to 70% of the cells are fusiform. This phenomena can be the result of the fusiform cells transforming into round cells during the stationary growth phase. There seems to be no differentiation between Groups 2, 3 and 4 in Figure 6.8.

The chlorophyll content of *C. pyrenoidosa* in runs F-7 and F-8 of Group 2 were analyzed. Chlorophyll $a$ and $b$, which are the active chlorophyll in the green algae, and pheophytin, which is the inactive form of the chlorophyll, were determined. The results of the analysis are in Figure 6.9. The data were determined only during the exponential growth phase. Chlorophyll $a$ has the highest cell concentration and shows a constant decrease in cell concentration. Chlorophyll $b$ initially is the next highest in concentration in the algal cell and decreases at a lower rate than chlorophyll $a$. Pheophytin is initially the lowest in cell concentration but the concentration increases with time to a maximum at 52 hours which is greater than the chlorophyll $b$ concentration. The pheophytin concentration decreases thereafter to a point where it is equivalent to the concentration of chlorophyll $b$ at 87 hours.

6.4-4  **The Physical and Chemical Dynamics in the Medium during *C. pyrenoidosa* Growth**

During the course of the dynamic growth of *C. pyrenoidosa*, several physical and chemical parameters in the medium were measured. The physical parameter of light intensity was measured at a point within the algal suspension. The concentration of the chemical parameters of
Figure 6.9 Dynamics of Pigment Content of *C. pyrenoidosa* in Batch Culture
o-PO$_4$, NO$_3$ and COD were determined after medium was filtered through a 0.45 um membrane filter.

The light energy that penetrated through the algal cell suspension was measured with a solar cell at an internal position 6 cm. from the fermentor vessel wall during the dynamic growth of *C. pyrenoidosa*. The results at both of the light intensity levels that were used are presented in Figure 6.10. Groups 1 and 2 are at the low light intensity which are initially 1.86 x 10$^4$ ft-candles. Groups 3 and 4 were at the high light intensity which was initially at 2.36 x 10$^4$ ft-candles. The initial phases of growth have a relatively constant intensity value but decreases rapidly as the cell population increases. The low light intensity levels off at 0.32 x 10$^4$ ft-candles while the high intensity light level seems to level off at 0.95 x 10$^4$ ft-candles. In many cases the volume of suspension taken from the fermentor vessel dropped the liquid level below the solar cell and little data could be taken after 100 hours.

The dynamics of the o-PO$_4$ concentration that remain in the medium after filtering through a 0.45 um membrane filter during the growth of *C. pyrenoidosa* are presented in Figure 6.11 for all four Groups. The concentration of o-PO$_4$ is presented as a weight fraction of the total P in the unfiltered medium. There are three distinct curves in the plot. Groups 1 and 2, in which no NaOH was added, and Group 4, in which NaOH was added slowly show that all the o-PO$_4$ passes through the membrane filter at time zero while only a 0.85 fraction of the o-PO$_4$ does for Group 3 in which NaOH was added rapidly. A 0.15 fraction of the P is filtered out with the filter. The filtered P is
Figure 6.10 Light Intensity Measured Inside Fermentor Vessel
associated with the inorganic particulates that were precipitated with the addition of NaOH. The curves of Groups 2, 3 and 4 merge to form a single curve at about 55 hours. Group 1 data lags the other data by 15 hours. The depletion of o-PO$_4$ in the filtered medium occurs in the range of 60 to 65 hours for Groups 2, 3 and 4. Group 1 data can be estimated to be depleted at 70 to 80 hours. The data thereafter show no significant o-PO$_4$ in the medium. Further there is no difference between the o-PO$_4$ and the Tot-P in the filtered medium throughout the growth experiments.

The uptake of o-PO$_4$ from the medium by *C. pyrenoidosa* is presented in Figure 6.12. The uptake rate/cell was determined from the slope of the curve of a run group in Figure 6.11 divided by the cell concentration of the run group. The slope is equivalent to the uptake rate of o-PO$_4$ from the medium. This uptake rate does not account for the o-PO$_4$ taken directly from the inorganic particulate P. The uptake rates level off between 30 and 40 hours. Run Groups 3 and 4 fall from a high rate to level off a $2.85 \times 10^{-10}$ mg P/cell·hr. Run Group 1 does not show as high of an initial uptake rate though there is no data in the 10-20 hour range to be conclusive. The uptake rate levels off at $1.6 \times 10^{-10}$ mg P/cell·hr. Run Group 3 shows an initial low uptake rate that increases with time to a rate of $2.4 \times 10^{-10}$ mg P/cell·hr. The uptake rates fall to zero when the o-PO$_4$ is depleted from the medium.

The concentration of NO$_3$-N and the COD after the medium is filtered through a 0.45 μm membrane filter was followed during the growth of *C. pyrenoidosa* in run F-15 of Group 3. The results are
Figure 6.11 Dynamics of Orthophosphate Concentration in Medium Filtered with GF/D Filter During Growth of *C. pyrenoidosa* in a Batch Culture
Figure 6.12 Phosphorus Uptake Rate in Batch Culture
in Figure 6.13. The COD decreases slowly during the exponential
growth phase from 265 to 240 mg COD/l. The COD decreases more rapidly
after the cessation of exponential growth during the transition to
the stationary growth phase and is 180 mg COD/l at 175 hours with no
indication of a steady state. The NO₃-N concentration shows a con-
tinuously increasing rate of N depletion from the initiation of the
run through the stationary growth phase at 325 hours. In terms of
total N taken up per cell, the amount of N taken up by the cells
divided by the average cell population of Group 3, there is a dramatic
drop in the N taken up as the exponential growth phase continues till
it ceases with a minimum value of 0.34 x 10⁻⁵ mg N/cell at 100 hours.
Thereafter the N taken up increased slowly and was approaching a
steady state value of 0.45 x 10⁻⁵ mg N/cell during the stationary
growth phase.

6.4-5 Inorganic Particulates—Composition
and Dynamics

A presentation of data concerning the composition and dynamics of
the inorganic particulates formed after the addition of NaOH is re-
quired in order to assess the influence of the inorganic particulate
P on the P compounds of C. pyrenoidosa which were extracted with
boiling water and cold TCA. There will be presented data concerning
P, Fe and Ca composition of the precipitate as well as the size
distribution of the precipitate. The ability of the inorganic
particulates to release o-PO₄ to the medium was investigated.
Finally there is reported limited data concerning Fe dynamics during
the growth of C. pyrenoidosa.
Figure 6.13 Dynamics of NO₃⁻N and COD of Medium and N-Uptake by C. pyrenoidosa in Batch Culture - Run F-15
The dry weight of the inorganic precipitate which was separated from the medium with the GF/D glass filter is shown in Figure 6.14 as a function of the time after the addition of NaOH to the medium. This figure has been previously discussed, but it should be repeated that the dry weight decreases with time till a steady state value of 1.25 mg/l is approached. At time equal zero, the dry weight was found to be 32.54 mg/l when the pH was still very high (11.5) and the formation of CaCO₃ is very probable. The precipitate was found to contain P, Fe and Ca. In Table 6.7, the following composition of the precipitate on membrane filters of different pore size was found for run F-18 at 74 hours. The medium was first filtered with a 0.45 µm membrane filter followed by a 0.025 µm filter. The concentration of o-PO₄ that is in solution after filtration through various pore-sized glass and membrane filters is shown in Table 6.8 for run F-18 at 100 hours. The data for run F-18 in Tables 6.7 and 6.8 were taken during the dry weight steady state of the particulates. I can be seen in Table 6.7 that P and Fe are primarily associated with the precipitates that are filtered out with the 0.45 µm filter. Calcium is primarily associated with precipitate filtered with 0.025 µm. Table

Table 6.7 Percipitate Composition of Inorganic Particulates in Run F018

<table>
<thead>
<tr>
<th>Particle Size (µm)</th>
<th>Ca (mg/l)</th>
<th>P (mg/l)</th>
<th>Fe (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total in Fermentor</td>
<td>~6.8</td>
<td>1.265</td>
<td>.886</td>
</tr>
<tr>
<td>≥ 0.45</td>
<td>.016</td>
<td>.090</td>
<td>.339</td>
</tr>
<tr>
<td>0.45 to 0.025</td>
<td>.140</td>
<td>.001</td>
<td>.010</td>
</tr>
</tbody>
</table>
Figure 6.14  Dry Weight Dynamics of Precipitate Filtered with GF/D Filter in a Batch Fermentor
6.8 shows that P in the precipitate is filtered out with the GF/D filter and that smaller filters successively remove an additional 10% of P from the medium as the pore size is reduced 2 orders of magnitude. Most of the additional P removal was with the 0.025 μm membrane filter.

Table 6.8 Filterability of o-PO₄ Associated with Inorganic Particulates of Run F-18

<table>
<thead>
<tr>
<th>Filter Pore Size (μm)</th>
<th>mg o-PO₄-P/l after filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total in Fermentor</td>
<td>1.265</td>
</tr>
<tr>
<td>2.7 (GF/D)</td>
<td>1.164</td>
</tr>
<tr>
<td>0.7 (GF/F)</td>
<td>1.160</td>
</tr>
<tr>
<td>.45</td>
<td>1.169</td>
</tr>
<tr>
<td>.22</td>
<td>1.157</td>
</tr>
<tr>
<td>.025</td>
<td>1.151</td>
</tr>
</tbody>
</table>

Figure 6.15 presents the particulate Fe and P concentration as a function of time. Iron was found to be filtered from the medium with equal efficiency by both the GF/D filter and the 0.45 μm membrane filter. The three curves are presented in Figure 6.15. The data of the particulate concentration of P and Fe is plotted as the weight fraction of the Tot-P and Tot-Fe in the fermentor. The particulate Fe shows an initial fast decline for the first 30 hours from a particulate Fe weight fraction of 0.98. The particulate Fe weight fraction decreases at a slower constant rate after 30 hours. The particulate P on the GF/D filter falls very rapidly in the first few hours from a particulate P weight fraction of 0.838. At 15 hours a constant decrease of the particulate weight fraction is established. The amount of P collected by the 0.45 μm membrane filter shows no trends but does
Figure 6.15 Weight Fraction Dynamics of Phosphorus and Iron in Precipitate in Batch Fermentor
show that a significant portion of P passes through the GF/D filter to be collected on the 0.45 μm membrane filter. The data in Figure 6.15 indicates that the particulate dry weight concentration should be constantly declining, but data points in Figure 6.14 seem to contradict Figure 6.15.

Phosphorus in the suspended solids of the medium that were filtered out with the GF/D filter was found to be partitioned into two parts: P which is only soluble in cold TCA and P which is soluble in both cold TCA and boiling water. Figure 6.16 shows the dynamics of the two forms of P as a function of time after the addition of NaOH. No solubility difference was found in the P concentration of a sample analyzed for Tot-P or for o-PO₄ by stannous chloride molybdenum blue analytical methods. The solubilized P in each extractant has a low acid hydrolysis stability. The same two P solubility types are extracted from C. pyrenoidosa and thus are valuable for the interpretation of data in which the P compounds are extracted from both the inorganic and the algal particulates. The difference between the amount of P extracted by cold TCA, which is equal to the total P in the inorganic particulates collected (same as the Tot-P curve in Figure 6.15), and the amount of P extracted with boiling water is the P that is only soluble in cold TCA. The difference is plotted in Figure 6.16. The data show an initial drop in the P weight fraction of the Tot-P in the fermentor for all three plots for the first 15 hours. After 15 hours the decline in concentration is at a constant rate. The P that is water soluble declines at a more rapid rate than P that is only soluble in cold TCA.
Figure 6.16 Solubility of Precipitate Phosphorus in Hot Water and Cold TCA as Function of Time in Batch Fermentor
The determination of the composition of the inorganic particulates, the molar ratios of P of the three curves plotted in Figure 6.16 to the Tot-Fe in the inorganic floc is shown in Figure 6.17. The ratio for the Tot-P/Fe increases at a constantly increasing rate as would be indicated by the ratioing of the Fe and P curves of Figure 6.15. For P which is soluble in both cold TCA and boiling water, the P/Fe ratio is constant at 0.305. For P soluble only in TCA, the curve shows an initial constant ratio of 0.215 but increases after 95 hours.

An attribute of the inorganic floc was the ease of solubilizing o-PO$_4$. The inorganic particulates were collected on a 0.025 µm membrane filter. The floc on the filter was washed with BBM, minus EDTA, Fe and o-PO$_4$, as it passed through the filter. The pH was 7.5 in order to simulate environment in fermentor during algal growth when o-PO$_4$ is depleted. The wash was collected and analyzed for o-PO$_4$ and Fe. The Fe analysis yielded only traces of Fe. Figure 6.18 presents the results of the o-PO$_4$ analysis. The inorganic particulates were analyzed for runs F-20 and F-21 at various times during the runs. The points on the curves are the total o-PO$_4$ collected per 100 ml of BBM that passed through the floc collected on filter. It was found that, as the floc ages, the curve goes to a lower equilibrium value of the total P solubilized. Further, as the floc ages, the curves approach each other, note curves at 144 and 153 hours. One curve, 30 hours, F-21, does not follow the pattern. In reviewing the data, the Tot-P of the floc used for the weight fraction calculations seemed to be two times higher than one would expect when it was compared to other data. If calculations were based on the general trend of the
Figure 6.17 Dynamics of the Molar Ratio of P/Fe in Precipitate Collected on GF/D Filter in Batch Fermentor
Figure 6.18  P Extraction from Precipitate from Batch Fermentor with BBM (Minus O-PO₄)

\[ \text{Run No.} \]
- \( F-20 \)
- \( F-21 \)

0 Hours x 1/2 - Run F-20

47 Hours - Run F-20
75 Hours - Run F-21
144 Hours - Run F-20
153 Hours - Run 21
30 Hours - Run F-21
particulate Tot-P, the curve would fall in the region above run F-20, 47 hours. If the curves are extrapolated to an equilibrium value (excluding 0 hours, run F-20, the values would fall between 0.1 to 0.2 weight fraction of Tot-P of the particulates with at least 80% of the equilibrium value solubilized in 3 hours.

The dynamics of particulate Fe was determined during *C. pyrenoidosa* growth. The GF/D filters, used to determine dry weight, were ashed. The residue on the filter was extracted with acid and the extractant was analyzed for Fe that was in the algal cells and in the inorganic particulates. Figure 6.19 presents the results of this analysis for the growth runs F-11 to F-25. The data scatter during the initial growth phase of the alga due primarily to the scatter of the dry weight of the precipitated solids before the inoculation of the growth population of *C. pyrenoidosa* increases. Data reaches a peak of 1.05 mg Fe/l at 70 hours which is 5 hours after the o-PO$_4$ is depleted in the medium. It cannot be determined if all the Fe is filtered out but the total Fe in experimental runs was found to be in the range of 0.8 to 1.2 mg Fe/l. One additional point at 200 hours, run F-25, shows that part of the particulate Fe does go back into solution as the Fe weight on the GF/D filter falls to 0.38 mg Fe/l.

**6.4-6 P Compartment Dynamics of *C. pyrenoidosa***

This section presents the dynamics of P in the *C. pyrenoidosa* cells in batch culture. The P of the alga was extracted with cold TCA and boiling water. The extracts were subjected to two or three levels of acid hydrolysis severity to release o-PO$_4$ from solubilized P compounds for P analysis. In addition the total P of *C. pyrenoidosa*
Figure 6.19 Iron Filtered with *C. pyrenoidosa* During Batch Growth
was determined. This section will define P compartments that are obtained from the data and the effect of the inorganic particulate P on these compartments. Finally comparison of P compartments between run groups are made.

6.4-6A General

The cold TCA and boiling water extraction cause cell death. The acidity of TCA and high temperatures of water extraction inactivated cellular enzymes. Upon cell death the permeability of the cell wall is increased. Thus cell compounds that are in the soluble state can diffuse out. Under acidic or high temperature conditions, additional compounds will dissolve and diffuse out of the cell (126).

The integrity of the cell wall during extraction with both cold TCA and boiling water was found to be a function of the cell growth phase. The *C. pyrenoidosa* cells were counted before collection on GF/D filter pad, the cells were ground with the filter in a teflon pestle grinder with extractant and the extraction procedures of the algal cells were carried out. The algal cells were counted after the grinding and after the extraction. Table 6.9 summarizes the results. Grinding breaks only 10% of algal cells during the stationary growth phase and no more than 10% during exponential phase. The extraction of the algal cells during exponential growth phase does not increase the number of cells broken. Fifty percent of the intact cells, extracted during the transition to the stationary growth phase, are found broken after the extraction procedure is completed. The number of cell wall remnants was found to be higher in the latter cell counts.
Table 6.9 Cell Wall Stability to Extraction Procedures

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Water Extract</th>
<th>TCA Extract</th>
<th>Growth Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Grinding</td>
<td>90</td>
<td>90</td>
<td>Stationary (F-25, 200 hours)</td>
</tr>
<tr>
<td>After Extraction</td>
<td>90</td>
<td>90</td>
<td>Exponential (F-25, 60 hours)</td>
</tr>
<tr>
<td>After Extraction</td>
<td>49</td>
<td>43</td>
<td>Transition to stationary (F-25, 100 hours)</td>
</tr>
</tbody>
</table>

The influence of broken cells on the P extraction and the resulting concentration of P in P compartments was not assessed.

The P extraction data was utilized to obtain the different P compartments of *C. pyrenoidosa*. Table 6.10 lists both the original cell P Data Groups and the Cell P Compartment Groups which were determined from the original cell P data. Data Groups 1 to 7 are original cell P data analytically determined of which Groups 2, 3 and 5 are also cell P compartment data. For data Group 3 o-PO₄ released from the boiling water extract which was hydrolyzed for 20 minutes in 1 N H₂SO₄ and analytically determined by stannous chloride coloration is equivalent to o-PO₄ determined by the hydrazine-molybdenum blue analytical technique. This was observed in run F-24 when both techniques were used on the same samples. Groups 8 to 14 use the differences in the data of Groups 1 to 7 to generate the cell P compartments as identified in Table 6.10. The P compartments in the algal cell can be generalized in the following way. For each extractant there are soluble and insoluble P components. The soluble P fraction
<table>
<thead>
<tr>
<th>Data Group No.</th>
<th>Data Group Code</th>
<th>Original Compartment</th>
<th>Cell P Data</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P(Tot-Part)</td>
<td>x</td>
<td></td>
<td>Total P in particulates collected on GF/D filter -determined by the P difference of the Tot-P of the medium before filtration by the GF/D filter and Tot-P after filtration</td>
</tr>
<tr>
<td>2</td>
<td>P(L-H₂O)</td>
<td>x</td>
<td>x</td>
<td>Low acid stable P extracted by boiling water -determined by H₂O(Ext)PO₄(SC)</td>
</tr>
<tr>
<td>3</td>
<td>P(L&amp;M-H₂O)</td>
<td>x</td>
<td>x</td>
<td>Low acid stable P plus medium acid stable P extracted by boiling water -determined by H₂O(Ext)PO₄(SC), H₂SO₄ or H₂O(Ext)PO₄(HM)</td>
</tr>
<tr>
<td>4</td>
<td>P(Tot-H₂O)</td>
<td>x</td>
<td></td>
<td>Total P in the boiling water extract -determined by H₂O(Ext)Tot-P</td>
</tr>
<tr>
<td>5</td>
<td>P(L-TCA)</td>
<td>x</td>
<td>x</td>
<td>Low acid stable P extracted by cold TCA -determined by TCA(Ext)PO₄(SC)</td>
</tr>
<tr>
<td>6</td>
<td>P(L&amp;M-TCA)</td>
<td>x</td>
<td></td>
<td>Low acid stable P plus medium acid stable P extracted by cold TCA -determined by TCA(Ext)PO₄(SC)HCl</td>
</tr>
<tr>
<td>7</td>
<td>P(Tot-TCA)</td>
<td>x</td>
<td></td>
<td>Total P in cold TCA extract -determined by TCA(Ext)Tot-P</td>
</tr>
<tr>
<td>8</td>
<td>P(Ins-H₂O)</td>
<td>x</td>
<td></td>
<td>Particulate P insoluble in boiling water -determined by P(Tot-Part) minus P(Tot-H₂O)</td>
</tr>
<tr>
<td>9</td>
<td>P(H-H₂O)</td>
<td>x</td>
<td></td>
<td>High acid stable P extracted by boiling water -determined by P(Tot-H₂O) minus P(L&amp;M-H₂O)</td>
</tr>
<tr>
<td>10</td>
<td>P(M-H₂O)</td>
<td>x</td>
<td></td>
<td>Medium acid stable P extracted by boiling water -determined by P(L&amp;M-H₂O) minus P(L-H₂O)</td>
</tr>
</tbody>
</table>
Table 6.10 (contd.)

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Code</th>
<th>Cell P Data</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>P(Ins-TCA)</td>
<td>x</td>
<td>Particulate P insoluble in cole TCA - determined by P(Tot-Part) minus P(Tot-TCA)</td>
</tr>
<tr>
<td>12</td>
<td>P(H-TCA)</td>
<td>x</td>
<td>High acid stable P extracted by cole TCA - determined by P(Tot-TCA) minus P(L&amp;M-TCA)</td>
</tr>
<tr>
<td>13</td>
<td>P(H&amp;M-TCA)</td>
<td>x</td>
<td>High acid stable P and medium acid stable P extracted by cold TCA - determined by P(Tot-TCA) minus P(L-TCA)</td>
</tr>
<tr>
<td>14</td>
<td>P(M-TCA)</td>
<td>x</td>
<td>Medium acid stable P extracted by cold TCA - determined by P(L&amp;M-TCA) minus P(L-TCA)</td>
</tr>
</tbody>
</table>

Legend

- **H2O(Ext)** - Particulates on GF/D filter extracted with boiling water - see Section 5.4-4B
- **TCA(Ext)** - Particulates on GF/D filter extracted with cold TCA - see Section 5.4-4B
- **PO4(SC)** - Stannous chloride-molybdenum blue determined of o-PO4 - see Section 5.4-4C
- **PO4(HM)** - Hydrazine-molybdenum blue determination of o-PO4 - see Section 5.4-4C
- **Tot-P** - Potassium persulfate digestion for total P - see Section 5.4-4C
- **H2SO4** - Acid hydrolysis in 1N H2SO4 at 100°C for 15 min - see Section 5.4-4C
- **HCl** - Acid hydrolysis in 1N HCl at 100°C for 7 min - see Section 5.4-4C
is divided into low, medium and high acid stable P. Inorganic particulate P is also extracted by cold TCA and boiling water. In Figure 6.16 it was found that boiling water extracts are approximately 50% of the inorganic P while cold TCA extracts all the P from the inorganic particulates. Later discussion will show that TCA soluble P is precipitated and that no boiling water soluble P was present in run F-24. The inorganic particulate o-PO$_4$ that is extracted would be defined as either TCA soluble, low acid stable P and water soluble, low acid stable P (see Table 6.10). For cold TCA extracted P, the inorganic particulate P would contribute the same amount of o-PO$_4$ to the original Data Groups P(Tot-Part), P(L-TCA), P(L&M-TCA) and P(Tot-TCA). Therefore the differences between these Data Groups, the P Compartment Data Groups P(Ins-TCA), P(H-TCA), P(H&M-TCA) and P(M-TCA), are not affected by the inorganic particulate P for boiling water extracted P, the inorganic particulate P would contribute the same amount of o-PO$_4$ to the original Data Groups P(L-H$_2$O), P(L&M-H$_2$O) and P(Tot-H$_2$O). Therefore, P Compartments Data Groups P(H-H$_2$O) and P(M-H$_2$O) are not affected by the inorganic particulate P. The P Compartments Data Groups that are affected by inorganic particulate P are P(L-H$_2$O), P(L&M-H$_2$O) and P(Ins-H$_2$O). If the inorganic particulate P does not contain boiling water soluble P then the P compartment Data Groups that are not affected by the inorganic P are P(L-H$_2$), P(L&M-H$_2$O), P(H-H$_2$O) and P(M-H$_2$O) while only P Compartment Data Group P(Ins-H$_2$O) is affected.
6.4-6B Identification of P Compounds in the P Compartments

The P compounds that were extracted from the *C. pyrenoidosa* have been separated according to acid lability of the compound. Table 6.10 summarized these groups from the boiling water extract and the cold TCA extract. A search of the literature gives a general overview of the P compounds that reside in each compartment. In Section 3.5, a general review of these compounds was made. The P compounds in the TCA extraction are the nucleosides, nucleotides, nucleotide sugars, poly-P and sugar phosphates (215). Meyer and Bartlett (154) did a more extensive identification of the P compounds that were isolated from horse blood granulocytes and lymphocytes cells. Summarizing the articles of Leloir and Cardini (140) and Lindberg and Ernster (141), the P compounds in the three TCA soluble P compartments are as follows. The low stable P, P(L-TCA), contains o-PO_4, acetyl phosphate, ribose-l-phosphate, carbamyl phosphate and partially hydrolyzed creatine and arginine phosphate. These P compounds constitute the precursor pool P and a large part of the intermediate pool P. The medium stable acid P, P(M-TCA), would contain unconverted creatine and arginine phosphates, Poly-P "A," the nucleotide polyphosphates (some of which lose all but the last phosphate group), glucose-l-phosphate and the partial hydrolysis of phosphoenolpyruvate, glycer-aldehyde phosphate, dihydroxyacetone phosphate, the phosphate groups in the l-position of the hexose diphosphates and diphosphoglyceric acid. Part of the storage pool P and another significant portion of the intermediate pool P are found in the medium acid stable P.
compartment, \( P(M-TCA) \). The high acid stable \( P, P(H-TCA) \), would be all the true phosphate esters, such as, glucose-6-phosphate, ribose-5-phosphate, glycerol phosphate, AMP, UMP, CMP, and GMP (the monophosphate nucleotides) with a part of the intermediate \( P \) pool. This can be primarily described as the functional pool \( P \). The TCA insoluble \( P, P(Ins-TCA) \), would be the phospholipids, RNA-P, DNA-P, phosphoproteins and Poly P "B," "C" and "D" (114). The \( P \) compounds contained in the insoluble \( P \) compartment, \( P(Ins-TCA) \), is the structural pool \( P \) and the storage pool \( P \).

