ATP Measurements in Laboratory Cultures and
Field Populations of Lake Plankton

By

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By

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ATP measurements in laboratory unialgal cultures and
lake plankton were made to investigate the use of ATP as a
biomass and activity parameter. No account was taken of
the bacterial population in the unialgal cultures or the
lake plankton. Although the algal cultures were not axenic,
efforts were made to minimize bacterial growth. ATP was
analysed using the luciferin-luciferase firefly reaction.
The ATP analysis proved to be sensitive and reliable for
quantitative determination of cellular ATP concentrations.

Good correlation was observed between ATP and
chlorophyll a, dry weight, and cell number. ATP to chloro-
phyll a ratios were relatively constant, ranging from 0.09
to 0.35. These values agree with those reported by other
investigators. ATP to dry weight ratios were also rela-
tively constant. The presence of detrital material in
laboratory and field samples reduced the significance of
any particular ATP to dry weight ratio.

The ATP concentration was observed to be relatively constant under both light and dark conditions. Maximum cellular ATP concentrations occurred at a pH level at or in the range of the normal pH of the organisms tested.

Rapid decreases in ATP concentration occurred immediately after addition of toxic substances. Results of one experiment indicate that toxic substances, at least in the first few hours, cause a reduction in ATP per cell rather than a reduction in viable biomass.

Additions of nutrients to nutrient-deficient cultures resulted in rapid increases in ATP concentration.

The advantages of ATP over existing biomass parameters are manifold. The results of this research indicate that ATP analysis is applicable as a biomass parameter for aquatic systems. It is also a sensitive activity parameter which can be applied to toxicity bioassays. These results also indicate that ATP could be used as a qualitative activity parameter in limiting nutrient bioassays.

CHAPTER I.   INTRODUCTION

Pollution of surface waters is well recognized as a serious problem in the United States and other highly developed countries. The effects of pollution range from the death of aquatic organisms as a result of oxygen depletion from organic discharges or poisoning from toxic industrial waters to over population with nuisance organisms as a result of enrichment from nutrient discharges. To properly measure and evaluate the effects of various pollutants on a particular water, reliable measures of biomass and activity are needed. The biomass parameters most often used in lake and stream studies are chlorophyll concentration, suspended solids, or plankton count. The application, theory and problems associated with these parameters are discussed in Chapter III, but it suffices to say here that better measures of phytoplankton biomass and activity are needed.

The deficiencies of present methodologies are evidenced by the many conflicting reports in the literature concerning limiting nutrients and the effects of municipal and industrial discharges on a body of water. In 1969 a Provisional Algal Assay Procedure (Joint Industry-Government Task Force on Eutrophication 1969) was developed to

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standardize existing methods of investigating phytoplankton responses in natural waters. However, this procedure has not been wholly successful probably because it utilizes inadequate biomass parameters.

An adequate biomass parameter must have a relatively constant cellular concentration under most environmental conditions, and it must not be associated with non-living material. Cellular biochemistry offers a number of possibilities for measurement of activity and biomass. One biochemical parameter that seems to offer an appropriate measure of biomass and metabolic activity is adenosine triphosphate (ATP). The overall objective of this study was to investigate the use of adenosine triphosphate (ATP) as a measure of phytoplankton biomass and metabolic activity. Biomass parameter evaluation was divided into two phases. First, the ATP content of batch unialgal cultures was measured and correlated with traditional biomass parameters. In the second phase, the ATP content of natural lake phytoplankton was measured and correlated with biomass parameters. ATP as a measure of metabolic activity was evaluated by observing the ATP changes in laboratory algal cultures and lake phytoplankton which were subjected to varying environmental conditions.

Correlation of cellular ATP concentration with current biomass parameters would indicate the validity of using ATP to measure phytoplankton biomass. The lack of response of

cellular ATP to various environmental conditions would
indicate the stability of cellular ATP to minor environ-
mental changes. The response of cellular ATP to additions
of nutrients and toxic substances would indicate its useful-
ness as a rapid bioassay parameter.

CHAPTER II.   MECHANISM OF ATP METABOLISM IN ALGAE

ATP has been called the "energy currency" of living
cells.   It occurs in all living cells.   ATP is a nucleotide
containing adenine, a 6-amino derivative of purine, D-ribose,
a 5-carbon sugar, and three phosphate groups as shown in
Figure 1.   The ATP molecule within the cell is highly
charged with negative charges concentrated around the
polyphosphate structure.   Most of the ATP in cells is
present as a Mg^{+2} complex.

It is common practice to refer to ATP as a "high
energy" phosphate compound.   However, as shown in Table 1,
ATP has an intermediate energy value when compared with
other phosphate compounds.   It is this intermediate position
which makes ATP so important.

TABLE 1

STANDARD FREE ENERGY OF HYDROLYSIS OF
COMMON INTRACELLULAR PHOSPHATE COMPOUNDS

	$\Delta G°$ Kcal/Mole
Phosphoenolpyruvate	-14.80
1,3-Diphoyphoglycerate	-11.80
Phosphocreative	-10.30
Acelyl phosphate	-10.10
Phosphoarginine	- 7.70
ATP	- 7.30
Glucose 1-phosphate	- 5.00
Fructose 6-phoaphate	- 3.80
Glucose 6-phosphate	- 3.30
Glycerol 1-phosphate	- 2.20

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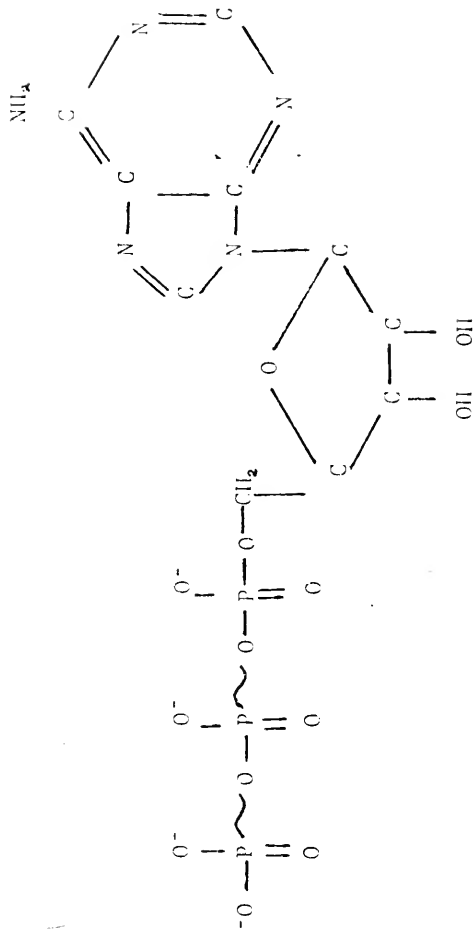
FIGURE 1. CHEMICAL STRUCTURE OF ATP

The function of the ATP-ADP (adenosine diphosphate) system is to act as an intermediate between high-energy phosphate compounds and low-energy phosphate compounds. One set of enzymes helps transfer phosphate from high-energy compounds to ADP, forming ATP; while another set of enzymes help transfer the terminal phosphate group of ATP to low-energy phosphate acceptors. No enzymes exist that can transfer phosphate groups directly from high-energy donors to low-energy acceptors. Thus, all high-energy phosphate transfers must use the ATP-ADP system.

Inside living cells there is usually very little ATP since it is continually being used up and remade. The energy in the energy-rich bonds of ATP is used to do cellular work. It is used to synthesize large molecules from simple subunits, to transport substances in and out of the cell, and to perform mechanical work.

In algae, ATP is formed from two basic processes: photosynthesis and respiration. Eucaryotic algal cells contain chloroplasts, where photosynthesis occurs, and mitochondria, where respiration occurs. Procaryotic cells, like the blue-green algae, do not have chloroplasts and mitochondria. Although eucaryotic and procaryotic cells differ in structure, the chemical reactions occurring in photosynthesis and respiration are similar.

Photosynthesis consists of both light and dark reactions; the light reactions drive the entire process. In the light reactions, water is split, ATP is formed, and

oxygen is liberated. The dark reactions use carbon dioxide
to form simple sugars, some of which are converted into
glucose as the product of photosynthesis. In photosynthesis,
chlorophyll molecules absorb light energy and give off high-
energy electrons. It is believed that molecules of ferredoxin,
a protein which contains iron, pick up the high-energy
electrons. The energy gained by ferredoxin is released in a
series of electron transfers. This released energy is used
to form ATP. The electrons, after losing their energy, return
to the chlorophyll molecule again. This process is called
cyclic photophosphorylation. Another process called non-
cyclic photophosphorylation also occurs and is similar to
the cyclic process except the original electrons do not return
to the chlorophyll molecule. Instead, the electrons are
transferred to NADP (nicotinamide adenine dinucleotide
phosphate) which subsequently is reduced. Electrons from
the hydroxyl groups of water are transferred to the chlorophyll
molecules, filling the electron gaps. Thus the primary
purpose of photosynthesis is to form ATP and reduced NADP,
with oxygen evolved as a by product. Under normal condi-
tions one would expect non-cyclic photophosphorylation to
occur since this process forms ATP and reducing power (in
the form of reduced NADP). Both ATP and the reducing power
are used in the dark reactions to form glucose from CO_2.
The role of cyclic photophosphorylation is not completely
understood. Some believe it is a shunt mechanism used to

form ATP without formation of reduced NADP. It appears that light-induced cyclic electron flow is used to generate ATP at whatever rate is required by the cell, without the necessity of generating reduced NADP or of evolving molecular oxygen.

ATP production from respiration is basically the same for algae and other microorganisms. Catabolic processes which terminate in the Krebs Cycle product ATP. For example, one molecule of glucose is transformed into two molecules of pyruvic acid, forming two molecules of ATP. These two molecules of pyruvic acid are broken down into six molecules of carbon dioxide and water, liberating 36 molecules of ATP. Thus, a net ATP production of 38 molecules of ATP results from the oxidative breakdown of one glucose molecule.

CHAPTER III.   LITERATURE REVIEW

Introduction

Vollenweider (1970) defines eutrophication as any-thing that accelerates the nutrient loading, increases the nutrient level and directly increases water productivity. He explains that a mere increase in the nutrient level is not important if it does not produce unpleasant or undesirable effects on the metabolism of the water. Vollenweider's definition emphasizes the two primary constituents of eutrophication - the addition of nutrients to a water and the concomitant response of the phytoplankton. Measuring the standing phytoplankton biomass and its response to nutrient additions is becoming increasingly important; yet, there is little consensus as to which parameter or method is best to use. A variety of methods is presently used to measure biomass and its response to nutrient additions, including pigment concentration, particulate organic carbon concentration, cell number or areal units, packed cell volume and dry weight, and DNA concentration. While commonly used each suffers from certain deficiencies. The following sections will briefly describe the merits and drawbacks of each.

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Biomass Parameters

Pigment Concentration

The algal pigments consist primarily of the chloro-
phylls, the carotenoids, and the phycolilins. Chlorophylls
are lipo-soluble molecules characterized by strong absorp-
tion of red (650 - 680 mμ) and blue light (400 - 450 mμ)
and by their red fluorescence in organic solvents. Chloro-
phylls are divided into three groups: chlorophyll *a* which
is present in all photosynthesizing cells; chlorophyll *b*,
an oxidized derivative of chlorophyll *a*, which is found in
green algae; and chlorophyll *c* which is found in brown
algae and diatoms. Carotenoids are C_{40} hydrocarbons with
numerous conjugated double bonds. There are two major
groups of carotenoids: the carotenes, which are pure
hydrocarbons, and the xanthophylls, which contain from 1
to 8 hydroxyl groups making them more polar. The carote-
noids serve as accessory pigments, extending the range of
visible light useful in photosynthesis. They are usually
yellow or orange and absorb light in the blue-violet region
of the spectrum. All photosynthesizing cells contain
carotenoids. Odum and Nixon (1970) have studied the role
of carotenoids in photorespiration. Odum *et al* (In Press)
note that the carotenoid-chlorophyll ratio may help measure
the relative use of solar energy in photosynthesis and
photorespiration. The third major class of pigments are
the phycobilins which are related structurally to the

chlorophylls. They consist of an open conjugated system
of four pyrrol rings, the fundamental structure of the bilin
pigments, so called because they were discovered in bile.
The phycobilins (algal bilins) are water-soluble pigments
consisting of the phycoerythrins and the physocyanins. Both
pigments are found in the red marine algae (Rhodophyceae)
and the primitive blue-green algae (Cyanophyceae).
Phycoerythrin, present primarily in red marine algae, absorbs
light in the middle of the visible spectrum. Phycocyanin,
present primarily in blue-green algae, absorbs red light at
about 630 mμ.

Chlorophyll is the major pigment of algae and occurs
in all species. Because of its universal distribution,
chlorophyll a is often used to estimate phytoplankton
biomass. Weber and McFarland (1969) and Keup and Stewart
(1966) used chlorophyll a as an estimate of the phytoplankton
standing crop and found it to correlate well with carbon,
nitrogen, and phosphorous content.

One problem with using chlorophyll as a measure of
photoplankton biomass is that the chlorophyll concentration
varies with environmental conditions. Spoehr and Milner
(1949) measured extremes of 0.1 percent and 6 percent for
chlorophyll content as percent of dry weight. Usually the
chlorophyll content is about 0.5 to 1.5 percent of the dry
weight (Round 1967). Studies on algae cultured under
controlled laboratory conditions demonstrate that mineral
nutrition, light intensity, and cell age affect the cellular

chlorophyll concentration. Emerson (1929) found a direct
correlation between photosynthetic activity and chlorophyll
content in *Chlorella pyrenoidosa* and concluded that a
reduced chlorophyll content was the only factor responsible
for the reduced photosynthesis of iron-deficient cells.
Sargent (1940) noted that chlorophyll constituted 6.6 per-
cent of the dry weight of "shade-grown" *Chlorella* cells
but only 3.3 percent of "sun-grown" cells. Myers (1946)
found that chlorophyll per milliliter of packed cells was
inversely related to light intensity, but he also found a
direct relationship between light intensity and cell size.
Fogg (1965) observed that the cellular chlorophyll concen-
tration decreased with cell age and nutrient deficiency.
Ryther and Yentsch (1957) found that marine phytoplankton
at light saturation has a reasonably constant assimilation
ratio of 3.7 grams of carbon assimilated per hour per gram
of chlorophyll. Calculated production rates based on this
ratio and on chlorophyll-light measurements were similar
to those obtained by simultaneous use of the light-and-dark
bottle oxygen method. Bain (1968) used a similar method
to predict primary production in San Francisco Bay. Some
controversy exists as to whether chlorophyll *a* can best be
used to measure biomass or primary production (Odum *et al*
1958).

The analytical technique for extracting and calcu-
lating chlorophyll concentration suffers from many weak-
nesses. Strickland and Parsons (1968) note that the only

rapid chemical method known for estimating living plant matter in the particulate organic matter of sea water is to determine the characteristic pigments. However, they also note that the amount of organic material associated with a given plant pigment is variable, depending on the class of algae and the nutritional state. A factor ranging from 25 to 100 is used to convert chlorophyll a concentration to total plant carbon. According to Strickland and Parsons (1968) extraction with 90 percent acetone gives results undoubtedly low in many instances because of the presence of plant cells that are not fully extracted. These authors state that some species may retain 50 percent or more of the pigments in their cells. Bogarad (1962) notes that it is generally more difficult to extract chlorophylls from algae than from higher plants; he adds that extraction with hot or cold methanol is usually more effective than acetone. Another weakness of the extraction process is that inactive chlorophyll and degradation products may be determined along with the active chlorophyll of the living phytoplanktons. Glooschenko and Moore (1971) analyzed Lake Ontario water samples for chlorophyll a and its degradation products, pheophorbide a and pheophytin a. These authors found the degradation products made up less than 20 percent of total chlorophyll a until the decline of the spring growth, after which degradation products accounted for up to 100 percent measured chlorophyll a in some regions of the lake. These studies indicate the necessity of correcting chlorophyll a data for degradation products.

Particulate Organic Carbon

Particulate organic carbon is often used as a measure of phytoplankton biomass. Menzel and Ryther (1964) measured the composition of particulate matter in the western North Atlantic to determine the relationship between carbon, nitrogen, phosphorus, and chlorophyll. Regression of phosphorus or chlorophyll vs. nitrogen or carbon, when extrapolated back toward the origin, indicated appreciable amounts of nitrogen and carbon in the absence of phosphorus and chlorophyll. In contrast, the regression of phosphorus vs. chlorophyll had its intercept at the origin. The authors concluded that chlorophyll and phosphorus are decomposed or mineralized at about the same rate, while carbon and nitrogen are more refractory. Menzel and Goering (1966) studied the decomposition of particular matter from surface and deep Atlantic waters. Changes in carbon were related to the initial biomass of phytoplankton. They found that the living carbon represents a variable fraction of the total organic particulate matter present in surface waters. Superimposed on this is detrital carbon which is refractory to decomposition. Parsons and Strickland (1962), Holm-Hansen (1969) and others have also found variable amounts of refractory particulate organic carbon in phytoplankton samples. Because of this refractory nature of carbon its use as an estimate of biomass is questionable.

Cell Number

Early work on algal physiology utilized the cell number, determined by counting cells with a hemocytometer, whipple disc, or a Segwick-Rafter Cell (Myers 1962). With unialgal cultures the cell count can be relatively meaningful because of the approximate uniformity of the cell size and volume. Cell counts on a heterogeneous culture of algae are tedious and open to misinterpretations. Oswald and Gaonkar (1969) in a review of the Provisional Algae Assay Procedure (Joint Industry-Government Task Force on Eutrophication 1969) note the difficulty in counting cells in mixed populations: taking the size of *Chlorella pyrenoidosa* as unity, the size of *Scenedesmus obliguus* is 1.2, *Chlamydomouas* 6.9, *Euglena gracilis* 54, *Euglena viridis* 84, and a *Phacus* sp. 71. Besides this variability among species, cell size differs with both age and nutritional state of each species. *Euglena* cells tend to become larger with age while *Chlorella* and *Scendesmus* cells become smaller. Nutrient starved *Euglena* usually decrease in size while nutrient starved *Chlorella* increase in size. There is also a difficulty in counting filamentous and agglomerated cells like *Anabaena* and *Microcystis*. Oswald and Gaonkar also note that many algae are photo regulated. *Chlorella*, for example, accumulates nutrients during the day and divides during the night; thus, unless cells are counted at the same time each day, the number of cells could vary significantly.

Aside from the problem of size, there are difficulties with counting technique. Hee (1954) studied variability in cell counts using the hemocytometer, a popular and simple cell counting device. He found that for *Euglena* the variation in cell counts exceeds 30 percent when the number of cells counted in the hemocytometer grid is less than 30, but that clumping occurs when the number of cells exceed 100. For *Scenedesmus* he found that a minimum of about 15 percent variation occurs when the number of cells counted in the five central squares exceeds 80. The variation is a minimum for *Chlorella* when the cells in the five squares exceed 100.

Finally, a problem exists with respect to count variations among technicians (Oswald and Gaonkar 1969). Although this problem occurs with all laboratory techniques, it is particularly acute for cell counting since personal judgement and knowledge of algae identification play a major role.

Packed Cell Volume and Dry Weight

Oswald and Gaonkar (1969) recommended the volumetric technique, commonly known as the packed-cell volume, be added to the Provisional Algal Assay Procedure as a method for evaluating algal growth. As Oswald (1967) noted, the volumetric (packed-cell volume) technique is a non-destructive procedure in which the sample need not be discarded after analysis and can be used for further analysis.

According to Oswald, the packed volume usually contains about 14 percent dry weight of algae and the approximate algal content in dry weight is 1400 times the packed cell volume plus or minus 400. Myers (1962) observed that sufficient centrifugal force and time must be used to obtain a constant and minimum packed cell volume. A major objection to the packed cell volume as a measure of biomass is that it measures detrital as well as living matter. Lee *et al* (1971) found a significant amount of detrital material in unialgal cultures of *Selenastrum capricornutum* after only four days of growth in batch systems. Thus, quantitative interpretation of packed cell volume results can be misleading.

DNA

Deoxyribonucleic acid (DNA) consists of two polynucleotide chains twisted upon each other to form a double helix; it is the genetic substance which contains the hereditary information of the cell. The use of DNA as a measure of phytoplankton biomass has been investigated by Holm-Hansen and colleagues (1968). After measuring concentrations in the Atlantic and Pacific Oceans and comparing them with measurements of chlorophyll and organic carbon, the authors concluded that there is either a considerable quantity of living material that is high in DNA or that DNA is associated with particulate, non-living material. Holm-Hansen (1969) measured ATP, chlorophyll, DNA, and organic

carbon off the coast of California; he converted ATP, chlorophyll, and DNA concentrations to organic carbon using conversion factors of 250, 50, and 100 respectively as shown in Table 2.

TABLE 2

COMPARISON OF BIOMASS ESTIMATED BY ATP, CHLOROPHYLL, DNA AND ORGANIC CARBON

Biomass (µg c/liter)	Depth (m)			
	0	50	100	200
Direct Examination	13	25	3.8	1.1
ATP	24	22	5.6	1.9
Chlorophyll	14	22	5.0	0.2
DNA	250	200	95	50

The biomass estimates as determined from DNA measurements are, according to Holm-Hansen, "impossibly high." He concluded that DNA is an excellent biomass parameter for laboratory cultures but not for work in the natural environment.

None of the methods discussed in this section are very good estimates of biomass. With biomass, nothing is constant or absolute; all parameters vary and this variation makes it difficult to say which is the best measure of biomass.

Limiting Nutrient Bioassay Methods

Many methods are used to determine which physical or chemical conditions limit phytoplankton growth. One general approach is to monitor the phytoplankton response over a

period of time after enriching the sample with some nutrient.
Although popular, this method depends on the response para-
meter measured and may give misleading results if another
nutrient or condition becomes limiting. Response parameters
include the various biomass measures and ^{14}C assimilation
rates. The former are usually insensitive to short term
changes while insufficient time for response to enrichment
may give inaccurate results with the latter. Other nutrient
bioassay procedures are based on analysis of planktonic
constituents (e.g., adaptive enzymes, percent nitrogen or
phosphorus) which vary in response to nutritional conditions.

Carbon-14 Uptake

Carbon assimilation rates, as measured by the fixation
of carbon-14, are often used as a measure of algal growth
in bioassays (Goldman 1960, 1961, 1962, 1964, 1965, 1967;
Goldman and Carter 1965; McAllister *et al* 1964; Menzel and
Ryther 1960, 1961; Putnam 1966). Primary production
measured by the carbon-14 method is a function of the environ-
mental conditions prevailing at the time of incubation.
Light intensity, for instance, is an important factor in
the amount of carbon fixed. After incubation formaldehyde
is usually added to kill the organisms, but Strickland
and Parsons (1968) note that even small concentrations of
formaldehyde may affect the excretion or loss of organic
material from delicate algae. *In situ* measurements of
productivity require two to three hours of incubation, thus

limiting the number of sampling stations that can be tested
in one day. On the other hand, laboratory incubation using
a constant light intensity gives more precise but inaccurate
results. Oswald and Gaonkar (1969) state that the technique
of radiocarbon measurements as set forth in PAAP seems
needlessly complex, delicate, and subject to error in the
hands of inexperienced personnel.

Goldman (1960, 1962, 1963) used carbon-14 uptake for
bioassays of limiting nutrients. Carbon-14 was added to a
phytoplankton culture to which nutrients were added.
Periodically, subsamples were removed and the uptake of
carbon-14 measured. This method measured the total carbon
taken up from the beginning of the experiment. An alternate
method consisted of adding nutrients to an unlabeled phyto-
plankton culture and periodically removing a subsample,
adding carbon-14 to it, incubating for three to six hours, and
then measuring the amount of carbon taken up during the
incubation period. Interpretation of the results of these
methods can be misleading due to the inherent variation in
measuring carbon-14 uptake. Since net productivity is not
being measured by this method, it is questionable what
exactly the results mean; is an increase in biomass also
occurring?