The \( P \) compounds extracted from \textit{C. pyrenoidosa} with boiling water have not been studied to the extent of the TCA \( P \) compounds. Nelson and Fitzgerald (64) concluded that the \( \text{o-PO}_4 \) in the water extract was primarily from the acid soluble and insoluble Poly-P in the algal cells. Rhee (184,185) found that the \( \text{o-PO}_4 \) in the water extract correlated closely to Poly-P "A," "B" and "C" with the water extract \( \text{o-PO}_4 \) higher in all cases. The higher \( \text{o-PO}_4 \) could be associated with other \( P \) compounds that are extracted (215). The conversion of all the extracted Poly-P to \( \text{o-PO}_4 \) during the extraction process is not documented. The conclusion from an article by Sutherland and Wilkerson (215) is that the water extraction of cells gives the same spectrum of \( P \) compounds plus Poly-P "A," "B" and "C" while Poly-P "D" may not be extracted. Poly-P "D" is bound with RNA (34) and more severe conditions are required to release the Poly-P in comparison with Poly-P "B" and "C" (114). Therefore utilizing the hydrolysis information of the \( P \) compounds extracted with TCA, the \( P \) compounds in the water extract compartments in Table 6.10 can be speculated. The low
acid stable P, P(L-H₂O), would include all the P compounds of the low acid stable P, P(L-TCA), in the TCA extracts, the TCA soluble Poly-P "A," the TCA insoluble Poly-P "B" and "C." The low acid stable P compartment, P(L-H₂O), contains the precursor pool P part of the intermediate pool P and a large portion of the storage pool P. The certainty that all the Poly-P cited above appears in the low acid stable P compartment, P(L-H₂O), is not supported by Goodman et al. (79). Part of the Poly-P(Poly-P "A") could appear in the medium acid stable P, P(M-H₂O). The medium acid stable P, P(M-H₂O), is assumed to include all the P compounds found in the TCA medium acid stable P, P(M-TCA), minus all or part of the Poly-P "A." The creatine and arginine phosphates are totally in this compartment. The water extraction is neutral in pH and creatine and arginine phosphate are more stable (241). There is uncertainty whether the nucleotide poly-P will reside in the low, P(L-H₂O), or middle acid stable P, P(M-H₂O). The high acid stable P of the water extract, P(H-H₂O), would contain the same P compounds of the high acid stable P in the TCA extract, P(H-TCA), which is the functional pool P. The water insoluble P, P(Ins-H₂O), would contain the phospholipids, RNA-P, DNA-P and Poly-P "D" which is the same as the TCA insoluble P, P(Ins-TCA), minus the Poly-P "B" and "C." The water insoluble P compartment, P(Ins-H₂O), is the structural pool P plus a small portion of the storage pool P. Speculation of additional P compounds that are only soluble in one extract and not the other is difficult to determine because of the lack of analysis of boiling water extract P.
6.4-6C Influence of Inorganic Particulate P or P Compartments Extracted from C. pyrenoidosa

In order to interpret the P dynamic data of C. pyrenoidosa, the availability of $o$-$PO_4$ from the inorganic particulate P for algal uptake needs to be assessed by utilizing the literature and the data that has been presented in Figures 6.14 to 6.19.

The conditions of precipitate formation for Group 3 runs was the addition of 15 ml of 5 N NaOH within 10 seconds to the medium in the fermentor apparatus concentrations of inorganic nutrients in the medium are found in Table 6.1 and Table C.1. The medium DO was in equilibrium with CO$_2$ enriched air. The pH immediately after the base addition was approximately 11.5 and fell to a pH of 7.5 within an hour as the hydroxide ion was neutralized by acidic carbon dioxide in the medium. The addition of an equal number of NaOH milliequivalents to the Group 4 run (P—24) was made over a three hour period. The pH did not increase over 7.5 during the addition. NaOH was not added to run Groups 1 and 2. The pH of the medium in equilibrium with CO$_2$-enriched air was 6.4 to 6.8.

The references which discuss the solubilities of inorganic particulate P generally deal with ideal systems which do not have competing coordinating ions or complexing species that increase solubility. The BBM medium, that provides the nutrients for growth of C. pyrenoidosa in this research, is complex and has many chemical species that complex with Fe, P and Ca. Therefore the interpretation of the dynamics of inorganic particulate P, which are presented in Figures 6.14 to 6.19 depend on utilizing the literature data which was generated in ideal systems. The first area of discussion will deal with
the inorganic species that exist in oxygenated medium at pH 6.5, at pH 11.5 after fast NaOH addition, at pH 7.5 after fast NaOH addition and at pH 7.5 after slow addition of NaOH. The availability of the inorganic particulate P for uptake by *C. pyrenoidosa* is assessed. In the slightly acidic oxygenated medium (pH-6.5) which is in Group 1 and 2 runs, iron exists in ferric (+3) state. The solubility data of Stumm and Morgan (214), Singer and Stumm (204) and Bohn and Pech (18), predict that the only inorganic particulate species that can exist is ferric hydroxide, Fe(OH)$_3$, at the concentrations of P($4.5 \times 10^{-5}$ moles/l), Mg ($3.4 \times 10^{-4}$ moles/l), Fe ($1.8 \times 10^{-5}$ moles/l) and Ca ($1.7 \times 10^{-4}$ moles/l) in a medium in equilibrium with CO$_2$ enriched air when no NaOH was added. Ferric phosphate precipitate does not exist. The medium contains 0.15 g moles/l EDTA which complexes with Fe$^{+3}$ to maintain iron in solution. When EDTA has a mole ratio to Fe$^{+3}$ of at least 3 to 1 for a pH less than 8.0, all the Fe$^{+3}$ is complexed with EDTA and remains in solution (182,205). The BBM has a mole ratio of EDTA to Fe of 10:1. The observations were noted in section 6.4-2C that there was no evidence of Fe or P precipitated particulates before the runs were initiated or evidence of Fe on dry weight glass filter pads after the ashing process. Under the conditions where all the ferric iron was complexed with EDTA, Zarnowski (248) found that *C. pyrenoidosa* was not inhibited in ultimate yield or growth rate.

Starting with medium conditions described in the previous paragraph for Group 1 and 2 runs, 15 ml 5 N NaOH was added rapidly to the medium. This procedure was carried out for Group 3 runs and the non-growth runs F-19 to F-22. The pH of the medium immediately rose to
pH 11.5 after the base addition. In the absence of EDTA, the stable precipitates are CaCO₃, Fe(OH)₃ as determined from solubility products (204,214). Nutrient metals, Zn, Cu, Co Mn and Mg will precipitate as hydroxides at pH 11.5 (see calculations in Appendix E). EDTA can complex with all of these heavy metals. For pH greater than 8, Fe has less affinity for EDTA than Mg (182). There is enough EDTA to satisfy only 50% of Mg (see Table C.1) without combining with the other heavy metals. Thus the other metals as well as the remaining uncomplexed Mg can form precipitates. Phosphorus can be found in the precipitate in the form of calcium phosphates, hydroxyapatite (Ca₅(OH)(PO₄)₃), and dicalcium phosphate (CaHPO₄), (37) which are found to form on the surface of solid CaCO₃ at high pH. Though CaHPO₄ is unstable at high pH it will form within 10 seconds after o-PO₄ is adsorbed onto CaCO₃ surface (37,60) and then form the more stable hydroxyapatite which is found to have a very low solubility product at high pH (214). The o-PO₄ does not adsorb to form ferric oxyhydroxide-orthophosphate complex because the pH of 11.5 is above the isoelectric point of 8.5 of ferric hydroxide (246). The non-growth run data in Figure 6.15, which presents the composition of the floc, indicates that at pH 11.5 approximately 98% of the Fe and 85% of the P are in the precipitate on the GF/D glass filters and that Ca (Table 6.7) is found in the precipitate. Analysis for Mn, Cu, Mg was not made. The dry weight of the floc was 32.54 mg/l at pH 11.5 (see section 6.4-5) immediately after addition of NaOH. Assuming that all the EDTA is combined with Mg, and that Mn, Cu, Fe and the remainder of the Mg are in the form of hydroxides, that P is in the hydroxyapatite form, Ca₅OH(PO₄)₃, and that
the remainder of the Ca is in the form of CaCO₃, the dry weight is calculated to be 33.36 mg dry weight (see Appendix E). This is considered good agreement of inorganic particulate dry weight and that the composition summarized above is valid. The 2.5% decrease in calculated dry weight over observed may be due to medium nutrient concentrations different than listed in Table C.1 and the passing of some of the CaCO₃ and metal hydroxides through the filter. The precipitated o-PO₄, as hydroxyapatite, will be soluble in acidic solutions (cold TCA) but only a trace is soluble in boiling H₂O extractant (pH=7.0). But data in Figure 6.16 was not determined at zero time and data in the plot cannot be extrapolated to determine the values. If CaHPO₄ was still the precipitated form of P, the P would be soluble in both cold TCA and boiling water (214). The evaluation of the availability of P or Fe for uptake by C. pyrenoidosa is not required since the algal cells were not inoculated at this condition. With the continuous addition of CO₂ enriched air to the fermentor medium, the pH falls to 7.5 within one hour.

The inorganic particulates go through a dynamic change as the pH of the system falls to 7.5. The solubility of the hydroxides of Zn, Co, Cu, Mg, Mn and CaCO₃ increases and they become totally soluble. But ferric hydroxide solubility decreases. Hydroxyapatite solubility increases but it is still essentially insoluble (214). This phenomenon is reflected in the decrease in the inorganic particulate dry weight in Figure 6.14. The data in Figure 6.15 indicate a sharp decrease in the concentration of the inorganic particulate P in the first 10 hours. The solubility of the ferric hydroxide decreases as
the pH changes from 11.5 to 7.5 the Fe concentration in the inorganic particulates in Figure 6.15 drops at a less rapid rate than P over a thirty hour range. Since the affinity of EDTA for Fe increases below a pH of 8.0, the particulate Fe is solubilized (129, 182). As indicated earlier all the Fe\(^{3+}\) should be complexed if the mole ratio of EDTA to Fe is greater than 3. But if the EDTA is bound to all the heavy metal nutrients in the medium, and also is bound to 30% of the Mg, the mole ratio of free EDTA to Fe is approximately 1:1. The partial binding of EDTA to Mg can be possible since the pH of 7.5 is very near pH 8.0. For a mole ratio of EDTA to Fe of 1:1, it can be estimated from the work of Jacobson (107) that approximately 24% of the Fe\(^{3+}\) will precipitate as Fe(OH)\(_3\). Since the pH is below the isoelectric point of 8.5 for Fe(OH)\(_3\), o-PO\(_4\) will coordinate with the solid phase to form ferric hydroxy-orthophosphate complex (246) which is in equilibrium with the o-PO\(_4\) concentration of the medium.

The rates of the initial decrease in the dry weight of the precipitate over the first 10 hours in Figure 6.14 can be attributed primarily to the solution of CaCO\(_3\) and the metal hydroxides. This would account for 70% of the particulate dry weight decrease. The decrease in dry weight from 10 to 20 hours will depend on the formation of the Fe-EDTA complex. This does not account for the total dry weight decrease. From the data in Figure 6.16, which gives the solubility of P in cold TCA and in boiling water, and from the discussion above, the molecular formulae that incorporate Ca, P and Fe can be suggested. Hydroxyapatite is only soluble in acid (214), thus the data in Figure 6.16 for P soluble only in cold TCA is the concentration of P in
hydroxyapatite, \( \text{Ca}_5(\text{OH})(\text{PO}_4)_3 \). Stamm and Kohlschutter (209) found that \( \text{o-PO}_4 \) associated with \( \text{Fe(OH)}_3 \) was liberated by washing with neutral water solutions. The boiling water extraction is neutral and liberates \( \text{o-PO}_4 \) from the inorganic solids. The \( \text{o-PO}_4 \) can also be liberated by cold TCA. The structure of the ferric hydroxide-orthophosphate complex in general is \( \text{Fe}_x(\text{OH})_3(x-y)(\text{PO}_4)_y \). Figure 6.19 gives the molar ratio of \( \text{P}/\text{Fe} \) for P extracted from the inorganic particulates by boiling water as 0.32. This molar ratio is approximately 1 to 3 and would give a ferric hydroxy-orthophosphate formula of \( \text{Fe}_3(\text{OH})_6\text{PO}_4 \). Using the solubility of P in cold TCA and in boiling water from Figure 6.16, the amount of P is partitioned between hydroxyapatite and ferric hydroxy-orthophosphate complex. At thirty hours the calculation of the dry weight contributed by these two chemical species is 1.71 mg dry weight/l (see Appendix E). The data of Figure 6.14 give a dry weight concentration of 3.0 mg dry weight/l. The difference may be due to the slow rate of solution of the hydroxides of \( \text{Cu}^{2+} \), \( \text{Zn}^{2+} \), \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) formed at pH 11.5. No data from the literature was found to assess the rates of solution of these compounds.

For the dynamics of the inorganic particulates of the non-growth runs, there is a less rapid decrease in the particulate P and Fe after thirty hours (see Figures 6.15 to 6.17) even though the dry weight concentration continues to decrease through fifty hours. With the ferric hydroxy-orthophosphate complex and hydroxyapatite as the chemical forms in the precipitate, the estimate of the dry weight concentration at eight hours from the data in Figures 6.16 and 6.17
is 1.30 mg dry weight/l (see Appendix E) compared to 1.25 mg dry weight/l in Figure 6.14. Data in Figures 6.15 and 6.16 indicate a steady decrease in the particulate concentrations of Fe and P that are soluble in both cold TCA and boiling water (the components of the ferrichydroxy-orthophosphate complex), but a low rate of decrease in P soluble only in cold TCA (component of hydroxyapatite). This indicates a higher stability of the latter species of the precipitate in the medium. Figure 6.17 shows a constant molar ratio of P (boiling water soluble)/Fe over the time that the data was experimentally taken. The other P/Fe molar ratios increase after 100 hours. This indicates that as Fe solubilizes, the boiling water soluble P solubilizes in a 3 to 1 mole ratio. Such a direct relationship would be found in the ferrichydroxy-orthophosphate complex. The P (only soluble in cold TCA)/Fe molar ratio increases after 100 hours. The P associated with the hydroxyapatite is stable. The above phenomenon is supported in the literature. Fresh precipitated ferric hydroxide has a solubility product which allows more solubilization of Fe and thus the liberation of the coordinated o-PO₄ (135). Griffin and Jurinak (81) found that hydroxyapatite was stable at pH 8.4. In the absence of o-PO₄ in solution with the hydroxyapatite, their equation predicts that only 20% of the bound P will solubilize in 100 hours at 23°C.

In Group 4 run data, a different procedure for the addition of NaOH to the medium was followed. The 15 ml 5N NaOH was diluted to 125 ml with distilled water and added at a rate of 0.7 ml/min. The pH of the medium was never above 7.5. The solubility product of hydroxyapatite (214) indicates that it forms a precipitate under these
medium conditions. Ferric hydroxy-orthophosphate complex solubility indicates the potential to form a precipitate (214). Without the cycling of the pH through a high value, Mg cannot compete for EDTA successfully as indicated earlier (182). Therefore the mole ratio of EDTA/Fe would be greater than 3 to 1 and all the Fe is complexed with EDTA in the soluble form. Indications from the cellular P extraction data of run F-24 are that hydroxyapatite is formed after the inoculum is added. Figures 6.30 and 6.38 are plots of the dynamics of original Data Groups P(Tot-Part), P(L-H2) and P(L&M-H2) and P compartment Data Groups P(Ins-H2O), P(H-H2O) and P(M-H2O) of run F-24 and a Group 3 run, F-25. The curves to note are the total particulate P, P(Tot-Part), and the boiling water insoluble P, P(Ins-H2O). Previous discussion indicates that both Ca5OH(PO4)3 and Fe(OH)3 are precipitated for Group 3 runs. Figure 6.15 indicates that a P weight fraction of 0.11 of the Tot-P in the fermentor is found in the precipitate at 70 hours after the NaOH is added to the medium. The P(Tot-Part) in Figure 6.38 gives a value of 0.13 for the P weight fraction immediately before and after the medium is inoculated with C. pyrenoidosa. The algal cells contribute an insignificant amount of P at this point of growth. The P(Ins-H2O) in Figure 6.30 shows a 0.05 P weight fraction. For boiling water insoluble P, P(Ins-H2O), in the non-growth runs, Figure 6.16 shows a value of 0.05 weight fraction at 70 hours after the addition of NaOH to the medium. For run F-24 at time equal to zero before the algal cell inoculum was added to the medium, no particulate P was filtered out of the medium with a 0.45 μm membrane filter. But 10 hours after the addition of the algal cell inoculum, the level of P concentration of
P(Ins-H$_2$O) increased sharply to 0.05 P weight fraction and P(Tot-Part) increases to a value slightly higher than P(Ins-H$_2$O). These observations indicate that hydroxyapatite precipitates from a saturated solution and accumulates to a P weight fraction that is found in Figure 6.16 for P soluble only in cold TCA. Termine and Posner (233) indicate that biological macromolecules initiate the precipitation of hydroxyapatite. Further no ferric hydroxy-orthophosphate complex formation is evidenced.

The importance of identifying the inorganic particulate forms of P is for the determination of the availability of the inorganic particulate P for uptake by C. pyrenoidosa. Hydroxyapatites are found in skeletal animals in the bones and teeth. About 86% of the P in the adult human body is apatite. In this biological system the stability of the hydroxyapatite crystal is obviously of great importance. Thus rapid biological breakdown is absent. But water leaches out hydroxyapatite crystals from bone. Atkins and Jurikiak (233) found that, in absence of o-PO$_4$ in a medium, hydroxyapatite released o-PO$_4$ by solution at a slow rate, approximately 20% of the crystal P is released in 100 hours. Goltermann et al. (78) found that the green alga, Scenedesmus obliquus, was able to utilize 33.6% of the P in hydroxyapatite in 192 hours when used as the sole source of P. Therefore for the C. pyrenoidosa growth experiments that were conducted, hydroxyapatite is not solubilized except at a slow rate after the depletion of o-PO$_4$ from the medium. The solubilized P would contribute only 1% of the total P to the algal compartments. There is no acid environment to solubilize the hydroxyapatite since the medium pH is 7.5. The
pH near the surface of the algal cell is higher than the medium due to the depletion of CO₂ by the alga.

The P associated with the ferric hydroxide yields a different result in terms of P availability for uptake by C. pyrenoidosa. Experiments were run to determine the desorption of o-PO₄ from the floc formed in the non-growth runs F-20 and F-21. Figure 6.18 presents the results as a function of the floc age. All the data obtained in Group 3 growth runs (except run F-17) were taken at least 70 hours after NaOH was added to the medium. Thus, as seen in Figure 6.18, the amount of o-PO₄ eluted from the floc was nearly constant. All data for run F-17 was taken between 15 and 50 hours. Therefore for run F-17 the amount of o-PO₄ in the desorbing liquid was dependent on the age of the precipitate.

In addition to the above information, two phenomena are found from the experiments that would help to evaluate the availability of the o-PO₄ which is complexed with ferric hydroxide. Figure 6.19 indicates that the amount of Fe filtered with the GF/D filter increases as the cell number increases up to the level equivalent to the Fe in the medium. The amount of Fe/cell ranges from 8.5 x 10⁻⁶ μg/cell at 30 hours are 1 to 3 orders of magnitude higher than the published values. C. pyrenoidosa was found to contain 5.6 x 10⁻⁹ μg Fe/cell by Wiessner (243) and 3.7 x 10⁻⁷ μg/cell by Knauss and Porter (118). The literature indicate that ferric hydroxide can precipitate or Fe(OH)₃ particulates can be attracted to cell surfaces. Harvey (86) found that ferric hydroxide precipitated on diatoms while McCrae and Edwards (143,144) determined that solid
Fe(OH)$_3$ was attracted and precipitated on bacterial cell surfaces at pH 7.6. Kennedy et al. (117) found that bacterial cells could be immobilized on metal hydroxide surfaces. The data in Figure 6.19 indicate that Fe would have come from both the particulate Fe and Fe complexed with EDTA. Marshall (149) found that bacteria attracted more clay particulates during the exponential growth. The observation could account for the drop in the amount of Fe found on the GF/D filter at 200 hours during stationary growth. In addition, algal can release organic chemicals that act as chelating agents for Fe (103). If the o-PO$_4$ complexed with ferric hydroxide is attracted to the cell surface, it would be available for uptake since the pH at the cell surface has a high pH. The high pH reduces the ability of ferric hydroxide to complex o-PO$_4$. In Figure 6.12, which is the o-PO$_4$ uptake/cell, Group 3 runs show very low o-PO$_4$ uptake rates in the first 20 hours of the growth in comparison to Groups 1, 2 and 4 runs. The phenomenon that may happen is that the particulate o-PO$_4$, complexed with Fe(OH)$_3$ is attracted to the *C. pyrenoidosa* cell surface and releases o-PO$_4$ to be taken up by the cell. This phenomenon would yield the response of uptake/cell in Figure 6.12 if the true uptake rate of the cell was not lower for these runs. The latter assumption seems valid since there were the same environmental conditions for Groups 3 and 4. Fitzgerald (63) found that algal cells attained the same growth rates and maximum yields when 85, 50 and 0% of the o-PO$_4$ was complexed with Fe(OH)$_3$. If the ferric hydroxide-orthophosphate complex is not attracted, o-PO$_4$ is released after or as the medium o-PO$_4$ decreases to zero. The desorption rate is first relative to
the growth of the alga as determined from Figure 6.18.

6.4-6D Dynamics of the Phosphorus Compartment of C. pyrenoidosa

General

This section summarizes the dynamic behavior of the P compartments which were extracted from C. pyrenoidosa. The discussion is introduced with general observations concerning the experimental data of run Groups 1 to 4. The discussion includes the application of the conclusions concerning the availability of P from hydroxyapatite and from ferric hydroxy-orthophosphate complex for uptake by the alga.

The P compartment dynamic data used for this discussion are plotted in Figures 6.24 to 6.35. The data are plotted on two type figures—P/cell vs. Time and Fraction of Tot-P in Medium vs. Time. One type plot was not able to present data in a meaningful way over all growth phases. For the growth time ranging from 0 to 80 hours, P/cell vs. Time gives the best indication of the P compartment dynamics while the Fraction of Tot-P in Medium vs. Time presents the P compartment data best for 60 hours and beyond. During the span of time that overlaps, the dynamics of each plot are nearly the same. The P compartment data for Run F-25 is significantly different from the remainder of the Group 3 runs and is plotted separately. Run F-25 was included into Group 3 based on the similarity of the cell population and dry weight dynamics to the other runs in Group 3 (see Figure 6.3).

The C. pyrenoidosa data show major concentration level variations and minor fluctuations as plotted in Figures 6.24 to 6.39. The major variations are significant and are due to physiological changes
during the growth of the *C. pyrenoidosa* culture. The minor fluctuations may be the result of error of the analytical techniques, synchronous growth or cell physiological changes. The maximum standard deviation of the differences between analytically determined values is 0.009 mg P/l. The percent standard deviation can be 100% for the first 10 hours of growth, less than 20% for the next 10 hours and less than 5-10% for the time greater than 20 hours. Some of the minor fluctuations may be significant. To test this assumption, it would require several replications of the growth runs. Six replications were made in Group 3 runs (excluding run F-25). Most of the runs in Group 3 contain only a few data sets, 2 to 4, of the P extractions as compared to runs F-24 and F-25 with 11 and 9 data sets in each run. Statistical analysis is not possible.

The presentation of the dynamic nature of the P compartments of *C. pyrenoidosa* is difficult when using Figures 6.24 to 6.39. The dynamic presentation was simplified into a block diagram form in Figures 6.20 to 6.23. Group 1 runs were not put into this format because there are too few data sets to make an analysis. The concentration levels of the cellular P compartments of Group 1 from Figures 6.24 and 6.25 though will be utilized.

The inorganic particulate P, hydroxyapatite and ferric hydroxy-orthophosphate complex, contributed an uncertain error to several of the P compartment data groups as previously discussed. These data groups are not entirely useless. The uncertainty of when the o-PO$_4$ was released from the ferric hydroxy-orthophosphate complex before the depletion of the o-PO$_4$ from the medium, made P Compartment Data Groups
P(L-H₂), P(L&M-H₂O) and P(Ins-TCA) useless for Group 3 runs and run F-25. After the depletion time was passed, it was assumed that no o-PO₄ was complexed with ferric hydroxide and therefore Data Groups P(L-H₂O), P(L&M-H₂O) and P(Ins-TCA) were valid. Since no ferric hydroxy-orthophosphate complex was formed in run F-24, P Compartment Data Groups P(L-H₂), P(L&M-H₂O) and P(Ins-TCA) were valid over the entire growth time. The stability of the hydroxyapatite in the presence of soluble o-PO₄ and the low rate of solution when o-PO₄ is not present in the medium makes it possible to use P Compartment Data Groups P(L-TCA) and P(Ins-H₂O) throughout the growth period. Caution must be taken in using Data Groups P(L-TCA) and P(Ins-H₂O) during the time before o-PO₄ depletion.