Many basic questions concerning the carbon-14 method
must be answered. Is there any luxury uptake of carbon;
how much fixed ^{14}C is excreted, how much is internally
recycled? What is the difference between ^{12}C and ^{14}C

uptake? Strickland and Parsons (1968) use the factor of
1.05 to account for this difference, but note that this
value is uncertain.

Enzymatic and Extractive Techniques

Enzymatic and extractive methods of analysis have
been used to study limiting nutrient concentrations in algal
cultures (Fitzgerald 1966, 1969; Shapiro and Ribeiro 1965).
Surplus phosphorus, the internal concentration in excess of
the amount needed for maximum growth, can be extracted from
algae by boiling the algal sample in water for 60 minutes
and measuring the orthophosphate in the extract. Fitzgerald
(1969) used this procedure and demonstrated that algae limited
by phosphorus contain little or no extractable phosphate
while algae grown with surplus phosphorus released more than
0.08 mg PO_4-P/100 mg algae. Gerloff and Skoog (1954, 1957)
used a tissue analysis method to evaluate nutrient availa-
bility in Wisconsin lakes for the growth of *Microcystis
aeruginosa*. Tissue analysis was also used to measure the
nutrient availability for the growth of angiosperm aquatic
plants (Gerloff and Kromholz 1966). The tissue analysis
method requires the establishment of a critical level for
each element. The critical level is the minimum tissue
content in a particular species that is required for maximum
growth. Tissue contents below the critical concentration
are associated with deficiencies of that element resulting
in less than maximum yields. This method requires the

determination of the critical level for each species
encountered; the maximum growth rate for each species is
a function of many conditions besides nutrient levels (light,
temperature, and pH for instance), and may be difficult to
evaluate. In mixed phytoplankton populations the complexity
of critical levels tend to make the method impractical.
Gerloff and Skoog (1954, 1957), using tissue analysis on
Microystis aerugiuosa in Wisconsin lakes, concluded that
nitrogen was more likely to become growth limiting than was
phosphorus. However, Gerloff and Krombholz (1966), using
tissue analysis on angiosperm aquatic plants in the same
Wisconsin lakes, concluded that phosphorus was more likely
to limit higher aquatic plant growth than nitrogen. These
results demonstrate the species-dependency of the tissue
analysis technique, and highlight the qualitative and
relative nature of the method.

Alkaline phosphatase activity is another measure of
algal phosphorus nutrition. When algae are phosphorus
limited, the alkaline phosphatase activity per unit weight
is as much as 25 times that of algae grown with surplus
phosphate (Fitzgerald 1966, 1969). Fitzgerald cautions that
the effect of the local environment such as recent rains
or unusual circulation patterns in lakes cause changes in
the distribution of algae and must be considered when
interpreting nutritional data. He also notes that results
vary according to the species of algae under consideration.

Fitzgerald (1968) found that the rate of NH_4-N absorption by algae in the dark is 4-5 times greater for algae which are nitrogen limited compared to plants with available nitrogen. The comparative rate of ammonium nitrogen absorption in the dark of algae containing surplus nitrogen versus algae limited by available nitrogen was measured. The test consisted of placing 5-20 mg (dry weight) of algae, washed in nitrogen-free medium, into 10-30 ml of Gorham's medium (minus N) and adding 0.1 mg ammonium nitrogen. After one hour incubation at 25° C in the dark the ammonium nitrogen content of the supernatant was compared to controls not containing algae. The procedure is based on the fact that nitrogen-starved cells can assimilate ammonium-nitrogen in the dark while normal cells require light and carbon dioxide (Wetherell 1958). It is believed that nitrogen-starved cells have a carbohydrate reserve which is lacking in normal cells and nitrogen-deficient cells assimilate ammonium-nitrogen until their carbohydrate reserves are exhausted (Syrett 1962).

All of the above techniques whether they be extractive or enzymatic are best interpreted when applied to unialgal test cultures rather than natural waters. These methods give results which are relative and qualitative, and standardization is difficult, that is, a given number by itself means little and interpreting what it means is no easy task.

ATP

In the past, most studies on the ATP pool in micro-
organisms were confined to cultures of bacteria grown on
synthetic substrates. By using pure cultures of bacteria
in a defined stage of growth, biochemists have been able
to study the role of ATP in the bioenergetics of the cell.
Recently, however, some researchers have proposed that ATP
could be used to measure microbial biomass. Determination
of microbial biomass by measurement of ATP depends upon
the assumptions that ATP is not associated with non-living
particulate material and that the ratio of ATP to cell
carbon is fairly constant (Holm-Hansen and Booth 1966).
It is also important that the cellular ATP pool does not
vary substantially under different environmental conditions.

Forrest and Walker (1965) observed that the ATP pool
in starved cultures of *Streptococcus faecalis* remained
constant for almost three hours under endogenous conditions.
They concluded that an energy balance kept the ATP pool
constant until all the stored substrate was utilized. They
also found that the length of time the ATP pool remained
constant during endogenous conditions was proportional to
the initial substrate concentration.

Bauchop and Elsden (1960), working with three species
of bacteria, showed that under anaerobic conditions the
amount of ATP synthesized was proportional to new cell
yield. Elsden (1963) reported that the "ATP growth

coefficient," that is, the grams of new cells produced per
mole of ATP synthesized, was approximately constant for all
the organisms studied. D'Eustachio *et al* (1968) reported
that cell counts based upon ATP concentration were linearly
correlated to standard plate count. D'Eustachio and Levin
(1967), studying the ATP pool in three aerobic bacterial
species during lag, exponential and stationary growth phases,
found that *E coli* had a relatively constant level of ATP
throughout all growth phases. *Pseudomonas fluorescens* and
Bacillus subtillis were also fairly constant except for a
small increase in ATP pool during exponential growth.

Holm-Hansen and his co-workers have used ATP to
measure phytoplankton in the ocean (Holm-Hansen and Booth
1966; Hamilton and Holm-Hansen 1967; Holm-Hansen, Sutcliffe
and Sharp 1968; Holm-Hansen 1969, 1970). Experiments have
shown that ATP is not associated with non-living material
(Holm-Hansen and Booth 1966). These experiments included
killing of various algae and bacteria with heat, repeated
freezing, or cyanide. The measured residual ATP was
negligible. The ATP content in three cultures of bacteria
studied averaged between 0.1 and 0.2 percent of the dry
weight while the content in eight species of algae ranged
from 0.003 to 0.016 percent of the dry weight. (Holm-Hansen
and Booth 1966.) These were maximum variations in ATP over
a wide variety of growth conditions and stages in batch
cultures.

Hamilton and Holm-Hansen (1967) determined the ATP content of seven marine bacterial isolates cultured in both batch and chemostat conditions. The range of ATP in the chemostat grown cells was 0.5 to 6.5 x 10^{-9} µg ATP/cell, or 0.3 to 1.1 percent of the cell carbon. Senescent cells in batch cultures and starved cells in general had an ATP content about one-fifth that of exponentially growing cells. Averaging a representative number of observations from the chemostat and batch grown cells, the authors calculated the average ATP of these bacteria to be 1.5 x 10^{-9} µg ATP/cell. On a per unit cellular carbon basis the ATP was calculated to be 0.4 percent of the cell carbon.

To determine whether the cellular ATP of algae changed with light and dark periods, Holm-Hansen (1970) studied the response of algal cells to periods of light and dark. As shown in Figure 2, an initial decrease in ATP pool size occurred in the dark followed by a gradual increase to approximately the initial ATP pool level. His test, however, only covered a time scale of 22 minutes and doesn't conclusively demonstrate the constant nature of cellular ATP under light and dark conditions.

Holm-Hansen (1969) measured the total particulate carbon, nitrogen, ATP, DNA, and chlorophyll in profiles to 600 meters and 1000 meters off the coast of southern California. As shown in Table 2, the biomass estimates based on ATP measurements are in good agreement with those based on chlorophyll data and direct microscopic measurements.
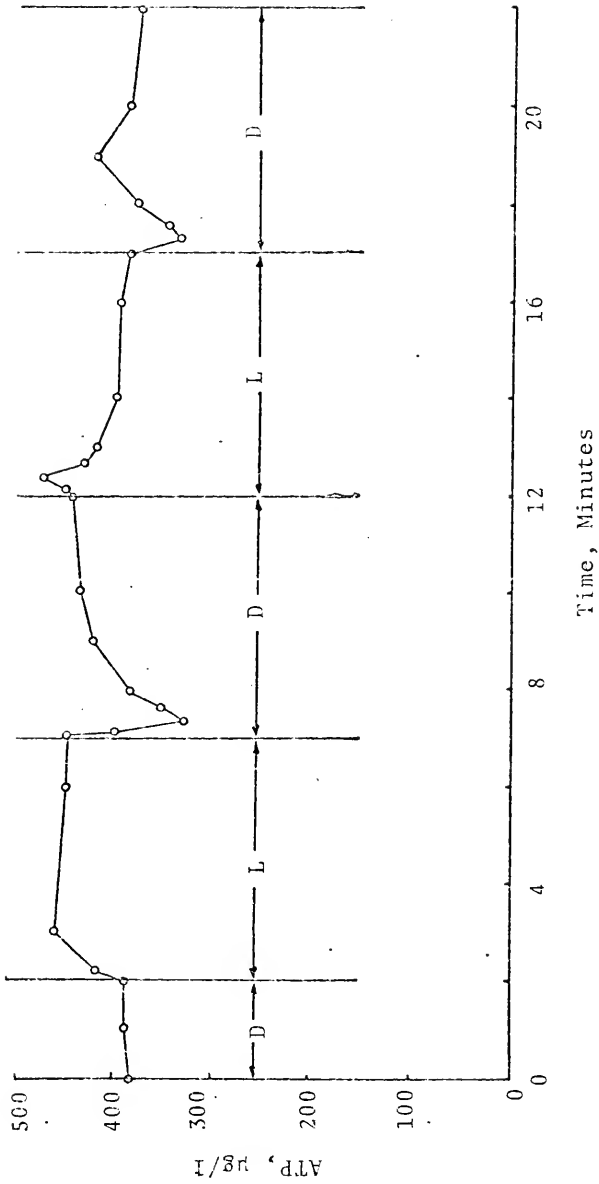
FIGURE 2. CELLULAR CONTENTS OF ATP IN *EUGLENA GRACILIS* DURING ALTERNATING PERIODS
OF LIGHT AND DARK (Holm-Hansen 1970)

Holm-Hansen concluded that there is a considerable uniformity
in algal ATP concentration over the size range from 1 pg
c/cell to 215,000 pg c/cell. Further the average algal
ATP value (0.35 percent of the organic carbon) is close to
those concentrations reported for bacteria.

Holm-Hansen (1970) investigated the cellular ATP
content in 30 different algal cultures under different
environmental conditions. As shown in Figure 3, the average
concentration of ATP as a percent of the cellular organic
carbon is 0.35 percent during exponential growth. Extreme
nitrogen deficiency in cultures dropped the ATP level to
35 percent of that found during exponential growth for
Skeletonema costatum, to 46 percent for *Monochnysis lutheri*,
and to 14 percent for *Dunaliella tertiolecta*. Phosphate
deficient cultures of *M. lutheri* showed that ATP dropped
to about 0.05 percent of the carbon content, but that it
increased to 0.15 percent one day after phosphate was added.
Holm-Hansen maintains that although ATP pool size varies
significantly under extreme nutrient deficient conditions,
these conditions would rarely be found in nature. He notes
that the apparent change in ATP concentration could result
from a significant amount of detrital carbon in his cultures,
giving the appearance of a drop in cellular ATP.

The ATP content of microorganisms calculated as
percent of dry weight seems relatively constant. Table 3
shows some of the values reported for bacteria and algae.
For natural lake and ocean samples of ATP per dry weight
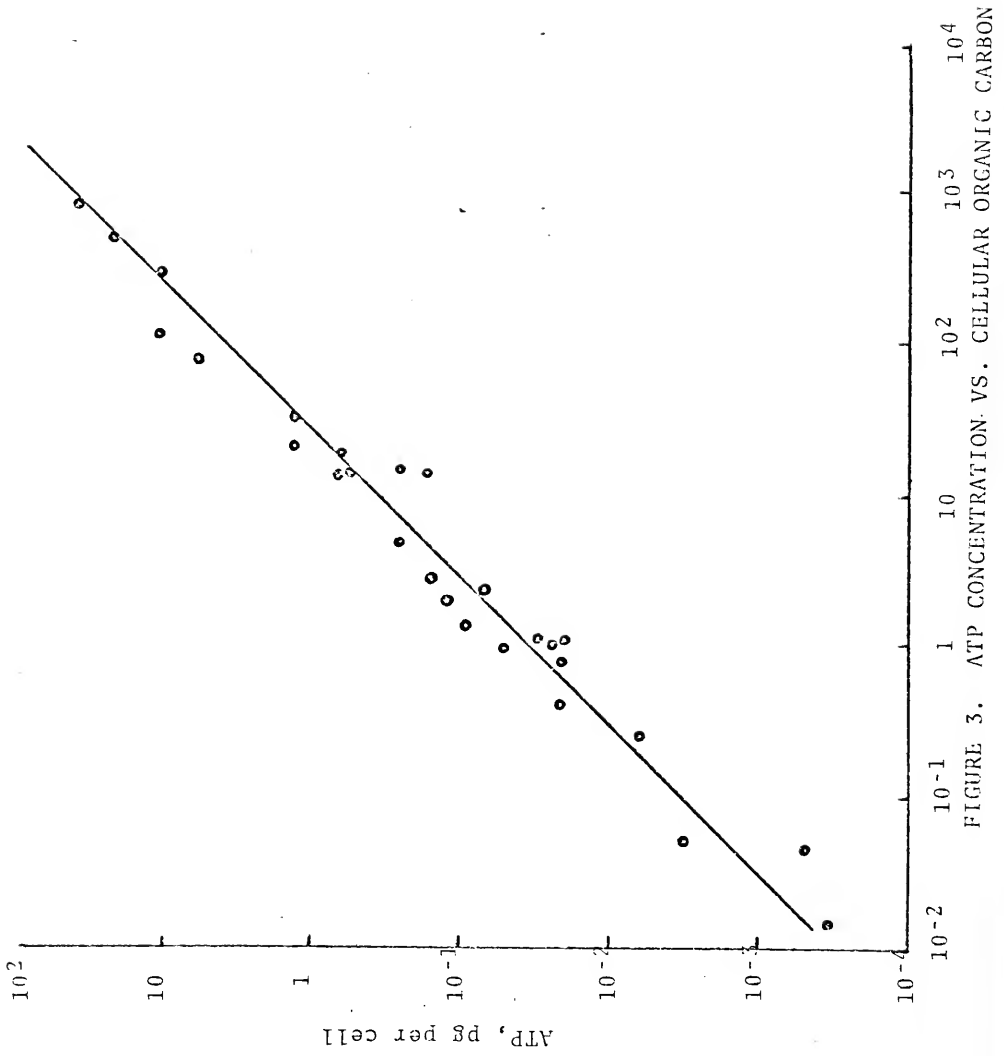
FIGURE 3. ATP CONCENTRATION VS. CELLULAR ORGANIC CARBON

TABLE 5

ATP AS PERCENT DRY WEIGHT

Organism	µg ATP/mg Dry Weight	Reference
Chlorella unalgaris (algae)	2.0	Syrett (1958)
S. cerevisial (bacteria)	0.4	Grylls (1961)
E. coli (bacteria)	3.0	Lehninger (1965)
Strept. faecalis (bacteria)	2.0 - 12.0	Forest (1965)
Selenastrum capricornutum (algae)		Lee (1971)
- phosphorus = 0.62 mg/l	3.1	
- phosphorus = 0.062 mg/l	1.4	
- phosphorus = 3.1 mg/l	3.4	

value may be meaningless because of the presence of detrital material. Holm-Hansen (private communication) does not believe it is feasible to report ATP/dry weight ratios and reports most of his values as percent of cellular carbon or as ATP per cell.

For algal samples the ratio of ATP to chlorophyll seems to have some meaning. As shown in Table 4, the ATP to chlorophyll ratio is relatively constant even under different nutritional and physical conditions. Using the ratios of ATP to chlorophyll seems more reliable than using the ATP to dry weight ratio, especially for natural populations. Even in unialgal cultures the ATP to dry weight ratio may not be reliable. Lee *et al* (1971), working with batch cultures of *Selenastrum capricornutum*, only calculated the ATP to dry weight ratio up to four days of batch growth. After four days they observed cell debris and dead cells, making the dry weight meaningless.

Lehninger (1971) estimated that the turnover time of the *E. coli* ATP pool is only a fraction of a second. Forrest and Walker (1965) have found the turnover time in *Streptococcus faecalis* to be about 5 seconds. In light of the rapid turnover rate of cellular ATP, many researchers have studied the response of cellular ATP to different environmental conditions.

Forrest (1965) showed that the ATP pool in *Strep faecalis* increased after addition of glucose and later returned to its endogenous level when the substrate was

TABLE 4

ATP TO CHLOROPHYLL RATIO FOR VARIOUS ALGAE
UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Organism	Condition	μg ATP/mg Chlor.*	Reference
Dunaliella tertiolecta (marine)	N-starved	t= 6 days 0.60 t=11 days 0.41 t=26 days 0.50	Holm-Hansen (1970)
Monochrysis lutheri (marine)	N-starved	t= 7 days 0.10 t=11 days 0.25 t=19 days 0.22 t=26 days 0.47	
Skeletonema costatum (marine)	N-starved	t= 6 days 0.25 t= 8 days 0.31 t=12 days 0.56	
Dunaliella tertiolecta (marine)	P-starved Fed at t=12 days	t= 5 days 0.10 t=10 days 0.08 t=13 days 0.26 t=17 days 0.10 t=18 days 0.10	
Monochrysis lutheri (marine)	P-starved Fed at t=19 days	t= 1 day 0.20 t=15 days 0.04 t=21 days 0.05 t=23 days 0.06	
Euglena gracilis (freshwater)	Light Dark Exp.	t= 3 min. 0.11 t=22 min. 0.09	
Phytoplankton from seawater	Depth = 0 meters Depth = 50 meters Depth = 100 meters	0.36 0.22 0.22	Holm-Hansen (1969)

* All values interpreted from graphs

used up. Working with *E. coli*, Wimpenny (1967) also noted
an increase in ATP pool size when glucose or pyruvate was
added.

Patterson *et al* (1969) and Patterson (1970), working
with bench scale activated sludge units, studied the
response of the ATP pool to changes in the incubation tempera-
ture and pH, to extended anaerobiosis, to starvation and
enrichment, and to inhibition by nickel, chromium, and
chromate. He also studied the ATP response of activated
sludge to additions of toxic materials including mercury,
copper, and cyanide. Maximum ATP pool occurred in the pH
range of 7.5 to 8.0, the normal operating range of the
units used in his research. Within 15 minutes after addition
of a lethal dose of mercuric chloride to activated sludge,
no ATP was detected.

Brezonik and Patterson (1971), reporting on the
effects of environmental stress on ATP in activated sludge,
observed increased ATP pool levels following addition of
substrate. They noted, however, that previous studies on
endogenous ATP pool indicated that only a small fraction
of activated sludge is viable. Patterson *et al* (1970)
reported 15 to 20 percent viability in a full scale activated
sludge unit and 35 to 40 percent in a laboratory unit.
Brezonik and Patterson questioned whether the increased ATP
pool levels following substrate addition reflect an increase
in cell population or an increase in ATP/cell. Recalculating
the ATP response on a viable fraction basis, Brezonik and

Patterson, showed that substrate addition can effect an increase in ATP per cell.

Coomb *et al* (1967a) subjected cultures of the marine diatom *Cylindrotheca fusiforms* to 24 hours of darkness followed by re-illumination at a high light intensity and addition of silicon. During the time of silicon uptake, the ATP pool size decreased with a subsequent increase when the silicon uptake ceased. Although this indicated that ATP participates in silicon metabolism of cell wall formation, the presence of other cellular activities such as cell division could also account for the decrease in ATP pool size. Coomb *et al* (1967b) also studied the same phenomena using *Navicula pelliculosa*, a diatom which does not divide when subjected to a period of silicon starvation. Addition of silicon induces synchronous uptake of silicon and wall formation. When silicon was added, a rapid temporary increase in the ATP pool occurred followed by a sharp decrease. The ATP pool increased slowly throughout the remainder of the silicon uptake period. Similar changes occurred in a synchronous culture kept in the dark.

Santarius and Heber (1965) studied the changes in ATP, ADP, AMP and inorganic phosphate in leaf cells. Leaves were exposed to light and dark, killed and fractionated into a chloroplastic and a residual fraction. When the chloroplast was exposed to light, the ATP increased rapidly and it decreased rapidly when the. chloroplast was placed in the dark. The ATP pool responded

in a similar manner in the cytoplasmic fraction. The ADP change was opposite to the ATP change and the AMP change followed that of ADP. From these results, Santarius and Heber concluded that the controlling factor for inhibition of glycolysis and respiration by light is the increased ratio of ATP to ADP rather than a drop in the orthophosphate concentration.

Because of the energy balance occurring within cells, ATP seems to be relatively constant under normal environmental conditions. This constant ATP pool may allow the use of ATP as a measure of microbial biomass. On the other hand, the rapid turnover time and sensitivity of ATP to environmental stress may permit the use of ATP analysis as a rapid bioassay method.

CHAPTER IV. EXPERIMENTAL METHODS AND MATERIALS

Analytical Techniques

The procedures used in this study were basically those commonly used to analyze the biological and chemical constituents of aquatic systems. Analysis of ATP is discussed separately in Chapter V.

Chemical oxygen demand (COD) was measured using the dichromate reflux method described in Standard Methods (APHA 1971). Distilled water blanks were analyzed simultaneously. Samples were not filtered prior to analysis thus the reported values represent combined soluble and suspended particulate chemical oxygen demand.

The dry weight of the algal cultures was determined using the gravimetric procedure recommended in the Provisional Algal Assay Procedure (Joint Industry-Government Task Force on Eutrophication 1969). A measured portion of algal suspension was filtered through a tared type AA millipore filter with a 0.80 micron pore size. The filters were dryed for several hours at 90° C in an oven, then they were placed in a desiccator to cool. The filters were weighed on a Mettler balance.

Turbidity was measured in Jackson Turbidity Units
(JTU) using a Hach Turbidimeter. Absorbance was measured
as described in the Provisional Algal Assay Procedure
(Joint Industry-Government Task Force on Eutrophication
1969). A Bausch and Lomb Spectronic 20 spectrophotometer
at a wavelength of 600 nm was used. Conductivity was
measured as described in Standard Methods (APHA 1971) using
a Beckman conductivity bridge.

Ortho-phosphate was measured by the single reagent
molybdenum blue method of Murphy and Riley (1962) adapted
to the Technicon Autoanalyzer. Total phosphorus was
measured by autoclaving samples at 15 psi for one hour in
the presence of potassium per sulfate and sulfuric acid.
The phosphorus concentration was measured using a Klett-
Sumerson photoelectric colorimeter.

The modified brucine method of Jenkins and Medsker
(1964) adapted to the Technicon Autoanalyzer was used to
measure nitrate. This procedure was essentially identical
to the automated method described by Kahn and Brezenski
(1967).

Alkalinity was measured by potentiometric titration
to pH 4.5 with 0.01 N H_2SO_4 as described in Standard Methods
(APHA 1971).

Chlorophyll a was extracted and measured by the
procedure originally described by Richards and Thompson

(1952) and Crietz and Richards (1955). The equations of Parsons and Strickland (1963) were employed to calculate the chlorophyll *a* concentration.

A modification of the carbon-14 procedure developed by Steeman-Nielsen (1951) was used to measure primary production. Rather than inoculating 300 ml samples with 5 micro-curies of ^{14}C, 30 ml samples were inoculated with 0.5 micro-curie of ^{14}C. After incubation for three hours in a constant temperature light box, aliquots were filtered, dried, and counted in an internal proportional counter. A method study was performed to determine the statistical accuracy of this modified procedure. Replication of this procedure yields a relative standard deviation of five percent.

Alkaline phosphatase activity of the algal cultures was measured using a modification of the method of Fitzgerald and Nelson (1966). Instead of using Gorham's medium as suggested, PAAP medium was used since all algal cultures were grown on PAAP medium.

Cell numbers were determined using a hemocyto-meter.

Experimental Materials

Four laboratory batch unialgal cultures were operated for a period of seven months. The following

algal species were grown: *Anabaena flos-aquae*,[1] *Micro-cystis aeruginosa*,[1] *Selenastrum capricornutum*,[1] and *Chlorella sp.* Initially, chemostat units were set up using *Anabaena flos-aquae, Selenastrum capricornutum,* and *Navicula minima*,[2] a diatom. A detention time of three days was maintained by feeding three liters of PAAP medium to the nine liter chemostats. A gradual wash-out of the algal cells occurred, necessitating a change in detention time from three days to nine days. After one month of operation all three chemostats were contaminated with *Chlorella* and new culture material had to be ordered from the National Eutrophication Research Center in Oregon and from the Starr Collection in Indiana. At this time a decision was made to change to batch cultures.

Batch unialgal cultures of *Anabaena, Microcystis, Selenastrum,* and *Navicula* were grown. However, *Navicula* continued to be contaminated with *Chlorella*. Finally, *Navicula* cells declined in numbers and had to be discarded. A unialgal culture of *Chlorella* was substituted for the diatom.

The batch units contained four liters of culture material which were kept well-mixed with magnetic stirrers. Gro-Lux[3] fluorescent lamps were used to illuminate the

[1]Obtained from the National Eutrophication Research Program, Pacific Northwest Water Laboratory, Corvallis, Oregon 97350.

[2]Obtained from Dr.R.Starr, Dept. of Botany, Univ. of Indiana, Bloomington, Indiana.

[3]Sylvania Electric Products Inc., Danvers, Mass.

cultures. *Selenastrum* and *Chlorella* received about 350 ft-c while *Anabaena* and *Microcystis* received about 200 ft-c. Each day 200 ml of culture material was removed from each unit and 200 ml of fresh PAAP medium was added. The PAAP medium was sterilized before it was fed to the units. Because of the poor buffer capacity of PAAP medium, the pH of the cultures often increased to a pH of 10 or greater, indicating a carbon-limiting condition. Carbon dioxide was occassionally bubbled into the units to lower the pH and add carbon. Frequent visible changes in the algal pigments of the units occurred, especially when the pH increased. At no time was a steady-state condition obtained.

CHAPTER V.   METHODS OF ATP ANALYSIS

McElroy (1947) found that luminescence in fireflies has an absolute requirement for ATP. Light production by firefly lantern extract (FLE) depends on the presence of luciferin, the enzyme luciferase, oxygen, magnesium ions, and ATP (McElroy 1947; McElroy and Strehler 1954). Hastings (1968) has reviewed the biochemistry of luminescent reactions in detail. Equations 1-3 illustrate the chemical reactions involved in the firefly light production process.

$$Lh_2 + ATP + E \overset{Mg^{++}}{\underset{}{\rightleftarrows}} E - LH_2 - AMP + PP_i \tag{1}$$

$$E - LH_2 - AMP + O_2 \rightleftarrows E - L - O^* + Product \tag{2}$$

$$E - L - O^* \rightleftarrows E - L - O^- + hv \tag{3}$$
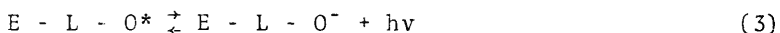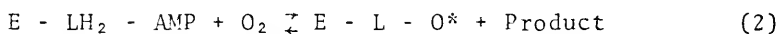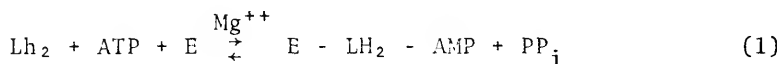
Luciferin and ATP, catalyzed by luciferase, react to form an enzyme-luciferin-adenosine monophosphate complex (luciferyl-adenylate) and inorganic pyrophosphate (PP_i). Rapid oxidation to oxyluciferyl-adenylate occurs (Equation 2). This is an excited state and is immediately followed (Equation 3) by the release of a quantum of light (Hopkins, Selinger *et al* 1968). One light quantum is emitted for each luciferin molecule oxidized (Seliger and McElroy 1959, 1960).

The mixing of ATP with firefly lantern extract
results in a flash of light which rapidly declines to a
uniform level. Addition of arsenate or phosphate buffer
to the firefly reaction decreases the initial light flash
and produces an intermediate level of light which decays
exponentially with time. Arsenate buffer is now routinely
added to many commercially available firefly lantern .
extracts used for ATP analysis.

ATP is the only nucleoside triphosphate that will
react with purified extracts of firefly lantern to produce
luminescence. The crude extracts of firefly lantern,
however, contain transphosphorylase enzymes, resulting in
light emission in the presence of high energy phosphate
molecules other than ATP (Balfour and Samson 1959). To
determine which high energy phosphate molecules might cause
light emission with firefly extract, Holm-Hansen and Booth
(1966) tested thirteen intracellular compounds including
adenylic acid, guanylic acid, cozymase, glucose-1-phosphate,
fructose-1-6-diphosphate, phosphocreative, adenosine, thia-
mine pyrophosphate, coenzyme-A, sodium glass, adenosine
diphosphate (ADP), cytidine-5-triphosphate (CTP) and inosine-
5-triphosphate (ITP). Of these compounds, only ADP, CTP,
and ITP affected light emissions. The amount of light
resulting from addition of ADP was less than 1 percent of
an equivalent amount of ATP. Both CTP and ITP stimulated
light production equivalent to that of ATP. Franzen and

Binkley (1961) reported cellular ATP values 10 to 25 per-
cent higher using the firefly method than when determined
by chromatographic techniques. The fact that some light
emission may be due to nucleoside triphosphates other than
ATP is probably not significant because of the relative
abundance of cellular ATP with respect to these other
compounds.

Since one quantum of light is emitted for each
molecule of ATP that is hydrolyzed (Seliger and McElroy
1960), the amount of ATP in a sample is directly proportional
to the total amount of light emitted by the enzyme mixture.
ATP was measured based on this principle.

Lyophilized aqueous extracts of firefly lantern[1]
were stored desiccated at -20° C until used. Each vial
contains the extract from 50 mg of firefly lanterns with
magnesium arsenate buffer added. The contents of each vial
was rehydrated with 35 ml of deionized distilled water.
After standing at room temperature for 1/2 hour, the enzyme
mixture was filtered (Whatman No. 3) and the filtrate
incubated in ice water for 3 to 4 hours. The crystalline
disodium salt of ATP was used to prepare standard solutions.
A stock solution was prepared in tris buffer (0.025 M, pH
7.75), and poured into individual test tubes which were
capped and stored at -20° C until needed. Storage for as
long as two months showed no change in standard ATP

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[1] FLE-50, Sigma Chemical Company, St. Louis, Missouri.

concentration (Patterson 1970). When ATP standards were
needed a test tube of stock solution was thawed and diluted
with tris buffer to the desired concentrations.

Samples to be analyzed for ATP were filtered through
47-mm membrane filters (pore size 0.80 μ). As soon as no
liquid remained above the filter, the filter was quickly
removed and placed in a test tube containing 9 ml of boiling
tris buffer (0.025 M, pH 7.75). The test tube was held in
a boiling water bath for 10 minutes, with occassional shaking
to disperse clumped cells. The test tube was rapidly cooled
and brought up to a volume of 10 ml with additional tris
buffer. The test tube was centrifuged (10 minutes at
2,250 rpm) to bring down cell debris, and the supernatant
was poured into a clean test tube. The test tube was then
frozen and stored at -20° C for future analysis.

This method of ATP extraction depends upon bringing
all of the ATP into solution. Other extraction methods
have been used. Holm-Hansen and Booth (1966) tested ATP
extraction using perchloric acid, boiling ethanol, boiling,
water, and boiling tris buffer. Perchloric acid extraction
inhibited the luciferase reaction because of its neutral
pH. Extraction by the last three solvents all gave satis-
factory results. Forest (1965) used sulfuric acid to
extract ATP and D'Eustachio and Levin (1967) used sonic
disruption to extract ATP. Patterson (1970) compared
sonication to boiling tris extraction and found it was

equally effective in extracting ATP. DuPont de Nemours
Company (Inc.) recommends ATP be extracted with either
butanol or dimethylsulfoxide (DMSO) for use with their
Luminescence Biometer.[2]

A sensitive method of measuring light emission is
required in the ATP assay. Holm-Hansen and Booth (1966)
used a photomultiplier tube and amplifier connected to a
recorder. Others (Forrest 1965; Lyman and DeVincenzo 1967)
have also used similar instrumentation. Another instrument
used to measure ATP is the DuPont Luminescence Biometer.
The DuPont Luminescence Biometer consists of a photomulti-
plier tube and solid-state electronics that converts the
analog signal to a digital readout proportional to the
maximum intensity of the light flash. The instrument is
calibrated for each reaction mixture so that the ATP con-
centration is read out directly. Purified luciferin-
luciferase is used without arsenate buffer to give a high
initial light flash and a rapid luminescent die-off. At
the start of this research the Biometer was used to measure
ATP concentration. During a two month investigation
period difficulties were encountered with calibration of the
instrument and read-out reproducibility. The Biometer is
also sensitive to fluctuations in line voltage entering the
instrument. Lack of reproducibility was probably caused by
by a combination of the complex solid-state electronics

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[2]DuPont 760 Luminescence Biometer, DuPont de Nemours
and Company, Wilmington, Delaware.

and the sample size and injection procedure. The DuPont
procedure calls for injection of a 0.01 ml aliquot of sample
into a vial containing 0.1 ml of enzyme mixture. Although
a precision microliter syringe was used to inject the
sample, it is doubtful whether exactly 0.01 ml was delivered
each time at the same rate. Small droplets would sometimes
cling to the needle point, indicating a lack of precision
in the injection procedure. In general, the Biometer was
found to be an unsatisfactory method of measuring ATP.

Conventional liquid scintillation spectrometers have
been used to measure luminescence (Cole, Wimpenny, *et al.*
1967; Patterson 1970). A liquid scintillation spectrometer[3]
was used in this study. Instrument gain was set at 53, with
a window opening of 50 to 1,000. The spectrometer was set
in a repeat count mode, with each 6-second counting interval
separated by a 7.5 second data print-out sequence.

Prior to each ATP analysis the background light
emission from the luciferin-luciferase firefly extract was
measured. Exactly 1.0 ml of the enzyme preparation was
pipetted into a scintillation vial. Normal background
emission was 10 to 20 counts in the 6-second counting
interval. Patterson (1970) found that higher counts
indicated glassware contamination. All glassware was washed

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[3]Packard Tri-Carb Model 2002, Packard Instrument
Company, Downers Grove, Illinois.

in an automatic dishwasher using hot soapy water, a hot
rinse, and a final rinse with distilled water. Following
this, the glassware was boiled for at least one hour in an
acid bath and rinsed three times in deionized, distilled
water.

After determining the background emission, exactly
0.5 ml of ATP standard or sample was pipetted into the
enzyme mixture and the vial was swirled to thoroughly mix
the contents. Exactly 11.0 seconds after ATP addition, the
vial was placed into the scintillation counting chamber.
This procedure was followed because of the exponential decay
of the luminescence, necessitating careful control over the
addition of ATP to the enzyme mixture and initiation of the
counting sequence.

Patterson (1970), using the scintillation counter to
measure ATP, analyzed the data graphically because of the
random variability of any 6-second count. The 6-second
emission counts were plotted on semi-log paper versus
elapsed reaction time. Good correlation between counts and
standard ATP concentrations were observed for reaction times
of one minute or more. The line of best fit to the data
points was extrapolated back to the initial data point and
the count at one-minute was read from this line. This
graphical technique was used in this research. Figure 4
shows lumincence decay curves for several ATP concentrations.
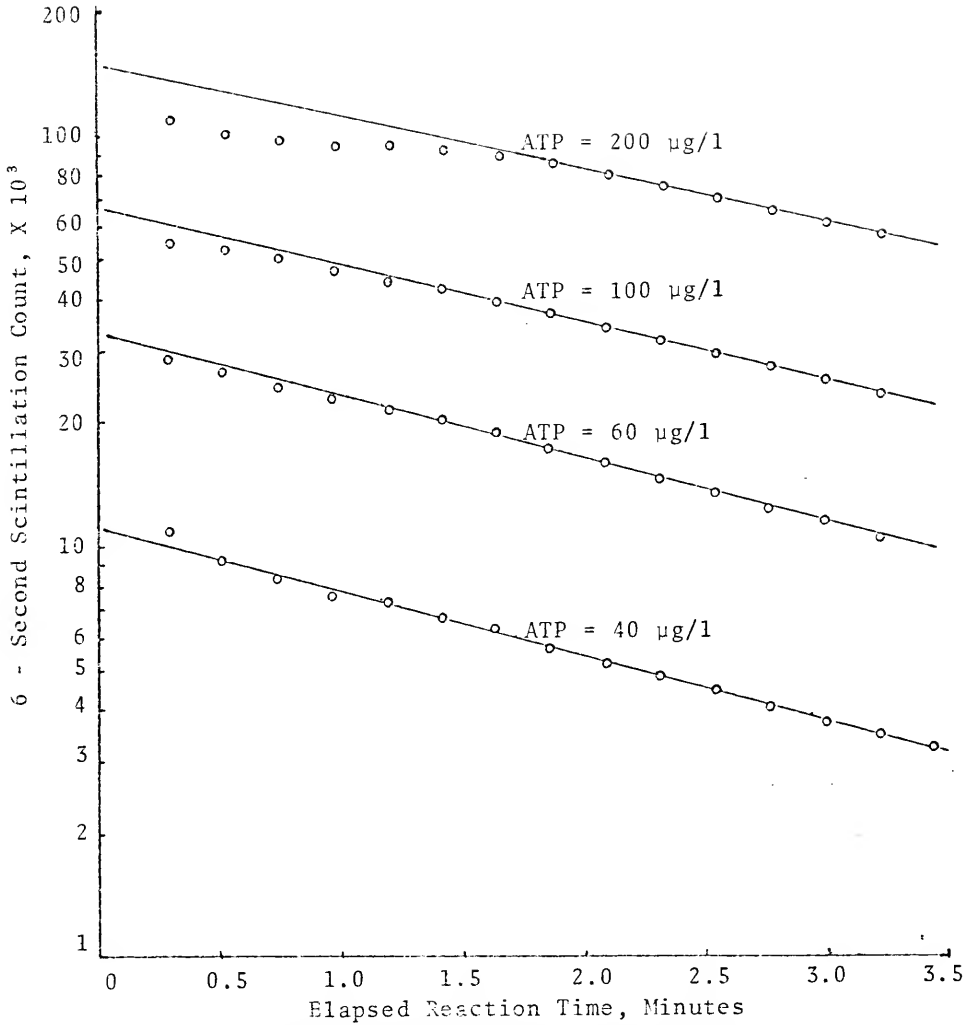
FIGURE 4.   LUMINESCENCE DECAY OR ATP FIREFLY LANTERN
EXTRACT REACTION WITH TIME (Patterson 1970)

The effects of aging on both ATP standard solutions
and firefly lantern extract were studied (Patterson 1970).
As shown in Table 5, the background emission of the luciferin-
luciferase mixture decreased rapidly during a 24-hour incuba-
tion period. Two sets of ATP standards were also made up:
one set was frozen, then thawed just prior to analysis;
the other was held in an ice water bath with the firefly
mixture. The light emission from the freshly thawed
standards decreased with the time of incubation, indicating
a slight loss of enzyme activity over the 24-hour period.
Light emission from the ATP solution kept in an ice bath
decreased rapidly, indicating a loss of ATP from the
standard solutions during the incubation period. In light
of these results, standards were always measured immediately
after preparation, and samples were thawed just prior to
analysis.

The effect of tris buffer temperature on ATP extraction
was also studied by Patterson (1970). He found (Figure 5)
that a temperature reduction of a few degrees below boiling
caused a significant decrease in the amount of ATP extracted.
For this reason, all extractions were performed in a rapidly
boiling water bath. However, it was often found that water
which appeared to be boiling actually had a temperature of
90-95°C. Thus a thermometer was always kept in the boiling
tris buffer to assure that the temperature remained at 100°C.

TABLE 5

EFFECT OF INCUBATION ON FIREFLY EXTRACT
AND ATP LIGHT EMISSION (6-SEC COUNT)
(Patterson 1970)

Extract Age, Hours	Extract Background Counts	Six Second Count					
		Fresh ATP, µg/1			Aged ATP,* µg/1		
		20	100	500	20	100	500
2	355	11,200	118,000	430,000	- - - - -	- - - - - - -	- - - - -
6	153	7,500	121,000	420,000	5,050	107,000	418,000
12	63	- - - - -	- - - - - - -	- - - - - - -	1,070	92,000	383,000
24	15	3,350	85,000	400,000	25	56,200	324,000

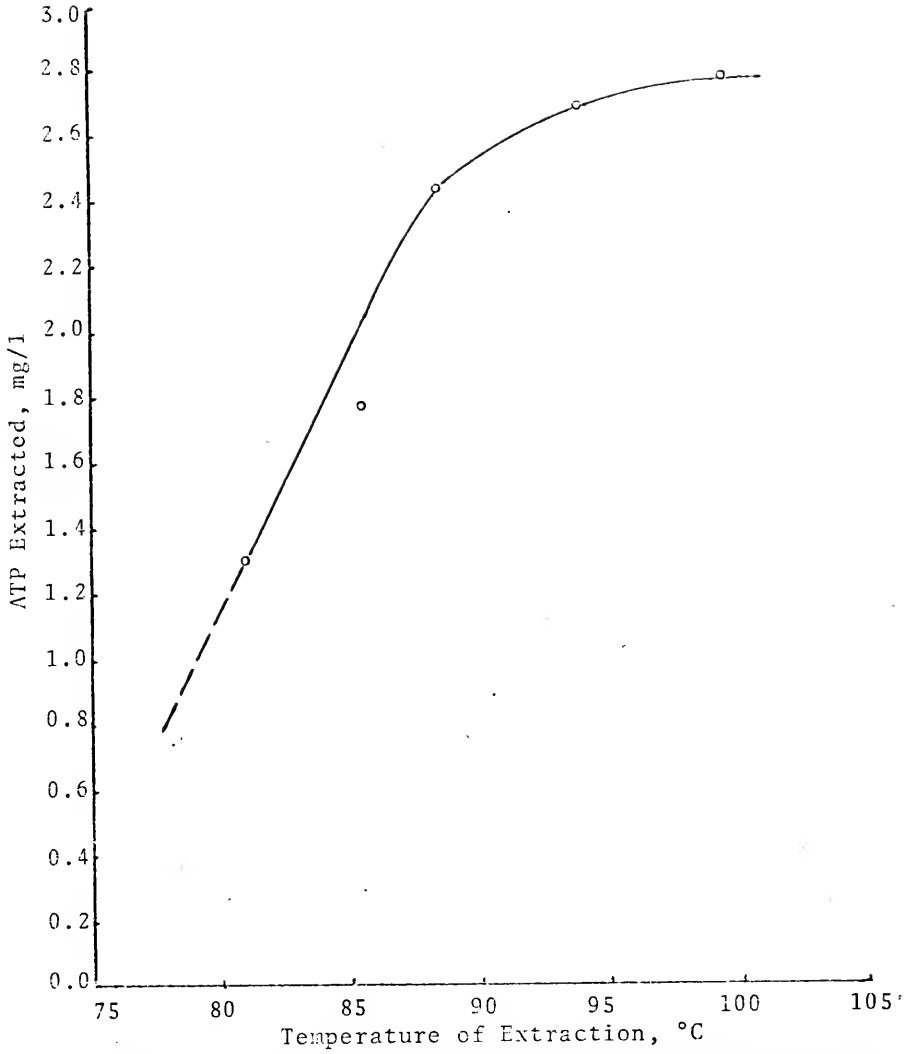* ATP age was one hour less than extract age

FIGURE 5.   DEPENDENCE OF QUANTITATIVE ACTIVATED SLUDGE
ATP EXTRACTION ON TEMPERATURE OF EXTRACTION SOLUTION
(Patterson 1970)

Rhodes and McElroy (1958) investigated the sensitivity of the firefly reaction to pH and found that luminescence increased rapidly from pH 2.0 to 3.8, decreased from pH 2.8 to 3.8, and increased from pH 4.8 to 7.0. The rate of light emission is much more rapid at pH 7.6 than at pH 9.4 (Seliger and McElroy 1960). Holm-Hansen (1966) used a reaction mixture buffered at pH 7.75. For this research, tris buffer at a pH of 7.75 was used.

Figure 6 shows a typical standard ATP curve. Depending on the enzyme age and concentration, the standard curve may be linear or non-linear. Results of recent measurements of ATP standards indicate that plotting the points on log-log graph paper gives a straight line of best fit.

A sample of *Selenastrum* was diluted into five portions and the ATP of each portion was measured. This experiment was run to determine whether the sampling procedure was valid. As shown in Figure 7, excellent correlation between ATP content and *Selenastrum* concentration was observed.