The block diagrams of the dynamic behavior of the P compartments of *C. pyrenoidosa* need explanation concerning the method of construction and the method of interpretation. Each P Compartment Data Group is represented by a block in each of the growth time segments. Data Groups P(L&M-H₂O) and P(H&M-TCA) are the combination of Data Groups P(H₂O) and P(L-H₂O) and Data Groups P(H-TCA) and P(M-TCA) respectively. The time span of the segment is the time between significant changes in the P dynamics. There is an uncertainty to the beginning and the end of each time segment since the experimental acquisition of the data sets was repeated every 10 to 20 hours. The error of the time of the onset of a rate change in a P compartment was assumed to be ±5 hours.

For each run the incorporation of the two type plots of the P dynamic data, into the block diagram was based on reasoning given earlier. The P/cell vs Time gives the best indication of the dynamics
in the first 80 hours of growth while the Fraction of Tot-P in Medium vs Time is the most effective form over the 60 hour and beyond range. The shift from one plot to the other was based on the dynamics of the Fraction of Tot-P in Medium plot. All the curves of the P compartment data groups reach an initial peak in the range of 60 to 70 hours. The time of the peak is the point at which the use of the plots is shifted to construct the block diagram.

The P/cell vs Time plot gives the net flow of P into or out of a P compartment. Neither the fate of P leaving a compartment nor the source of the P into a compartment can be determined. Further the uptake of $\text{o-PO}_4$ from the medium is a continuous process. Whether the $\text{o-PO}_4$ is distributed to each P compartment or passed through one or more compartments cannot be assessed. A complication of interpreting the data is cell division. If no P is brought into a P compartment, the concentration in the compartment will be half as the cell number doubles due to cell division. If P is lost from the compartment, the rate of loss of P from compartment in the figure is greater than the loss due only to cell division. There are other conditions. The rate of loss can be less than the cell division loss if the P into the compartment is not sufficient to replace P lost by cell division. If there is satisfactory P replacement, the rate of change of P in a compartment is zero. Finally when there is more P entering a compartment than is needed to replace P lost by cell division, the compartment concentration increases with time. Table 6.11 lists each of these events with a symbol that is used in the block diagrams, Figures 6.20 to 6.23. In the block diagrams an arrow indicates the direction of the P flow of a
Table 6.11 Symbols Used in Figures 6.20 to 6.23 Concerning the Net Flow of P of a Compartment.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>Concentration loss in a cellular P compartment at a rate greater than can be accounted for by cell division. Net loss from P compartment.</td>
</tr>
<tr>
<td>0</td>
<td>Concentration loss in a cellular P compartment at a rate equivalent to loss by cell division. Net input to P compartment is zero.</td>
</tr>
<tr>
<td>1</td>
<td>Concentration loss in a cellular P compartment at a rate less than can be accounted for by cell division. Net input to the P compartment.</td>
</tr>
<tr>
<td>2</td>
<td>There is no concentration loss or gain in P compartment. Net input to P compartment greater than 1.</td>
</tr>
<tr>
<td>3</td>
<td>There is a concentration gain in the P compartment. Net input to P compartment is greater than 2.</td>
</tr>
</tbody>
</table>

compartment, there is a number symbol from Table 6.11 and the rate of change of P/cell is given. For those compartments that contain inorganic particulate P, the concentration loss in the compartment of the particulate P is equivalent to that due to cell division. No rates could be measured but assumptions of P flow magnitude could be made and the appropriate symbols were used. For blocks that are crossed out, no data was available or data was too scattered to be meaningful. Blocks that contain a zero have compartment P concentrations that were not detectable.

The shift to the Fraction Tot-P in Medium vs Time changes the presentation of the P dynamics. Since all $\text{o-PO}_4$ now is in the particulate fraction, gross flow of P can be followed from one P compartment
to another. The flow of P is not normalized to a per cell basis. The gross P flow gives a better view of what is happening in the particulate fraction. To determine the per cell basis, the P rates of change need to be divided by the cell number. It was felt that the way it is presented gives the best feeling of the dynamics. The same procedure could have been applied in the first growth hours. But the rates of change of the Fraction Tot-P in Medium of each compartment was so low initially that rate differences would be difficult to determine. Further as the growth reaches the point near \( {\text{o-P}}_{\text{4}} \) depletion from the medium, the rate of change of P in each compartment are very rapid. There are too few data points to determine the rates of change of P to adequately evaluate the flow of P into or out of a P compartment. In the block diagrams over the later time period, the number associated with each block is the rate of change per hour of the P compartment per liter of medium. These rates give the true magnitude of the rate of change. The arrows in the diagram indicate the flow of P to and from the P compartment.

The presentation of the dynamic data in the block diagrams of Figures 6.20 to 6.23 and in Figures 6.24 to 6.39 in a very detailed discussion can be done but the data are of limited value without further experimental duplication. But the general dynamics are of great value. The discussion will be limited to general observations of the dynamic behavior of the P compartments. There are two observations concerning the data from the experimental analysis point of view that need to be stated because they affect the conclusions made from the experimental data. In the first ten hours of the growth it has been
stated that the error of the P concentration can be as high as 100%. Therefore if there is going to be a large deviation in the P compartment dynamics it would be found in this region. Second, there is still a question of repeatability of P between the compartments of both the TCA and the water extracts. For example, there is a chance that the TCA medium acid stable P will be counted as TCA low stable due to the variation in time before the coloration of the sample, in temperatures in the extract before the analysis and in pH. These are subject to change when there is an incorporation of a new analytical technique which changes the integration of all the procedures. This will be reflected in the more sensitive low and medium acid stable P compartments. Therefore the P Compartment Data Groups P(H&M-TCA) and P(L&M-H$_2$O) which are the sum of two other P compartments are included in all plots. The P data for some of the run groups were only obtained in this form. Further, these combined data groups add a check to the data.

Results Presented in the Block Diagrams
(Figures 6.20 to 6.23)

The first part of the discussion will cover the general dynamics of all the P compartments and the deviations between the groups as shown in Figures 6.20 to 6.23. The later discussion will compare the TCA to water compartments based on the similarity of the P compounds in compartments of each extract as outlined in Section 6.4-6B.

In the first hours after C. pyrenoidosa is inoculated into the growth culture during the lag growth phase, there is a rapid increase in the P concentration of most of the cellular compartments. There is
### Water P Compartments

<table>
<thead>
<tr>
<th>P Group</th>
<th>Time (Hrs)</th>
<th>Phosphorus Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(Ins-H₂O)</td>
<td>0-20</td>
<td>60-70: <strong>-0.005</strong></td>
</tr>
<tr>
<td></td>
<td>20-30</td>
<td>70-80: +0.003</td>
</tr>
<tr>
<td></td>
<td>30-60</td>
<td>80-100: +0.002</td>
</tr>
<tr>
<td>P(H₂O)</td>
<td></td>
<td>60-70: <strong>-0.005</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>70-80: +0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80-100: +0.002</td>
</tr>
<tr>
<td>P(L₅M-H₂O)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* \(+0.23 \times 10^{-10}\) MgP/Cell·Hr

**\(-0.005\) Mg/Liter·Hr

Figure 6.20 Block Diagram of P Flow for Run Group 2
### Water P Compartments

<table>
<thead>
<tr>
<th>P Group</th>
<th>Time (Hrs)</th>
<th>0-5</th>
<th>5-25</th>
<th>25-40</th>
<th>40-60</th>
<th>60-70</th>
<th>70-80</th>
<th>80-100</th>
<th>100-120</th>
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<tbody>
<tr>
<td>P(Ins-H₂O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.01</td>
<td>+0.004</td>
<td>+0.005</td>
<td>+0.009</td>
</tr>
<tr>
<td>P(H₂O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+0.20</td>
<td>-0.011</td>
<td>-0.002</td>
</tr>
<tr>
<td>P(M-H₂O)</td>
<td>+1.4</td>
<td></td>
<td>-0.65</td>
<td></td>
<td></td>
<td>-0.03</td>
<td></td>
<td></td>
<td>+0.005</td>
<td></td>
</tr>
<tr>
<td>P(L-H₂O)</td>
<td>+1.9</td>
<td></td>
<td>-0.08</td>
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<td>-0.08</td>
<td>+0.38</td>
<td>-0.67</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>P Group</th>
<th>Time (Hrs)</th>
<th>0-5</th>
<th>5-10</th>
<th>10-25</th>
<th>25-50</th>
<th>50-60</th>
<th>60-70</th>
<th>70-80</th>
<th>80-100</th>
<th>100-120</th>
<th>120-150</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(Ins-TCA)</td>
<td></td>
<td>+4.7</td>
<td>+8.4</td>
<td></td>
<td>-4.4</td>
<td></td>
<td></td>
<td>+0.01</td>
<td>+0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(H-TCA)</td>
<td>+5.6</td>
<td></td>
<td>-4.1</td>
<td>-0.35</td>
<td>+0.1</td>
<td></td>
<td></td>
<td></td>
<td>+0.013</td>
<td>+0.003</td>
<td>+0.001</td>
</tr>
<tr>
<td>P(M-TCA)</td>
<td></td>
<td></td>
<td></td>
<td>-0.13</td>
<td>+0.12</td>
<td>+0.57</td>
<td></td>
<td></td>
<td>+0.011</td>
<td>+0.002</td>
<td>-0.001</td>
</tr>
<tr>
<td>P(L-TCA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+0.009</td>
<td>+0.001</td>
<td>+0.003</td>
</tr>
</tbody>
</table>

* +1.4 x 10⁻¹¹ Mg P/Cell·Hr

** -0.11 Mg P/Liter·Hr

Figure 6.21 Block Diagram of P Flow for Run Group 4
Figure 6.22 Block Diagram of P Flow for Run Group 3
**Water P Compartments**

<table>
<thead>
<tr>
<th>P Group</th>
<th>Time (Hrs)</th>
<th>0-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-60</th>
<th>60-70</th>
<th>70-80</th>
<th>80-90</th>
<th>90-110</th>
<th>110-200</th>
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</thead>
<tbody>
<tr>
<td>P(Ins-H₂O)</td>
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<td>☒</td>
<td>☒</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>P(H₂O)</td>
<td></td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>P(L₂O)</td>
<td></td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
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</tr>
</tbody>
</table>

**TCA P Compartments**

<table>
<thead>
<tr>
<th>P Group</th>
<th>Time (Hrs)</th>
<th>0-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-60</th>
<th>60-80</th>
<th>80-90</th>
<th>90-110</th>
<th>110-200</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(Ins-TCA)</td>
<td></td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>P(H-TCA)</td>
<td></td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>P(L-TCA)</td>
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<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td></td>
</tr>
</tbody>
</table>

Phosphate Depletion

- 0-20: +0.011
- 20-30: +0.004
- 30-40: -0.002
- 40-60: +0.009
- 60-70: -0.002
- 70-80: +0.003
- 80-90: +0.002
- 90-110: +0.003
- 110-200: +0.003

* +0.38 x 10⁻¹⁶ Mg P/Cell·Hr

** -0.005 Mg P/Liter·Hr

Figure 6.23 Block Diagram of P Flow for Run F-25
no detectable input to some compartments of which P Compartment Data Group P(H-H₂O) is the most evident. For the water extracted compartments, the low and medium acid stable compartments, the increase is less at pH 6.5 for Group 2 run in Figure 6.20 than pH 7.5 in Figures 6.21 and 6.22 but the higher growth temperature of run F-25 in Figure 6.23 also gives a low P input rate. Rapid input of P into TCA compartments also occurs. For the available data the input rates are higher for the P compartments that are soluble in TCA and water. Further the input rates are higher in the TCA compartments than the water compartments. After the period of rapid increase in the P concentration of the compartments, there is either zero flow of P or a flow of P into the compartments below the replacement rate for the loss of P due to cell division. The TCA compartments show a decrease in P concentrations as a result of a net flow of P from the compartment. In run F-25, Figure 6.23, the P Compartment Data Group P(H-TCA) was the exception. This compartment did not have a rapid input of P after inoculation of the run. As the first part of the exponential growth phase progressed, there is almost a general phenomenon in all compartments of the increase in the P concentration as the flow of P into the compartments is above the replacement rate. A majority of the compartments reach a maximum input P rate during the exponential growth phase before the time of o-PO₄ depletion from the medium. The maximum input rate is followed by a rapid decline in the P concentrations which is generally near the rate equivalent to the loss due to cell division.

After the depletion of o-PO₄ in the medium, the second part of the exponential growth phase begins. The data are presented in terms
of net flow of P between compartments. During the first 10 hours after the depletion, there is a net flow of P from the insoluble P and a net loss from the sum of the soluble low and medium acid stable P in the water extract compartments. Then there is a reversal of P flow back to the insoluble P from the soluble compartments of the water extract except Run Group 3 in Figure 6.22 which continues to lose P into the transition to stationary growth phase. The reversal comes before the end of the exponential growth phase. The flow of P to the water insoluble P for Run Group 3 lags the other run groups. There is a net flow of P to the insoluble and low acid stable P of the TCA compartments from the high and medium acid stable P in the first ten hours after the depletion of O-PO$_4$. Then there is flow away from the insoluble P but not as high of a rate of P as entered the compartment. The flow continues up to the end of the exponential growth phase and on into the first hours of the transition to stationary growth phase. The flow is generally to the medium and high acid stable P of the TCA extract.

Flow of P, after the exponential growth ceases, is more random between run groups and occurs at low rates.

Results Presented in Figures 6.24 to 6.38.

The P compounds in TCA and water extracts were discussed in Section 6.4-6B. There is similarity in compounds between some of the corresponding acid stable P compartments of the TCA extract and the water extract. The remainder of this chapter concerns the relationships in the P data in Figures 6.24 to 6.38. Each comparable P compartment data group for each extract will be discussed separately.
Figure 6.24 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Per Cell Content - Group 1
Figure 6.25 Dynamics of P Extracted from *C. pyrenoidosa* in Batch Culture with Water Based on Weight Fraction of Tot-P in Medium - Group 1
Figure 6.26 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Per Cell Content - Group 2
Figure 6.27 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Weight Fraction of Tot-P in Medium - Group 2
Figure 6.28  Dynamics of P Extracted from C. Pyrenoidosa in Batch Culture with Water Based on Cell Content - Run F-24 - Group 4
Figure 6.29 Dynamics of P Extracted from *C. pyrenoidosa* in Batch Culture with TCA Based on Cell Content - Run F-24 - Groups 4
Figure 6.30 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Weight Fraction of Tot-P Medium - Fun F-24 - Group 4
Figure 6.31 Dynamics of P Extracted from C. Pyrenoidosa in Batch Culture with TCA Based on Weight Fraction of Tot-P in Medium - F-24 - Group 4
Figure 6.32 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Cell Content - Group 3
Figure 6.33 Dynamics of P Extracted from C. Pyrenoidosa in Batch Culture with TCA Based on Cell Content - Group 3
Figure 6.34 Dynamics of P Extraction from C. pyrenoidosa in Batch Culture with Water Based on Weight Fraction of Tot-P in Medium — Group 3
Figure 6.35 Dynamics of P Extraction from *C. pyrenoidosa* in Batch Culture with TCA Based on Weight Fraction of Tot-P in Medium - Group 3
Figure 6.36 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with Water Based on Cell Content - Run F-25
Figure 6.37  Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with TCA Based on Cell Content - Run F-25
Figure 6.38 Dynamics of P Extracted from C. Pyrenoidosa in Batch Culture with Water Based on Weight Fraction of Tot-P in Medium - Run F-25
Figure 6.39 Dynamics of P Extracted from C. pyrenoidosa in Batch Culture with TCA Based on Weight Fraction of Tot-P in Medium - Run F-25
The first P compartments to compare is the low acid stable soluble P in the water extract, \( P_{(L-H_2O)} \), in Figures 6.27, 6.30, 6.34 and 6.38, and in the TCA extract \( P_{(L-TCA)} \), in Figures 6.29, 6.31 and 6.39. The concentration of P in the water extract should be higher or equal to the TCA extract as previously assumed in Section 6.4-6B. No comparisons can be made for the concentrations in the algal cell before the depletion of \( o-PO_4 \) in the medium because of the influence of the inorganic particulate P. After the depletion of \( o-PO_4 \) from the medium, valid comparisons can be made. It is found that the concentration of P in all the water extract compartments is higher than the TCA extract within a run group. The dynamics of the water extracts are similar with the highest concentrations belonging to run F-25 in Figure 6.38 followed by Run Group 3 in Figure 6.34 and then Run Group 4 in Figure 6.30. All the concentrations decline in the stationary growth phase with Run Group 3 falling first followed by run F-25 with Run Group 4 showing signs of decline at the end of the data. The TCA extract concentrations of P are highest for run F-25 in Figure 6.39 followed by Run Group 4 in Figure 6.31. The P concentrations of run F-25 and Run Group 4 begin to fall after the end of the second part of the exponential growth phase.

The medium acid stable P compartments in water extract, \( P_{(M-H_2O)} \), in Figures 6.28, 6.30, 6.32, 6.34 6.36 and 6.38 and in TCA, \( P_{(M-TCA)} \), in Figures 6.29, 6.31, 6.37 and 6.39. From the discussion in Section 6.4-6B, the concentration of P in the TCA extract is higher or equivalent to the P in the water extract. The water extract P concentration, \( P_{(M-H_2O)} \), falls from a high value during the lag phase for
Run Group 3 in Figure 6.32 and Run Group 4 in Figure 6.28 while run F-25 in Figure 6.36 has no detectable concentration. The TCA extract, P(M-TCA), shows a similar declining P concentration but the concentration level is much lower for Run Group 4 in Figure 6.29 and run F-25 in Figure 6.37. There is no data for Run Group 3 before the depletion of o-PO₄. In the 30 to 40 hour range, the medium acid stable P in both extracts reach a minimum concentration before the concentration increases and, only now, does run F-25 in Figure 6.37 begin again to show a P concentration. The water extract, P(M-H₂O), for Run Group 4 in Figure 6.28 continues to decline from the initial maximum value and does not increase. The TCA extract, P(M-TCA), reaches zero concentration before the rise in Figures 6.29 (Group 4) and 6.37 (Run F-25). In the same figures the P concentration rises for the water and the TCA extracts to a maximum value just before or at the point of o-PO₄ depletion from the medium and then falls rapidly. The concentration of P in the TCA extract is lower than the water extract through all the stages of the growth up to this point. This is opposite to the initial assumption.

Run Group 1 in Figures 6.24 and 6.25 and run Group 2 in Figures 6.26 and 6.27 have the low and medium acid stable P which is soluble in the water extract combined, P(L&M-H₂O), P which can be compared to Run Group 4 in Figures 6.28 and 6.30. Both Run Group 2 in Figure 6.26 and Run Group 4 in Figure 6.28 show a decrease in the P concentration from a high value in the lag phase. Run Group 1 in Figure 6.24 does not have as much data to determine the dynamics during this phase. All the run groups show decline in concentration into the first part of the
exponential growth phase. A minimum value is reached before the concentration rises to a maximum in all three groups at a point just before or at the time of o-PO$_4$ depletion. The concentration level is highest for Run Group 2 in Figure 6.26 followed by Run Group 1 in Figure 6.24 and then Run Group 4 in Figure 6.28.

After the depletion of o-PO$_4$ from the medium, the concentration of P in the medium acid stable compartments in the TCA and water extracts is at a maximum and rapidly declines during the first 10 hours of the second part of the exponential growth phase. For run F-25 in Figures 6.38 and 6.39, both extracts plunge to a concentration of zero after the maximum though the TCA extract rises during the stationary growth phase at 200 hours. For Run Group 3 in Figure 6.34 the water extract reaches a maximum and falls to a constant level of P concentration above zero. There is no data for the TCA extract in Run Group 3. Run Group 4 presents a different dynamic course. The maximum reached in the water extract in Figure 6.30 is much lower than the other runs and does decrease in concentration until after the exponential growth is ended. The TCA extract in Figure 6.31 does go through a maximum after which there is a decrease of the P concentration to a minimum near zero. But in Figure 6.31 the TCA concentration rises again at the end of the exponential growth phase and then it again falls to zero. Overall the concentration levels of the water extract is higher than the TCA extract except for Run Group 4.

The combined P Compartment, P(L&M-H$_2$O), can be compared on only a limited basis between Run Group 2 in Figure 6.27 and Run Group 4 in Figure 6.30. Run Group 1 has no data after o-PO$_4$ depletion. Both run
groups fall from a maximum value after the end of the first part of
the exponential growth phase. The P concentration of P(LM-H₂O) in
Run Group is higher than Run Group 4.

The high acid stable P are P(H-TCA) in TCA extract in Figures
6.29, 6.31, 6.37 and 6.39 and P(H-H₂O) in Figures 6.24 to 6.28, 6.30,
6.32, 6.34, 6.36 and 6.38. The two groups show a great deal of sim-
ilarity. The discussion in Section 6.4-6B indicates that the concen-
trations of P in the TCA and water extracts should be equivalent. For
Group 4, Figure 6.29, the concentration of P in the lag phase is not
detectable. Run Group 3 has no data. The TCA extract of Run Group 4
in Figure 6.24 though is very high and falls rapidly to reach a minimum
at the time when the exponential growth phase initiates. In Figure
6.26 Run Group 2, water extract does not go through the radical con-
centration increase like Run Group 4 and rises more slowly to a peak
at the beginning of the exponential growth phase. The concentration
then falls and reaches a minimum value. The P concentration for all
other data begins to rise from zero at the beginning of the exponential
growth phase. All the Run Groups 3 and 4 and Run F-25 (pH=7.5) tend to
plateau during the first part of the exponential growth phase up to the
time when o-PO₄ is depleted when the concentration rapidly falls. In
Run Groups 1 and 2 (pH=6.5) in Figures 6.24 and 6.26 there is no
plateauing of the P concentration. The concentration rises to a maximum
value at the time of o-PO₄ depletion and then the concentration rapidly
decreases. The P concentrations of the TCA and water extracts are
nearly equal with the water extracts slightly higher.
The P Compartment P(H&M-TCA) is in Figures 6.29, 6.31, 6.33, 6.35, 6.37 and 6.39. It is the sum of P(H-TCA and P(M-TCA) of the TCA extracts. The dynamics of the P concentrations for Run Groups 3 (Figure 6.33) and 4 (Figure 6.29) and run F-25 (Figure 6.37) are the same. Each shows a declining P concentration during the lag phase and reaches a minimum value in the 30 to 40 hour time during the first hours of exponential growth. The P concentration rises to a maximum at or before the time of o-PO₄ depletion and then the concentration rapidly decreases. This preceding discussion is the example of where the analytical procedure did not give the correct distribution of P between Data Groups P(H-TCA) and P(M-TCA) while the sum shows very similar behavior for all the run groups.

After the depletion of o-PO₄ in the medium, the high acid stable P of the water extracts in Figures 6.38 and 6.34 continue to rise to reach a maximum at 10 to 20 hours later. At the same time the high acid stable P in the TCA extracts of run F-25 in Figure 6.39 and Run Group 3 in Figure 6.35 are at a peak concentration at the depletion time and decline in concentration for the next 10 to 20 hours to a minimum value. Thereafter the TCA extract increases in concentration while run F-25 levels off. The water extract concentration decreases during the rest of the growth time after the maximum in all the run groups. The dynamics during this growth stage are definitely different between the water and the TCA extracts as compared to the dynamics before o-PO₄ depletion from the medium.

The dynamics of Data Group P(H&M-TCA), the sum of the medium and high acid stable soluble P of the TCA extract, begins at a maximum at
the beginning of the second part of the exponential growth phase in all run groups in Figures 6.31, 6.35 and 6.39. During the next 10 hours of the second part of the exponential growth phase, the P concentration in all the compartments fall rapidly and reach a minimum value. In run F-25 in Figure 6.39, the TCA extract levels off at a constant value. For Run Group 3 in Figure 6.35 and run F-25, the TCA extract concentrations rise and level off at a constant concentration at the end of the exponential growth phase. The concentration levels are nearly equal for all the run groups.

The final P compartment is the insoluble P of the P Compartment Data Groups P(Ins-H₂O) in Figures 6.24 to 6.28, 6.30, 6.34, 6.36 and 6.38 and P(Ins-TCA) in Figures 6.29, 6.31, 6.35, 6.37 and 6.39. The dynamic data is very limited before the depletion of the o-PO₄. The water extract plots are useless because of the interference of hydroxyapatite in Run Groups 3 and 4 and run F-25. Run Groups 1 and 2 Figures 6.24 and 6.26 respectively indicate that the water insoluble P reaches a peak in the early stages of the exponential growth phase and the concentration declines thereafter. The P concentration of Run Group 2 is higher than Run Group 1.

There is sufficient data of insoluble P in the TCA and water extracts after the depletion of o-PO₄. The TCA extract, P(Ins-TCA), continues to rise after the beginning of the second part of the exponential growth phase. The concentration peaks about 10 hours later. The peak concentration is highest for run F-25 in Figure 6.39 followed by Run Groups 3 (Figure 6.39 and than 4 (Figure 6.31). The water extract is at a peak for all three run groups at the time of
the $\text{o-PO}_4$ depletion. The concentration falls and reaches a minimum in 10 hours for run F-25 (Figure 6.38) and Run Group 4 (Figure 6.30), while Run Group 3 (Figure 6.34) continues to fall for another 30 hours till it reaches a minimum. After the minimum the water extract concentrations rise continuously for the remainder of the growth data. The TCA extracts fall after the peak values for about 10 hours at which point the exponential growth phase ceases. The concentration remains the same or rises till the end of the growth data. The concentrations are approximately equal for all three run groups. The dynamics of the insoluble P in the water and the TCA extracts are definitely different with the TCA concentrations always higher than the water extracts. But, if the growth time was extended beyond the 200 hour mark, the trend shows that the concentrations of the water and TCA insoluble P are approaching each other.