Using the method of ATP analysis described in this chapter, replication of ATP samples gave results with a relative standard deviation of less than 5 percent.
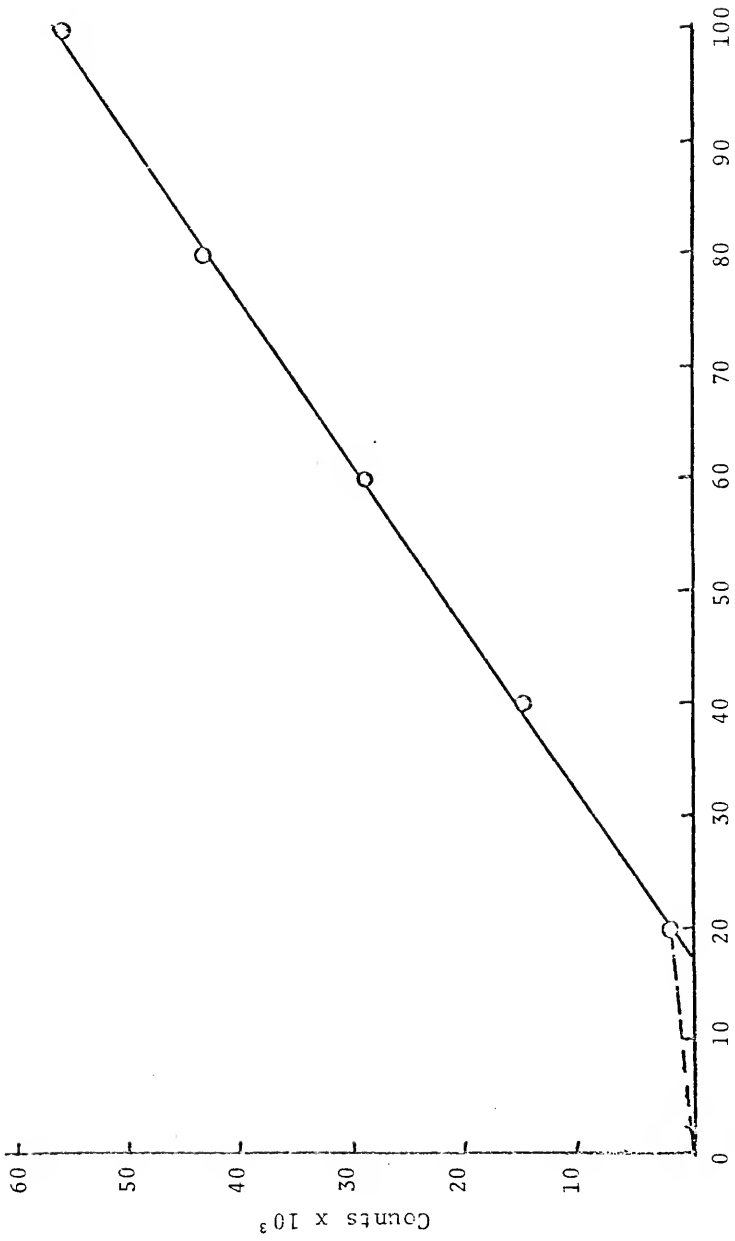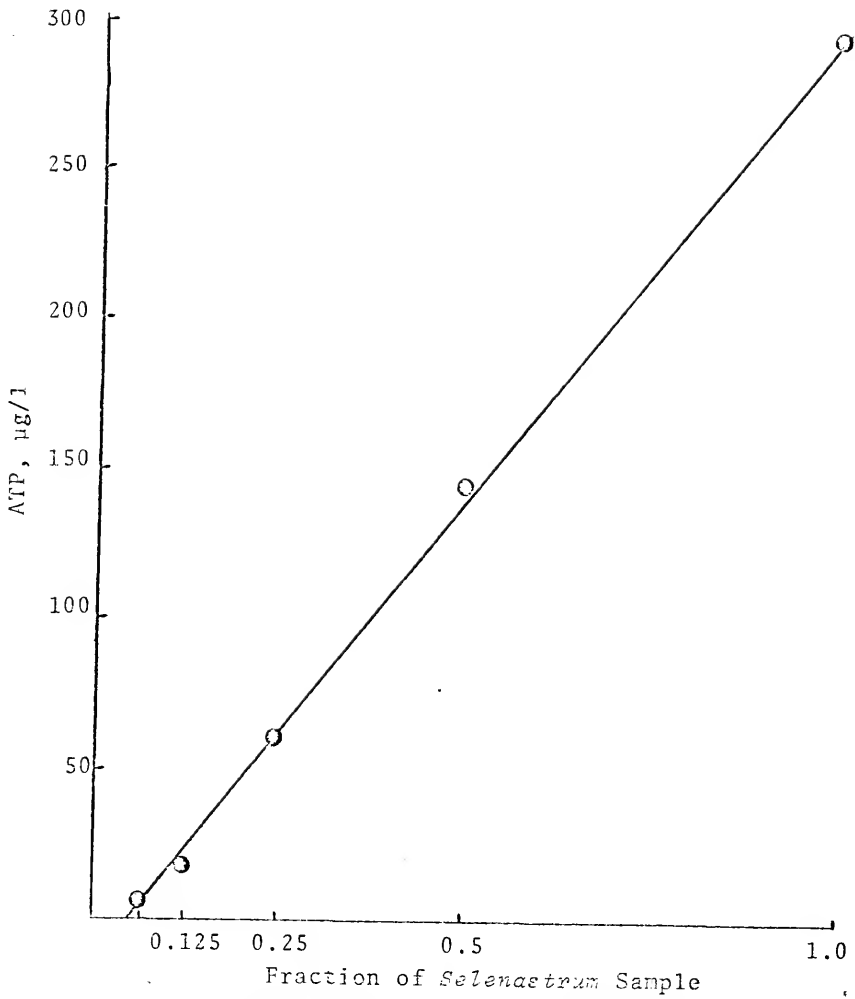
FIGURE 6. STANDARD ATP CURVE

FIGURE 7.   CORRELATION OF ATP WITH *SELENASTRUM* CONCENTRATION

CHAPTER VI.   EXPERIMENTAL RESULTS

The study of ATP in phytoplankton was divided into
two phases. The first phase consisted of monitoring the
ATP pool in batch grown unialgal cultures and natural lake
populations. In the second phase, samples from the unialgal
cultures and from lake waters were subjected to various
environmental conditions such as pH, light and addition of
nutrients and toxic substances. The ATP response to these
conditions was measured.

ATP - Biomass Results

Batch cultures of *Anabaena flos-aque, Selenastrum
capricornutum, Microcystis aeruginosa,* and *Chlorella sp.*
were grown in PAAP media for a period of approximately three
months. Twice each week a 200 ml sample was taken from each
culture and analyzed for ATP and other biomass parameters
including chlorophyll *a*, dry weight, and absorbance. These
experiments were performed to determine whether the cellular
ATP concentrations in batch unialgal cultures would correlate
with current biomass parameters (chlorophyll *a*, dry weight,
etc.). Samples of phytoplankton from local lakes were also
collected and analyzed for ATP, chlorophyll *a*, dry weight,
and absorbance. The correlation of ATP with current biomass

parameters was determined to test the usefulness of ATP measurements in natural phytoplankton populations. No attempt was made to quantitatively measure the bacteria population in the batch cultures or the lake samples.

ATP vs. Chlorophyll *a*

Figures 1 to 3 present ATP vs. chlorophyll *a* values for the batch algal cultures. The slopes of the linear regression lines for *Selenastrum* and *Chlorella* are similar; thus Figure 8 contains the data for both these organisms. Although the slopes of the linear regression lines are similar for *Anabaena* and *Microcystis*, the ATP and chlorophyll *a* values are plotted separately for reasons of clarity and because of the much larger range of chlorophyll *a* values observed in the *Anabaena* culture.

Although there is a wide degree of scatter about the regression lines for all algal species, a definite relation-ship exists. Correlation coefficients for ATP vs. chlorophyll *a* concentrations are relatively high as shown in Table 6. Correlation is a measure of the degree to which variables vary together, that is, it is a measure of the intensity of association. The sample correlation coefficient, r, given in Table 6 is an estimate of the population coefficient, ρ. Table 6 gives the 95 percent and 99 percent confidence intervals for the population correlation coefficient. Note that a correlation does not exist if the population correlation coefficient is zero. Thus, to be significant

57
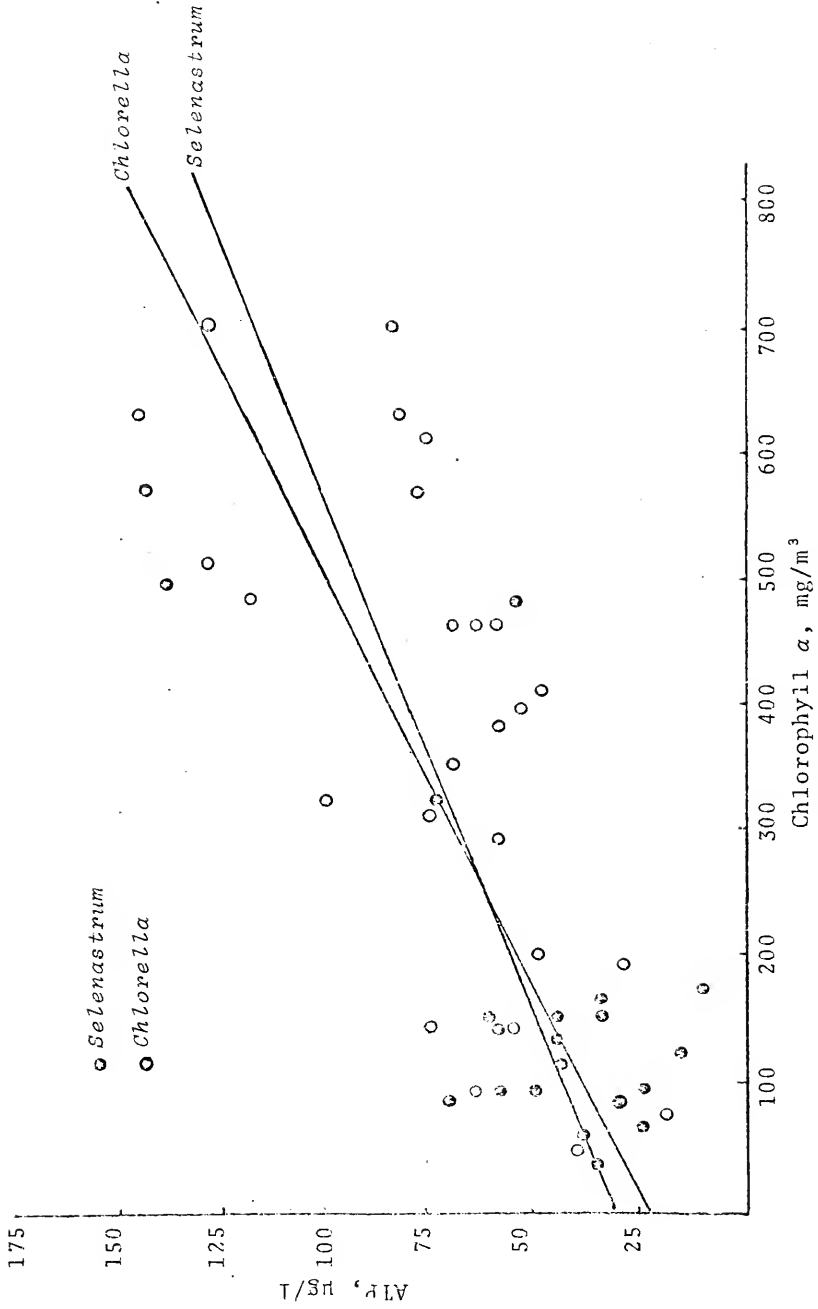
FIGURE 8. ATP VS. CHLOROPHYLL A OF *SELENASTRUM* AND *CHLORELLA*
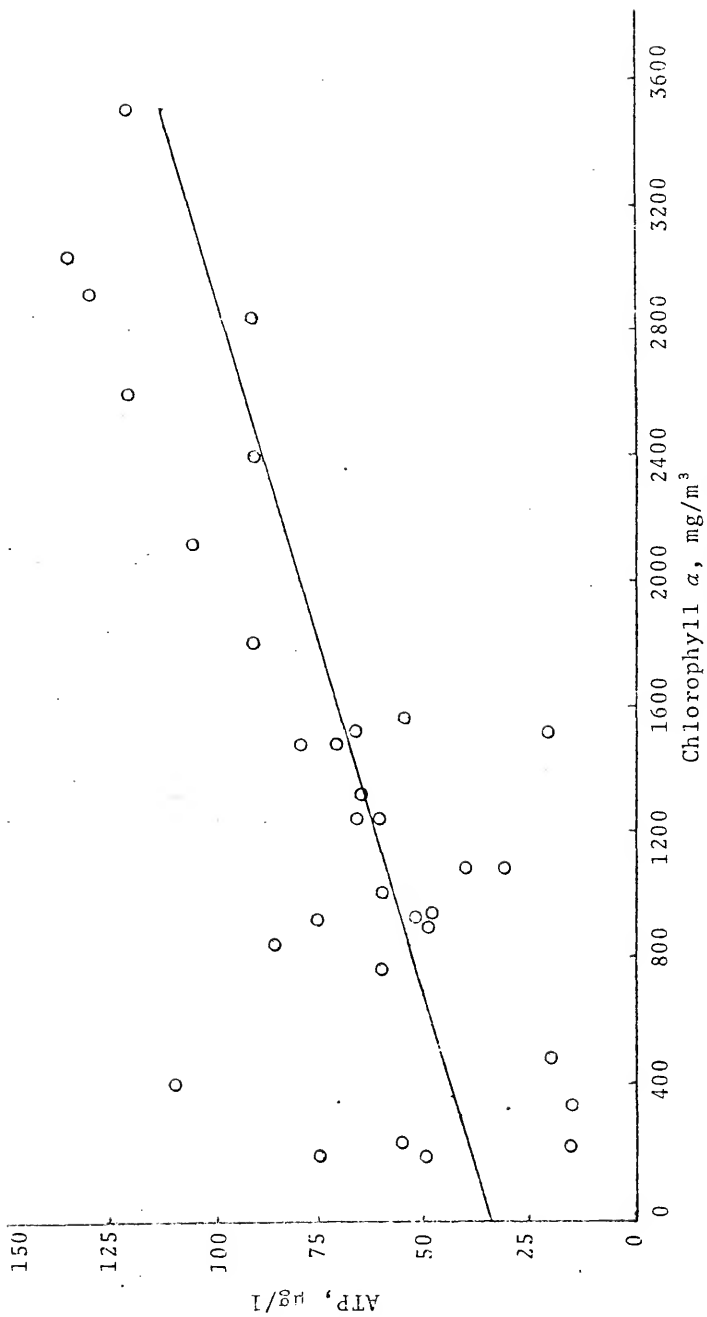
FIGURE 9. ATP VS. CHLOROPHYLL A FOR *ANABAENA*
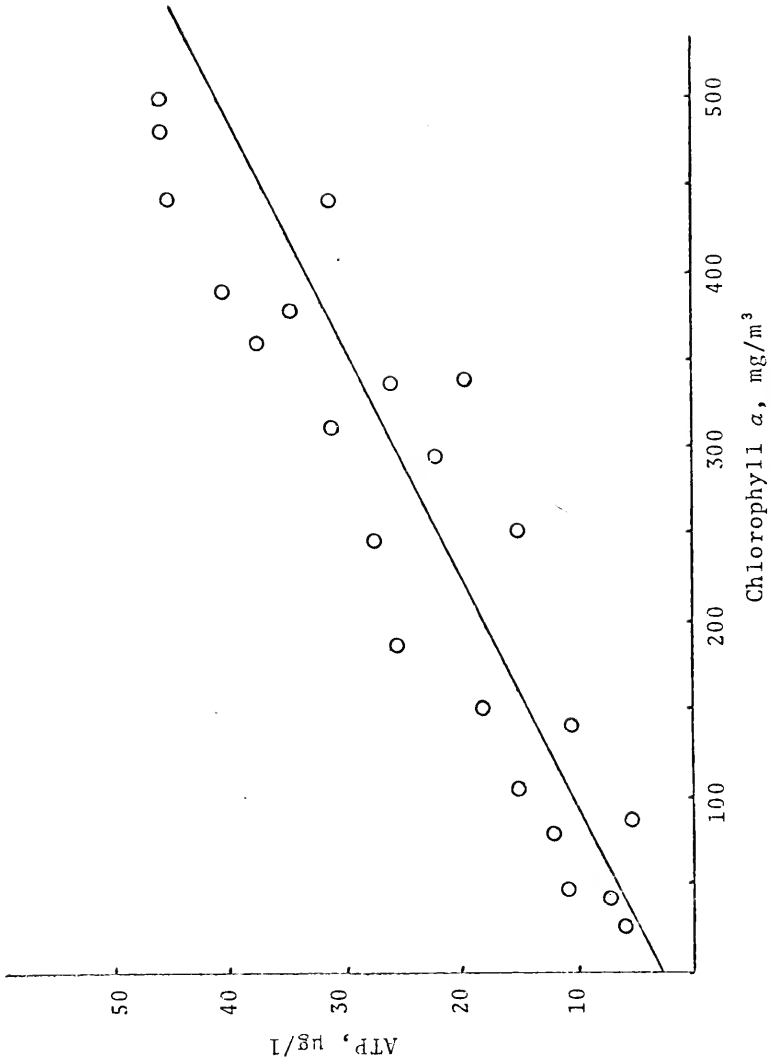
FIGURE 10. ATP VS. CHLOROPHYLL *A* FOR *MICROCYSTIS*

TABLE 6

ATP VS. CHLOROPHYLL *A* CORRELATION COEFFICIENTS FOR
UNIALGAL CULTURES

Algae	Sample Correlation Coefficient, r	95% Confidence Interval, ρ	99% Confidence Interval, ρ
Anabaena	0.707	0.45 - 0.83	0.35 - 0.87
Selenastrum	0.539	0.15 - 0.76	0.01 - 0.82
Microcystis	0.683	0.36 - 0.84	0.28 - 0.87
Chlorella	0.725	0.45 - 0.87	0.31 - 0.89

at a particular confidence level, the confidence interval must not contain zero. As shown in Table 6, the sample correlation coefficients for all algal species are significant at the 99 percent confidence level, but the correlation for *Selenastrum*, which had the lowest correlation ($r = 0.539$) is barely significant at the 99 percent confidence level since its confidence interval approaches zero at the lower limit.

To study further the relationship between ATP and chlorophyll a, a dense culture of *Microcystis* was diluted into 10 portions, incubated for 24-hours, and analyzed for ATP and chlorophyll a. A nearly perfect linear relationship was obtained (Figure 11). These results demonstrate a strict ATP to chlorophyll a relationship in the absence of varying conditions of pH, light intensity, and nutrient state.

To determine the relationship of ATP to chlorophyll a in natural lake phytoplankton, measurements were made on three Florida lakes: Bivens Arm, Newnan's Lake, and Anderson-Cue Lake. These lakes were selected to provide a wide range of biomass values. Bivens Arm is a hypereutrophic lake, Newnan's Lake is eutrophic, and Anderson-Cue Lake is oligotrophic. Chemical and biological characteristics of these lakes are given in Table 7. Results of the lake measurements are shown in Figure 12, and the correlation coefficients and confidence intervals are given in Table 8. Correlation of ATP with chlorophyll a for all

62
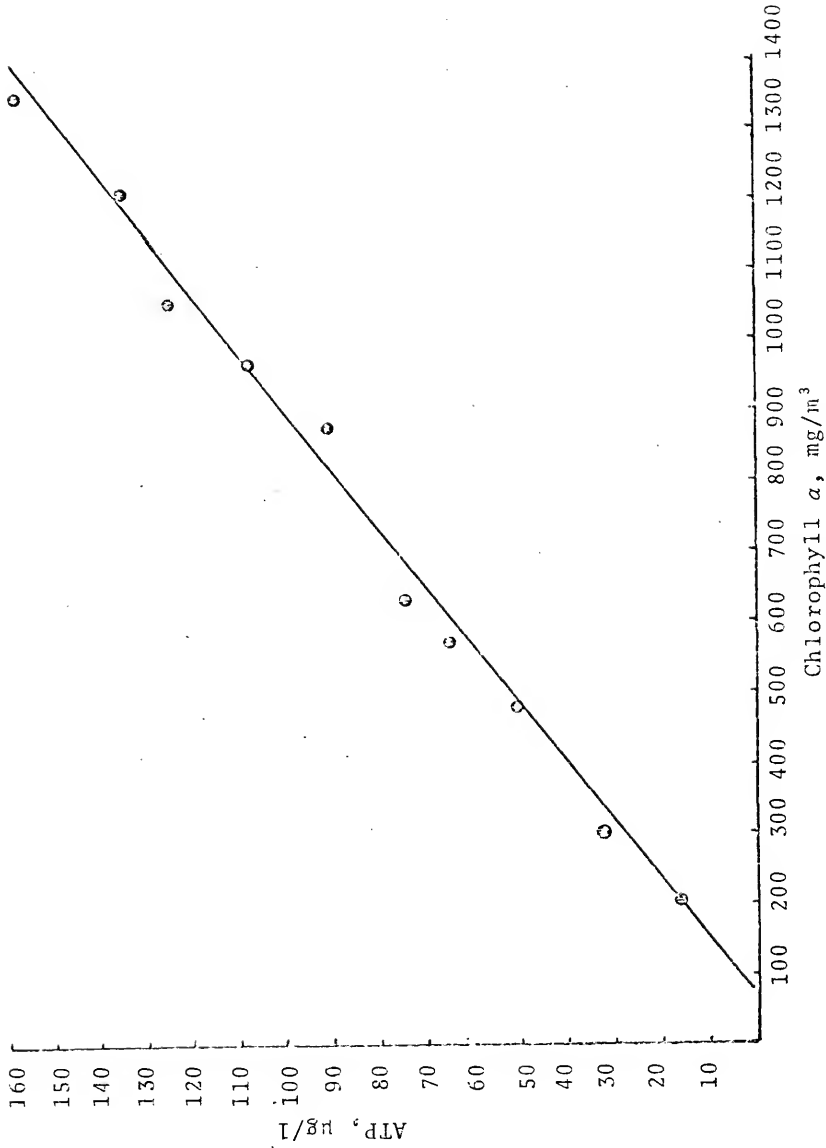
FIGURE 11. ATP VS. CHLOROPHYLL A OF DILUTED *MICROCYSTIS*

TABLE 7

CHEMICAL AND BIOLOGICAL CHARACTERISTICS
OF EXPERIMENTAL LAKES[1]

	Bivens Arm	Newnan's Lake	Andersen-Cue
pH	8.99	7.78	4.76
Acidity (mg/1 $CaCO_3$)	0.0	1.01	2.68
Alkalinity (mg/1 $CaCO_3$)	105.0	7.3	0.0
Specific Conductance (umho cm^{-1})	253.8	59.8	38.2
Color (mg Pt/1 at pH 8.3)	42.1	188.9	3.0
Total Organic Nitrogen (mg/1)	1.87	1.41	0.28
NH_3 (mg/1)	0.19	0.36	0.14
NO_3 (mg/1)	0.05	0.07	0.09
Ortho-PO_4, (mg/1)	0.225	0.012	0.002
Total-PO_4, (mg/1)	0.546	0.110	0.018
Turbidity (JTU)	10.2	4.2	1.0
Primary Production (mg C/m^3-hr)	251.7	102.5	1.1
Chlorophyll a (mg/m^3)	56.03	47.39	3.17

[1] Average of data collected from June 1968 to April 1970 (Shannon 1970)

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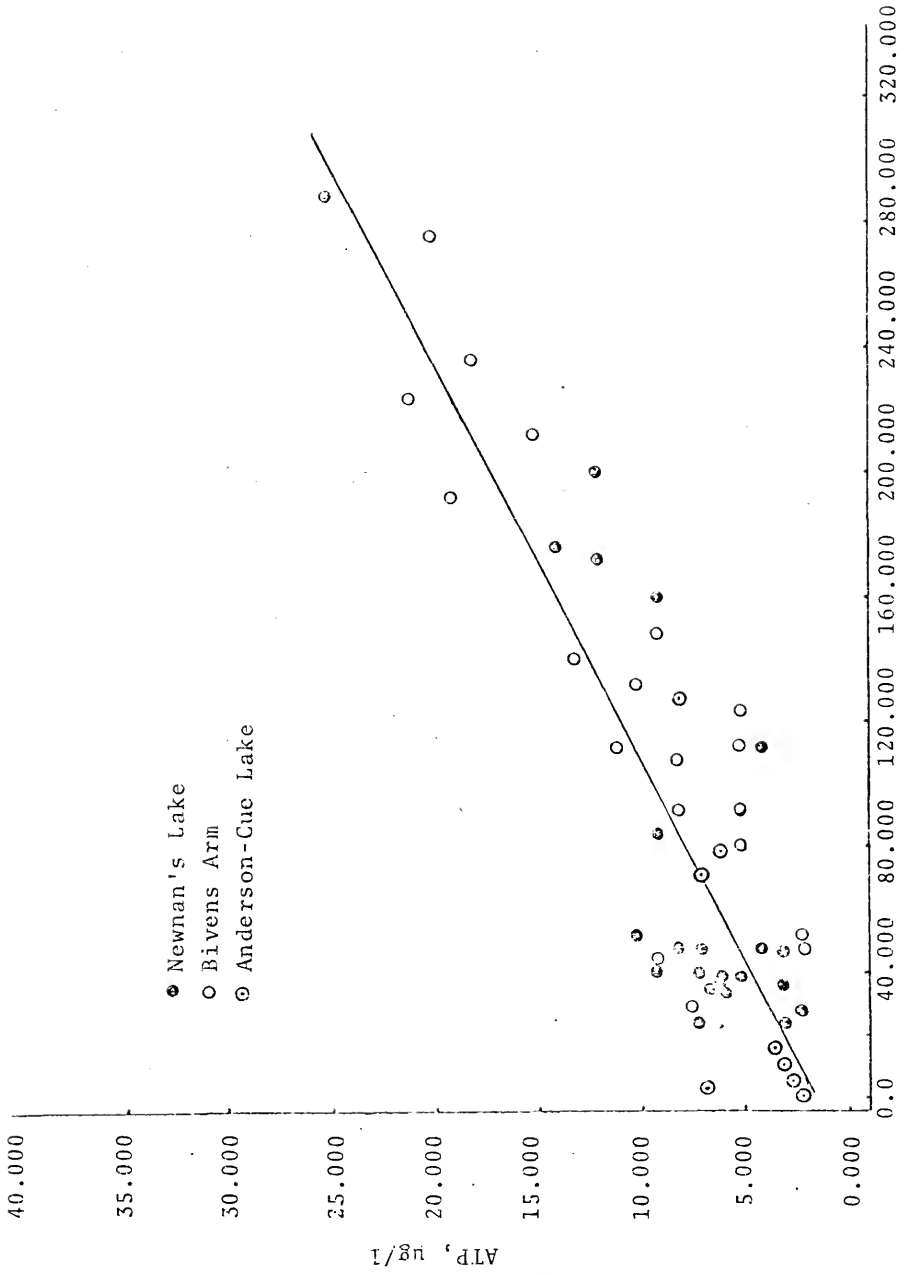
FIGURE 12. ATP VS. CHLOROPHYLL A FOR LAKE PHYTOPLANKTON

TABLE 8

ATP VS. CHLOROPHYLL *A* CORRELATION COEFFICIENTS FOR
LAKE PHYTOPLANKTON

Lake	Sample Correlation Coefficient, r	95% Confidence Interval, ρ	99% Confidence Interval, ρ
Bivens Arm	0.869	0.70 - 0.94	0.66 - 0.96
Newnan's Lake	0.718	0.42 - 0.86	0.28 - 0.90
Anderson-Cue Lake	0.790	0.20 - 0.94	-0.10 - 0.97
All Lakes	0.830	0.73 - 0.92	0.68 - 0.94

three lakes is significant at the 95 percent confidence
level. Bivens Arm and Newnan's Lake are significant at
the 99 percent confidence level. However, the correlation
is not very good for Anderson-Cue Lake alone as evidenced
by its large confidence interval and its not being signifi-
cant at the 99 percent confidence level. The poor correla-
tion of ATP with chlorophyll *a* in Anderson-Cue is probably
a result of the small range of values measured. The
correlation coefficient of the combined lake values is 0.830.
These high correlation values indicate the excellent associa-
tion of ATP with chlorophyll *a* in lake phytoplankton.

The ATP to chlorophyll *a* ratios for the laboratory
algal cultures and the lake samples were calculated and
are given in Table 9. *Anabaena* and *Microcystis* have lower
ATP to chlorophyll *a* ratios than *Selenastrum* and *Chlorella*.
This could be explained by the fact that *Anabaena* and

TABLE 9

ATP TO CHLOROPHYLL *A* RATIOS FOR ALGAL CULTURES
AND LAKE PHYTOPLANKTON

	ATP/Chlorophyll *a*
Anabaena	0.09
Microcystis	0.09
Selenastrum	0.35
Chlorella	0.29
Bivens Arm	0.09
Newnan's Lake	0.11
Anderson-Cue Lake	0.69
All Algae	0.20
All Lakes	0.25
Algae and Lakes	0.21

Microcystis, both blue-green algae, are procaryotic cells with a pigment system containing chlorophyll *a*, phycocyanin, and trace amounts of phycoerythrin. A very efficient energy transfer, approaching 100 percent, occurs from phycocyanin to chlorophyll *a* (Brock 1970). Thus the amount of chlorophyll *a* required by a blue-green alga may be less than that required by a green alga. In blue-green algae the photosynthetic pigments occur in organized internal membranes unlike eucaryotic algae where the pigments occur in membrane-bound chloroplasts. This difference in cellular organization could also account for the observed differences in the ATP to chlorophyll *a* ratio.

Bivens Arm and Newnan's Lake both have lower ATP to chlorophyll *a* ratios than Anderson-Cue Lake. One would almost expect the opposite, considering that Bivens Arm and Newnan's Lake probably contain a larger bacterial population than Anderson-Cue Lake. However, other factors must be considered. For instance, both Bivens Arm and Newnan's Lake contain large populations of blue-green algae while Anderson-Cue Lake does not. A large population of blue-green algae, according to the above data, would give a lower ATP to chlorophyll *a* ratio. Also, the much higher turbidity and color in Bivens Arm and Newnan's Lake probably induce a higher cellular chlorophyll content in order to utilize the subdued light entering the water.

Other experiments were performed to determine whether
the ATP to chlorophyll *a* ratio varied with nutritional state
and cell age. Aliquots from each of the unialgal cultures
were transfered to flasks containing fresh media. One flask
contained PAAP medium with all nutrients present, one
contained PAAP medium without phosphorus, and one contained
PAAP medium without nitrogen. The ATP to chlorophyll *a*
ratio increased as the nutritional content of the medium
decreased and the cell age increased (Figure 13). In most
cases, the ATP to chlorophyll *a* ratio remained relatively
constant for the first few days then increased. This
resulted from an initial increase in chlorophyll *a* and ATP
concentrations followed by a decrease in chlorophyll *a* as
the cultures aged and the nutrient content of the medium
decreased. Fogg (1965) observed that the cellular chlorophyll
concentration decreased with cell age and nutrient deficiency.
Thus, variations in the ATP to chlorophyll ratio may be a
result of natural fluctuations in cellular chlorophyll rather
than changes in cellular ATP.

ATP vs. Dry Weight

ATP and dry weight values of the batch algal cultures
are shown in Figure 14. All four algal species have similar
linear regression lines indicating a relatively constant
association between ATP and dry weight. Correlation
coefficients and their confidence intervals are given in
Table 10. The sample correlation coefficients for *Anabaena*,
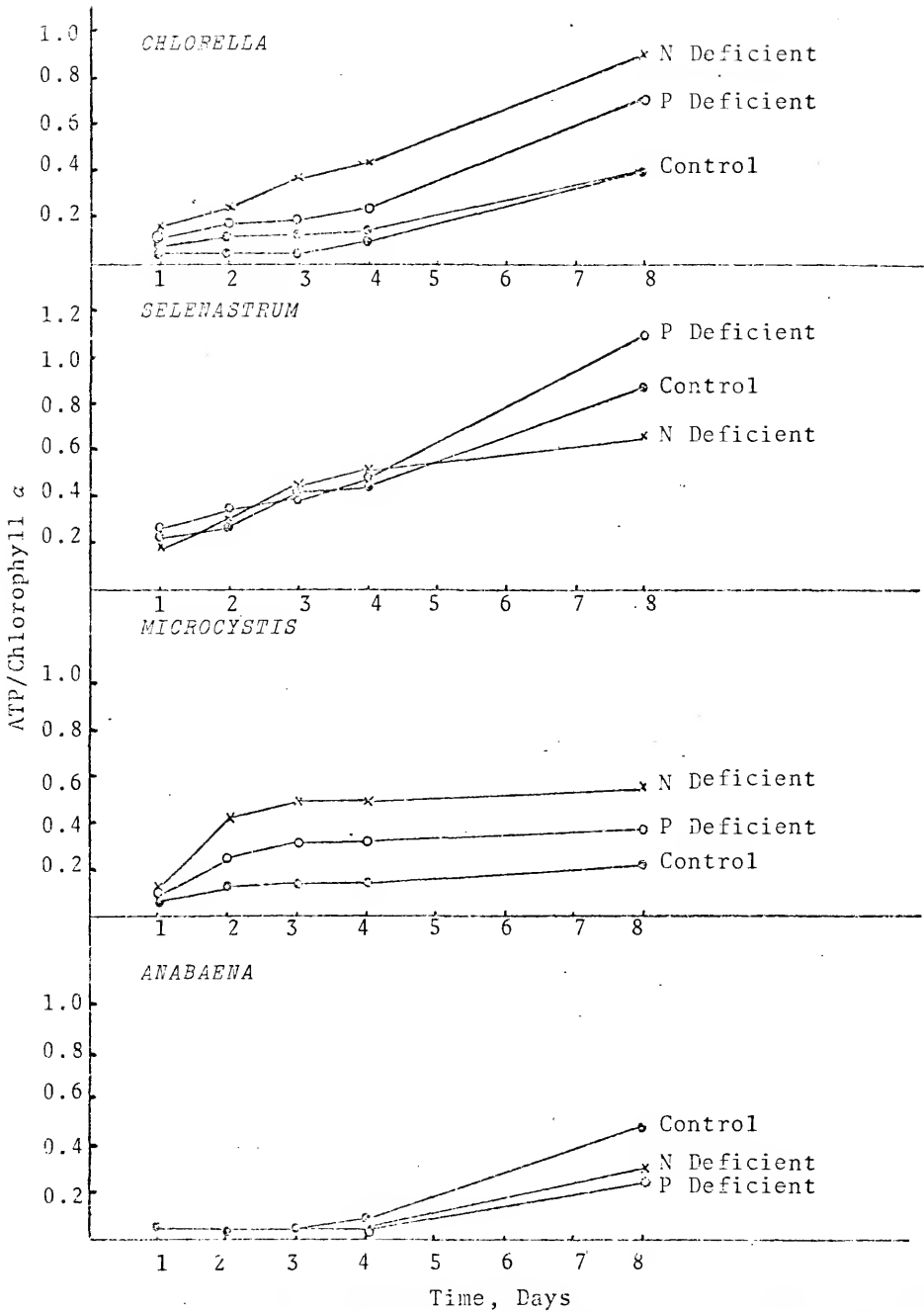
FIGURE 13. VARIATION OF ATP/CHLOROPHYLL a RATIO WITH CELL AGE
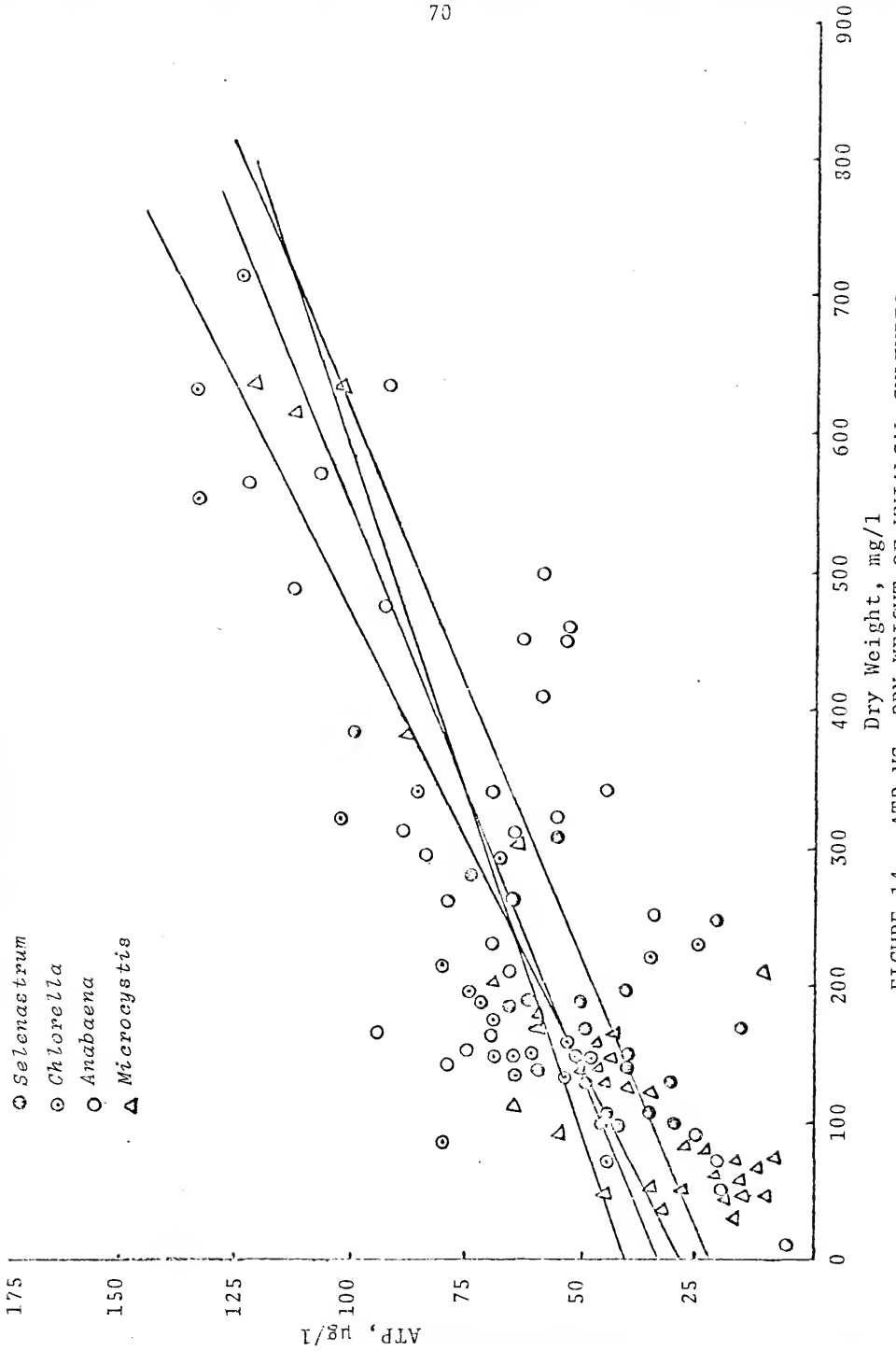
FIGURE 14. ATP VS. DRY WEIGHT OF UNIALGAL CULTURES

TABLE 10

ATP VS. DRY WEIGHT CORRELATION COEFFICIENTS
FOR UNIALGAL CULTURES

Algae	Sample Correlation Coefficient, r	95% Confidence Interval, ρ	99% Confidence Interval, ρ
Anabaena	0.607	0.29 - 0.78	0.16 - 0.82
Selenastrum	0.244	-0.18 - 0.60	-0.33 - 0.68
Microcystis	0.718	0.41 - 0.87	0.27 - 0.91
Chlorella	0.860	0.65 - 0.94	0.56 - 0.85
All Algae	0.726	0.61 - 0.79	0.56 - 0.82

Microcystis, and *Chlorella* are relatively good ranging from
0.607 to 0.860, with all three significant at the 99 percent
confidence level. Correlation at ATP with dry weight for
Selenastrum is poor and not significant even at the 95
percent level. The poor correlation of *Selenastrum* probably
resulted primarily from the small range of values measured.
All of the algae together have a correlation coefficient of
0.726 which is significant at the 99 percent confidence
level. In general, good correlation exists between ATP
and dry weight in the unialgal cultures.

The ATP to dry weight measurements for Bivens Arm,
Newnan's Lake, and Anderson-Cue Lake are shown in Figure 15.
Correlation coefficients for the three lakes are all
significant at the 99 percent confidence level (Table 11).
Good correlation exists between ATP and dry weight for all
three lakes, with Anderson-Cue Lake having the best
correlation.

ATP to dry weight ratios for the algal cultures and
the lakes were calculated and are presented in Table 12.
All four algal cultures have relatively constant ATP to dry
weight ratios ranging from 0.24 to 0.38 µg/mg. However, the
ATP to dry weight ratios for the three lakes vary from 0.12
to 0.96 µg/mg. Bivens Arm and Newnan's Lake have a much
lower average ATP to dry weight ratio than Anderson-Cue Lake.
These low ratios are evidently the result of a high
concentration of detrital matter which also explains the

73
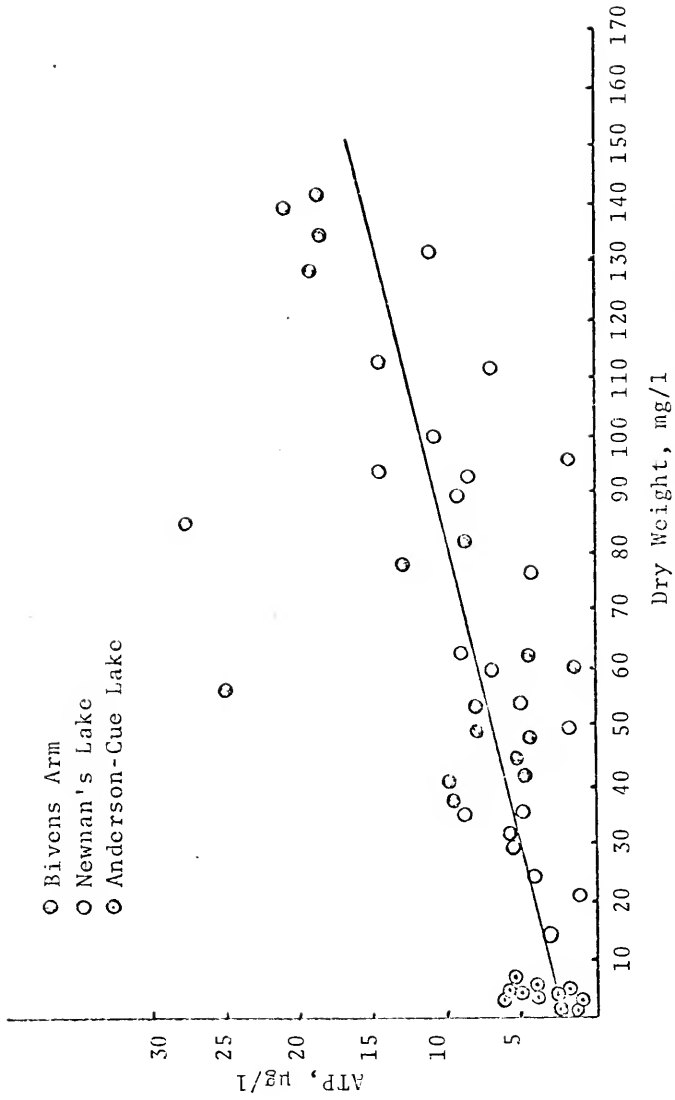
FIGURE 15. ATP VS. DRY WEIGHT FOR LAKE PHYTOPLANKTON

Dry Weight, mg/l

ATP, µg/l

⊙ Bivens Arm
⊙ Newnan's Lake
⊙ Anderson-Cue Lake

TABLE 11

ATP TO DRY WEIGHT CORRELATION COEFFICIENTS
FOR LAKE PHYTOPLANKTON

Lake	Correlation Coefficient, r	95% Confidence Interval, ρ	99% Confidence Interval, ρ
Bivens Arm	0.649	0.20 - 0.82	0.15 - 0.87
Newnan's Lake	0.673	0.23 - 0.83	0.18 - 0.88
Anderson-Cue Lake	0.790	0.36 - 0.93	0.14 - 0.95

TABLE 12

ATP TO DRY WEIGHT RATIO'S FOR ALGAL CULTURES
AND LAKE PHYTOPLANKTON

	ATP/dry weight
Anabaena	0.26
Microcystis	0.36
Selenastrum	0.24
Chlorella	0.38
Bivens Arm	0.13
Newnan's Lake	0.12
Anderson-Cue Lake	0.96

poorer correlation of ATP with dry weight in these lakes.
Table 13 compares the turbidity of each lake with the
measured ATP to dry weight ratio. Both Bivens Arm, and
Newnan's Lake have large amounts of turbidity. Bivens
Arm, for example, has an average turbidity of 10.2 JTU's
compared to Anderson-Cue's average of 1.0 JTU.

TABLE 13

COMPARISON OF ATP/DRY WEIGHT RATIO WITH AVERAGE
TURBIDITY OF EXPERIMENTAL LAKES

Lake	ATP/Dry Weight	Average Turbidity[1] (JTU)
Bivens Arm	0.13	10.2
Newnan's Lake	0.12	4.2
Anderson-Cue Lake	0.96	1.0

[1]Shannon, 1970

ATP vs. Cell Number

Another common biomass measurement is the cell
number. A dense culture of *Microcystis* was diluted into
ten portions, incubated for 24-hours, and analyzed for ATP
and cell number. Figure 16 shows that a linear correlation
exists between ATP concentration and cell number. The ATP
per cell for *Microcystis* ranged from 5.0×10^{-8} to 6.25×10^{-8} μg/cell. It should be noted that cell number, because
of differences in size between various algal species, would
not give a useful correlation with ATP in mixed populations.
These results are presented only to illustrate the good
correlation between ATP and cell number in unialgal cultures.

ATP vs. Absorbance

Absorbance is a common means of measuring relative
algal densities in laboratory cultures (Joint Industry-
Government Task Force on Eutrophication 1969). The absorbance
of the four algal cultures was routinely measured along with
ATP and the values are plotted vs. each other in Figure 17.
Correlation coefficients for ATP vs. absorbance are given
in Table 14. Except for *Microcystis* the correlation of ATP
with absorbance is poor. A high correlation between dry
weight and absorbance exists, indicating that scattering
rather than absorbance is really being measured. It is
surprising that better correlations were not obtained, espe-
cially for such dispersed algae as *Chlorella*. The reason
lies partially in the small range of chlorophyll a values
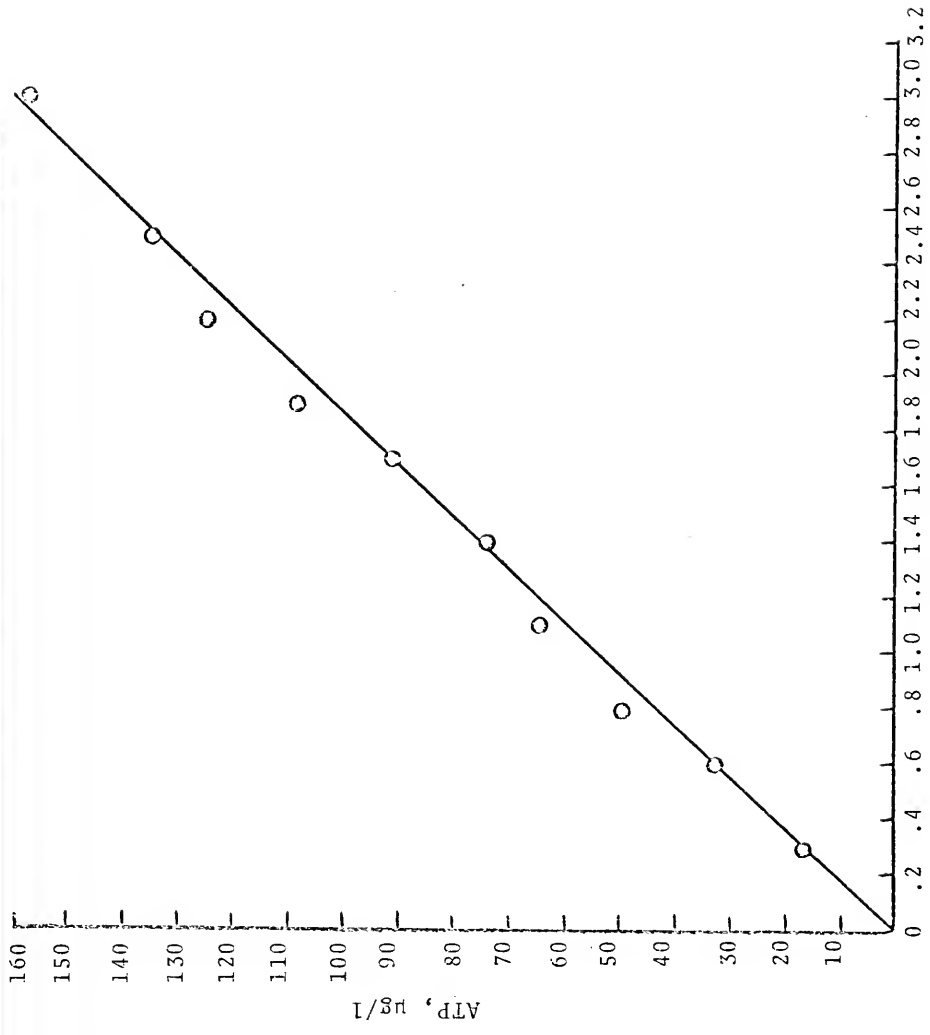observed.