6.5 Concluding Remarks

To conclude the presentation of the P dynamic results, an observation must be made. There are concentration level differences between the same P compartments of the different run groups and between the compared TCA and water P compartments. Some of the differences may not be significant. The only test of significance is replicate growth runs which were not made. But there are also similarities and differences in the concentration levels and dynamics of the P compartments that are of significance.
CHAPTER VII

DISCUSSION OF RESULTS

7.1 General

This chapter discusses the results of the C. pyrenoidosa growth experiments. The chapter is organized into three sections in order to discuss the results given in Chapter VI. The first section covers results which show that the measured growth characteristics of C. pyrenoidosa are reasonable and the data are comparable to data found in the literature. This would include the specific growth rate, cell size, iodine stain intensity, and o-PO₄ uptake rate. The second section deals with the primary goal of the research which was outlined in Chapter IV which dealt with the P compartments that were extracted from C. pyrenoidosa by cold TCA and boiling water during dynamic growth. The final section will discuss observations that were made which deviated from the majority of comparable observations found in the literature. This would include algal cell shape, specific growth rate increase at time of o-PO₄ depletion from the medium and the dynamics of the COD and NO₃ in medium. The final section will also discuss procedures to prevent formation of phosphate precipitates in alkaline medium.
7.2 Growth Characteristics of C. pyrenoidosa

The specific growth rates of C. pyrenoidosa based on cell population, total cell volume and cell dry weight during exponential growth are listed in Table 6.5 for Run Groups 1, 2, 3 and 4. All three specific growth rates must be approximately equal in balanced growth (96). This is true for Run Groups 2, 3 and 4 if only the first part of the exponential growth phase is considered. Run Group 1 does not show this equivalence. The cell population specific growth rate for the first part of the exponential growth phase is lower than the cell volume specific growth rate. There is no explanation for this unbalanced growth. Run Group 1 had no energy source (sucrose) present in the medium. In batch cultures Krauss (120) found a specific growth rate of .090 hr\(^{-1}\) at 25°C under continuous light for C. pyrenoidosa (Emerson strain) which is the same strain used in this experimental work. This is higher than the approximately 0.067 hr\(^{-1}\) obtained in this experiment for Run Groups 2, 3 and 4. The difference is not drastic since there are many Emerson strain C. pyrenoidosa in the Indiana University Algae Collection of R. C. Starr (211) and each may show a slightly different growth response. Further different growth conditions can influence the growth rate difference. Sarmjima and Myers (194) found that the specific growth rate of C. pyrenoidosa is the same in a medium in which the sucrose is present and absent in acid medium under continuous light at 25°C and CO\(_2\) enriched air. The specific growth rates based on cell volumes of Run Groups 1 (no sucrose) and 2 (with sucrose) are equal under equivalent medium conditions. Krauss found that the optimum growth temperature is 25-26°C for C. pyrenoidosa (Emerson
strain). All the runs in this experimental work are at 25-26°C except run F-25 which was 30°C. The latter run should have a lower specific growth rate, but the experimental data does not indicate a lower rate and was included in Run Group 3 in Figure 6.3 growth data. But as will be discussed in Section 7.3, run F-25 P compartment dynamics are different from the rest of the Run Group 3 data.

The plots of C. pyrenoidosa growth are in Figures 6.1 to 6.5 for Run Groups 1 to 4. Growth based on cell dry weight and total cell volume in Figure 3.1 follow typical growth curves in batch culture. There are remnants of the lag phase, an exponential phase, transition to stationary phase, and, if growth continued, the stationary phase. The outstanding feature of the cell population growth curve is a two part exponential growth phase. The first part followed by a second part of a higher specific growth rate. The point of increased slope occurs when o-PO$_4$ is depleted from the medium. At a later time the exponential phase stops. At this later point, the exponential phases of both the cell volume and dry weight growth curves also cease.

Growth curves of o-PO$_4$-limited batch cultures of Scenedesmus specialis (185) and Anabaena variabilis (89) are similar to the total cell volume and dry weight curves of this experimental work. The exponential growth continues after o-PO$_4$ depletion from the medium. These phenomena are also confirmed in other works for nitrate (32,223) and phosphate (137, 184). There is no evidence in the literature of the increased specific growth rate at the point of o-PO$_4$ depletion in the medium. Discussion of these phenomena is found in Section 7.4.
Physical characteristics of the *C. pyrenoidosa* cell changed during the growth cycle. The cell weight is constant during exponential growth when the slope of dry weight and population growth are parallel (96), and declines as the exponential growth ceases. Figure 6.6 shows a similar phenomenon of cell weight for *C. pyrenoidosa* but the decline in cell weight occurs at the point of \( \Delta \text{PO}_4 \) depletion from the medium and not at the end of the exponential growth phase. The decline continues till exponential growth ceases, after which cell weight increases. The cell weight parallels the dynamics of cell volume change as shown in Figure 6.7, though Run Group 1 indicates increasing cell weight during the first part of the exponential phase. During P deficient growth the increase in cell weight and size is the result of accumulation of carbohydrates in the cell (127). The experimental data reflect these phenomena. The intensity of the iodine stain is an indication of cell carbohydrate since iodine preferentially stains carbohydrates (147). Table 6.6 notes a high stain intensity initially during the growth cycle and decreases as the exponential phase progresses. At the end of the exponential growth phase, the cells are extremely light in color indicating a loss of carbohydrates in cell. As the stationary phase progresses the stain intensity becomes very dark indicating an accumulation of carbohydrate (139). Healy (89) found the same characteristic carbohydrate concentration change in the alga, *Anabaena variabilis*, in P- limited batch cultures. The carbohydrate concentration of the bacterium, *Aerobacter aerogenes* increases as the growth decreases in P- limited cultures (46). The accumulation of carbohydrate would expand the cell giving it both
increased size and weight. The increase of cell carbohydrate after o-PO$_4$ depletion from the medium lowers the cell density. This phenomenon is ecologically advantageous. The lower density would decrease the settling rate of the cell and allow it to remain in the photic zone longer in water systems (244). The increase in cell volume of Run Group 1 during the exponential phase in Figure 6.7 indicates that the cell was not in a steady state during exponential phase, i.e., as much as can be allowed in dynamic growth. The cell densities are different when cell volumes of run P-25 (Run Group 3) and Run Group 2 are nearly equal during exponential growth but at different cell weight levels in Figure 6.6. This may still be the remnant of the inorganic particulates of Run Group 3 that are not totally corrected or that the density of *C. pyrenoidosa* cell is greater at pH 7.5 than 6.5. Though Run Group 4 shows a high cell weight and would support the latter conclusion, there was no data of the cell volume for Run Group 4 to reinforce this conclusion. A higher cell density is indicative of low internal cell storage products (244).

The number of daughter cells in *C. pyrenoidosa* changes as cell division occurs under cell P-sufficiency and cell P-deficiency. Table 6.6 indicates that 4 to 8 daughter cells are formed during the first part of the exponential phase. After o-PO$_4$ depletion from the medium, the number of daughter cells are predominantly two with four daughter cells in the minority. Hase et al. (87) report an average of 6.5 daughter cells under P-sufficiency and 3.5 daughter cells in P-free medium for *C. pyrenoidosa*. 
The chlorophyll content of *C. pyrenoidosa* during the growth cycle of Run Group 2 is in Figure 6.9. The rate of decline in cell concentration of chlorophyll $a$ and $b$ is less than would be expected by cell division. Thus chlorophyll is being produced and does not correlate with the specific growth rate. The decline begins almost from the beginning of the exponential growth phase and continues to decline to the end of the exponential phase. *Anabaena varabilis* shows the same pattern of chlorophyll $a$ concentration loss both in the exponential phase up to the point of $o$-$PO_4$ depletion in the medium. Chlorophyll is expected to decline when there is cell nutrient deficiency because the photosynthesis rate declines (90). The increase of pheophytin concentration as the first of the exponential growth phase progresses shows inactivation of chlorophyll. The inactivation ceases at the end of the first exponential phase. Thereafter the concentration loss is due to cell division alone. The increase of pheophytin can be due to high light intensity inactivation (167). The plot of light intensity in the *C. pyrenoidosa* growth culture is shown in Figure 6.10. The maximum light intensity for both high light (23,000 fc, Run Groups 3 and 4) and low light intensity (18,500 fc, Run Groups 1 and 2) are above the maximum light intensity of 12,000 fc above which inhibition of photosynthesis is found (167). At intensities above 12,000 irreversible damage to the chlorophyll takes place within a few hours. The greater the intensity over the maximum intensity, the shorter the time of exposure of chlorophyll before irreversible injury takes place. The result of this injury should be found in the build-up of pheophytin in the cell (167). But the previous comparisons of
specific growth rate, cell physical parameters, and dynamics of chlorophyll \( a \) during growth cycle indicate that the \textit{C. pyrenoidosa} cell does not show inhibition. The maximum determined chlorophyll \( a \) concentration for \textit{C. pyrenoidosa} in Figure 6.9 is approximately 25\% of the value of \textit{C. vannelli} which was obtained under 2700 fc light intensity (120). The higher light intensity of this experimental work, 10,000 to 13,000 fc, would result in a lower chlorophyll \( a \) concentration (120). Therefore, the measurement of the internal light intensity if the fermentor is in error. The internal solar cell was enclosed in a Pyrex glass tube. The error, on the high end, can be attributed to the calibration method of the solar cell. The light intensity source used for the calibration was measured with a Luna Pro light meter that was set to measure direct light. The internal solar cell calibration was made when the cell was not enclosed in the Pyrex tube. The solar cell arrangement for the calibration was such that it emulated the light meter cell. The glass tube acts as a lens to concentrate light from \( 180^\circ \) onto the solar cell. Thus the cell will give a higher light intensity in the fermentor than actually exists. With the existence of inactive chlorophyll in the algal cell, the light intensity in the fermentor could be closer to 10,000 to 12,000 fc for the lower light intensity and 11,000 to 13,000 fc for the high intensity. The minimum light intensity in the fermentor when the maximum cell density occurs is estimated to be above the saturation light intensity of 500 fc (120).

The concentration of the extracellular Org-P compounds would be revealed in Figure 6.11, particularly after the depletion of \( o\text{-PO}_4 \).
from the medium. The analytical techniques did not indicate that Org-P compounds existed that are stable during the molybdenum blue determination of o-PO$_4$$_4$. During the time up to depletion, there also was no difference in the amount of o-PO$_4$$_4$ and Tot-P as determined by the analytical techniques. This observation is confirmed by Lean and Nalewajko (137) for C. pyrenoidosa. The maximum amount of Org-P was found to be at most 0.020 mg P/l which is slightly higher than the residual P (~0.010 mg P/l) determined after the depletion of o-PO$_4$$_4$ from the medium. They did not determine the acid stability of the released Org-P.

The uptake rate of o-PO$_4$$_4$ for Run Groups 1 to 4 is shown in Figure 6.12. The maximum uptake rates of Run Groups 1, 2 and 4, in which there was no evidence of ferric hydroxide formation, are comparable of Anabaena variabilis (10$^{-10}$ mg P/cell·h) (189) from a batch culture at pH 7.5. The data in the literature was converted to cell basis from biomass basis. The low rate of uptake of o-PO$_4$$_4$ for Run Group 3 was discussed in Section 6.4-6C. Run Group 3 was found to have ferric hydroxide precipitate in the medium. There is a possibility of N-limited growth for Run Groups 1 and 2, since the N/P ratio is approximately 30 (186). This does not appear in the data of Figure 6.12. The uptake rates of o-PO$_4$$_4$ for Run Groups 2 and 4 which was a N/P ratio of 93 fall upon each other. Rhee (186) cited that if the N/P ratio is less than 30 the growth is N-limited and the uptake rate of o-PO$_4$$_4$ for N-limited growth is depressed by a factor of 8 from the uptake rate of P-limited growth.
The discussion in this section gives support to the conclusion that the growth of *C. pyrenoidosa* in this research work is comparable to other growth data under conditions of P-limitation, continuous saturated light, at 25°C in axenic culture.

### 7.3 P Dynamics of *C. pyrenoidosa*

This section discusses the P dynamics of the TCA and water extract compartments of *C. pyrenoidosa* in batch culture in which P is stoichiometrically limiting the growth. Several aspects of the data are to be explored utilizing the general dynamics that were found in Figures 6.20 to 6.38 and summarized in Section 6.4-6C. Do the TCA compartments that have been isolated in this experimental work show the dynamics that have been found in other research? Section 6.4-6B gave a summary of the water extracted intracellular P compounds that are to be expected in each compartment based on the literature. Do the experimental water extracted P compartments give the expected results? Does the addition of the water extracted P compartments increase the knowledge of the P dynamics in *C. pyrenoidosa*? A major goal of the research was a determination of a P compartment of the water and TCA extracts that is proportional to the growth rate. Does the inorganic particulate P interfere with the determination of P in the compartments? Finally, is the phosphate in the hydroxyapatite available for *C. pyrenoidosa* in the course of the growth cycle in this experimental work?

There is much literature concerning the internal P dynamics of microbes. But before application of these results, there is a need
to determine the prehistory of the cells that were inoculated into the growth medium. The principle variables to ascertain are the N/P ratio of the inoculum medium, the stage of growth of the inoculated cells. The N/P ratio of the inoculated cells must be greater than 30 or else the cell is N-limited when in the stationary growth phase and is not at the minimum P concentration (186). Under N-limitation the cell accumulates P as surplus P. The minimum cell P in P-limited cultures occurs during the stationary growth phase. Literature values of cells that are not at the minimum cell P and were transferred to P-free medium can be applied to the internal P dynamics of C. pyrenoidosa after the depletion of o-PO$_4^-$ from the medium. In both the literature and the experimental cells, the internal cell P over the minimum cell P is utilized for cell growth. Exponential growth dynamics in which all the nutrients are in excess can be applied to the first part of exponential growth phase.

The rapid increase of the P that was found in most of the TCA and water P compartments in Figures 6.20 to 6.23 during the lag growth phase is to be expected because of the rapid uptake of o-PO$_4^-$ that was found. The rapid uptake is typical for P-deficient C. pyrenoidosa (83,90,110,127). The available experimental data does indicate that the increase is to be in the insoluble P, and in the medium and low acid stable P of the water extract, and in the insoluble P and the medium acid stable P of the TCA extract. For P-deficient cells transferred to a medium with o-PO$_4^-$, Aitchison and Butt (2) found both an increase in the TCA soluble and insoluble Poly-P in
C. vulgaris while Sicko-Goad and Jensen (203) found that there was an increase in the total P of both the TCA soluble P, lipid-P and a fraction that includes RNA-P, Poly-P, and DNA-P for the alga Plectonema boryanum. Both observations were made in the first few hours after the cells were inoculated. The increase in the TCA soluble P, primarily Poly-P "A," should reside in the TCA medium acid stable P compartment. But experimental data show that it is in either the medium or high acid stable compartment. The answer may lie in the high error of P analytical determination in this region where P concentrations are low. The P concentration in a compartment is determined by subtraction. If one analytical procedure is in error, the P would be shifted to a higher or a lower acid stable compartment.

The increase in the TCA insoluble P would be the increase in Poly-P "B," "C" and "C." The water soluble low and medium acid stable P increase. The low acid stable P would be the result of the formation of Poly-P "B" and "C" and the medium acid stable P increase would be the result of the formation of Poly-P "A" and intermediate pool P. The increase in the water insoluble P would be due to the formation of Poly-P "D", RNA-P, DNA-P and lipid-P. The uptake rate into the water insoluble P was less than the TCA insoluble P as would be expected if Poly-P "B" and "C" are removed by water extraction.

The rapid plunge in concentration of the TCA soluble P from the medium stable P in the 10 to 20 hour range is due to rapid loss of soluble Poly-P "A." Aitchison and Butt (2) noted that the TCA soluble Poly-P "A" in C. vulgaris goes through a maximum at 10 hours after the inoculation into a medium containing $\text{O}_4\text{PO}_4$. There is a rapid
decrease in the TCA soluble Poly-P "A" in the next 10 hour span. Their data also indicates that the Tot-P in the TCA extract and TCA insoluble P go through a maximum and fall in a similar manner but to a lesser degree. This phenomenon is reflected in this research effort in the TCA insoluble P. Further, the low and medium acid stable P of the water extract indicate a reduction in cell concentration (input to compartment less than loss due to cell division) after reaching a peak concentration mimicking the decrease in the Poly-P "A" (TCA soluble), "B" and "C" (TCA insoluble). The reduction in the concentration occurs at the beginning of the exponential growth phase. The reduction in the concentration is the result of reduced uptake of o-PO\textsubscript{4} (184) and increased demand of P for energy transfer and cell synthesis during exponential growth.

During the first part of the exponential growth phase, all nutrients are supplied in the growth experiments in excess of the limiting or saturation concentration. Under this condition the P content/cell is nearly constant (127). Therefore there is a lot of data, with a different prehistory than the work carried out here, that meet the criteria of a maximum P concentration. In Run Groups 1 and 2 in Figures 6.24 and 6.26 where the inorganic particulate P is not present, the Tot-P/cell is relatively constant during the first part of the exponential growth phase. The P concentration levels/cell of the P compartments of TCA and water extract are found to generally increase to a maximum before the depletion of o-PO\textsubscript{4} for the medium acid stable P of water and TCA extracts while high acid stable P plateaus at a relatively constant value through the first part of the
exponential growth phase. There was no experimental data obtained for TCA low acid stable P but the water low acid stable P cell concentration declines through the exponential phase as does the TCA insoluble P. In order to satisfy the constant cell P, there must be a decrease in the P concentration of some cell compartments if others increase in concentration RNA-P/cell must increase for protein synthesis (96) during exponential growth from the low value of the inoculated cells. With increased requirements of synthesis of cell material, the phosphorylated P compounds, such as sugar esters from the metabolic pathways, and the functional compounds, such as ATP, CTP, GTP, etc., increases in cell concentration. In batch culture of *C. vulgaris*, cell concentration of P, both TCA soluble and insoluble Poly-P falls as the exponential growth phase continues (2,34). The observed phenomena would give rise to the rapid loss of the cell P from the water insoluble P. Because of the changing environment in the medium during exponential growth in the batch fermentor, Herbert (96) did not expect the dynamics of the internal cell components to be at a steady state. The results of this research effort concurs with the above observation for the P compartments. There was an exception. For the high acid stable P in both TCA and water extracts generally give rise to a relatively stable cell P concentration. Run Group 1 shows a slightly different dynamics while Run Group 2 shows a saddle shaped curve. The second peak being similar to Run Group 1. It was generally concluded that the functional pool P resided in the high acid stable P. Holm-Hanson (in Kuhl, 127) observed that ATP levels remain constant during exponential growth and decreased under P-limitation.
ATP is considered the major functional P component (145).

The source of P entering a compartment or the fate of P leaving a compartment cannot be speculated with the experimental data. Many other researchers have tried to define the P flow during exponential growth (2, 34, 158, 161).

The conclusion in the P dynamics of TCA and water extracts during the growth of *C. pyrenoidosa* before the o-PO$_4$ depletion is that the dynamics in general are as would be expected. The TCA and water extracts were compared within a run group at the same acid stability level (see Figures 6.24 to 6.39). There was only sufficient data to compare the medium and high acid stable P. The dynamics compared favorably. For medium acid stable P, it was expected that the TCA extract would be greater than or equal to the water extract. The opposite was found to be true. This observation seems to reinforce the possibility that Poly-P "A" is in the water extract medium acid stable P compartment. But this may not be true if the following contributes a significant amount of o-PO$_4$ to the medium acid stable P in the water extract. The hydrolysis of the P compounds by boiling in acid was different in the length of boiling time. The water extract was boiled 15 minutes while the TCA extract was boiled for 7 minutes. This would increase the o-PO$_4$ in the water extract from P compounds partially hydrolyzed by the 7 minute boiling time of the TCA extract. But the high acid stable P for the water extract was only slightly greater than the TCA extract. The P concentrations were expected to be equivalent. If there were a great shift in P in the water extract from the high to medium acid stable P due to the longer hydrolysis time, it would then
be expected to see that TCA P concentration greater than the water extract for the high acid stable P. Thus additional P compounds that would be a part of the initially assumed water insoluble P (see Section 6.4-6B) would be found in the high acid stable P.

After the depletion of o-PO$_4$ from the medium, all the P is in the particulate fraction. The data are presented as a fraction of the Tot-P in the fermentor as compared to P/cell before the o-PO$_4$ depletion. The P dynamics of the algal cells in the literature, that is used in this part of the discussion, has a prehistory of being grown in excess o-PO$_4$ before being transferred to fresh P-free medium in batch culture. The P dynamics of *E. coli* bacterium was followed in P-limited batch culture from the later stages of the exponential growth phase into the P-deficient growth phase.

In the first 10 hours after the depletion of o-PO$_4$, there is a flow of P from the high and medium acid to the insoluble and low acid stable P compartments in the TCA extract. The flow then reverses. The P flow is from the insoluble P compartment to the medium, high and low acid stable P till the end of the exponential growth. The data of Rhee (185) indicate that this is typical for *Scenedesmus specialis*. In this article, the lipid-P, DNA-P, Poly-P "B," rise in concentration while the RNA-P**Poly-P "D" complex, protein-P and Poly-P "C" remain constant for the first 20 hours. The sum of the concentrations show a net increase in the TCA insoluble P while there is a large decrease in the Poly-P "A," the medium acid stable P. In block diagrams flow of P through intermediate pool P and precursor pool P for the synthesis of structural P is required, thus the flow of
to the low acid stable P. The flow of P from the functional pool P of the high acid stable P is of importance since ATP is a pathway through which P is transported to and from TCA insoluble Poly-P (247).

Twenty to thirty hours after the depletion of o-PO₄, the TCA insoluble P in Rhee's data decline as the RNA-P**Poly-P "D" complex and Poly-P "B" and "C" decline, but lipid-P and DNA-P continue to increase. The data of Rhee show a loss of P in his plotted P compounds which is not replaced by sufficient increase in other P compounds. Rhee does not show the functional and intermediate pool P data which one or both pools absorb the excess P. Both the high and medium acid stable P in the experimental work of this dissertation, which contain the functional and intermediate P pools, show increased P concentration at the end of the exponential growth phase. There are two other research efforts concerning internal cell of the P dynamics. Miyachi and Tamiya (161) studied _C. pyrenoidosa_ and Aitchison and Butt (2) studied _C. vulgaris_. Neither study showed the increase in the TCA insoluble P in the early stage of growth after the cells were transferred from cultures with excess o-PO₄. Each study showed the immediate decline in the TCA insoluble P concentration. Harold (83) found the work of Miyachi and his colleagues confusing in the data that they present.

Water extract shows different dynamics of P flow. There was a P flow from the insoluble P and a net flow from the low and medium acid stable P to the high acid stable P in the first 10 hours after the depletion of o-PO₄. Then the flow of P reverses with P flowing from high acid stable P to both low acid stable P and insoluble P. The
Rhee data (185) cannot explain the dynamics of the water extract compartments. The data seem to be in opposition to the TCA extract based on the distribution of the P compounds to the compartments concluded in Section 6.4-6B. The water insoluble P which is assumed to contain primarily DNAP, RNA-P, Poly-P "D," lipid-P and protein-P, show a net loss of P. Any combination of the above compounds to give a net loss of P from the water compartment gives a different dynamic characteristic than was found for the same P compounds determined by the TCA extract. The loss of P from the low acid stable water P would come from the Poly-P "B" and "C." The increase in medium and high acid stable P could be the accumulation of functional and intermediate pool P as the conduit of P from the breakdown of Poly-P. When the reversal of P flow occurs in the water extract, there is a flow to the insoluble P, primarily to DNA-P, RNA-P, Lipid-P, and protein-P (161), and the low acid stable P for the intermediate and precursor pool P which reside in this compartment. The above discussion gives a reverse view of the dynamics of the structural and functional pool P, but the dynamics of the intermediate and the precursor pool P seem to be similar in both the TCA and water extract. The explanation of the contradiction in the water insoluble and the high acid stable P would be that water extracts one of the assumed structural P compounds and is found in the high acid stable P. It is unlikely that DNA-P or protein-P are extracted because of the severe conditions required to solubilize them (158).

The exponential growth, the flow of P in the TCA extracts is random with no general pattern among the P compartments. The water
extract indicates a repeatable pattern after the exponential growth phase. The P flow is from the soluble compartments to the insoluble P. As the cell reaches its minimum P concentration, the P components of importance to the survival of the cell receive the P. The most important is DNA-P which is water insoluble. All the other P compartments will have residual amounts present in order to maintain the very low metabolic activity required for cell survival in stationary growth.

After exponential growth, the Poly-P cell concentration becomes negligible for P-deficient cells (83,127,185). Therefore the P concentration in the comparable acid stability levels of the TCA and water extracts should approach each in the later stages of the growth experiments (see Figures 6.24 to 6.39). The dynamics were similar for the low and medium acid stable P with the P concentration of the water extract higher than the TCA extract. But as the 200th hour of growth approached, the low acid stable P of Run Group 4 are approximately equal with the water extract slightly higher as both decline in concentration rapidly toward a low concentration. Run Group 3 and run F-25 show the water extract concentration considerably higher. Run Group 3 shows no rapid P concentration loss in both the low and medium acid stable P. Further they do not approach each other. Run F-25 is similar to Run Group 3 but both extracts begin to show concentration decrease at the end of the data. The large concentration differences cannot be tied to the inorganic particulates because it would contribute more P to the TCA extract than the water extract. The concentrations of the medium acid stable P for both the water and the TCA
extracts approach are zero concentration at the end of the growth experiment.

The high acid stable P and the insoluble P have different dynamics between extraction methods. The water extract concentration for the high acid stable P is higher than the TCA concentration but the reverse is true for the insoluble P. The concentration of the high acid stable P approach each other near the end of the growth data as the water extract concentration declines. Following the previous reasoning that the high acid stable P of the water extract solubilizes a previously assumed insoluble P, the phenomena cited above is not related to the functional pool P because it would be in the TCA extract. Therefore the difference is related to this unidentified P compound which shows that it is not required for cell survival in stationary growth. It is not possible to identify the compound. The insoluble P concentration is increasing in concentration and rising toward the TCA insoluble P concentration at the end of the growth experiments. The concentrations do not stabilize. If they did stabilize, there might be the possibility to determine the availability of $\text{O-PO}_4$ from hydroxyapatite.

The questions posed at the beginning of this section can now be answered. The TCA P compartments that were isolated in this experimental work from *C. pyrenoidosa* during the growth cycle show the dynamics that were found by other experimenters.

The water P compartments though gave the expected dynamics during the first part of the exponential growth phase based on the P compounds summarized in Section 6.5-6B. But after $\text{O-PO}_4$ depletion, the
dynamics for the insoluble and the high acid stable P gave drastically different dynamics while the low and medium acid stable P dynamics were as expected. The concentration levels of low acid stable P show that the water extract was significantly higher than the TCA extract when they should be equal or TCA be higher by amount of concentration of the inorganic particulate P, hydroxyapatite. There were indications during the first part of the exponential growth phase that part of the assumed insoluble P was extracted by water and was determined in the high acid stable soluble P. The difference seemed to be emphasized in the growth phase after $\alpha$-PO$_4$ depletion. It had been noted earlier that after depletion the cells of *C. pyrenoidosa* ruptured at a high percentage during extraction by both TCA and water. The cell wall and membrane, if intact, act as a filter during the extraction process. Whether cell rupture increased the ability of the water extraction to remove an assumed insoluble P cannot be answered. But there seemed to be no effect on the TCA P extraction.

The high acid stable P in both TCA and water extract of *C. pyrenoidosa* gives an elevated constant P concentration during the first part of the exponential growth phase. This appears when the P concentration is based on a per cell basis. All other P compartments did not show such a behavior. The presence of the inorganic particulate P in the medium may have prevented the TCA and water low acid stable P and the water insoluble P and Tot-P/cell from exhibiting this phenomenon. But, since the inorganic particulates are difficult to eliminate from the medium and that these same particulates exist in natural water systems, it is of great value to find a P compartment
that responded as the high acid stable p compartment does. Whether concentration of P in the compartment is proportional to the maximum specific growth rate was not determined.

7.4. Other Points of Discussion on Dynamic Growth of C. pyrenoidosa

Further discussion of results is required to cover data taken during the growth of C. pyrenoidosa which have not been confirmed in the literature. These results fall into the following areas: cell shape, the associated increase in the specific growth rate and the percentage of non-spherical algal cells increase during the exponential phase; the reduction in COD in medium during the growth cycle; the increased uptake of NO₃ during the stationary phase; and procedures to prevent the formation of ferric hydroxides and hydroxyapatite.

In reviews of the literature which cover P nutrition in algae (90,127) there was no information concerning the change in the shape of C. pyrenoidosa or any other algal cell after o-PO₄ was depleted from the medium. Further no discussion of an increase in the specific growth rate was noted at time of o-PO₄ depletion. The results of this research in Figure 6.9 show a definite correlation between the increase in the percent non-spherical cells (fusiform in shape) and the time after the depletion of o-PO₄ from the medium. During the first part of the exponential growth phase, there were no more than 10% of the algal cells in the fusiform shape. Table 6.6 also indicates that the number of daughter cells formed during this period was 4 or 8. But as the percent fusiform cells increase, the number of daughter cells
decrease to 2 to 4 with the fusiform cells arising from binary division. Hase et al. (87) notes that daughter cells decrease to an average of 3.5 during P depletion for *C. pyrenoidosa*. But the average for this research is very near 2 since there is 90-100% of the fusiform at the end of exponential growth. Under o-PO$_4$ limitation there are found compositional changes in the cell membrane in bacteria. Tempest et al. (219) found that the phospholipids in the cell membrane were replaced by non-phosphorus lipid polymers for *Bacillus subtilis var. niger* under o-PO$_4$ limitation. Forsberg et al. (67) found the same phenomena in *Bacillus licheniformis* under o-PO$_4$ limitation. They also noted that the cells were irregular spheres in shape as compared to rods when the cells were P-sufficient. Both organisms have rigid cell walls. The mechanisms for the cell shape change were changes in the protein and wall synthesis. The reduction in the amount of teichoic acid (phospholipid) in the cell membrane and increase of teichuronic acid (non-P lipid) in cell membrane was speculated to decrease the number of sites of wall formation. The change in membrane composition could result in a maledistribution of wall formation sites which led to portions of cell with different wall thickness and thus different wall rigidity causing cell shape changes. The cell plasma membrane regulates all passage of nutrients into and out of the cell. The cell wall is external to the cell plasma membrane (208). Therefore compositional changes in cell membrane will regulate cell wall structure. Bailey and Northcot (9) found phospholipids in the cell membrane of the green alga *Hydrodictyon africanum*. A mechanism can be assumed to be parallel for *C. pyrenoidosa* which also has a rigid
cell wall. With the reduction in the number of daughter cells formed under $\text{o-PO}_4$ limitation the increased synthesis of both cell wall and membrane to form 4 and 8 daughter cells is not required before the daughter cells are liberated. There is less total surface area for two fusiform cells than 4 or 8 spherical cells enclosing same total volume. Further the maledistribution of cell wall formation sites due to reduced phospholipid in the membrane could lead to different cell wall thickness and the formation of the irregular fusiform cells that were observed with the thicker walls parallel to the major axis. The strength of this mechanistic argument is supported by the following investigations. Fuhs (68) noted that under P-deficient growth lipid-P associated with chloroplasts decreased in the diatoms Cyclotella nana and Thalassiosira fluviatilis. The reduction of chloroplast lipid-P would be correlated to decreased chlorophyll content that occurs during P starvation (90). Rhee (184,185) found lipid-P decreased with growth rate for Scenedesmus specialis. With these observations the speculation of P flow would be from phospholipid membranes. Very little research work concerning algal cell walls or membranes has been carried out and no work concerning phospholipid concentration in the cell plasma membrane has been found to support decreased P in the cell membrane. Two experimental observations during the growth of C. pyrenoidosa are important. Table 6.9 notes the increased cell rupturing during TCA and water extraction after $\text{o-PO}_4$ is depleted from the medium. The conclusion is a change in cell wall because of the reduction of strength. The second observation is that the fusiform cell shape of C. pyrenoidosa returns to a spherical shape when
in the stationary phase (see Figure 6.9). Forsberg et al. (67) observed that Bacillus licheniformis returned to rod shaped from irregular spheres under P depletion if wall synthesis and protein formation continued. C. pyrenoidosa continued to show growth activity. Figure 6.3 shows both slow increase in cell number, cell dry weight and cell size plus both the uptake of COD components and NO₃. The protein synthesis and wall formation continue in C. pyrenoidosa. The continued activity will allow for the formation of the uniform cell wall thickness and the formation of a spherical cell. The change in cell shape under P-deficient growth has ecological importance. A spherical cell has the lowest surface to volume ratio. A nonspherical cell has increased surface area and the possibility of more active sites for o-PO₄ uptake, in comparison to a single spherical cell enclosing the same volume. Further the cell shape change can reduce the settling velocity of the cell from the photic zone in a natural water system (244).

There was found to be associated with the increase in the percent fusiform C. pyrenoidosa cells, an increased specific growth rate for cell number at the point of o-PO₄ depletion from the medium. The increase in the rate is immediate while the rate of increase in percent fusiform cells is gradual (see Figures 6.1, 6.3, 6.5 and 6.9). The specific growth rates for the total cell volume and dry weight concentrations do not increase. Thus the increase in the specific growth rate is due only to increased cell division with no increase in rate of biomass production. Under P-sufficient conditions, the cell cycle of C. pyrenoidosa cell development goes through the
succession of first 2 immature daughter cells followed by further division to 4 and 8 immature daughter cells before they are released as mature cells (159). It is obvious that the formation of 2 daughter cells is faster if the time to cell maturity after formation is equal for 2, 4 or 8 daughter cells. The gradual increase in the fusiform cells formed by the binary division shows that upon o-PO₄ depletion there is no synchronous or immediate formation of fusiform cells. Since there is a distribution of cell ages (not synchronous growth) at the time of o-PO₄ depletion, then there is a distribution in time of formation and of release of fusiform daughter cells.

The COD in the medium after 0.45 μm membrane filtration does not discriminate the type of compounds present. The analytical result is the net value of all the compounds in the medium. During the first part of the exponential phase, there is a decrease in the COD value from the initial value of the sucrose and EDTA in the medium. There is a more rapid decrease in COD during the transition to stationary growth phase. But studies of the release of organic compounds during the exponential growth phase (103,147,168,169) for C. pyrenoidosa under continuous light and aerobic conditions indicate that up to 10% of the carbon that is fixed is released by the cell and 5% of the fixed carbon during stationary phase. From the cell dry weight for the experiments carried out in this work in Figure 6.3 the extracellular organic compounds would be in the range of 8 mg/l at the point of o-PO₄ depletion, 40 mg/l at end of exponential phase and 80 mg/l during the stationary phase. The organic compounds that are released during the exponential growth phase are generally volatile organic
acids, such as glycollic, acetic, formic acids (147), and higher molecular weight organic compounds during the stationary growth, such as polysaccharides (169). These observations would indicate that COD would increase throughout the growth cycle. This is counter to observations that were made during the experimental work: The differences during the exponential phase may be the stripping of the volatile acids with the air that is bubbled through the medium during the growth cycle. The experiments in the literature in which the extracellular products were determined were in flask cultures, thus reducing the chance of loss of the volatile acids. This still does not explain the reduction of the COD found in the experiments. Sucrose was found not to support the growth of _C. pyrenoidosa_ (194). EDTA has been found to be metabolically broken down by _Scenedesmus_ in order to utilize the bound Fe (121), but another study indicates that EDTA need not be broken down to obtain the Fe (179). There is no way of determining the extent of the uptake of either EDTA or sucrose, and how much of the original organic compounds were replaced by released extracellular organic compounds to give a net COD loss during exponential growth. But this is a speculation of a mechanism for the results.

There is continued loss of COD after the exponential phase at an accelerated rate for the growth experiment of _C. pyrenoidosa_. The literature indicates that the release rate of organic compounds is lowered and shifts to the higher molecular weight compounds (93,169). Thus the accelerated net loss of COD would mean accelerated uptake of sucrose, EDTA and other nonvolatile compounds that were released earlier in the growth cycle to maintain the experimental loss rate.
The much lower COD in the medium cannot be totally accounted for by the metabolizing of EDTA, so some of the sucrose must be metabolized or no EDTA is metabolized and the total decrease in the COD is accounted for by sucrose uptake. With the depletion of o-PO$_4$ from medium and reduced cellular P, the use of heterotrophic growth (utilization of organic energy) may conserve cellular P for more vital compounds, e.g., DNA. There is continued growth activity as noted in the growth curves in Figure 6.3. Both the cell population and cell dry weight continue to increase slowly after the exponential phase. No experimental work has been found to confirm this phenomenon.

The decrease in the NO$_3$ concentration in the medium was followed during the growth cycle of $C$. pyrenoidosa and shown in Figure 6.14. The loss of NO$_3$ from medium was converted to NO$_3$ uptake per cell. The rate did reach a minimum at the end of the exponential growth phase but the uptake rate increased slowly thereafter. This indicates increased cell concentration. This phenomenon is the parallel of the accumulation of P in the alga Scenedesmus specialis when growth was under N-deficient conditions (186).

The reduction of the inorganic particulate P is important in order to improve the results of the extraction of P compounds from the cells. The discussion of the dynamics of the P inorganic particulates in Chapter 6 indicated the problems of the absorbed o-PO$_4$ on the ferric hydroxide particulates and the o-PO$_4$ in the hydroxyapatite at pH 7.5. Further the prevention of the formation of ferric hydroxide was the presence of sufficient EDTA (a molar ratio of EDTA:Fe of greater than 3:1) in the medium. Iron did not precipitate. If the pH of the
medium did not rise above a pH of 7.5. The difficulty with the elimination of the hydroxyapatite formation in basic solutions is the need to reduce the concentration of Ca to approximately $10^{-8}$ M for a $\text{PO}_4$ concentration range of $10^{-4}$ to $10^{-6}$ M. Many of the research studies (91,184,185,186) to which the discussion refers in this section do not meet the criteria for the elimination of the hydroxyapatite formation. The need is for a complexing agent to bind Ca in the pH range of the experimental work, 6.0 to 8.0. The complexing agent must be able to release Ca to meet the metabolic requirements of *C. pyrenoidosa*. The alternative is a method to solubilize the hydroxyapatite that is filtered out with the cells without extraction of P compounds from the isolated cells. The method would be based on acid, since a low pH is favorable for the solution of hydroxyapatite (214).
1. The growth dynamics of axenic \textit{C. pyrenoidosa} in a batch fermentor under P stoichiometric limitation was observed to correspond to data in the literature for the following parameters:
- cell population, total dry weight, total cell volume,
- maximum specific growth rate
- cell size (volume) dynamics
- cell carbohydrate concentration dynamics (iodine stain)
- cell chlorophyll $a$ and $b$, pheophytin concentration dynamics
- uptake rate of $\text{O}_4 \text{PO}_4$ per cell
- dissolved Org-P dynamics in the medium
- reduction in the number of daughter cells after the depletion of $\text{O}_4 \text{PO}_4$ from the medium
- dynamics of the TCA extracted P compartments.

2. There is a constant concentration of P per cell in the high acid stable solution compartment for both the TCA, $P(H-TCA)$, and the water extracts, $P(H-H_2O)$, during the first part of the exponential growth phase of \textit{C. pyrenoidosa} at pH 7.5 in the presence of the inorganic particulate P. This compartment contains the functional pool P (nucleotides) and some of the intermediate pool P (phosphate esters).
3. The dynamics of the P compartments of the water extract from *C. pyrenoidosa* supports the conclusion drawn from the literature, that not all the o-PO$_4^-$ from Poly-P is found in the low acid stable P compartment which was assumed by Nelson and Fitzgerald (64). Only Poly-P "B" and "C" seem to be present in the low acid stable P of the water extract.

4. Basing initial assumptions of the relationship between the TCA and water extracts of *C. pyrenoidosa* on the literature, the data obtained for the high acid stable soluble P and the insoluble P indicate significant differences between extracts not accounted for by the assumptions primarily after the o-PO$_4^-$ depletion from the medium. It was concluded that the boiling water extract solubilizes a structural P compound that was found in the high acid stable soluble P compartment.

5. Further, there is a significantly higher water over TCA extract concentration in the low acid stable solution P during the stationary growth phase when both extracts (as cited in reported literature) are either equal or the TCA extract concentration is to be higher because of the inorganic particulate P.

6. The change in the cell shape of *C. pyrenoidosa* from round to fusiform correlates with: 1) the depletion of o-PO$_4^-$ from the medium, 2) the formation of 2 daughter cells rather than 4 or 8, 3) the significant increase in the frequency of cell rupture during the TCA and water extractions and 4) the increase in the cell population specific growth rate. The phenomenon of cell shape change as associated with the reduced rate of cell wall formation.
7. The cell population specific growth rate of *C. pyrenoidosa* increases after the depletion of o-PO₄ from the medium because of the more rapid formation and release of 2 daughter cells which occurs at this time as compared with the formation of 4 or 8 daughter cells before the depletion of o-PO₄.

8. The reduction of COD in the medium during the growth of *C. pyrenoidosa* in batch culture is due to uptake of sucrose from the medium.

9. The increased uptake of NO₃ by *C. pyrenoidosa* after the end of the exponential growth phase indicates a luxury uptake of N under growth stress conditions similar to the luxury uptake of o-PO₄.

10. No conclusion could be drawn from the compartmentalized P dynamic data of *C. pyrenoidosa* in batch culture concerning the availability of o-PO₄ from hydroxyapatite.

11. Ferric hydroxide, Fe(OH)₃, is found at pH 7.5 after the fast addition of NaOH, but it is not found after the slow addition of NaOH at pH 7.5 and at pH 6.5.

12. Orthophosphate coordinates with Fe(OH)₃ at pH 7.5 in the growth medium used in the following molecular ratio: Fe₃(OH)₆PO₄.

13. Hydroxyapatite, Ca₅OH(PO₄)₃, is found at pH 7.5 after the fast and slow addition of NaOH to the growth medium.

14. The precipitation of hydroxyapatite, after the slow addition of NaOH to the growth medium, is initiated by the presence of *C. pyrenoidosa* in the exponential growth phase.

15. At pH 7.5, o-PO₄ is released from the ferric hydroxide-orthophosphate complex at a rate faster than required for cell
growth of *C. pyrenoidosa* when o-PO$_4$ is absent from the medium in a non-growth culture.

16. At pH 7.5, Fe, up to 1.0 mg/l, is filtered from the medium in proportion to the population of exponentially growing *C. pyrenoidosa* cells in an extreme excess of the cell requirements. The amount of Fe filtered from the medium per cell decreases as *C. pyrenoidosa* cells are in stationary growth phase. The phenomenon may be associated with the activity of the cell surface.

17. The internal solar cell measuring the light intensity in the experimental apparatus is in error due to the calibration procedure.

18. Objectives were achieved from the observations, enumeration and description of the dynamics of *C. pyrenoidosa* growth in this work on which an effective experimental program can be designed to achieve original research goals.
Experimental Approach. Changes in approach are required in order to meet the original research goals:

1. The results of this research work have indicated that the P concentration of the high acid stable compartment for both the water and the TCA extracts correlated with the growth rate of C. pyrenoidosa in dynamic growth culture at 1.4 mg PO₄-P/l in the medium. The consistency of this result at lower concentration levels, where the maximum growth rate is limited, needs to be examined in both dynamic and steady-state cultures.

2. Since a major component of the high acid stable P in the TCA and water extracts of C. pyrenoidosa is the functional pool P, a determination of the contribution of ATP, CTP, GTP, etc., should be made and determine if the concentration levels, particularly ATP, correlates with the growth rate of C. pyrenoidosa in the same manner as the high acid stable P.

3. Since a model of the P dynamics of C. pyrenoidosa based on a Grenney type model cannot utilize the intracellular P data from analytical techniques in this work, a new model will need to be developed that can incorporate the data that is generated.
Analytical Procedures. New analytical procedures are required to fulfill the experimental approach.

1. Only one P extraction procedure can be used if increased significance for the results is desired. An increased number of samplings per day and duplication per sample are required.

2. A more thorough identification of P compounds in each P compartment of the water extract is required in order to fulfill recommendation 3 in the previous section.

3. A complexing agent, not toxic to \textit{C. pyrenoidosa}, is needed in order to bind Ca to prevent the precipitation of hydroxyapatite.

Analytical Techniques. The completed work raised several questions that are not supported adequately with experimental data. Techniques are required to obtain that data.

1. Procedures are required to determine if Fe precipitates onto the surface of \textit{C. pyrenoidosa} during exponential growth and is released under stationary growth conditions.

2. A procedure is required to determine if phospholipids are present in the cell wall membrane of \textit{C. pyrenoidosa} when the cell is at minimum cell P.

3. Procedures are required to determine if $o$-PO$_4$ is definitely released from inorganic particulates, hydroxyapatite and ferric hydroxide-orthophosphate, either \textit{C. pyrenoidosa} by cell action or solution.
APPENDIX A

NOMENCLATURE

A  Major diameter of oblong cell
a  Interfacial area between the gas and the liquid phases per unit volume of liquid
B  Minor diameter of oblong cell
b  Cellular concentration of vitamin B\textsubscript{12}
b\textsubscript{0}  Minimum cellular concentration of vitamin B\textsubscript{12}
C  Concentration of dissolved oxygen in the bulk liquid phase
\(c^*\)  Concentration of dissolved oxygen in the liquid at the gas-liquid interface that is in equilibrium with the bulk gas phase concentration of oxygen and related by Henry's Law
D  Diameter of spherical cell
F(S')  The nutrient saturation curve for the limiting nutrient in the ecosystem (Michaelis-Menten)
H  Henry's Law constant
In  Inhibitor concentration
K  A constant which is equal to the substrate concentration at one-half maximum growth rate in nutrient saturation curve.
K\textsubscript{La}  Volumetric oxygen transfer coefficient
k\textsubscript{L}  Mass transfer coefficient in the liquid phase
k\textsubscript{1}  Reaction rate constant for P exchange rate between P compartments
k\textsubscript{2}  Constant
N  Cell number concentration
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_R$</td>
<td>Concentration of RNA in cell</td>
</tr>
<tr>
<td>$N_{RO}$</td>
<td>Minimum concentration of RNA in cell</td>
</tr>
<tr>
<td>$n$</td>
<td>Volumetric oxygen transfer rate</td>
</tr>
<tr>
<td>$n_c$</td>
<td>Cellular nitrogen to carbon ratio</td>
</tr>
<tr>
<td>$n_{cO}$</td>
<td>Minimum cellular nitrogen to carbon ratio</td>
</tr>
<tr>
<td>$P(I)$</td>
<td>The light intensity saturation curve that accounts for the light variation due to water depth and the light variation during the day cycle</td>
</tr>
<tr>
<td>$PP$</td>
<td>Particulate phosphorus concentration</td>
</tr>
<tr>
<td>$p$</td>
<td>Cellular concentration of Poly-P</td>
</tr>
<tr>
<td>$Pa$</td>
<td>Partial pressure of solute in the gas phase</td>
</tr>
<tr>
<td>$q$</td>
<td>Cellular concentration of P</td>
</tr>
<tr>
<td>$q_o$</td>
<td>Minimum cellular concentration of P under $o-PO_4$ limitation</td>
</tr>
<tr>
<td>$R$</td>
<td>Respiration rate of algae and bacteria measured in situ</td>
</tr>
<tr>
<td>$R_i$</td>
<td>P exchange rates between P compartments</td>
</tr>
<tr>
<td>$S$</td>
<td>Nutrient or substrate concentration</td>
</tr>
<tr>
<td>$S_e$</td>
<td>Effective concentration of substrate in the cell influenced by the external substrate concentration</td>
</tr>
<tr>
<td>$S'$</td>
<td>Sum of the P in solution as $o-PO_4$ plus the P concentration in the algal cell that is stored as surplus</td>
</tr>
<tr>
<td>$SRP$</td>
<td>Soluble reactive P concentration</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
</tr>
<tr>
<td>$V$</td>
<td>Cell volume concentration</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Volume of single cell</td>
</tr>
<tr>
<td>$V_u$</td>
<td>Uptake rate of substrate by cell</td>
</tr>
<tr>
<td>$X$</td>
<td>Cell dry weight concentration</td>
</tr>
</tbody>
</table>
\[ \alpha \] Photosynthesis rate of algae measured in situ

\[ D \] Molecular diffusivity coefficient

\[ \mu_x, \mu_n, \mu_y \] Specific growth rate in terms of cell dry weight, cell number, cell volume respectively

\[ \mu_{x, \text{max}} \] A constant that is the maximum growth rate based on dry weight for increasing concentrations of substrate in Michaelis-Menten equation
APPENDIX B

EQUIPMENT CALIBRATION

B.1 Calibration of the Solar Cells

Light intensity was measured with Centralab Type 52CL solar cells. Two cells were calibrated under known light intensity conditions. The cells were placed in a box which allowed no light to enter. At one end one inch of length of a 15 watt General Electric Cool White fluorescent bulb was exposed. The intensity of light decreased with distance from the light. This intensity was measured at various distances with a Gossen Luna-Pro light meter set for direct light. Using this distance versus light intensity calibration, the voltage output of each cell was determined for a range of light intensities. Each cell's voltage output was amplified 20x by a Dana Model 2210 Data Amplifier and connected to the Beckman Type R Dynograph on which the amplifier was set at 1 volt/cm. The voltage across the input to the recorder was measured by a Tektronix Model DM 501 Digital Multimeter. The results of the calibration are shown in Figure B.1. The calibrations were carried out at room temperature which approximated the temperature in the fermentor, but it was noted that the output of the cell increases with temperature and its variation could be significant for temperatures that vary more that 10°C from the calibration temperature.
Figure B.1 Calibration Curve for the Internal and External Solar Cells on Fermentor Apparatus
B.2 Thermocouple Calibration

The temperatures of the fermentor vessel medium, the room temperature, and the cooling water to the constant temperature bath were measured with iron-constantan thermocouples referenced against an ice bath. The millivolt output of the thermocouples was amplified 1000x with a Dana Model 2210 Data Amplifier, whose output was recorded on a Beckman 8-channel, Type R Dynograph on which the amplifier was set at 100 mv/cm and observed on a Tektronix DM501 Digital Multimeter.

The thermocouples were calibrated in the temperature range of operation (10 to 35°C). All the thermocouples were placed in a temperature bath that maintained a temperature of ±1°C and monitored with a mercury thermometer which could be read to ±0.05°C. The output of the thermocouples was recorded with the amplifier and recorder in operating using the digital voltmeter. Figure B.2 shows the calibration curves.