FIGURE 16.   ATP VS. CELL COUNT FOR *MICROCYSTIS*
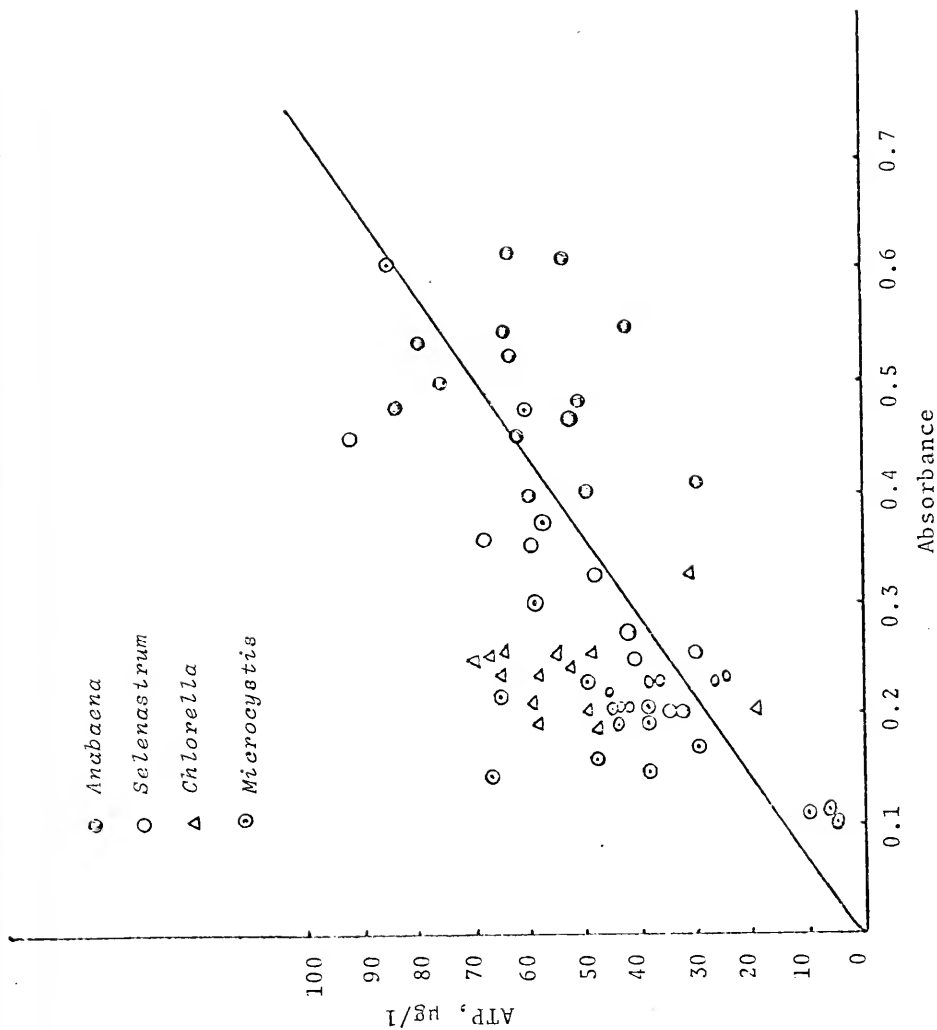
FIGURE 17. ATP VS. ABSORBANCE OF UNIALGAL CULTURES

TABLE 14

ATP TO ABSORBANCE CORRELATION COEFFICIENTS
FOR UNIALGAL CULTURES

Algae	Correlation Coefficient, r	95% Confidence Interval, ρ	99% Confidence Interval, ρ
Anabaena	0.069	-0.42 - 0.48	-0.51 - 0.66
Microcystis	0.676	0.36 - 0.84	0.15 - 0.90
Selenastrum	0.236	-0.29 - 0.70	-0.47 - 0.75
Chlorella	0.152	-0.37 - 0.60	-0.53 - 0.71

Zooplankton Study

In order to determine the effect of zooplankton ATP
on the total measured ATP in natural plankton populations,
the ATP of zooplankton from a local lake was measured.
Zooplankton from Newnan's Lake was collected using a zoo-
plankton net. The zooplankton consisted almost exclusively
of Cyclops whose average dimensions were 1.4 mm by 0.3 mm.
Using these average dimensions and assuming a specific
gravity of 1.05, the ATP to dry weight ratio for zooplankton
was calculated to be 0.1 µg/mg. This value is slightly lower
than the average value reported in this research for algal
cultures and much lower than the average value reported for
bacterial cultures (1.5 µg/mg). Based on the lower ATP to
dry weight ratio of zooplankton and their relatively small
number in most lakes, it seems that the ATP in zooplankton
would not significantly add to the total ATP measured in a
lake. Thus, these results indicate that ATP from zooplankton
would not significantly affect the phytoplankton biomass
estimated using ATP concentration.

ATP - Response Results

The response of ATP to different environmental
conditions was measured to determine the usefulness of ATP
analysis as a rapid bioassay technique and to assess the
stability of ATP in reference to its application to
measurement of phytoplankton biomass. Laboratory algal

cultures and lake phytoplankton were subjected to varying
light periods, different pH levels, and additions of
nutrients and toxins.

Light-Dark Study

In order to be used as a reliable estimate of phyto-
plankton biomass, the cellular ATP concentration must not
vary under light and dark conditions. To determine whether
the cellular ATP concentration remained relatively constant
under light and dark conditions the following experiment
was performed. Aliquots of *Selenastrum* were placed alter-
nately in the light and the dark for a period of ten hours
and the ATP content was monitored. The response of ATP in
Selenastrum cells to light and dark periods is shown in
Figure 18. Fluctuations in the ATP content occurred when-
ever the algae were changed from either light to dark of
from dark to light. Within 15 to 20 minutes after each
transition the ATP concentration returned to the initial
ATP level. Holm-Hansen (1970) obtained similar results
working with marine algae, and found ATP concentrations to
be maintained at fairly uniform levels in both light and
dark. Holm-Hansen's experiment only covered a time span
of 22 minutes while the above mentioned experiment covered
a span of ten hours, indicating the long-term stability of
ATP under light and dark conditions. The ATP pool in algae
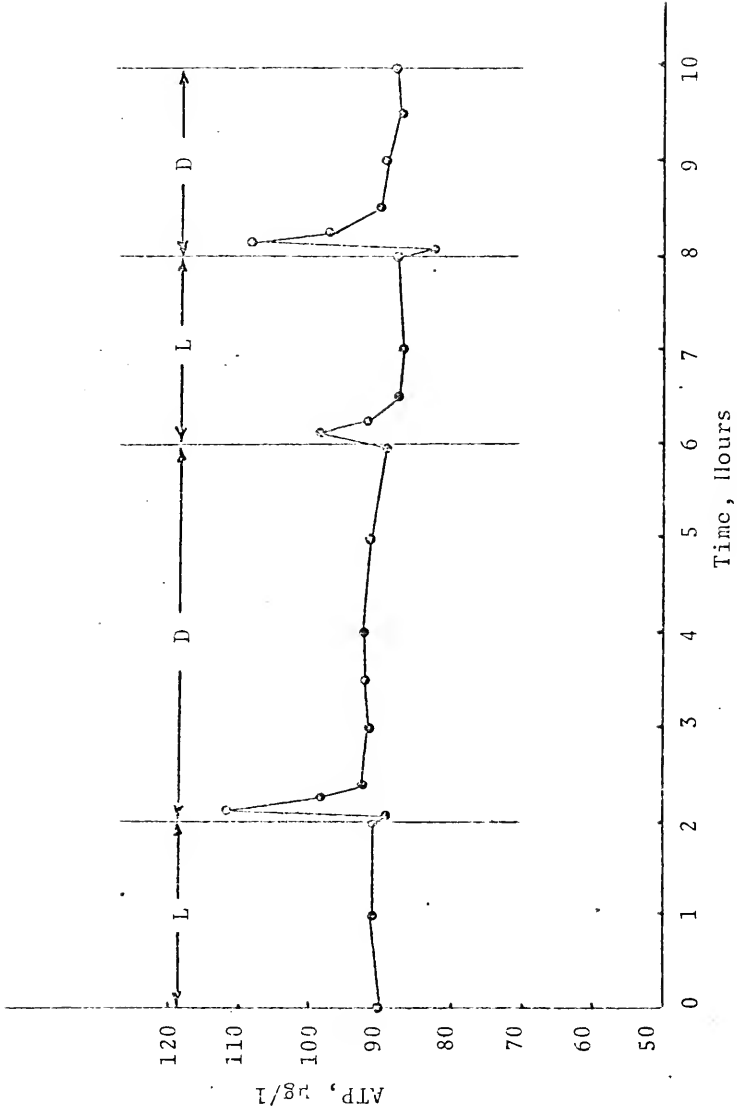is probably regulated by the interaction of the photosynthetic

FIGURE 18. RESPONSE OF ATP TO PERIODS OF LIGHT AND DARK

and respiratory mechanisms of the cell. ATP occurs in trace
amounts in the cell and has a rapid turnover rate. It is
probable that ATP is primarily regulated by respiration
rather than photosynthesis. In photosynthesis light energy
is used to form ATP which is almost immediately used form
simple sugars. These simple sugars enter the carbon cycle
and eventually supply all the energy needs of the cell.
However, the fluctuations in ATP levels observed at light
to dark transitions indicate a definite interaction and
dependence between photosynthesis and respiration.

pH Studies

The pH of natural waters is an important environ-
mental variable. The biota, productivity and solubilities
of nutrients and toxic substances are all somewhat dependent
on the pH of a specific water. To use ATP as a measure of
biomass, the relationship between ATP concentration and pH
must be known. Thus, the response of cellular ATP level to
different pH values was measured. Figure 19 shows the ATP
concentration measured in *Selenastrum* cells after incubation
for one hour at various pH levels. Maximum ATP concentration
occurred in the pH range of 7.5 to 8.0, the normal growing
range of the laboratory cultures. The ATP concentration
was greatly reduced in the pH range of 4.0 to 5.0. Moderate
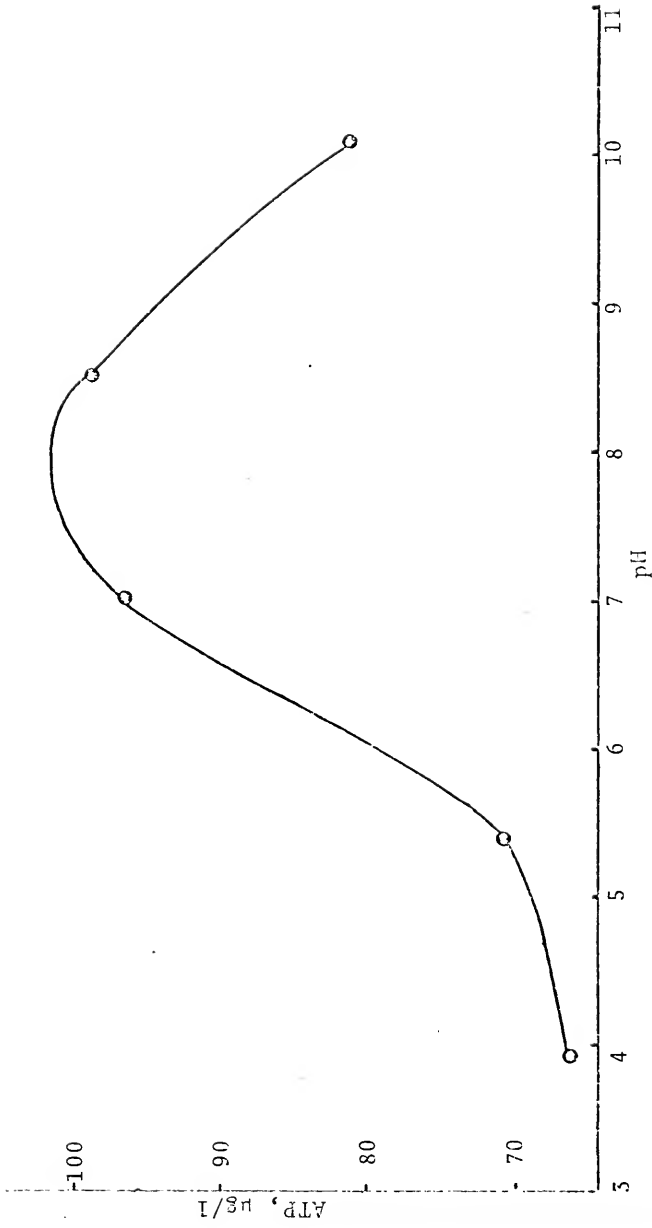reduction in ATP concentration occurred at a pH of 10.0.

FIGURE 19. EFFECT OF pH ON ATP CONTENT OF *SELENASTRUM* CELLS

The above experiment was repeated using lake water, in order to determine whether maximum ATP content always occurred within a neutral pH range such as pH 7 to 8, or whether the optimum ATP occurred at the normal ambient pH of the organisms. Figure 20 shows the ATP concentration measured in Anderson-Cue Lake water after incubation for one hour at various pH values. Maximum ATP concentration occurred at a pH of 4.6, the original pH of the lake water. The greatest reduction in ATP concentration occurred in the pH range of 9.0 to 11.0. A high pH lake water was tested in the same manner. Figure 21 shows the ATP concentration measured in Bivens Arm lake water after incubation for one hour at different pH levels. Maximum ATP concentration occurred at a pH of 9.0, the original pH of the lake water. Maximum reduction in ATP concentration occurred at a pH of 3.0.

It should be noted that the pH values reported are those of the incubation media or lakewater. It is probable that the cellular pH changed less than the media or lake water since the cell can control intracellular pH to some extent. The reduced ATP concentrations may indicate a reduction in viable cells or a shift in the energy balance of the cell as it attempts to maintain homeostasis. These results indicate that a shift from the organisms normal pH range causes a reduction in the ATP level, whether acidic, neutral, or basic.
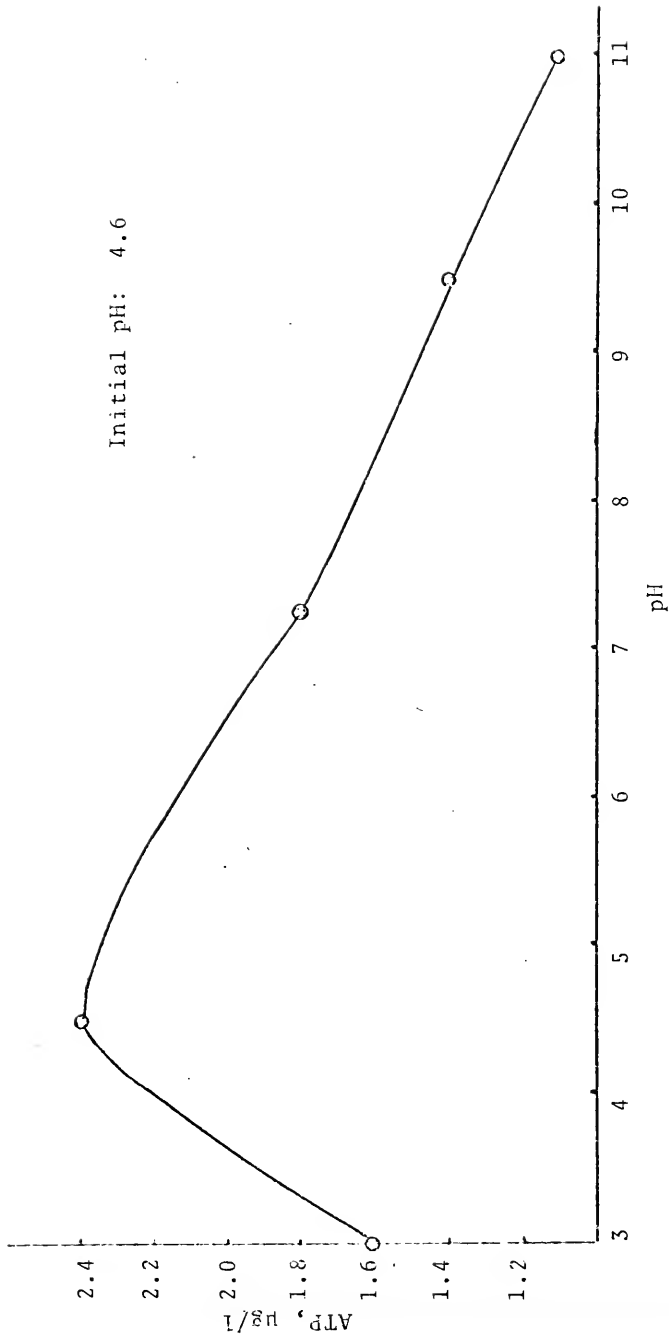
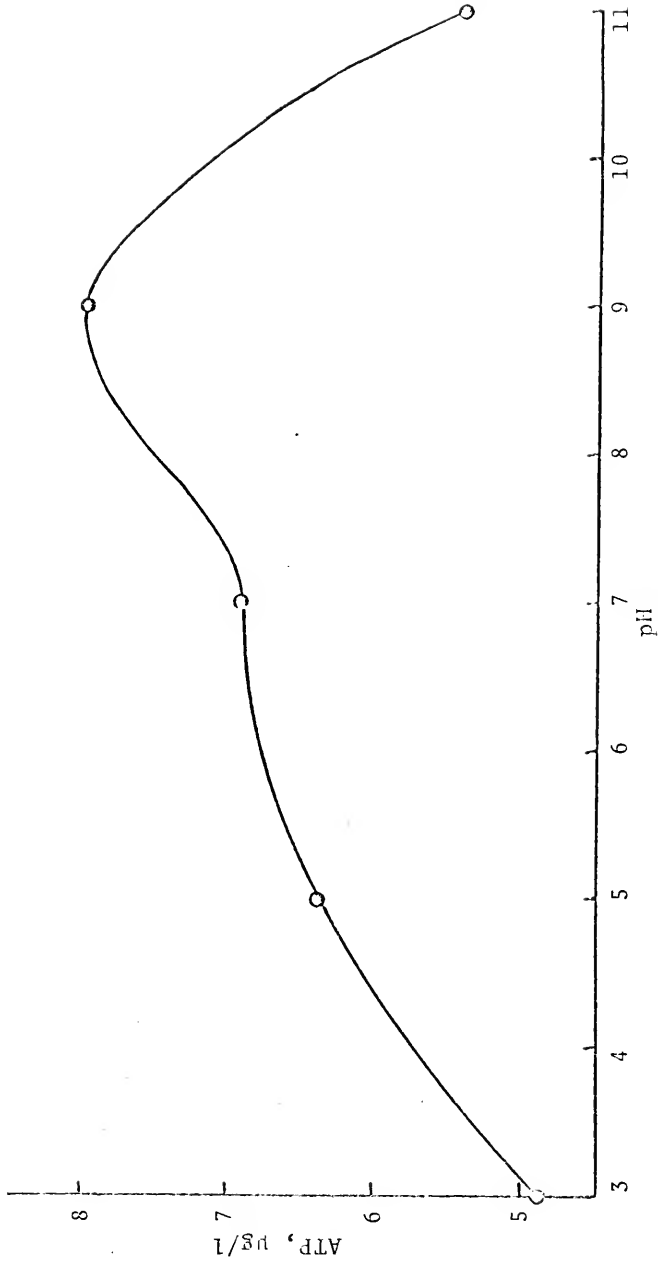FIGURE 20.   EFFECT OF pH ON ATP CONTENT OF ANDERSON-CUE LAKE WATER

Initial pH: 4.6

FIGURE 21. EFFECT OF pH ON ATP CONTENT OF BIVENS ARM LAKE WATER

Toxicity Studies

One of the greatest problems today is the intro-
duction of toxic materials into our waterways. In 1969
almost 70 percent of the reported fish kills were caused
by the discharge of industrial wastes. Wastes containing
concentrations of heavy metals may be toxic to aquatic
organisms and severely affect the water community. The
bioassay is an important tool in the investigation of
toxic materials because the results of such a study indicate
the degree of hazard to aquatic life of particular toxins.
From bioassay studies interpretations and recommendations
can be made concerning the level of discharge that can be
tolerated by the receiving water.

Mercury compounds are highly toxic to aquatic
communities. Mercuric chloride is used in disinfecting,
preserving, tanning, electroplating and many other processes.
Mercuro-organic compounds are used in herbicides, fungicides,
and medical treatment. They have been used extensively to
control slimes in paper mills. To study the effects of
mercuric ion on phytoplankton, aliquots of *Selenastrum* were
incubated with various concentrations of mercuric chloride
and the response of ATP, carbon-14 uptake, chlorophyll *a*,
and suspended solids was measured. Figure 22 shows the ATP
content in *Selenastrum* samples after three hours of incuba-
tion in the presence of various concentrations of mercuirc
chloride. The ATP toxicity pattern in Figure 22 shows a

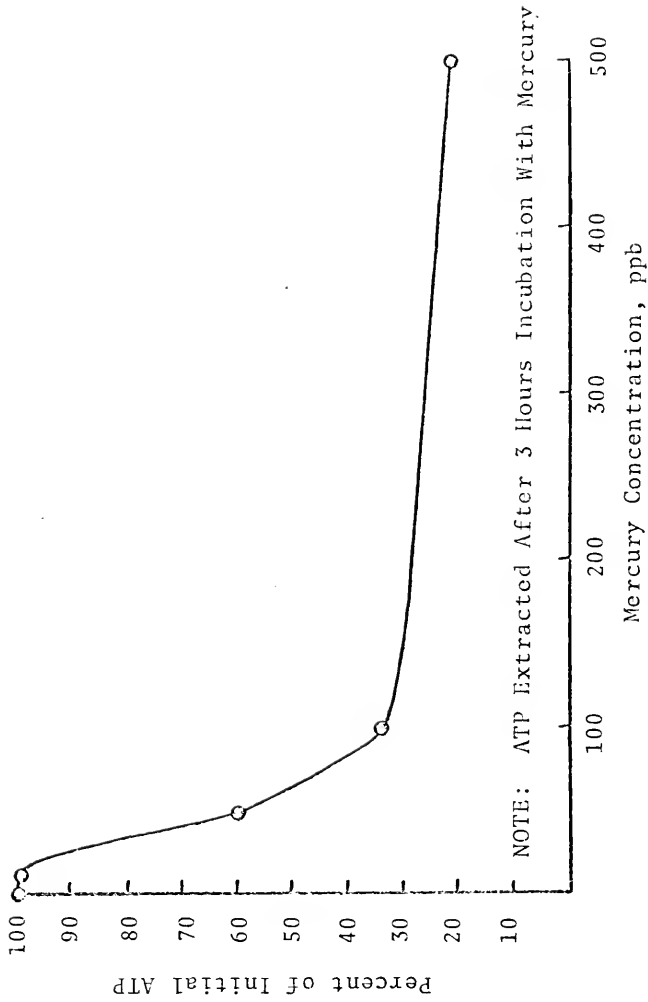NOTE: ATP Extracted After 3 Hours Incubation With Mercury.

FIGURE 22. ATP POOL RESPONSE TO INCUBATION WITH VARIOUS CONCENTRATIONS
OF MERCURY

rapid reduction of ATP for mercuric chloride concentrations of 50 ppb and 100 ppb, with a gradual further reduction for concentrations above 100 ppb. Table 15 shows the percent reduction in ATP, carbon-14 uptake, chlorophyll a, and suspended solids after one-hour incubation with 500 ppb of mercury.

TABLE 15

PERCENT REDUCTION AFTER ONE-HOUR INCUBATION WITH
MERCURIC CHLORIDE

Parameter	Percent Reduction
ATP	54
Carbon-14 Uptake	56
Chlorophyll a	20
Suspended Solids	10

From Table 15 it is apparent that ATP is a much more sensitive bioassay response parameter than chlorophyll a or suspended solids. However, ATP is about equal to or a little less sensitive than carbon-14 uptake as a response parameter.

The observed reduction in ATP may result from a reduction in the viable cells or it may result from a reduction in the cellular ATP pool. In an attempt to explain which mechanism was responsible for the reduction in ATP, a sample of *Chlorella* was incubated with 100 ppb of mercury for 80 minutes at which time half of the sample was centrifuged and the *Chlorella* cells were transferred to fresh PAAP media without mercury. The final volumes

of both samples were the same. As shown in Figure 23, the
ATP content of the cells transferred to fresh media immediately
increased from 104 µg/l to 192 µg/l while the ATP of the
sample with mercury remained constant. After five minutes
the ATP content of the transferred cells decreased to 125
µg/l, a concentration slightly below the initial concentra-
tion of the culture before addition of mercury. These
results indicate that most of the ATP reduction results from
a reduction in the cellular ATP pool rather than a reduction
in the viable cell population. The difference between the
initial ATP concentration (135 µg/l) and the final concentra-
tion of the transferred cells (125 µg/l) indicates that a
fraction of the algal cells were killed by the addition of
mercury. Eighty minutes was selected as the time to transfer
the poisoned *Chlorella* cells to fresh media because previous
experiments showed that maximum ATP reduction occurred in
the first 1 to 1 1/2 hours after addition of the poison. It
is probable that as the incubation time increases the
fraction of dead cells would increase. After two hours of
incubation the ATP content of the transferred cells decreased
slightly; this is probably a result of carry over of mercury
in the centrifuged cells.