B.3 Calibration of the Tachometer

The impeller speed was monitored with a Servo-Tek Products Co. DC generator. The output was passed through a first order filter with a 1.8 second time constant to eliminate the high rms fluctuation before it was connected to the Beckman 8-channel Type R Dynograph on which the amplifier was set at 2 volt/cm. The calibration curve was generated with a mechanical tachometer measuring the shaft speed and a Tektronix DM 501 Digital Multimeter monitored the input to the recorder which was running at the time to maintain an electrical load across the input. The calibration is in Figure B.3.
Figure B.2 Calibration Curve of Thermocouples on Fermentor Apparatus

Iron-Constantan Thermocouples
Referenced to 0°C
Dana Amplifier - 1000x
Beckman Recorder - 100 mv/cm

- Bath Temperature
- Fermentor Temperature
- Room Temperature
Figure B.3 Calibration of DC Generator as Tachometer for Impeller Rotation in Fermentor Apparatus
B.4 Calibration of the Dissolved Oxygen Probe

The New Brunswick Scientific Model M1016-0201 oxygen probe showed a decay in the output with time due to the depletion of the filling solution. Full scale value of the probe was set under a known mass rate of air through the fermentor vessel filled to a specified liquid level (3.2 gm/min, 17.5 liters liquid, 25°C, 450 RPM). Full scale reading was set by increasing the resistance across the probe output. The design of the oxygen probe output amplifier was found in the literature (113). It should be noted that the transistors in this amplifier are sensitive to temperature changes even though buried in a block of brass. It was required to maintain the transistors at an elevated temperature (40°C) using an on-off controlled heating tape which utilized a thermistor to monitor the temperature surrounding the brass block.

Calibration of the probe was conducted under simulated run conditions. First, the full scale was set as noted previously. Then, various ratios of oxygen and nitrogen were passed through the fermentor vessel at a total flow of 0.22 gm moles per minute (rotameter flows were determined as outlined in Section B.6 and the total pressure in the fermentor vessel was determined as shown in Section B.5). The amplifier output was passed through a first order filter with a 1.8 second time constant, amplified with a Dana Model 2210 Data Amplifier set at 100x, and connected across the input of a Beckman Type R Dynograph on which the amplifier was set at 500 mv/cm. The voltage across the input to the recorder was measured with a Tektronix DM501 Digital Multimeter with the recorder on. The calibration is shown in Figure B.4.
Figure B.4 Calibration of DO Probe
B.5 Determination of the Volumetric Oxygen Transfer Coefficient

The determination of the volumetric oxygen transfer coefficient for the fermentor vessel was made in order that calculations of the rates of transfer of oxygen to the liquid phase could be made. Further through generalized correlations, the volumetric transfer coefficient for carbon dioxide could be determined from the oxygen coefficient in order to calculate the rate of absorption of this gas into the liquid phase.

The volumetric oxygen transfer coefficient was calculated from the following equation (1,71):

$$K_{La} = \frac{n}{(c^*-c)}$$  \hspace{1cm} (B.1)

where:  

- $K_{La}$ - volumetric oxygen transfer coefficient, \(\text{cm}^2/\text{sec}\),  
- $a$ - interfacial area between the gas and the liquid phases per unit volume of liquid, \(\text{cm}^2/\text{liter}\),  
- $k_L$ - mass transfer coefficient in the liquid phase, \(\text{gm moles/sec} \cdot \text{cm}^2\),  
- $c$ - concentration of dissolved oxygen in the bulk liquid phase, \(\text{gm moles/liter}\),  
- $c^*$ - concentration of dissolved oxygen in the liquid at gas-liquid interface that is in equilibrium with the bulk gas phase concentration of oxygen and related by Henry's law, \(\text{gm moles/liter}\),  
- $n$ - volumetric oxygen transfer rate, \(\text{gm moles/liter} \cdot \text{sec}\)
The form of Henry's law, for the equilibrium relation between the partial pressure of a solute (oxygen or carbon dioxide) in the gas phase and the concentration in the liquid phase, can be expressed by the following equation, for dilute solutions (71):

$$P_a = H x_a$$  \hspace{1cm} (B.2)

where: \( P_a \) - partial pressure of solute in the gas phase (atm.), 
\( x_a \) - mole fraction of the solute in the liquid phase, 
\( H \) - Henry's law constant \( \left( \frac{\text{atm.}}{\text{mole frac.}} \right) \).

The Henry's law constants in a water-gas system at 25°C were interpolated to be \( 4.38 \times 10^4 \) and \( 0.163 \times 10^4 \) atm./mole frac. for oxygen and carbon dioxide respectively (71). For oxygen, the correction for dissolved solids is negligible (210). Henry's law also predicted free CO\(_2\) in water with dissolved solids. This was verified using the total inorganic carbon analysis technique in the liquid phase (see Section C.8). The partial pressure of carbon dioxide in the gas phase was verified by scrubbing a known portion of the gas phase with Ba(OH)\(_2\), thus precipitating BaCO\(_3\). The unreacted Ba(OH)\(_2\) was titrated with acid.

The volumetric transfer coefficient for carbon dioxide can be estimated from the volumetric oxygen transfer coefficient. It is known that the transfer coefficient, \( k'_{L} \), can be related to the molecular diffusivity coefficient, \( D \), in the following manner assuming that all operating conditions of the fermentor vessel, liquid phase conditions and gas phase conditions are the same. Since for one gas, i.e., oxygen (71),
\[ k_{L02} \propto \sqrt[3]{\frac{D_{O2}}{D_{CO2}}} \]  

\[ \frac{k_{L02}}{k_{LCO2}} = \left( \frac{D_{O2}}{D_{CO2}} \right)^{2/3} \]  

then ratioing this relationship to a similar one for carbon dioxide, you get the following equation:

Therefore, knowing the diffusivities for oxygen and carbon dioxide and the volumetric oxygen transfer coefficient, the volumetric carbon dioxide transfer coefficient can be estimated. At 25°C the diffusivities for oxygen and carbon dioxide in water are respectively 2.41 x 10^{-5} and 2.00 x 10^{-5} cm²/sec. (71).

The values on the right side of the Equation B.1 were taken from experimental observations. The volumetric oxygen transfer rate, \( n \), was obtained using the dissolved oxygen probe. The initial slope of the increase in dissolved oxygen concentration with time from 0.0 ppm was taken as the measure of the oxygen transfer rate. The calibration procedure began with the elimination of the oxygen in the liquid phase in the fermentor by passing nitrogen gas through the water till there was no detectable output from the dissolved oxygen probe. With the temperature of the water at 25°C and a liquid height of 39.4 cm (17.5 liters), air was passed through the liquid at various mass flow rates and impeller speeds. The dissolved oxygen concentration was zero. The interfacial concentration in the liquid phase, \( c^* \), was calculated using Henry's law, Equation B.2, and the partial pressure of oxygen in the air. The total pressure in the fermentor vessel is
higher than atmosphere pressure because of the pressure drop of the
exit gas through the check valve. A plot of the pressure drop versus
the mass flow rate through the valve is shown in Figure B.5. Further,
as the gas ascends through the liquid column the total pressure in
gas bubble decreases. Half the column height was added to the pres-
sure drop in the check valve to obtain the total pressure in the gas
bubble. It was assumed that the change in the mole fraction of
oxygen of the gas phase was negligible.

A plot of the volumetric oxygen transfer coefficient, $k_{La}$, versus
mass flow rate is shown in Figure B.6 with impeller speed as a
variable.

The response of the New Brunswick Scientific Model M1016-0201
oxygen probe is relatively slow and the rate data obtained may only
have been that of the probe response and not the response of the
liquid medium to the dissolution of oxygen from the dispersed gas
phase. The response of the probe to an ideal step change in oxygen
concentration from zero to oxygen saturation in a well stirred water
was observed to be 60 seconds for 80% of the final equilibrium probe
output at 25°C (105). The major resistance was in the filling
solution (113).

An estimate can be made of the volumetric oxygen transport
coefficient, $k_{La}$, from published correlations which use impeller speed,
gas rate, fermentor tank, impeller dimensions and liquid phase physi-
cal constants. The volumetric oxygen transport coefficient was cor-
related with power input to the fermentor vessel (1). The power input
was correlated with the impeller Reynolds Number (11). But the power
Figure B.5 Pressure Drop Calibration of Exit Gas Check Valve on Fermentor Apparatus
Figure B.6 Volumetric Oxygen Coefficient as Function of Impeller Speed and Gas Mass Rate in Fermentor Apparatus
from this correlation was increased for two impellers (11) and the power input from the Reynolds Number correlation must be reduced for the dispersed gas phase (11). The power for the two-phase system predicted by the correlation was lower than was observed. Power input to the impellers was monitored by amperage to a DC motor and power was reduced 15% as compared to 15 to 50% from the correlation. This power does not account for efficiency losses in the motor or the transmission of that power. The power reduction was the most uncertain of the values used in the calculations. Therefore, limits of this value were shown above. As a result of the calculations, the value predicted for the volumetric oxygen transport coefficient was approximately 50% lower for 300 and 450 rpm than calculated from observed oxygen concentration rate of change monitored by the oxygen probe; but was of the same or slightly above for the 600 rpm case. These values of the predicted oxygen transfer rate are slightly higher if one does not take into account the effect of all the probes and support rods in the vessel for which the correlations do not show cases. The magnitude of this can be seen in the Chemical Engineer's Handbook, Figure 19-29, pages 19-15 (177), curves 5 and 6. For the turbine impeller, with baffles, curve 6 is almost an order of magnitude higher in power input than the case of no baffles, curve 5, for the same rpm. These structures act as baffles and dissipate energy. Thus a reduced power should give lower coefficients than listed above. In conclusion, these lower predicted volumetric oxygen transport coefficients than those observed support the fact that the probe output was the response of the system and not of the probe itself.
B.6 Rotameter Calibration

The rotameters are Brooks E/C meters. The tube size and float for each gas is noted below:

<table>
<thead>
<tr>
<th>Gas</th>
<th>Tube Size No.</th>
<th>Float</th>
</tr>
</thead>
<tbody>
<tr>
<td>air</td>
<td>R-6-15B</td>
<td>glass</td>
</tr>
<tr>
<td>nitrogen</td>
<td>R-6-15A</td>
<td>glass</td>
</tr>
<tr>
<td>oxygen</td>
<td>R-6-15A</td>
<td>glass</td>
</tr>
<tr>
<td>carbon dioxide</td>
<td>R-2-15A</td>
<td>stainless steel</td>
</tr>
</tbody>
</table>

The rotameters were calibrated using a Precision Scientific Wet Test Meter, which had a capacity of 3 liters per revolution, and water displacement in an inverted 1000 ml volumetric flask. The thermal variables that were noted were the room temperature, which was assumed to be the temperature of the gas through the rotameter; and the temperature of the water in the wet test meter and inverted flask, which were used to correct the gas volume for the water vapor partial pressure in equilibrium water at those temperatures. The atmospheric pressure was read during the calibration period every 4 hours and the pressure of the gas in the rotameter was read using a mercury manometer (see Figures 4.11 and 4.12). The rotameter pressure was adjusted using a needle point valve downstream from the rotameter.

The accuracy of the rotameter varied primarily with the pressure on the gas in the meter, or the density of the gas. The gas density varied with the flow rate of the gas and the various downstream pressure drops. All these were variable in time and therefore the calibration needed to be general enough to account for them. Further temperature effects were accounted for in the density of the gas.

The effect of temperature on the viscosity of the gas is negligible
over the range of temperature (20 to 30°C). The gas viscosity is independent of gas pressure over the range of 1 to 2 atmospheres (57,23).

The accuracy of the rotameter calibration over the temperature and pressure ranges as mentioned above, is 2% except in the low range of the rotameter scale (<0.5 on rotameter scale).

The calibration curves are used in the following way. The temperature and pressure of the gas in the rotameter are taken and the gas density calculated. The calculated density is divided by the reference density for the rotameter (note each calibration curve). On Curve One for the rotameter, the ratio of mass rate to reference mass rate is read from the ordinate. Using the observed rotameter reading, the reference mass rate is obtained from Curve Two for the rotameter. Thus the actual mass flow rate can be calculated for the rotameter. Each rotameter for a particular gas has both Curve One and Curve Two. The calibration curves are found in Figures B.7 to B.12.
Figure B.7 Carbon Dioxide Rotameter Calibration Curve for Correction of Gas Density Different Than Standard Density - Curve 1
Figure B.8 Carbon Dioxide Standard Calibration Curve at Standard Density - Curve 2
Figure B.9 Air Rotameter Calibration Curve for Correction of Gas Density Different Than Standard Density - Curve 1

Standard Density = .002301 gm/cc
For Rotameter Scale Readings < 7.0
Curve 1
Rotameter Scale

Constant Density = .002301 gm/cc
Note: <2% Error for Rotameter
Readings <7.0

Curve 2

Figure B.10 Air Rotameter Standard Calibration Curve at Standard Density - Curve 2
Stand Density = 0.001752 gm/cc
- Nitrogen Rotameter
- Oxygen Rotameter

Note: Different Curves at Low Ratios

Curve 1

Figure B.11 Oxygen and Nitrogen Rotameter Calibration Curve for Correction of Gas Density Different Than Standard Density - Curve 1
Constant Density = .001752 gm/cc

Note: Rotameter Scale Reading

<3.1 Average of Several Observations

<3.1 Individual Observations

Curve 2

Figure B.12 Oxygen and Nitrogen Rotameter Standard Calibration Curve at Standard Density - Curve 2
APPENDIX C

DETAILED PROCEDURES

C.1 Medium Preparation

C.1-1 Bold's Basal Medium (15)

Six stock solutions were prepared with water as follows:

1. NaNO₃.........25. gm/l  4. K₂HPO₄............. 7.5 gm/l
2. CaCl₂·2H₂O.........2.5 gm/l  5. KH₂PO₄.............17.5 gm/l
3. MgSO₄·7H₂O..........7.5 gj/l  6. NaCl................. 2.5 gm/l

Four stock trace element solutions were prepared with distilled water as follows:

1. 56.7 gm Na₂EDTA and 5.92 gm NaOH per liter. (This is different from the original formulation of 50.0 gm EDTA and 31.0 gm KOH. Equal number of equivalents of Na were added as K in the original specification.)
2. 4.98 gm FeSO₄·7H₂O per liter as acidified water (acidified water: 1 ml concentrated H₂SO₄ in 999 ml water).
3. 11.42 gm H₃BO₃ per liter.
4. The following were added to 1 liter acidified water:
   a) ZnSO₄·7H₂O.............8.82 gm
   b) MnCl₂·4H₂O.............1.44 gm
   c) MoO₃(molybdic acid)...0.71 gm 0.835 gm)
   d) CuSO₄·5H₂O.............1.57 gm
   e) Co(NO₃)₂·6H₂O...........0.49 gm
To 500 ml water, 10 ml of each stock solution and 1 ml of each trace solution were added and diluted to 1 liter. When P was being limited, the potassium level was maintained by adding KCl (16.03 gm/l in stock solution). The potassium level was also maintained by added KNO₃ (75.83 gm/l) when a higher nitrate level was required. The final medium concentrations are listed in Table C.1.

C.1-2 Proteose Agar (211)

This medium was used to maintain the *Chlorella pyrenoidosa* reference culture. For each 1000 ml of medium the following was required:

- **Bold's Basal Medium** 1000 ml
- **Proteose peptone No. 3 (Difco)** 1.0 gm
- **Bacto-Agar (Difco)** 15.0 gm

Before autoclaving the agar medium, the medium was heated to boiling to dissolve all the agar.

C.1-3 Nutrient Agar

This medium was used for microbial contamination checks. For each 1000 ml of medium the following was required:

- **Bold's Basal Medium** 1000 ml
- **Glucose** 200 mg
- **Nutrient Agar (Difco)** 23 gm

Before autoclaving, the agar medium was heated to boiling to dissolve all the agar.
Table C.1 Concentrations of Nutrients in Bold's Basal Medium

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>mg/L</th>
<th>mllili moles/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>87.82</td>
<td>3.82</td>
</tr>
<tr>
<td>Ca</td>
<td>6.81</td>
<td>0.17</td>
</tr>
<tr>
<td>Mg</td>
<td>8.27</td>
<td>0.34</td>
</tr>
<tr>
<td>K</td>
<td>84.07</td>
<td>2.15</td>
</tr>
<tr>
<td>Fe</td>
<td>1.00</td>
<td>0.018</td>
</tr>
<tr>
<td>Zn</td>
<td>2.00</td>
<td>0.031</td>
</tr>
<tr>
<td>Mn</td>
<td>0.40</td>
<td>0.0073</td>
</tr>
<tr>
<td>Mo</td>
<td>0.56</td>
<td>0.0058</td>
</tr>
<tr>
<td>Cu</td>
<td>0.40</td>
<td>0.0063</td>
</tr>
<tr>
<td>Co</td>
<td>0.10</td>
<td>0.0017</td>
</tr>
<tr>
<td>B</td>
<td>2.00</td>
<td>0.18</td>
</tr>
<tr>
<td>N</td>
<td>41.18</td>
<td>2.94</td>
</tr>
<tr>
<td>P</td>
<td>53.28</td>
<td>1.72</td>
</tr>
<tr>
<td>S</td>
<td>12.70</td>
<td>0.40</td>
</tr>
<tr>
<td>Cl</td>
<td>27.76</td>
<td>0.78</td>
</tr>
<tr>
<td>EDTA</td>
<td>44.42</td>
<td>0.15</td>
</tr>
<tr>
<td>NO₃</td>
<td>182.30</td>
<td>2.95</td>
</tr>
<tr>
<td>SO₄</td>
<td>38.04</td>
<td>0.40</td>
</tr>
<tr>
<td>PO₄</td>
<td>163.36</td>
<td>1.72</td>
</tr>
</tbody>
</table>
C.2 Run Preparation

C.2-1 Preparation of Chlorella Pyrenoidosa Inoculum

1. The stock culture of *Chlorella pyrenoidosa* (211) was maintained on a proteose peptone agar at room temperature under subdued non-continuous light conditions (18 hour light: 6 hour dark cycle). This culture can be maintained for 3 to 4 months on the slant before a new agar medium is required.

2. A conditioning culture of *Chlorella pyrenoidosa* was prepared using Bold's Basal medium with either 2.5 or 5.0 mg P/l and the pH adjusted to 7.0 with NaOH. Two 250 ml cotton stoppered erlenmyer flasks were filled with 100 ml of the medium, sterilized and inoculated with a loopful of algal cells from the stock culture. These cultures were kept on a shaker table (New Brunswick Scientific, Model R-7) at 100 cycles per minute. The cultures were at room temperature under continuous illumination of 450 ft-candles from General Electric Cool White fluorescent bulbs. The conditioning culture was checked for contamination after 3 days. One-tenth ml of the culture was plated onto nutrient agar and was checked after two days for contamination. At this time the culture was ready for transfer.

3. An inoculum culture of *Chlorella pyrenoidosa* was prepared using Bold's Basal media with the P concentration of the run to be inoculated. Two 250 ml cotton stoppered erlenmyer flasks were filled with 150 ml of media, sterilized, and inoculated with 1 ml of the conditioning culture. The inoculum cultures were treated in the same manner as the conditioning cultures.
C.2-2 Preparation of the Pseudomonas Bacterium Inoculum

1. The stock culture of the isolated Pseudomonas was maintained on a nutrient agar medium at 4°C in a refrigerator. This culture can be maintained 3 or 4 months on the agar before a new agar medium is required.

2. An inoculum culture of Pseudomonas was prepared using Bold's Basal medium with 250 mg/1 sucrose and the pH adjusted to 7.0 with NaOH. Two 250 ml cotton stoppered erlenmyer flasks were filled with 100 ml of the medium, sterilized and inoculated with a loopful of the stock culture. These cultures were kept on a shaker table at 100 cycles per minute at room temperature. The inoculum culture was checked for contamination after 3 days. One-tenth milliliter of the culture was plated onto nutrient agar and the plate was checked for colony uniformity after 2 days. At this time the culture was ready for transfer.

C.2-3 Preparation of the Fermentor Vessel for a Run

1. The fermentor jar, head assembly and the baffles (all three to be subsequently called the fermentor vessel, a 20 liter VirTis Co. Model 43-120 vessel with magnetic drive) were washed thoroughly with water. Any surface dirt was removed with a plastic scrubber.

2. The fermentor vessel was filled with water and 200 ml of cleaning solution (chromic acid solution). This solution was allowed to sit for 1 hour to dissolve any traces of phosphorus, nitrogen, or organic compounds from fermentor vessel surfaces.

3. The fermentor vessel was thoroughly rinsed with water.
4. During the final assembly of the fermentor vessel, the head assembly was centered and the solar cell was adjusted to be perpendicular to the jar surface and placed at a point midway between adjacent baffles.

5. The vessel was filled with water for the medium preparation.

6. The proper amounts of nutrients and substrates were added.

7. Sterilization of the fermentor vessel followed.
   a. Screw clamps were fastened to all outlet and inlet tubes (except inlet gas, acid and base lines).
   b. A glass wool wad was placed in the gas outlet check valve. During a run this would prevent any contaminated condensate from flowing back through valve when open. Glass wool wads were placed in either or both the acid and the base lines to the vessel.
   c. A small plug was removed from the top of the vessel prior to sterilization to maintain pressure equalization. It was replaced after sterilization prior to removal of fermentor vessel from the autoclave (New Brunswick Scientific, Model AS, steam). The gas inlet tube was plugged.
   d. The exit gas line, inlet gas line and filter, and the above plug were sterilized with the fermentor vessel.
   e. Sterilization time cycle was as follows:
      - One hour heat up,
      - One hour at 121°C,
      - Two to three hour cooldown.
f. During the sterilization cycle, autoclave steam was bled through a bacterial filter both to allow the escape of inert gases and to sterilize the filter. After the steam to the autoclave was shut off the autoclave was pressurized during cooldown with nitrogen fed through the sterile bacterial filter at 15 psi. When the temperature of the autoclave was approximately 90°C, the pressure was slowly bled off.

g. After the autoclave was opened, the small plug was replaced in the top of the fermentor vessel and the air filter attached.

8. The fermentor vessel was attached to the magnetic drive and adjusted to a slow speed.

9. The glass tip of the harvesting line was placed in a 125 ml erlenmyer flask filled with pure methyl alcohol and sealed with a rubber stopper.

10. The air filter line was connected to the laboratory air source. The air rate was initially slow to maintain positive pressure on the fermentor vessel. Later, as the fermentor vessel cooled, the rate was increased. The inlet line was checked for leaks with a soap solution. The gas outlet line was attached and connected to the wet test meter.

11. Cooling water lines were attached to the fermentor vessel. The refrigeration unit to the constant temperature bath was turned on. The refrigeration unit was later turned off when cold tap water was able to supply enough cooling capacity to the constant temperature bath.
12. The fluorescent light manifold was placed around the fermentor vessel and the air fans were turned on. Near the end of the cooling period (approximately 2 hours) the lights were turned on in order to facilitate the balancing of the bulb heat load on the fermentor vessel.

13. The fermentor vessel thermocouple and electrical ground were attached. The external solar cell was taped to the side of the fermentor jar at mid-height and at the mid-point circumferentially of two adjacent illuminated fluorescent bulbs. Both the internal and external solar cells were attached to the recorder.

14. Adjustment of the temperature, agitation rate, air and CO₂ rates to values of the experimental run were made.

15. The pH control was through CO₂-HCO₃⁻-CO₃⁻ buffering. For pH above 7.0, either 15 ml of sterilized 5N NaOH was added through the inoculation port, which has been sterilized by covering with pure methyl alcohol for ten minutes, using a sterilized syringe; or 125 ml of 0.6N NaOH, which has been sterilized in a 125 ml separatory funnel, was metered into the fermentor vessel with the base pump at a rate of 0.65 ml per minute. The number of base equivalents are equal in each case. For pH less than 7.0, no base was added.

16. Final trimming of the pH was made by adjustment of the CO₂ rate.

18. One analysis cycle was conducted to obtain base line values. The _Chlorella pyrenoidosa_ inoculum culture was added using a sterile, 4 inch, 20 gage B-D Luer-Lok needle attached to 50 ml sterile syringe. The long needle was required to take up the inoculum
from a 250 ml erlenmyer flask. The inoculum was injected through the inoculation port, which was sterilized by covering with pure methyl alcohol for 10 minutes. Enough inoculum was added to give a cell count in the fermentor vessel of at least $1 \times 10^3$ cells/cc.

19. The fermentor vessel variables, inside and outside light intensity, agitation rate, and temperatures, were monitored continuously with a Beckman 8-channel, Type R Dynograph. Individual data points were taken on a Tektronics DM501 Digital Multimeter.

C.3 Integration of the Analyses During Experimental Run

The following is the procedure of one analysis cycle. See Figure 5.15 for the flow sheet of the overall analysis procedure.

1. The run variables were recorded:
   a. $\text{CO}_2$ and air rotameters, and wet test meter,
   b. Fermentor vessel and atmospheric pressures,
   c. Temperatures of the room, cooling water and fermentor vessel,
   d. Inside and outside light intensities.

Adjustment of the cooling capacity of the constant temperature bath was made at this time, if necessary. Water rate to the constant temperature bath was constant. If constant temperature bath required increased cooling capacity, the cooling water temperature was decreased by increasing the rate to the temperature bath. If this did not provide adequate cooling, then the refrigeration unit was turned on.