Another important toxic material is copper. Copper
sulfate is used extensively in industry for tanning,
electroplating, engraving, and pigment manufacture. It is
also used to control undesirable plankton growth in reser-
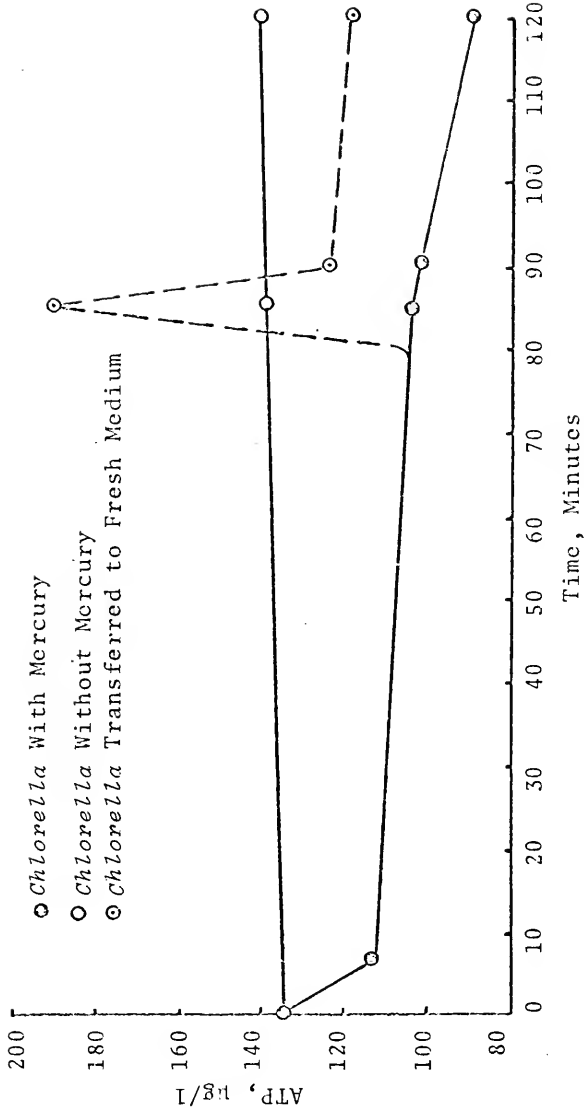voirs and lakes. In order to study the effect of copper on

FIGURE 23. RESPONSE OF MERCURY-POISONED *CHLORELLA* AFTER TRANSFER TO FRESH MEDIUM

natural phytoplankton populations, Bivens Arm water was
incubated with a concentration of 2 mg/l of copper sulfate.
Bivens Arm, at the time of sampling, contained a very dense
bloom of *Microcystis*. Figure 24 shows the response of ATP,
chlorophyll *a*, suspended solids, and COD to incubation with
copper sulfate. In the first three hours of incubation
the ATP content dropped by 45 percent while carbon-14 uptake,
suspended solids, chlorophyll *a*, and COD dropped 40 percent,
22 percent, 29 percent, and 21 percent respectively. The
ATP concentration decreased much more rapidly than all of
the other parameters with the exception of carbon-14 uptake.
Both ATP and carbon-14 uptake decreased rapidly after
addition of copper sulfate. The rapidity of the decrease
in ATP and its similarity to the decrease in carbon-14
uptake indicates that a decrease in cellular ATP occurs
initially rather than a decrease in living cells. These
results indicate that ATP analysis is a sensitive response
method that can be useful in routine toxicity bioassays.

Nutrient Studies

Besides bioassays for determining the effect of toxic
substances on aquatic communities, there is a need for
better limiting nutrient. Considerable controversy exists
today as to whether phosphorus, nitrogen, or carbon is the
primary limiting nutrient. These three nutrients were used
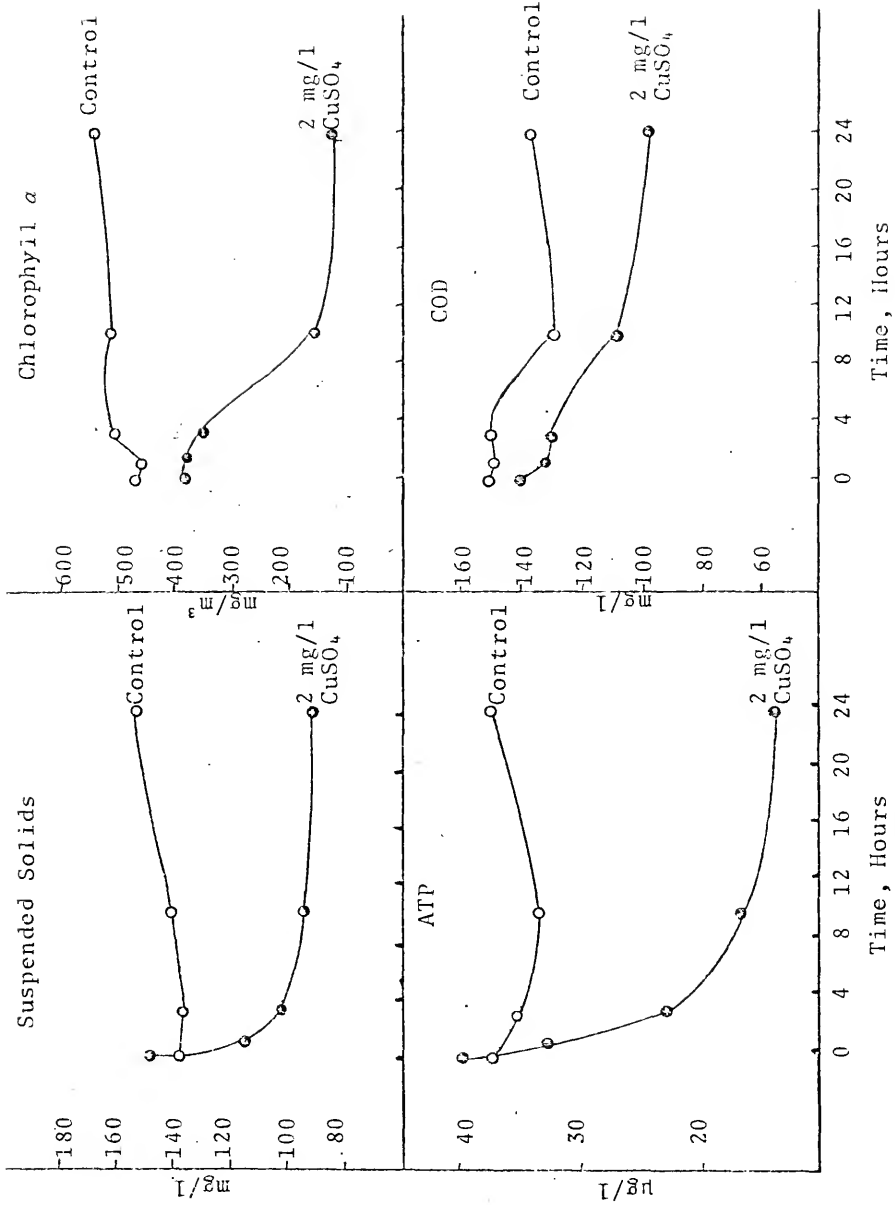to determine the usefulness of ATP analysis as a rapid
bioassay method.

FIGURE 24. RESPONSE OF BIOMASS PARAMETERS IN BIVENS ARM TO ADDITION OF COPPER

The effect of adding carbon dioxide to carbon-limited algae was determined by bubbling carbon dioxide gas into a batch culture of *Anabaena* and measuring the response in the ATP concentration. The *Anabaena* culture had been grown for three days on PAAP medium. Initially the pH of the culture was 7.6 but each day it increased until it reached 10.5 on the day of the experiment. At this pH the culture was carbon-limited, very dense, and a dull green. Past experiments indicated that carbon-limited *Anabaena* become dull green, but change to a bright green color approximately one day after adding carbon-dioxide. In this experiment the carbon-dioxide was bubbled into the culture for five seconds. The response of ATP to addition of carbon dioxide was dramatic as shown in Figure 25. Cellular ATP increased from 134 µg/l to 312 µg/l in three minutes. The ATP content was measured for 12 minutes and exhibited a tendency to rapidly increase and decrease. After six hours the ATP content still remained relatively constant at 312 µg/l. The significant increase in ATP concentration and its stability at the higher concentration may also indicate that the original sample at pH 10.5 may have been inhibited by the high pH. The higher ATP concentrations may be a result of increased cellular activity, utilizing the available carbon. Immediately after carbon dioxide addition the pH dropped to 5.8, but after six hours the pH increased to 8.6, indicating that the carbon was being utilized by the algae.
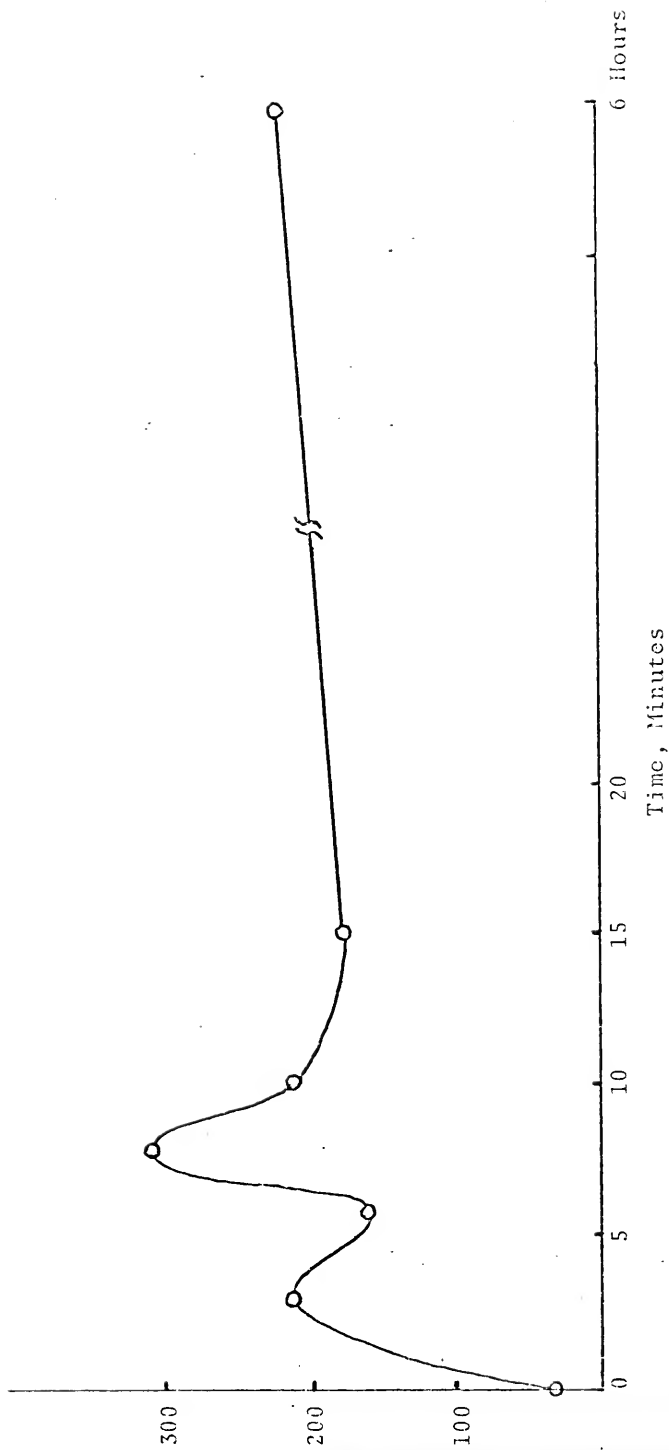
FIGURE 25. ATP RESPONSE IN *ANABAENA* TO ADDITION OF CARBON DIOXIDE

Another experiment on carbon dioxide addition was
performed with a natural population from Bivens Arm. The
Bivens Arm sample had a mixed algal population with a low
density. The initial pH of the sample was 9.0. Figure 26
shows the ATP response in Bivens Arm water to which carbon
dioxide was added. Like the algal culture, an initial
increase in ATP occurred. However, unlike the algal culture,
the ATP concentration decreased shortly thereafter and
continued to decrease, indicating inhibition or die-off of
the phytoplankton. After three minutes the pH dropped to
6.7 and after 23 minutes it dropped to 5.7. The decrease
in ATP is probably a result of the lowered pH of the lake
water. An increase in the pH to 7.6 was observed after
three hours, an indication that the lake water was recovering
from the carbon dioxide addition.

The response of ATP in phosphorus-deficient cultures
to the addition of phosphorus was also measured. Batch
cultures of *Anabaena*, *Microcystis*, *Selenastrum*, and *Chlorella*
were grown in duplicate for a period of five days. One
culture of each algal species contained regular PAAP medium
with 0.62 mg/l while the other culture contained a modified
PAAP medium without phorphorus. All cultures were relatively
dense having an absorbance ranging from 0.19 to 0.57. On
the fifth day 1.24 mg/l of phosphorus was added to each
culture and the ATP response measured. Table 16 gives the
percent increases of ATP after addition of phosphorus. All
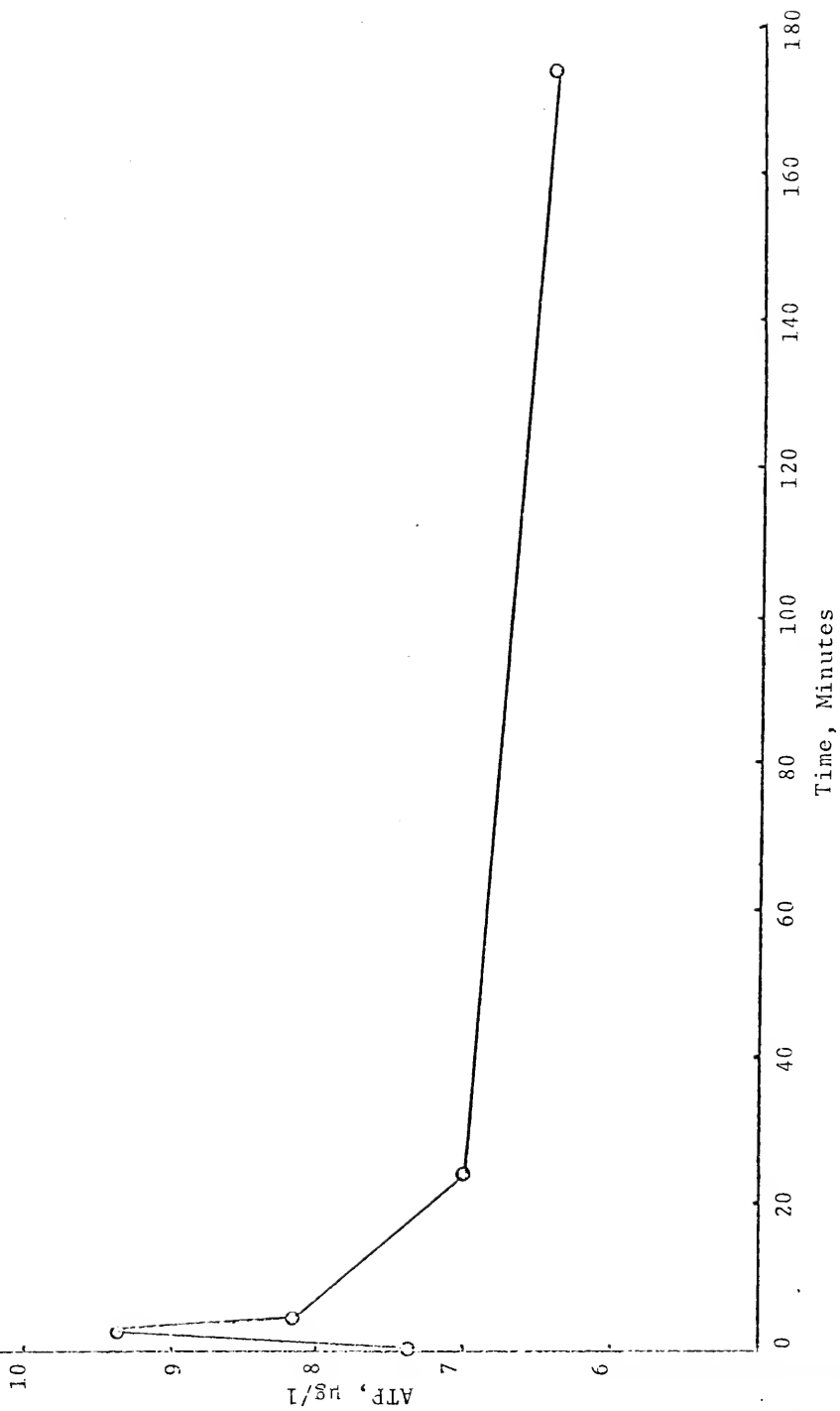
FIGURE 26. ATP RESPONSE OF BIVENS ARM TO ADDITION OF CARBON DIOXIDE

TABLE 16

RESPONSE OF CELLULAR ATP TO ADDITION OF PHOSPHATE

Algal Culture	Phosphate Concentration		Percent Increase in ATP[1]
	Initial	Final	
Anabaena[2]	.52	.12	10
Anabaena[3]	.12	.07	80
Selenastrum[2]	.28	.14	13
Selenastrum[3]	.05	.03	36
Chlorella[2]	.58	.12	5
Chlorella[3]	.02	.001	23
Microcystis[2]	.48	.03	12
Microcystis[3]	.16	.01	38

[1] Measured approximately 80 minutes after phosphorus addition

[2] Standard PAAP medium with 0.62 mg/1 phosphorus

[3] Modified PAAP medium without phosphorus

phosphorus-deficient cultures exhibited a greater ATP increase than the cultures with phosphorus. *Anabaena* showed the greatest difference in ATP response with the phosphorus deficient culture increasing eight times as much as the control culture. It is interesting to note that although both cultures of *Microcystis* had very low final concentrations of phosphorus, the ATP of the phosphorus-deficient culture increased approximately three times as much as the phosphorus-enriched culture. This would indicate that even though the phosphorus concentration of the phosphorus-enriched culture was low, the algal cells were not phosphorus limited, that is, at least not to the same extent.

The response patterns for each of the species are different as shown in Figures 27 to 30. *Anabaena* (Figure 27) exhibited an initial drop in ATP followed by an increase while *Microcystis* and *Selenastrum* (Figures 28 and 29 respectively) showed a rapid initial increase. *Chlorella* (Figure 30) displayed a gradual increase in ATP. All of the species, however, responded essentially in the same manner, that is, each exhibited an increase in ATP concentration.

To study the ATP response in nitrogen deficient cultures, *Anabaena* and *Selenastrum* were grown for five days in duplicate cultures. One culture contained PAAP medium with 14.0 mg/l of nitrogen as nitrate while the other contained a modified PAAP medium without nitrogen. On the
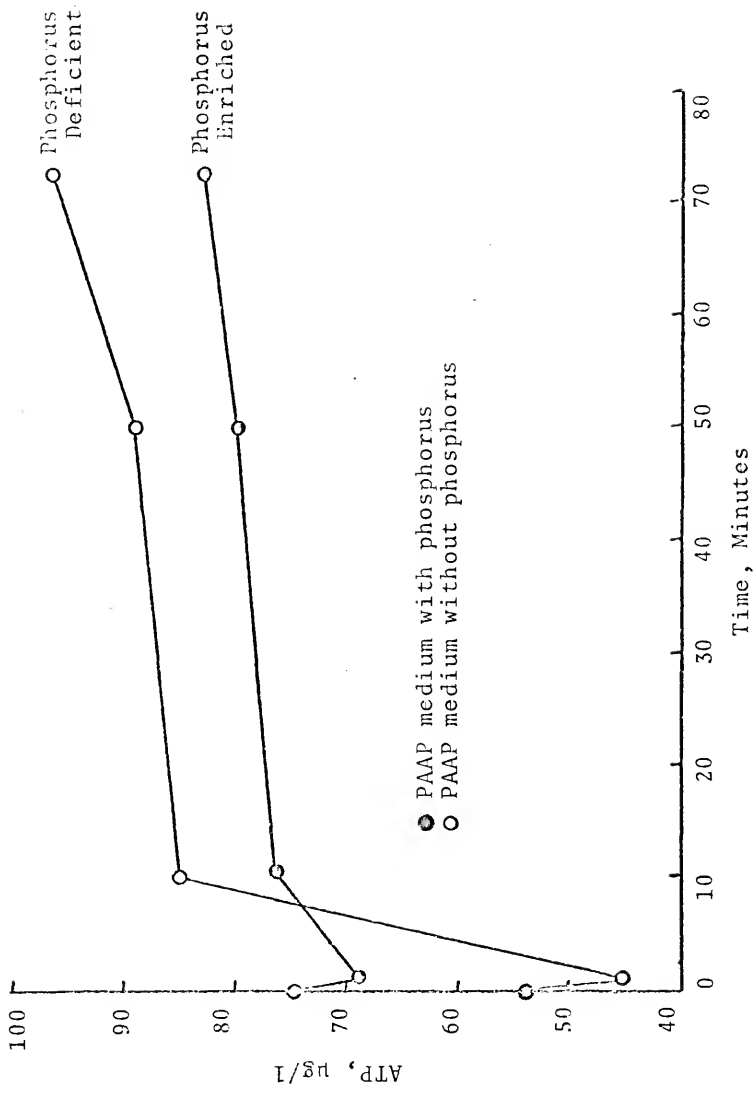
FIGURE 27. RESPONSE OF ATP IN *ANABAENA* TO ADDITION OF PHOSPHORUS
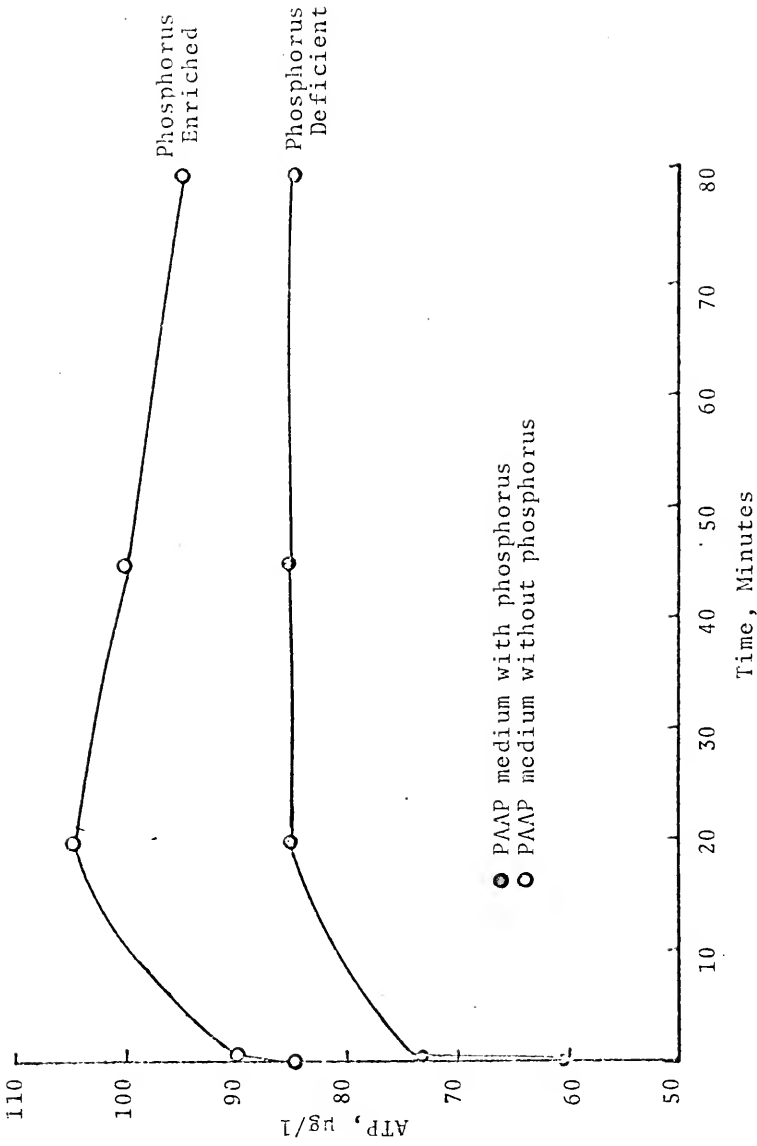
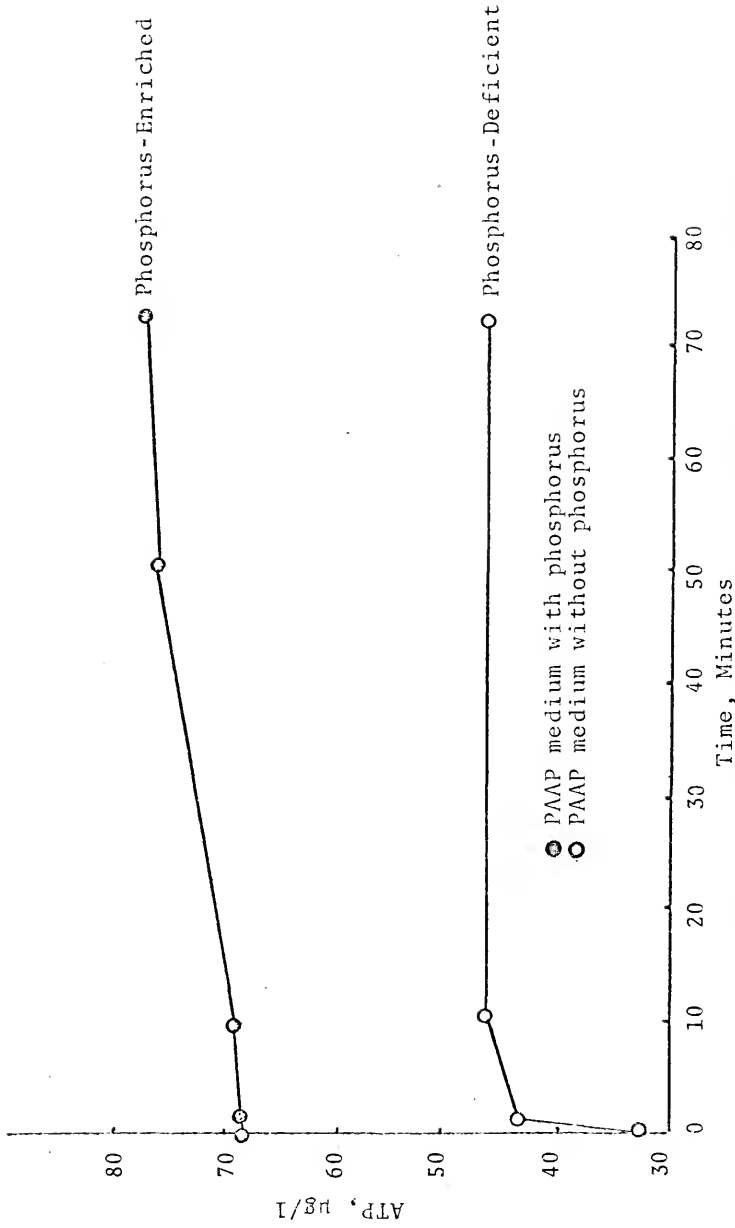FIGURE 28. RESPONSE OF ATP IN *MICROCYSTIS* TO ADDITION OF PHOSPHORUS