2. Microbial contamination of the fermentor vessel was checked. A sample of the medium in the fermentor vessel was withdrawn through
the harvesting line into a sterile cotton stoppered test tube. Before sampling, some of the media ran through the tube to eliminate any methyl alcohol and stagnant liquid in the tube. One-tenth ml was sterilly transfered to nutrient agar plates. After two days at room temperature, the plates were checked for signs of contamination.

3. If the fermentor vessel pH probe was not functioning, external pH measurement was necessary. A sample of the medium in the fermentor vessel was withdrawn through the harvesting line into a 100 ml beaker. With a magnetic stirrer at a low speed, the pH value was taken with a glass electrode (Leeds Northrup, Model 117169) and reference electrode (Leeds Northrup, Model 117417).

4. The population of Chlorella pyrenoidosa was determined. A sample of the media was withdrawn, as before. A known volume of the well mixed cell suspension was placed into 2-15 ml graduated centrifuge tubes. The cells were stained with 1 to 5 drops iodine solution (4 gm. I and 6 gm KI per 100 ml (82)) depending on the dilution. The stained cells were diluted with water so that the final population density was approximately 2x10⁶ cells/ml. Each tube was counted 8 times. Each tube was counted 20 times if the cell population was less than 1x10⁵ cells/ml. Counts were made on an American Optical Bright-Line counting chamber with Neubauer ruling with a volume of 10⁻⁴ cc and a chamber depth of 0.1 mm. placed under a microscope at 200x.

5. The algal cell volume was determined. The algal cell volume is calculated from the measured cell diameter obtained during the
population count. Two or three cells were selected randomly during a cell count for diameter measurement with a calibrated scale under a microscope. This process continued until ten or twenty measurements were made. If the cell was round the diameter was taken and the volume was calculated for a sphere (Equation 5.1); if the cell was oblong, or fusiform, both the major and the minor diameters were measured and the volume of the cell was calculated for a prolate spheroid (Equation 5.2). The determined volume of the cell at the time of observation is the average of the calculated cell volumes.

6. General observations of the cell morphology were made during the cell population count. The percentage of the C. pyrenoidosa that were fusiform were determined. The number of daughter cells produced from a single mother cell was counted. Finally, iodine the staining characteristics of the algal cell was summarized in terms of the color intensity and the cellular structures that are stained.

7. The analysis sample was withdrawn from the fermentor vessel through the harvesting line as before and collected in an erlenmyer flask. Only enough media was collected to complete one analysis cycle.

8. A sample was taken for total P analysis. If total iron and total calcium were determined, the samples were taken at this time. The erlenmyer flask was well mixed before the samples were withdrawn.
9. The dry weight of suspended particles were determined. Using 47 mm diameter Whatman GF/D glass fiber filter, particles with a diameter greater than 2.7 µm were filtered out. (These filters were fired at 550°C for 2 hours in a muffle oven. They were weighed on a Perkin Elmer Autobalance, Model AD-2 to ±0.005 mg. The filters were placed in individual plastic petri dishes and stored in a dessicator with anhydrous magnesium perchlorate as desiccant.) The suspended particles from a known volume were collected on the filter which was in a stainless steel Millipore 47 mm Pressure Filter Holder with a 100 ml capacity. The maximum amount of cells on the filter was 1x10^7 or no more than 500 ml of the medium was filtered. (This was dictated by the maximum dry weight limit on the filter of 2.5 mg for analysis in the Perkin Elmer Elemental Analyzer, Model 240.) The filter and particles were washed with 20 ml of distilled water to remove salts that would increase dry weight. The filter holder was closed and nitrogen gas was passed through the filter for 5 minutes to drain excess water. The pressure did not exceed 12 psi across filter. The nitrogen line contains an in-line filter with a 0.22 µm membrane filter to remove any particles from the gas. The dry weight filter was removed from the filter holder and placed in a glass petri dish which had the bottom covered with 9 mm glass beads to prevent the filter from sticking during drying. The filters were placed in an air-circulating oven for 1 hour at 105°C. The filter was removed, allowed to cool and placed into the plastic petri dish to be stored in a dessicator till weighed on the micro-balance. Dry weight was run in duplicate.
10. The filtrate from the GF/D filtration was saved while the wash was discarded. At times the filtrate was analyzed for total P and subsequently filtered through a Whatman GF/F glass filter which passed particles smaller than 0.7 μm. The filter was used to determine the dry weight of particles that passed through the GF/D filter. In this case the filter was treated in the same manner as the GF/D for dry weight analysis.

11. The filtrate from the GF/F filtration was saved. This portion was analyzed for total P.

12. Cells for the trichloroacetic acid (TCA) and boiling water extractions were collected separately on non-pretreated Whatman GF/D filters. The maximum amount of cells on the filter was $1 \times 10^7$ cells or a maximum of 500 ml of medium was filtered. The filter was washed with 20 ml of the boiling water extractant to remove P compounds external to the cell. Nitrogen was passed over the filter, as in the dry weight determination, for about 5 minutes. The filter was removed from the filter holder, placed in a plastic petri dish, and stored for approximately 30 minutes till it was required for the extraction procedure.

13. The filter for the boiling water extraction was ground in a Thomas Teflon Pestle Homogenizer, size B. The pestle was rotated at 950 rpm with a GKH 1/20 hp DC motor with a S-20 Motor Controller. The filter plus 10 ml of the extractant were placed into the grinding vessel. Grinding proceeded till filter was disintegrated. The pestle and the grinding vessel were thoroughly washed with 90 ml of extractant and combined with the ground filter in a
250 ml erlenmyer flask containing a few glass beads. The flask was fitted with a cold finger and the contents were boiled for one hour. After cooling the contents to room temperature, they were centrifuged at maximum rpm for 5 minutes in a bench top IEC Model CL centrifuge. Following the centrifuging, the extractant was filtered through a 0.45 µm MF-Millipore filter. All MF-Millipore filters were stored in distilled water at least 24 hours before use at a ratio of 50 ml water per filter. The filtrate was analyzed for total P, acid hydrolyzable P, and o-PO₄.

14. The filter for the TCA extraction was ground with an 8% (by wt.) TCA solution which was maintained at 0°C in either an ice bath or a refrigerator. In this procedure only 45 ml of extraction, 10 ml for grinding and 35 ml for washing of grinder, were used for grinding and washing of the pestle and the grinding vessel. The ground filter is kept at 0°C for 30 minutes, centrifuged, the supernatant collected and maintained at 0°C, and a second extraction of the ground filter followed using 45 ml of 8% TCA for 15 minutes at 0°C. The second supernatant was centrifuged, combined with the supernatant of the first extraction and filtered through a 0.45 µm MF-Millipore filter in a precooled filter holder. The filtrate was collected and diluted to 100 ml with fresh extractant. The filtrate was kept at 0°C till analyzed. The filtrate was analyzed for total P, acid hydrolyzable P, and o-PO₄.

15. The filtrates from steps 7 and 10 were passed through a 0.45 µm MF-Millipore filter. This filtrate was analyzed for total P,
acid, hydrolyzable P, o-PO₄, nitrate, COD, iron and calcium.

C.4 Phosphorus Analysis

C.4-1 Acid Digestion for Total Phosphorus (195)

1. Procedure

a. To a 100 ml sample, or sample diluted with water to 100 ml, in a 250 ml erlenmyer flask, 1 ml of concentrated H₂SO₄, 8 gm. K₂S₂O₈ and glass beads were added.

b. The sample was boiled gently for 1 1/2 hours on a hot plate till the sample was fuming H₂SO₄.

c. After the flask was cooled, the residue was dissolved in 25 ml of water.

d. The contents of the flask and the flask washings were transferred to a 100 ml volumetric flask for the coloration procedure.

e. A reagent blank was carried through same procedure.

C.4-2 Acid Hydrolysis for 7-Minute Acid-Hydrolyzable Phosphorus (184)

1. Reagents

a. Hydrochloric acid solution, 6N:

2. Procedure

a. To a 25 ml sample in a 100 ml volumetric flask 1 ml hydrochloric acid solution per 5 ml of sample was added to obtain a final concentration of 1N HCl.

b. The flask was initially heated over a Fisher burner to the boiling point (approximately 1 1/2 minutes).
c. The flask was transferred to a hot plate adjusted to maintain the flask at 100°C with a minimum boiling. The time on the hot plate plus the time over the burner was seven minutes.
d. The flask then was transferred to an ice bath.
e. After the flask reached room temperature, it was ready for the coloration procedure.
f. A reagent blank was carried through same procedure.

C.4-3 Acid Hydrolysis for 15-Minute Acid-Hydrolyzable Phosphorus (184)

1. Reagents
   a. Sulfuric acid solution, 6N.

2. Procedure
   The procedure is the same as above except that the total time on the burner and the hot plate was 15 minutes.

C.4-4 Phosphate Determination Using Hydrazine-Molybdenum Blue Method (3)

1. Apparatus
   a. Spectrometer: This was set at 830 nm.
   b. Steam bath.

2. Reagents
   a. Ammonium molybdate solute: 300 ml concentrated H₂SO₄ was added to 500 ml of water and allowed to cool. To this solution 20 gm (NH₄)₆Mo₇O₂₄·4H₂O was added and diluted to 1 liter.
   b. Hydrazine sulfate solution: 1.5 gm (NH₂)₂·H₂SO₄ was dissolved in water and diluted to 1 liter.
c. Ammonium molybdate-hydrazine sulfate solution: 250 ml of the ammonium molybdate solution was diluted to 600 ml with water. Then 100 ml of the hydrazine sulfate solution was added and diluted to 1 liter with water. This solution was prepared immediately before use.

d. Phosphorus standard solution: In 200 ml of water, 0.2292 gm Na$_2$HPO$_4$ was dissolved and diluted to 1.0 liter. This solution was preserved with 2 ml chloroform/l. (1 ml = 0.05 mg P)

e. Sodium sulfite solution: 100 gm Na$_2$SO$_3$ was dissolved in water and diluted to 1 liter.

3. Procedure

a. To a 100 ml volumetric flask, 25 ml of the sample was added. Then 1 ml of concentrated H$_2$SO$_4$ and 15 ml of sodium sulfite solution were added. No H$_2$SO$_4$ was added if sample was pre-treated by persulfate digestion.

b. The flask was boiled gently over a flame for 30 seconds and then transferred to a steam bath for 20 minutes. (The steam bath must be able to maintain a temperature of 95°C in the flask.)

c. The flask was quickly cooled to room temperature.

d. The flasks were brought up to the mark, mixed and absorbance measured at 830 nm. (The color was stable for 2 hours.)

e. A reagent blank was carried through the same procedure.

f. A calibration curve was prepared using the same procedure.

The calibration curve was checked to determine if 8% (by wt.)
trichloroacetic acid in the sample and a 25 ml sample from the persulfate digestion shifted the curve (see Figure C.1).

C.4-5 Phosphate Determination by Stannous Chloride Method (210)

1. Apparatus
   a. Spectrophotometer: This was set at 690 nm.

2. Reagents
   a. Phenolphthalein indicator solution: 1.0 gm of phenolphthalein was dissolved in a mixture of 80 ml of ethanol and 20 ml of water.
   b. Strong acid solution: To about 600 ml of water, 300 ml concentrated H₂SO₄ was added slowly. When cool, 4.0 ml HNO₃ was added and diluted to 1 liter.
   c. Ammonium molybdate solution: Cautiously 280 ml concentrated H₂SO₄ was added to 400 ml of water and cooled. Twenty-five grams (HN₄)₆Mo₇O₂₄·4H₂O was dissolved in 175 ml water. These two solutions were mixed and diluted with water to 1 liter.
   d. Stannous chloride reagent: 2.5 gm of a fresh supply of SnCl₂·2H₂O was dissolved in 100 ml of glycerol. The solution was heated in a water bath to hasten the dissolution.
   e. Standard Phosphate solution: See previous Reagents list.
   f. Sodium hydroxide solution, 5N.

3. Procedure
   a. Adjustment of the samples to a pH of approximately 7 was required before the color development procedure began. The procedure depended on the type of sample to be analyzed. One
Figure C.1 Calibration Curve of Absorbance for Hydrazine Molybdenum Blue Phosphate Determination
drop of phenolphthalein solution was added to the sample in a
100 ml volumetric flask. If the sample was first digested by
the persulfate method, 20 ml sodium hydroxide solution was
added and the same was back titrated with strong acid solution
to discharge the color. If a 25 ml sample was hydrolyzed in
the 7 or 15 minute procedure, 7 ml sodium hydroxide solution
was used. But if the sample contained 8% TCA, 9 ml were re-
quired. If the sample was not previously treated but con-
tained 8% TCA, only 5 ml were required. For each type sample a
calibration curve was made (see Figure C.2).

b. Since neutralization with base increases the temperature of
the samples, the flasks were placed in a water bath for at
least 15 minutes at a temperature of approximately 24.5°C.

c. To each flask were added in succession 4.0 ml of the molybdate
solution and 10 drops of the stannous chloride reagent. After
each addition the flask was thoroughly mixed. The flask was
brought to the mark with water and thoroughly mixed.

d. The samples were read after ten minutes in a spectrometer at
690 nm against a reagent blank. The temperature of the sample
was recorded. The rate of color development and the intensity
of the color depended on the temperature of the final solution,
each 1°C increase produced about a 1% increase in intensity.
Hence the samples, standards, and the reagents should be within
2°C of one another and at a temperature between 20 and 30°C. A
reagent blank was carried through for each type sample.
Figure C.2 Calibration Curve of Absorbance for Stannous Chloride - Molybdenum Blue Phosphate Determination
C.5 Chemical Oxygen Demand (COD) (210)

1. Apparatus

   a. Reflux apparatus consisted of 250 ml round bottom flasks with ground glass 24/40 neck, 300 mm jacket Liebig condenser with 24/40 ground glass joint and a Labconco Micro Kjeldahl Digestion rack.

2. Reagents

   a. Standard potassium dichromate solution. 0.250N: 12.259 gm $K_2Cr_2O_7$ primary standard grade was dissolved, previous dried at 103°C for 2 hours, in water and diluted to 1 liter.

   b. Sulfuric acid reagent: concentrated $H_2SO_4$ containing 22 gm $Ag_2SO_4$ per 9 lb bottle (1 to 2 days required for dissolution).

   c. Standard ferrous ammonium sulfate titrant, analytical grade crystals, 0.10N: 35 gm $Fe(NH_4)_2(SO_4)_2\cdot6H_2O$ dissolved in water. Twenty milliliters concentrated $H_2SO_4$ was added, cooled and diluted to 1 liter. This solution was standardized against the standard potassium dichromate solution before use.

   **STANDARDIZATION**—10.0 ml standard potassium dichromate solution was diluted to about 100 ml. Thirty milliliters concentrated $H_2SO_4$ was added and allowed to cool. This solution was titrated with the ferrous ammonium sulfate titrant after adding 2 to 3 drops (0.10-0.15 ml) ferroin indicator

   $$\text{Normality} = \frac{(mlK_2Cr_2O_7) \times (0.25)}{(mlFe(NH_4)_2(SO_4)_2)} \quad \text{(C.1)}$$
d. **Ferroin indicator solution**: 1.735 gm 1,10-phenanthroline dihydrate, together with 695 mg FeSO₄·7H₂O, were dissolved in water and diluted to 100 ml.

e. **Standard potassium acid phthalate solution**: 425.1 mg was dissolved in water and diluted to 1 liter for a 500 mg COD/l solution.

3. Procedure

   a. Four-tenths gram HgSO₄ was placed in the refluxing flask followed by a 20.0 ml sample. Then 10.0 ml standard potassium dichromate solution and glass beads were added. The flask was connected to the condenser. Slowly 30 ml of concentrated H₂SO₄ with Ag₂SO₄ was added through the open end of the condenser, mixing thoroughly by swirling during the addition. The reflux mixture was mixed thoroughly before heat was applied; if this is not done, local leading occurs in the bottom of the flask and the mixture may be blown out of the condenser. The open end of the condenser was covered with a 50 ml beaker to prevent dust from entering the flask and to prevent the blown flask contents contaminating other samples or analytical analyzes in the same hood.

   b. The mixture was refluxed for 2 hours, cooled and then the condenser was washed down with distilled water which was collected in the flask.

   c. The mixture was diluted to approximately 150 ml with water, cooled to room temperature and the excess dichromate titrated with standard ferrous ammonium sulfate, using ferroin indicator
(2-3 drops (0.1-0.15 ml) of indicator). The end point was the sharp color change from blue-green to reddish brown, even though the blue-green may reappear in a few minutes.

d. A blank, consisting of 20 ml of water as sample, was refluxed with the same reagents. The titrant was standardized, as previous discussed, before the unknown sample was analyzed.

4. Determination of standard solution. The technique and quality of the reagents was evaluated using various quantities of the standard potassium acid phthalate solution diluted to 20 ml, and run through the above analysis procedure.

5. Calculation

\[
\text{mgCOD/l} = \frac{(a-b)(N)(8000)}{(\text{ml sample})} \tag{C-2}
\]

where 
a - ml Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4}) used for the blank,
b - ml Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4}) used for the sample,
N - normality of Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4}) titrant.

C.6 Analyses of Iron and Calcium

The analytical techniques required for Fe and Ca were inadequate unless a pretreatment of the samples from the experimental runs was undertaken. First, part of the iron and the calcium were in particulate form and were required to be solubilized before the analytical procedures began for total iron and total calcium. The acidic persulfate digestion was too high content of salts and also contains H\textsubscript{2}SO\textsubscript{4} both of which interfere with the analyses. Second, the samples contained sucrose at 200 mg per liter. The sugar is reducible and
interferes with reduction of iron in the analysis. Further the sugar causes the burner of the Flame Photometer to clog and generates an unstable flame. Therefore the following pretreatment was followed before the analyses.

C. 6-1 Pretreatment (West Ashing) (210)

1. Apparatus
   a. steam bath
   b. Hot Plate

2. Procedure
   a. To a 100 ml sample in a 150 ml beaker, 2 ml of perchloric acid was added. (This amount of acid covers the bottom of the beaker completely when the beaker is empty.)
   b. The water was evaporated by placing the beaker on a steam bath. When the liquid level was approximately 10 ml, the beaker walls were washed down with water. The evaporation continued till all the water was vaporized.
   c. Nitric acid, 1 ml, was added to each beaker (larger volumes were required for high organic levels to dissipate color in the sample). The beaker was put on a hot plate and covered with a watch glass. (The hot plate was set high enough to boil the perchloric acid.)
   d. The beakers were removed after 30 minutes when sufficient acid refluxed over the sides of the beaker.

3. The beaker contents were dissolved with water and combined with the beaker washings in a 100 ml volumetric flask, filled to the mark and mixed.
f. The flask contents were divided so that 50 ml were available for the analysis of Fe and Ca.

g. A reagent blank was run under the same conditions.

C.6-2 Pretreatment of Suspended Solids on Membrane Filters

1. Apparatus
   a. Steam bath
   b. Muffle oven: Hoskins Electric Furnace, Model FD 2040

2. Procedure
   a. MF-Millipore membrane filter with the collected suspended solids was oven dried at 103°C for one hour.
   b. The filters were placed in porcelain crucibles, which were previously boiled in 1:1 mixture of water and concentrated HCl for 1 hour to remove traces of Fe and Ca.
   c. The crucibles were placed in a cold muffle oven and the oven was turned on. The filter and contents were ashed. During the oven warm-up period (approximately 1/2 hour) to the maximum temperature of 550°C where the temperature was maintained at this level for an additional 1/2 hour.
   d. The crucibles were removed from the oven and cooled. Then they were placed on a steam bath and the residue was treated with 2 ml concentrated HCl and 5 ml water to insure the dissolution of iron and calcium. The crucibles were kept on the steam bath for 1/2 hour.
   e. The crucible contents and washings were put in 100 ml volumetric flasks and diluted to the mark and mixed.
f. The flask contents were divided evenly for both the iron and the calcium analysis. No further additions of HCl were required for the Fe analysis procedure.

g. A filter with no collected solids was carried through the same procedure.

C.6-3 Pretreatment of Suspended Solids on Glass Filters

1. Apparatus

   a. Hot plate

2. Procedure

   a. Washed suspended solids which were collected on a Whatman GF/D glass fiber filter, were dried in an air circulating oven for one hour at 105°C.

   b. The filter was placed in a cold muffle oven and the oven was turned on. During the warmup period to 550°C (approximately 1/2 hour) and during the time the oven was maintained at this temperature (1/2 hour), the filter contents were ashed.

   c. After the filter was removed from the oven and cooled, it was placed in a 250 ml erlenmyer flask with 100 ml water and a few glass beads. The contents were shaken to break up the filter.

   d. Two milliliters of concentrated HCl and 4 ml of hydroxylamine solution (10 gm/100 ml water) were added to dissolve the Fe from the residues on the filter and to reduce the ferric iron to ferrous iron.

   e. The flask was placed on a hot plate set to maintain the temperature of the flask below the boiling point. The contents of the flask was evaporated to 50 ml.
f. The sample was filtered through a glass wool plug. The filtrate plus the flask washings were combined in a 250 ml erlenmyer flask.

g. The filtered glass fibers were transferred to the original erlenmyer flask and the steps e and f were repeated.

h. The filtrates and flask washings were combined in a 250 ml erlenmyer flask and boiled down to 15-20 ml on a hot plate.

g. The sample was analyzed for Fe as detailed in Section C.6-5. No further additions of HCl and hydroxylamine were necessary.

C.6-4 Iron Analysis—Phenanthroline Method (210)

1. Apparatus
   a. Hot plate
   b. Spectrophotometer: This was set at 510 nm.

2. Reagents
   a. Hydroxylamine solution: 10 gm NH₂OH·HCl was dissolved in 100 ml water.
   b. Ammonium acetate buffer solution: 250 gm NH₄C₂H₃O₂ were dissolved in water. 700 ml concentrated (glacial) acetic acid was added to form slightly more than 1 liter of solution.
   c. Phenanthroline solution: 100 mg 1,10-phenanthroline monohydrate was dissolved in 100 ml water. Two drops of concentrated HCl were added to hasten the solution.
   d. Stock iron solution: Twenty ml concentrated H₂SO₄ was added slowly to 50 ml water and 1.404 gm Fe(NH₄)₂(SO₄)₂·6H₂O was dissolved in this solution. Dropwise 0.1N KMnO₄ was added until a
faint pink color persisted. The solution was diluted to 1.0 liter with water.

e. **Standard iron solution:** This was prepared each time the standard was used. Five ml of stock iron solution was diluted to 1.0 liter with water for 1.0 ml = 1.0 µg Fe. Fifty ml of the stock iron solution was diluted to 1.0 liter with water for 1.0 ml = 10.0 µg Fe.

f. **Sodium hydroxide solution,** 6N.

3. **Procedure**

a. To a sample in a 250 ml erlenmyer flask, 2 ml of concentrated HCl, 4 ml of the hydroxylamine solution and a few glass beads were added.

b. To insure the dissolution of the iron, the flask was put on a hot plate and the contents were boiled down to 15-20 ml.

c. The flasks were cooled to room temperature and the contents and the flask washings were transferred to 100 ml volumetric flasks. (Do not have more than 80 ml of liquid.)

d. **Adjustment of the pH was necessary if the pretreatment was followed.** Depending on the amount of perchloric acid in the sample, add enough sodium hydroxide solution to bring the pH of the solution to approximately 3.3 (measured with pH meter). Equal amounts of the sodium hydroxide solution were added to all the samples as well as the reagent blank.

e. Ten ml of ammonium acetate buffer solution was added to the flask and mixed.
f. Two ml of phenanthroline solution was added, the flask diluted to the mark and mixed.

g. The absorbance was read after ten minutes at 510 nm.

h. A calibration curve was run at the same conditions that the samples were run. A set of standards were run that followed the pretreatment in Section C.6-1. The calibration curve is in Figure C.3

C.6-5 Calcium Analysis

1. Apparatus

   a. Spectrophotometer: Perkin-Elmer Atomic Absorption Spectrophotometer, Model 303 set at 212 nm.

2. Procedure

   a. Operating procedure of spectrophotometer was followed as outlined in the instructions manual (106).

   b. When running the samples, standard solutions of calcium with values above and below the unknown sample were run before and after the sample. A sample blank was analyzed.

C.7 Determination of Nitrate (Cadmium Reduction Method) (70)

1. Apparatus

   a. Reduction Column: This column was constructed from two pieces of glass tubing joined end to end. A 10 cm length of 3 cm ID tubing was joined to a 25 cm length of 3.5 mm ID tubing. Two ground glass stopcocks were attached to the free end of the 3.5 mm ID tubing. One was on-off control and the second was flow rate control.
Figure C.3 Calibration Curve of Absorbance for Phenanthroline Method of Determination of Iron
b. Spectrophotometer: This was set at 540 nm.

2. Reagents

a. Granulated cadmium: 40-60 mesh

b. Ammonium chloride-EDTA solution: 13.0 gm ammonium chloride and 1.7 gm disodium ethylenediamine tetracetate were dissolved in 900 ml of water. The pH was adjusted to 8.5 with concentrated ammonium hydroxide and diluted to 1 liter.

c. Dilute ammonium chloride-EDTA solution: 300 ml of ammonium chloride-EDTA solution was diluted to 500 ml with water.

d. Color reagent: 10.0 gm of sulfanilamide and 1.0 gm N(1-naphthyl)-ethylenediamine dihydrochloride were dissolved in a mixture of 100 ml of concentrated phosphoric acid and 800 ml of Water and diluted to 1 liter.

e. Dilute hydrochloric acid, 6N: 50 ml of concentrated HCl was diluted to 100 ml with water.

f. Copper sulfate solution, 2%: 20 gm of CuSO₄·5H₂O was dissolved in 50 ml of water and diluted to 1 liter.

g. Stock nitrate solution: 7.220 gm KNO₃ was dissolved in water and diluted to 1 liter. The solution was preserved with 2 ml of chloroform per liter. (1 ml = 1.00 mg N)

h. Standard nitrate solution: 10.0 ml of the stock nitrate solution was diluted to 1.0 liter. (1 ml = 0.01 mg N)

i. Stock nitrite solution: 6.072 gm KNO₂ was dissolved in water and diluted to 1.0 liter. The solution was preserved with 2 ml chloroform per liter and kept under refrigeration. (1 ml = 1.0 mg N)
j. **Standard nitrite solution:** 10.0 ml of the stock nitrite solution was diluted to 1.0 liter. \(1 \text{ ml} = 0.01 \text{ mg N}\)

k. **Cadmium-copper:**

1. Approximately 25 gm (new or used) cadmium granules were washed with dilute HCl and rinsed with water.

2. The cadmium was swirled in 100 ml portions of 2\% copper sulfate solution for five minutes or until the blue color was partially faded.

3. The copper-cadmium was washed with water (at least 10 times) to remove all the precipitated copper.

3. **Procedure—Preparation of the reaction column**

a. A glass plug was inserted in the bottom of the reduction column and filled with water. Sufficient cadmium-copper granules were added to produce a column 18.5 cm in length.

b. The water level was maintained above the granules to eliminate entrapment of air.

c. The column was washed with 200 ml of dilute ammonium chloride-EDTA solution.

d. The column was activated by passing 100 ml of a solution composed of 25 ml of a nitrate standard containing 1.0 mg per liter N and 75 ml of ammonium chloride-EDTA solution. A flow rate of 7-10 ml per minute was used.

4. **Analysis Procedure**

a. If the pH of the sample was below 5 or above 9, it was adjusted to between 5 and 9 with either concentrated HCl or concentrated \(\text{NH}_4\text{OH}\). This was done to insure that the sample was at pH 8.5
after the following step.

b. To a 25 ml sample or a sample that was diluted to 25 ml, was added 75 ml of an ammonium chloride-EDTA solution and mixed.

c. The sample was poured into the column and collected from the column at a rate of 7-10 ml per minute.

d. The first 25 ml was discarded and the rest of the sample was collected in the original sample flask. (Do not allow the liquid level to fall below cadmium-copper granules.)

e. Two milliliters of the color reagent was added to 50 ml of the sample and the color allowed to develop. After 30 minutes, the absorbance was read at 540 nm against a reagent blank.

f. A series of standards was carried through the procedure exactly as described for the samples. At least one nitrite standard was treated in the column to verify the efficiency of the column.

g. In the analysis, at least one nitrate standard was analyzed in order to check the reduction column performance against the standard curve. If the standard is significantly different, the cadmium should be copperized (step 2 k), the column repacked (step 3) and the standards rerun.

C.8 Analysis for Carbon Dioxide, Bicarbonate and Carbonate Ions in Water: Differential Titration Method (3)

1. Apparatus
   a. pH meter

2. Reagents
a. **Buffer solutions**: Buffers solutions were obtained corresponding to pH 8.5 and 5.0 for pH meter calibration.

b. **Sodium hydroxide standard solution**, 0.02N.

c. **Hydrochloric acid standard solution**, 0.02N.

(All the above solutions were obtained from the Ohio State University Reagent Stores.)

3. **Procedure**

a. Fifty milliliters of CO₂-free water (demineralized double distilled water that was boiled for 15 minutes) was adjusted to pH 8.5, as indicated by a pH meter, with standard NaOH solution.

b. This solution was titrated to pH 5.0 with standard HCl solution. The volume of HCl was recorded as A.

c. Twenty milliliters of standard NaOH solution was added, titrated with standard HCl solution and was recorded as B, the volume of acid required to change the pH from 8.5 to 5.0. The volume of acid required to titrate the 20 ml of NaOH solution to pH 8.5 was disregarded.

d. The volume of standard HCl corresponding to the CO₂ in 1 ml of standard NaOH solution was determined by the following equation and the value X was used in correcting the sample titration.

\[ X = \frac{(B-A)}{20}. \]  

(C.3)

e. Fifty milliliters of sample water was poured in to a 100 ml beaker and titrated with standard NaOH solution or standard HCl solution to pH 8.5. If NaOH solution was used, its volume was recorded as \( V_{1x} \).
Figure C.4 Calibration Curve of Absorbance for Cadmium Reduction Method for the Determination of Nitrate
f. The solution was titrated from pH 8.5 to pH 5.0 with standard HCl and the volume was recorded as $V_1$.

g. The sample was acidified by adding 20% of the volume of standard HCl used in the titration, but in no case was less than 5 drops used.

h. The solution was boiled vigorously for 2 minutes, cooled in ice bath to room temperature, titrated with standard NaOH solution to pH 8.5 and the volume of alkali required was recorded as $V_{2x}$.

i. The sample was titrated from pH 8.5 to pH 5.0 with standard HCl and the volume recorded as $V_2$.

4. Calculation

a. The total CO$_2$ content of the sample was calculated with the following equation:

$$\text{mg Total CO}_2/\text{l} = \frac{45,600 \times N}{S} [(V_1-V_{1x}x)-(V_2-V_{2x}x)]$$  \hspace{1cm} (C.4)

b. The concentrations of the various carbonate species were determined from table in Reference (3) which is a function of pH of the sample versus the mole fraction of the total CO$_2$. 
APPENDIX D

ADDITIONAL RESULTS

D.1 Weight Loss of Fired Glass Fiber Filters

Figure D.1 shows the weight loss of the GF/D glass fiber filters at 550°C in a muffle oven after first being prefired at either 500°C or 550°C. The weight loss is a function of the initial firing. Further, the weight loss rate decreases with time. This data was used to correct glass filter weights that were used in the determination of the volatile solids of filtered algal which is run for one hour at 550°C. The weight correction of Figure D.1 was too small to account for the weight losses that were found on the filters which were fired for volatile solids determination. After the weight correction was made, the weight loss of the filters with the filtered solids was greater than the dry weight of the collected solids. The excess weight loss did not correlate to the dry weight of the filtered solids. Table D.1 summarizes several examples.
Figure D.1 Weight Loss of GF/D Glass Fiber Filters at 550°C in Muffle Oven After Initial Firing for Conditioning as Function of Time.
<table>
<thead>
<tr>
<th>GF/D Filter No.</th>
<th>Original Weight of Standard Weight of GF/D Filter After Prefiring at 500°C for 2 Hours (mg)</th>
<th>Dry Weight of Filtered Solids (mg)</th>
<th>Weight Loss of Filter Fired at 500°C for 1 Hour (See Figure D.1) (mg)</th>
<th>Maximum Weight Loss Assuming All Filtered Solids Are Volatile Plus Filter Weight Loss (mg)</th>
<th>Determined Weight Loss (mg)</th>
<th>Excess Weight Loss (mg)</th>
<th>Excess Weight Loss as Percent of Dry Weight of Filtered Solids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+5.312</td>
<td>0.219</td>
<td>0.192</td>
<td>0.411</td>
<td>0.886</td>
<td>+0.475</td>
<td>217</td>
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<td>2</td>
<td>+0.310</td>
<td>0.537</td>
<td>0.192</td>
<td>0.729</td>
<td>1.066</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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<td>6</td>
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<td>10</td>
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<td>0.220</td>
<td>0.192</td>
<td>0.412</td>
<td>0.308</td>
<td>-0.106</td>
<td>--</td>
</tr>
<tr>
<td>14</td>
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<tr>
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<td>2.211</td>
<td>2.813</td>
<td>+0.602</td>
<td>30</td>
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</table>
D.2 Test Results for Identification of Pseudomonas Bacterium

A series of selective growth media was inoculated with a loopful of the bacterium to be identified. Table D.2 lists media that was inoculated with the unknown cells. One series was incubated at 25°C and a second series incubated at 37°C. After 48 and 96 hours the results were read.

Table D.2 Media and Results Used to Identify Unknown Bacterium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reaction (Growth or, Positive Reaction)</th>
<th>No Growth or, Negative Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hours 25°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Plate Count Agar</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Eosin Methylene Blue Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endo Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose, Lysine Deoxycholate Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hektoen Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella-Shigella Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vogel-Johnson Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas F Agar</td>
<td>+, UV-</td>
<td>+, UV-</td>
</tr>
<tr>
<td>Pseudomonas P Agar</td>
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<td>+, UV-</td>
</tr>
<tr>
<td>DNase Agar</td>
<td>+, HCl-</td>
<td>-</td>
</tr>
<tr>
<td>M-Enterococcus Agar</td>
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<tr>
<td>Pectate Agar</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Triple Sugar Iron Agar</td>
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<tr>
<td>Sulfate-Indole-Motility Agar</td>
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<tr>
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</tr>
<tr>
<td>Simmon's Citrate Agar</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red-Vogues</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proskaur Broth</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate Broth</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus Faecalis Broth</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea Broth</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tryptone Broth</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Arginine Decarboxylase Broth</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table D.2 (Continued)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reaction</th>
<th>(Growth or No Growth or)</th>
<th>48 hours</th>
<th>96 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>25°C</td>
</tr>
<tr>
<td>Ornithine Decarboxylase</td>
<td>Broth</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lysine Decarboxylase</td>
<td>Broth</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine Agar</td>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rhamnose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Adonitol Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Salecin Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Xylose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Mannose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol-Selenite Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Inositol Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Malonate Broth</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Raffinose Broth</td>
<td>+, Gas</td>
<td>-</td>
<td>+, Gas</td>
<td>-</td>
</tr>
<tr>
<td>Thiosulfate Citrate Bile Salts</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sucrose Agar</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Crystal Violet Agar</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**UV-** Fluorescence under UV Light  
**Gas-** Formation of Gas  
**HCl-** Precipitate Formation

In addition to the media listed above, a series of media was prepared in which 20 mg/l of an organic substrate was added to BBM that was adjusted pH=7.0. The series was used to determine if the unknown bacterium could utilize the substrate as an energy source. In Table D.3, the organic substrates are listed. After inoculation, the media were maintained at 25°C. Observations were made at 96 hours.
Table D.3 Determination of Organic Energy Substrate Utilization by Unknown Bacterium

<table>
<thead>
<tr>
<th>Organic Substrate</th>
<th>Reaction (+, Growth, - No Growth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>-</td>
</tr>
<tr>
<td>Trehelose</td>
<td>+ (?)</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Na Lactate</td>
<td>+</td>
</tr>
<tr>
<td>Na Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Na Succinate</td>
<td>-</td>
</tr>
<tr>
<td>Na Oxalate</td>
<td>-</td>
</tr>
<tr>
<td>Na Acetate</td>
<td>+</td>
</tr>
<tr>
<td>Erythritol</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
</tr>
<tr>
<td>Uridine</td>
<td>-</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>+ (?)</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>-</td>
</tr>
<tr>
<td>L-Listidine</td>
<td>+</td>
</tr>
<tr>
<td>L-Serine</td>
<td>-</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxy L-Proline</td>
<td>-</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>+</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>-</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>-</td>
</tr>
<tr>
<td>Na Tartrate</td>
<td>-</td>
</tr>
<tr>
<td>Na Propionate</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
</tr>
<tr>
<td>Na Benzoate</td>
<td>-</td>
</tr>
<tr>
<td>Hexanoic Acid</td>
<td>+</td>
</tr>
<tr>
<td>Na Butyrate</td>
<td>-</td>
</tr>
<tr>
<td>Na Ocanoate</td>
<td>+</td>
</tr>
</tbody>
</table>
APPENDIX E

CALCULATIONS OF PRECIPITATE FORMATION

The following Table E.1 is a summary of the heavy metal hydroxides, carbonates and calcium phosphates that may exist at pH 11.5 and 7.5.

Table E.1  Solubility Products (214)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ksp</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCO₃</td>
<td>4.7x10⁻⁹</td>
<td>100.09</td>
</tr>
<tr>
<td>Ca(OH)₂</td>
<td>1.3x10⁻¹³</td>
<td>74.1</td>
</tr>
<tr>
<td>CoCO₃</td>
<td>8.0x10⁻¹²</td>
<td>118.95</td>
</tr>
<tr>
<td>Co(OH)₂</td>
<td>2.5x10⁻¹⁶</td>
<td>92.96</td>
</tr>
<tr>
<td>Ca₅OH(PO₄)₃</td>
<td>2.52x10⁻⁵⁶</td>
<td>502.35</td>
</tr>
<tr>
<td>Cu(OH)₂</td>
<td>1.6x10⁻¹⁹</td>
<td>97.56</td>
</tr>
<tr>
<td>Fe(OH)₃</td>
<td>6x10⁻³⁸</td>
<td>106.87</td>
</tr>
<tr>
<td>MgCO₃</td>
<td>2.6x10⁻⁵</td>
<td>84.33</td>
</tr>
<tr>
<td>Mg(OH)₂</td>
<td>8.9x10⁻¹²</td>
<td>58.34</td>
</tr>
<tr>
<td>Mn(OH)₂</td>
<td>2x10⁻¹³</td>
<td>88.96</td>
</tr>
<tr>
<td>ZnCO₃</td>
<td>2.0x10⁻¹⁰</td>
<td>125.39</td>
</tr>
<tr>
<td>Zn(OH)₂</td>
<td>4.5x10⁻¹⁷</td>
<td>99.4</td>
</tr>
</tbody>
</table>

The following concentrations are found in the medium.

\[
\begin{align*}
[\text{Ca}] &= 1.7 \times 10^{-4} \text{ gm moles/liter} \\
[\text{Co}] &= 1.7 \times 10^{-6} \\
[\text{Cu}] &= 6.3 \times 10^{-6} \\
[\text{Fe}] &= 1.8 \times 10^{-5} \\
[\text{Mg}] &= 3.4 \times 10^{-4} \\
[\text{Mn}] &= 7.3 \times 10^{-6} \\
[\text{PO}_4] &= 4.5 \times 10^{-5} \\
[\text{Zn}] &= 3.1 \times 10^{-5}
\end{align*}
\]
The \( \text{OH}^- \) concentration is a function of pH.

\[ [\text{H}^+] [\text{OH}^-] = 10^{-14} \quad \text{or} \quad \text{pH} + \text{pOH} = 14.0 \]

For \( \text{pH} = 7.5 \) \[ \text{pOH} = 6.5 \quad \Rightarrow \quad [\text{OH}^-] = 3.16 \times 10^{-7} \text{ gm moles/liter} \]

For \( \text{pH} = 11.5 \) \[ \text{pOH} = 2.5 \quad \Rightarrow \quad [\text{OH}^-] = 3.16 \times 10^{-3} \text{ gm moles/liter} \]

The \( \text{CO}_3^- \) concentration is a function of pH and the partial pressure of \( \text{CO}_2 \) over water because of the equilibrium system of \( \text{CO}_2 - \text{HCO}_3^- - \text{CO}_3^- \). The equilibrium concentration of \( \text{CO}_2 \) in water for a partial pressure of \( \text{CO}_2 \) in air, \( \text{pa} = 0.005 \text{ ATM} \) is related by Henry's Law.

\[ \text{pa} = \text{H} \times \alpha \quad (B.2) \]

at \( 25^\circ \text{C} \) \[ H = 1.64 \times 10^3 \text{ ATM mole frac.} \]

\[ \frac{\text{CCO}_2 \text{ gm moles}}{\text{liter}} = \frac{\text{H} \times \alpha \times 55.6 \text{ gm moles H}_2\text{O}}{\text{liter}} \]

Therefore \( \text{CCO}_2 = \frac{\text{pa} \times 55.6 \text{ gm moles H}_2\text{O}}{\text{liter}} \]

\[ = 17 \times 10^{-5} \text{ gm moles CO}_2 \text{ liter} \]

At \( \text{pH} = 7.5 \) (See Section C.8) \[ \frac{[\text{CO}_2]}{[\text{CO}_3^-]} = 48.79 \]

Therefore \( [\text{CO}_3^-] = 3.48 \times 10^{-6} \text{ gm moles liter} \)

At \( \text{pH} = 11.5 \), the form of \( \text{CO}_2 \) in water is \( \text{HCO}_3^- \) (See Section C.8).

\[ \frac{[\text{HCO}_3^-]}{[\text{CO}_3^-]} = 0.067 \]

Therefore \( [\text{CO}_3^-] = 2.54 \times 10^{-3} \text{ gm moles liter} \)
The compounds in Table E.1 can be determined if they precipitate at pH 11.5 and 7.5. If the product of the concentrations of the ions of each compound are greater than \( K_{sp} \), a precipitate will form.

At pH = 11.5

\[
\begin{align*}
\text{[Ca][CO}_3^\text{2-}] &= [1.7 \times 10^{-4}][2.54 \times 10^{-3}] = 4.32 \times 10^{-7} > K_{sp}(\text{CaCO}_3) \\
\text{Precipitation (99%)} \\
\text{[Ca][OH}_2^\text{2-}] &= [1.7 \times 10^{-4}][3.16 \times 10^{-3}]^2 = 1.70 \times 10^{-9} < K_{sp}(\text{Ca(OH})_2) \\
\text{Precipitation (100%)} \\
\text{[Ca]_5^5[OH][PO}_4^3^\text{3-}] &= [1.7 \times 10^{-4}]^5[3.16 \times 10^{-3}]^3[4.5 \times 10^{-5}]^3 = 4.09 \times 10^{-35} > K_{sp}(\text{Ca}_5\text{OH(PO}_4^3)) \\
\text{Precipitation (100%)} \\
\text{[Cu][OH}_2^\text{2-}] &= [6.3 \times 10^{-6}][3.16 \times 10^{-3}]^2 = 6.29 \times 10^{-11} > K_{sp}(\text{Cu(OH})_2) \\
\text{Precipitation (100%)} \\
\text{[Co][CO}_3^\text{2-}] &= [1.7 \times 10^{-6}][2.54 \times 10^{-3}] = 4.32 \times 10^{-9} > K_{sp}(\text{CoCO}_3) \\
\text{Precipitation (100%)} \\
\text{[Co][OH}_2^\text{2-}] &= [1.7 \times 10^{-6}][3.16 \times 10^{-3}]^2 = 1.70 \times 10^{-11} > K_{sp}(\text{Co(OH})_2) \\
\text{Precipitation (100%)} \\
\text{[Fe][OH}_3^\text{3-}] &= [1.8 \times 10^{-5}][3.16 \times 10^{-3}]^3 = 5.68 \times 10^{-13} > K_{sp}(\text{Fe(OH})_3) \\
\text{Precipitation (100%)} \\
\text{[Mg][CO}_3^\text{2-}] &= [3.4 \times 10^{-4}][2.54 \times 10^{-3}] = 8.64 \times 10^{-7} < K_{sp}(\text{MgCO}_3) \\
\text{[Mg][OH}_2^\text{2-}] &= [3.4 \times 10^{-4}][3.16 \times 10^{-3}]^2 = 3.4 \times 10^{-9} > K_{sp}(\text{Mg(OH})_2) \\
\text{Precipitation (100%)} \\
\text{[Mn][OH}_2^\text{2-}] &= [7.3 \times 10^{-6}][3.16 \times 10^{-3}]^2 = 7.29 \times 10^{-11} > K_{sp}(\text{Mn(OH})_2) \\
\text{Precipitation (100%)} \\
\text{[Zn][CO}_3^\text{2-}] &= [3.1 \times 10^{-5}][2.54 \times 10^{-3}] = 7.87 \times 10^{-8} > K_{sp}(\text{ZnCO}_3) \\
\text{Precipitation (100%)} \\
\text{[Zn][OH}_2^\text{2-}] &= [3.1 \times 10^{-5}][3.16 \times 10^{-3}]^2 = 3.10 \times 10^{-10} > K_{sp}(\text{Zn(OH})_2) \\
\text{Precipitation (100%)} \\
\end{align*}
\]

At pH = 7.5

\[
\begin{align*}
\text{[Ca][CO}_3^\text{2-}] &= [1.7 \times 10^{-4}][3.48 \times 10^{-6}] = 5.92 \times 10^{-10} < K_{sp}(\text{CaCO}_3) \\
\text{[Ca][OH}_2^\text{2-}] &= [1.7 \times 10^{-4}][3.16 \times 10^{-7}]^2 = 1.70 \times 10^{-17} < K_{sp}(\text{Ca(OH})_2) \\
\end{align*}
\]
\([Ca]^{5}[OH][PO_4]^{3} = [1.7 \times 10^{-4}][3.16 \times 10^{-7}][4.5 \times 10^{-5}]^3 = 4.09 \times 10^{-39} > K_{sp} \) 

\((CaSOH(P_4)_3)\)

--- Precipitation (100%) ---

\([Co][C_2] = [1.7 \times 10^{-6}][3.48 \times 10^{-6}] = 5.92 \times 10^{-12} < K_{sp}(CoC_3)\)

\([Co][OH]^2 = [1.7 \times 10^{-6}][3.16 \times 10^{-7}]^2 = 1.7 \times 10^{-19} < K_{sp}(CO(OH)_2)\)

\([Cu][OH]^2 = 6.3 \times 10^{-6}[3.16 \times 10^{-7}]^2 = 6.29 \times 10^{-19} < K_{sp}(Cu(OH)_2)\)

\([Fe][OH]^{3} = [1.8 \times 10^{-5}][3.16 \times 10^{-7}]^3 = 5.68 \times 10^{-25} > K_{sp}(Fe(OH)_3)\)

--- Precipitation (100%) ---

\([Mg][C_2] = [3.4 \times 10^{-4}][3.48 \times 10^{-6}] = 1.18 \times 10^{-9} < K_{sp}(MgC_3)\)

\([Mg][OH]^2 = 3.4 \times 10^{-4}[3.16 \times 10^{-7}]^2 = 3.4 \times 10^{-17} < K_{sp}(Mg(OH)_2)\)

\([Mn][OH]^2 = [7.3 \times 10^{-6}][3.16 \times 10^{-7}] = 7.29 \times 10^{-19} < K_{sp}(Mn(OH)_2)\)

\([Zn][C_2] = [3.1 \times 10^{-5}][3.48 \times 10^{-6}] = 1.08 \times 10^{-10} < K_{sp}(ZnC_3)\)

\([Zn][OH]^2 = [3.1 \times 10^{-5}][3.16 \times 10^{-7}]^2 = 3.1 \times 10^{-18} < K_{sp}(Zn(OH)_2)\)

Weight of precipitate can be determined for the non-growth runs F-18 to F-22 (see Section 6.4-6C for assumptions)

At pH = 11.5, time = 0 hours

Precipitated compounds: CaCO_3, CaSOH(P_4)_3, Cu(OH)_2, Fe(OH)_3, Mg(CO_3), Mn(OH)_2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (gm moles/liter)</th>
<th>Factor</th>
<th>Weight Precipitate (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(OH)_3</td>
<td>1.8 \times 10^{-5}</td>
<td>0.98</td>
<td>1.89</td>
</tr>
<tr>
<td>Mg(OH)_2</td>
<td>3.4 \times 10^{-4}</td>
<td>0.50</td>
<td>9.86</td>
</tr>
<tr>
<td>Mn(OH)_2</td>
<td>7.3 \times 10^{-6}</td>
<td>1.0</td>
<td>0.65</td>
</tr>
<tr>
<td>Cu(OH)_2</td>
<td>6.3 \times 10^{-6}</td>
<td></td>
<td>0.61</td>
</tr>
</tbody>
</table>
Ca$_5$OH(PO$_4$)$_3$ [PO$_4$]$=4.5 \times 10^{-5}$ (85% of PO$_4$ collected on GF/D filter)

Remainder of Ca

CaCO$_3$ [Ca]$=1.7 \times 10^{-4}-0.63 \times 10^{-4}$

ZnCO$_3$ $3.1 \times 10^{-5}$
Zn(OH)$_2$
both can precipitate, use lower mol.wt. for conservative estimate

CoCO$_3$ $1.7 \times 10^{-6}$
Co(OH)$_2$
(same as above)

Total weight 33.36 mg liter

At pH = 7.5, time = 30 hours

Precipitated compounds: Fe$_3$(OH)$_6$PO$_4$, Ca$_5$OH(PO$_4$)$_3$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (gm moles liter$^{-1}$)</th>
<th>Factor</th>
<th>Weight Precipitate (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_3$(OH)$_6$PO$_4$</td>
<td>[PO$_4$]$=4.5 \times 10^{-5}$</td>
<td>0.08</td>
<td>1.31</td>
</tr>
<tr>
<td>Ca$_5$OH(PO$_4$)$_3$</td>
<td>[PO$_4$]$=4.5 \times 10^{-5}$</td>
<td>0.053</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Total Weight 1.71 mg liter
At pH = 7.5, 80 hours

Precipitated compounds: Fe$_3$(OH)$_6$PO$_4$, Ca$_5$OH(PO$_4$)$_3$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (gm moles/liter)</th>
<th>gm moles</th>
<th>Factor</th>
<th>Weight Precipitate (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_3$(OH)$_6$PO$_4$</td>
<td>[PO$_4$]$=4.5 \times 10^{-5}$</td>
<td>0.058</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Ca$_5$OH(PO$_4$)$_3$</td>
<td>[PO$_4$]$=4.5 \times 10^{-5}$</td>
<td>0.047</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

(5.8% of total P associated with Fe collected on 6F/D filter)

(4.7% of total P associated with Ca collected on 6F/D filter)

Total Weight 1.30
BIBLIOGRAPHY


229. Unpublished Data: Field Seasons 1974-1976, Center for Lake Erie Area Research, The Ohio State University, Columbus, Ohio.