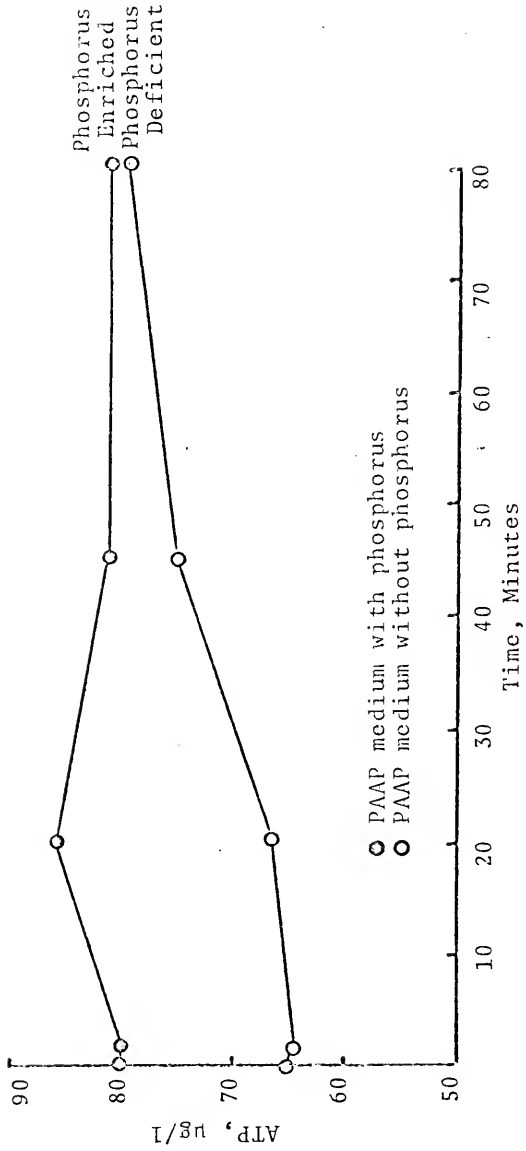FIGURE 29. RESPONSE OF ATP IN *SELENASTRUM* TO ADDITION OF PHOSPHORUS

FIGURE 30. RESPONSE OF ATP IN *CHLORELLA* TO ADDITION OF PHOSPHORUS

fifth day, 23 mg/1 of nitrogen as nitrate was added to each culture. Percent increases in ATP concentration after addition are given in Table 17. Like the phosphorus experiment, *Anabaena* exhibited the greatest difference in ATP response which is surprising because *Anabaena* is a nitrogen fixer while *Selenastrum* is not. The ATP response in nitrogen-deficient *Anabaena* was 46 times as great as that of the control. The ATP increase in the nitrogen-deficient *Selenastrum* was only twice that of the increase in the nitrogen-enriched culture. The response of the nitrogen-deficient *Anabaena* showed a gradual increase in ATP while the *Anabaena* control showed a rapid initial increase followed by a gradual decrease to approximately the original concentration (Figure 31). Both the nitrogen-deficient *Selenastrum* and the control responded rapidly as shown in Figure 32. Essentially, the ATP response to the addition of nitrogen was similar to that of phosphorus addition in that an increase in ATP concentration always occurred.

The percent increases in ATP concentrations reported here were calculated using the ATP values measured 70 to 80 minutes after the addition of nitrogen. This time interval was selected because the ATP content of both the nitrogen-deficient and the nitrogen-enriched cultures seemed to level off by this time. Different percent changes in ATP could be obtained simply by choosing a shorter or longer interval.
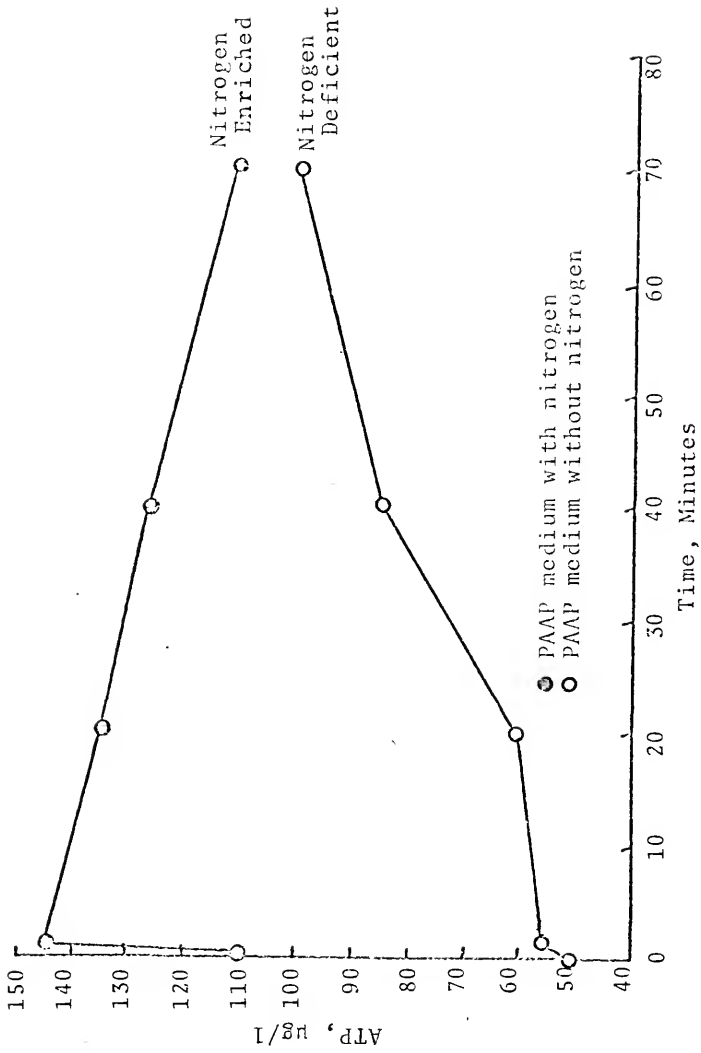
FIGURE 51. RESPONSE OF ATP IN *ANABAENA* TO ADDITION OF NITROGEN
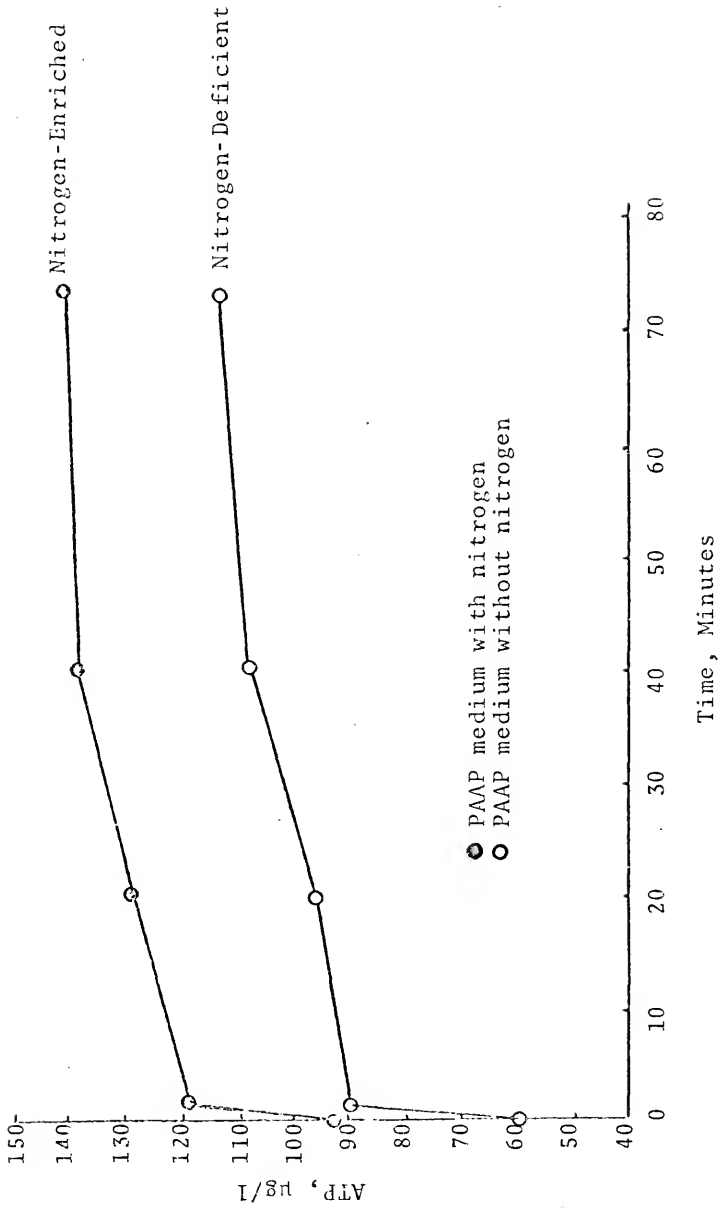
FIGURE 32. RESPONSE OF ATP IN *SELENASTRUM* TO ADDITION OF NITROGEN

TABLE 17

RESPONSE OF CELLULAR ATP TO NITRATE ADDITION

Algal Culture	Nitrate Concentration		Percent Increase in ATP[1]
	Initial	Final	
Anabaena[2]	6.70	1.4	2
Anabaena[3]	0.12	0.003	92
Selenastrum[2]	7.50	5.6	49
Selenastrum[3]	0.40	0.1	80

[1]Measured approximately 70 minutes after nitrogen addition

[2]Standard PAAP medium with 14.0 mg/l nitrogen

[3]Modified PAAP medium without nitrogen

For example, if the initial increase was used, the same response would be shown by both cultures in several instances.

This criticism is valid for the phosphorus experiments as well. The percent increases shown in Tables 16 and 17 for phosphorus and nitrogen respectively are presented merely to illustrate the differences observed in nutrient-deficient and nutrient-enriched cultures. The values presented in these tables indicate that ATP could be used as a rapid nutrient bioassay parameter. However, to be of practical value, the ATP response to nutrient addition should be measured over a period of a few hours and plotted against time. Plots of ATP vs. time would be a qualitative but relatively good tool in nutrient bioassays.

CHAPTER VII.   DISCUSSION

The experimental results presented in Chapter VII were briefly discussed as they were reported. In this chapter these results will be discussed in further detail and compared with work reported by other investigators.

Correlation of ATP with chlorophyll a for both unialgal samples and lake phytoplankton was good. The scatter of values around the linear regression line may be a result of changes in the cellular ATP pool but it is also likely that it may result from changes in cellular chlorophyll content. The lake samples were collected at different times of the day, during different seasons, and under different atmospheric conditions. Thus, the cellular chlorophyll may have varied diurnally. Ryther and Yentsch (1957) and Odum et al (1958) reported diurnal variations in chlorophyll concentrations. The chlorophyll concentrations may also have varied with nutritional state as reported by Fogg (1965) and others. Atmospheric conditions, such as a clear sky or an overcast sky, would also affect the chlorophyll concentration.

Holm-Hansen (1969) found good correlation between ATP and chlorophyll. He found that both ATP and chlorophyll gave good estimates of the cellular carbon in phytoplankton in seawater.

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The ratios of ATP to chlorophyll *a* ranged from 0.09
to 0.35 for unialgal cultures and from 0.09 to 0.69 for
lake phytoplankton. These values are in excellent agreement
with those reported by Holm-Hansen (1969, 1970). His
values ranged from 0.04 to 0.60 for five species of marine
algae and one species of freshwater algae, and from 0.22
to 0.36 for seawater phytoplankton.

Good correlation of ATP with dry weight of unialgal
cultures and lake phytoplankton was also observed. The
ATP to dry weight ratios ranged from 0.24 to 0.38 μg/mg for
unialgal cultures and from 0.12 to 0.96 μg/mg for lake phyto-
plankton. Holm-Hansen (1966) reported average ATP to dry
weight values ranging from 0.15 to 1.6 μg/mg dry weight
for various marine algae (Table 18).

TABLE 18

ATP TO DRY WEIGHT RATIO'S OF VARIOUS MARINE ALGAE
(Holm-Hansen 1966)

Organism	Average ATP/Dry Weight (μg/mg)
Skeletonema ccstatum	0.31
Amphidinium carteri	0.15
Dunaliella tertiolecta	1.5
Syracosphaera elongata	0.03
Monochrysis lutheri	0.28
Cyclotella nana	0.84
Ditylum brightwellii	1.2
Rhizosolenia sp.	1.6

Lee *et al* (1971) reported ATP to dry weight values of
Selenastrum capricornutum ranging from 1.4 to 3.4 µg/mg
dry weight.   These values are much higher than those
reported for this research.   However, the authors note that
they did not calculate the ATP to dry weight ratio after
four days of incubation since the biomass after four days
was known to consist of dead as well as living cell material.
Table 19 lists the ATP to dry weight ratios reported by
Lee *et al* (1971).   Three cultures were grown with three
different concentrations of phosphorus: 0.62 mg/1, 0.062
mg/1, and 3.1 mg/1.

TABLE 19

ATP TO DRY WEIGHT RATIO'S OF *SELENASTRUM* (Lee *et al* 1971)

Phosphorus Content (mg/1)	Incubation Time	ATP/Dry Weight (µg/mg)
0.62	4	3.1
	8	2.4
	16	0.7
	32	0.57
0.062	4	1.4
	8	0.47
3.1	4	3.4

The results in Table 19 indicate that the ATP to dry
weight ratio varies with nutritional state.   It is question-
able whether the decrease in the ATP to dry weight ratio
results primarily from a build-up of detrital material or
from a physiological change due to a decrease in nutrition.

Correlation of ATP with cell number was also good.
Holm-Hansen (1970) reported excellent correlation between
ATP/cell and organic carbon/cell. Lee *et al* (1971) reported
ATP contents ranging from 3.9 x 10^{-8} to 6.5 x 10^{-8} μg/cell
for *Selenastrum capricornutum* grown with 0.62 mg/1 phosphorus
and 1.9 x 10^{-8} to 2.1 x 10^{-8} μg/cell for *Selenastrum
capricornutum* grown with 0.062 mg/1 phosphorus.

Cellular regulation of ATP was demonstrated by
subjecting algal cells to alternating periods of light
and dark. The ATP concentration fluctuated but always
returned to its initial level. Santarius and Heber (1965)
showed that ATP increased rapidly when an isolated chloro-
plast was exposed to light. When placed in the dark, the
ATP decreased rapidly. They concluded that the ATP to ADP
ratio controlled respiration in plants. When photosynthesis
increases under high light intensity, it is believed that
photorespiration occurs to stabilize the cell (Lehninger
1970). According to Lehninger, photorespiration does not
occur in the mitochondria like regular respiration; it
seems to be a wasteful way of regulating cells which are
photosynthesizing too fast. Thus, evidence indicates that
ATP is regulated by an intricate balance between photo-
synthesis, photorespiration, and respiration.

The response of ATP to various incubation pH levels
indicates that cellular ATP decreases when an organism is
shifted to a pH level different than its normal level.

Patterson (1970) reported similar results for activated
sludge. He found the optimum ATP pool size occurred in the
pH range of 7.5 to 8.0, the normal operating range of the
activated sludge process.

Addition of toxic chemicals to unialgal cultures
and lake phytoplankton caused a rapid decrease in ATP
content. Patterson (1970) reported rapid decreases in ATP
of activated sludge incubated with various toxins including
nickel, chromium, chromate, mercury, copper, and cyanide.

Nutrient-deficient cultures of algae exhibited an
increase in ATP content when nutrients were added. Holm-
Hansen (1970) reported similar results for marine algae.
He found that the ATP content increased when nitorgen was
added to nitrogen-deficient cultures of *Monochrysis lutheri*
and *Skeletonema costatum*. He also reported an increase in
ATP content after addition of phosphorus to phosphorus-
deficient cultures of *Dunaliella tertiolecta* and *Monochrysis
lutheri*. Patterson (1970) reported an increase in ATP pool
size after addition of substrate to a starved activated
sludge. Brezonik and Patterson (1971) reported increased
ATP per cell upon addition of substrate to activated sludge.
The rapid increase in ATP content shown in Figures 27 to 32
indicate an increase in ATP per cell rather than an increase
in biomass. These results agree with those of Brezonik
and Patterson (1971).

CHAPTER VIII.   CONCLUSIONS AND RECOMMENDATIONS

Conclusions

It is difficult to evaluate ATP as a biomass para-
meter since it must be compared to current biomass para-
meters which, it is believed, are inadequate. There is no
absolute biomass parameter to which ATP can be compared.
However, working with the available biomass estimates,
some conclusions can be made.

Good correlation was observed between ATP and three
biomass parameters: chlorophyll a, dry weight, and cell
number. Cellular ATP did not vary under light and dark
conditions, and it was relatively constant under normal or
ambient pH conditions. It is proposed, therefore, that
ATP could be used as a biomass parameter in studies of
aquatic systems. One problem with ATP as a biomass para-
meter is that it is a measure of total biomass, not just
algal biomass. In measuring the ATP content of the unialgal
cultures and the lake phytoplankton, no account was taken of
the bacterial population. However, in many instances the
total biomass is as important or even more important than
algal biomass alone. The plankton community consists of
phytoplankton, bacteria (free-floating and attached to the
phytoplankton), and zooplankton. It seems reasonable that

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in pollution or trophic-state studies the biomass of the
total community is important, especially considering the
symbiotic relationship between bacteria and algae. Too
often, the phytoplankton, the bacteria, and the zooplankton
have been studied as separate entities when the total
community should have been studied.

If a distinction between algal and total biomass is
required, then the ATP to chlorophyll ratio can be
determined and a rough estimate of the autotrophic nature
of a water can be made. Taylor (1967) coined the term
"Autotrophic Index" as a measure of the self-feeding or
food-producing organisms. The "Autotrophic Index" is
defined as the ratio of biomass to chlorophyll. Although
the chlorophyll concentration varies considerably under
different conditions, it can be used to give a rough
estimate of the autotrophic level of a particular lake or
stream. Each water, however, because of differences in
color, turbidity, and nutritional state, should be monitored
to determine the average ambient ATP to chlorophyll ratio.
In many waters, the relative abundance of algae compared
to bacteria make the distinction between total biomass and
algal biomass almost insignificant.

The rapid response of ATP after the addition of toxic
substances indicates the feasibility of using ATP in toxicity
studies. The data presented in this thesis demonstrates
that cellular ATP responds almost immediately. It is

suggested that ATP is an excellent bioassay parameter for toxicity studies.

The response of ATP in nutrient-deficient cultures after addition of nutrients suggests that ATP could be used in limiting nutrient bioassays. However, the qualitative nature of the response must be considered. Like alkaline phosphatase activity, ATP activity is a relative measure of deficiency. Unlike alkaline phosphatase activity which is limited to phosphate-deficient cells, ATP activity can be used for many nutrients. It is suggested that ATP as a limiting nutrient bioassay parameter is an important tool which can be used in a qualitative fashion in nutrient bioassays.

Recommendations

The first recommendation of this report is that more theoretical work be performed to determine more about the role and response of ATP under different conditions and in different species. Chemostats should be used in order to maintain algal cultures in a defined state of growth.

Secondly, ATP analysis should be used in routine laboratory and field studies. ATP analysis has a great potential in research and in solving every day problems, but unless it is used in routine studies, it will never be anything but a potentially good tool.

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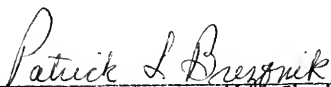Wetherell, D. F. 1958. Obligate phototrophy in *Chlamydomonas eugametos*. Physiol. Plantarum, 11: 260-274.

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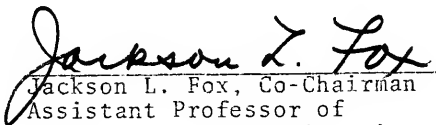 in the eutrophic zone. Limnol. Oceanog., 9: 519-524.

BIOGRAPHICAL SKETCH

Francis Xavier Browne was born October 11, 1943,
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Francis Xavier Browne is married to the former
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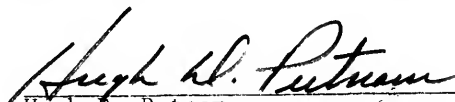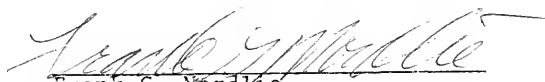and Oceanography.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Patrick L. Brezonik, Chairman
Associate Professor of
Environmental Engineering

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.
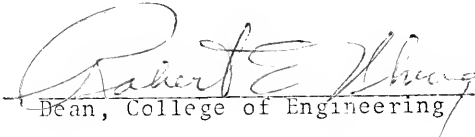
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Dean of the College of Engineering and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1971

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Dean, Graduate School