THE EFFECT OF ACIDIFICATION UPON THE BACTERIAL FLORA OF EXPERIMENTAL ENCLOSURES IN A SOFT WATER LAKE

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Robert D. Eloranta

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ABSTRACT

An attempt was made to characterize the bacterial populations in the water column of experimentally acidified enclosures in a soft water lake. The experiments were intended to assess the impact of acidification on aquatic heterotrophs, and to compare several methods of assessing heterotrophic activity. Size fractionated samples were analysed for adenylates in order to determine both relative standing crop from ATP concentrations and the physiological state from adenylate charge. Total bacterial numbers (epifluorescent counts) and bacterial production (dark $^{14}CO_2$ uptake) were also measured on several dates to determine if these methods yield comparable results. The bacterial standing crop, estimated from ATP, showed a bimodal response which was interpreted as an indication that several competing mechanisms regulate the bacterial standing crop.

Coincident bacterial standing crop maxima among the four acid treatments correlated well with periods of reduced solar irradiance. The negative correlation between solar irradiance and bacterial standing crop is attributed to direct inhibitory effects of visible light upon bacterial metabolism.

Useful adenylate charge datawere unobtainable; some difficulties are discussed.

Epifluorescent counts and heterotrophic $^{14}CO_2$ uptake were compared with ATP data. Disparity among the results of the methods is discussed.

INTRODUCTION

INTRODUCTION

The high incidence of acid rain has long been recognized as a serious environmental problem (Likens and Bormann 1974; Grahn et al. 1974; Dickson 1975; Dillon et al. 1977; Wright et al. 1975; Wright and Gjessing 1976). The acid nature of rain has been attributed primarily to the strong acid anions sulfate, nitrate, and chloride (Likens and Bormann 1974). Although these anions are naturally present in small amounts, the high levels associated with acid rain are from anthropogenic sources (Likens and Bormann 1974).

The most abundant anion in acid rain, sulfate, is produced during the combustion of fossil fuels containing sulfur and in the smelting of pyrite minerals in mining operations. The sulfur is released as sulfur dioxide which is further oxidized to sulfate in the atmosphere (Calvert 1976; NRCC 1977).

Nitrate is the second most prevalent anion in acid rain (Likens and Bormann 1974). It results from the atmospheric oxidation of nitrous oxides which are produced from both endogenous and atmospheric nitrogen during high temperature combustion (Gorham 1976).

Chloride is generally a less important anion in acid rain except in some areas of Great Britain where some coal reserves contain, in addition to sulfur, significant amounts of chloride (Gorham 1976).

The acidic anions released into the atmosphere are dispersed and distributed by air mass transport. The highly hydroscopic anions are then precipitated out of the atmosphere as acid rain, frequently at a

great distance from the source. Transport of acid pollutants across international borders often results in considerable economic and political damage (Odén 1976; Gorham and Gordon 1960; Royal Ministry for Foreign Affairs, Sweden 1971). The soft water lakes of the Precambrian shield areas of Scandinavia and Canada are particularly susceptible to the deleterious effects of acid rain due to their proximity to sources of acid pollutants and the low buffering capacity of podsol soils. These areas receive much of their yearly precipitation (and acid pollutants) in the winter snow. Acid anions accumulate in the snow pack until early spring when they are released in the first melt water (Schofield 1965). This phenomena concentrates a high portion of the yearly input of acid anions into a small volume of extremely acid run-off. This spring pulse of acid results in acute acid pollution and may be responsible for much of the ecological damage caused by acid rain.

The very acidic spring melt water also mobilizes toxic metals from soils by cation exchange. The aluminum concentrations observed in spring melt water are high enough to account for the spring fish kills observed in acidified streams (Schofield 1965). Thus the heavy metals mobilized from soils or lake sediments (Schindler et al. 1977) are a major factor contributing to the acidification problem.

Soft water lakes of the shield areas demonstrate reduced alkalinity and pH, altered chemistry, and disrupted ecosystems during the course of acidification (Hendrey et al. 1976); Gorham and Gordon 1960; Beamish

et al. 1975). A loss of productivity (oligotrophication) in acidified lakes has been reported (Grahn et al. 1974). The mechanism of this trend is, as yet, uncertain. Grahn et al. (1974) have suggested that it may be a consequence of reduced bacterial-mediated nutrient cycling.

Location

The Canadian Department of Fisheries and the Environment through the Freshwater Institute at Winnipeg, Manitoba, has established the Experimental Lakes Area (ELA) to study ecosystem response to pollutants by conducting whole lake experiments (Johnson and Vallentyne 1971). ELA is located 35 miles southeast of Kenora, Ontario, on the Canadian Precambrian Shield.

Recognizing acidification as a serious environmental problem in Canada, ELA has initiated a long-term lake acidification experiment (Schindler et al. 1977). Lakes 223 (Fig. 1) and 224, small oligotrophic soft water lakes, have been selected as experimental and control lakes respectively. Lake 223 is being acidified with sulfuric acid at the rate of 0.5 pH units per year to mimic acid rain-induced lake acidification. During the course of acidification extensive studies of lake biota and chemistry have been designed to document changes and offer insight into the problems associated with lake acidification in areas of high sulfur emissions such as those that occur near Sudbury, Ontario, and that are anticipated near the Alberta Oil Sands.

As a supplement to the whole lake experiment, experimental enclosures (Fig. 2) 10 meters in diameter and 2 meters deep were

Figure 1. Bathymetric map of Lake 223 showing the location of the experimental enclosures, upper left. From Schindler *et al.* (1977).

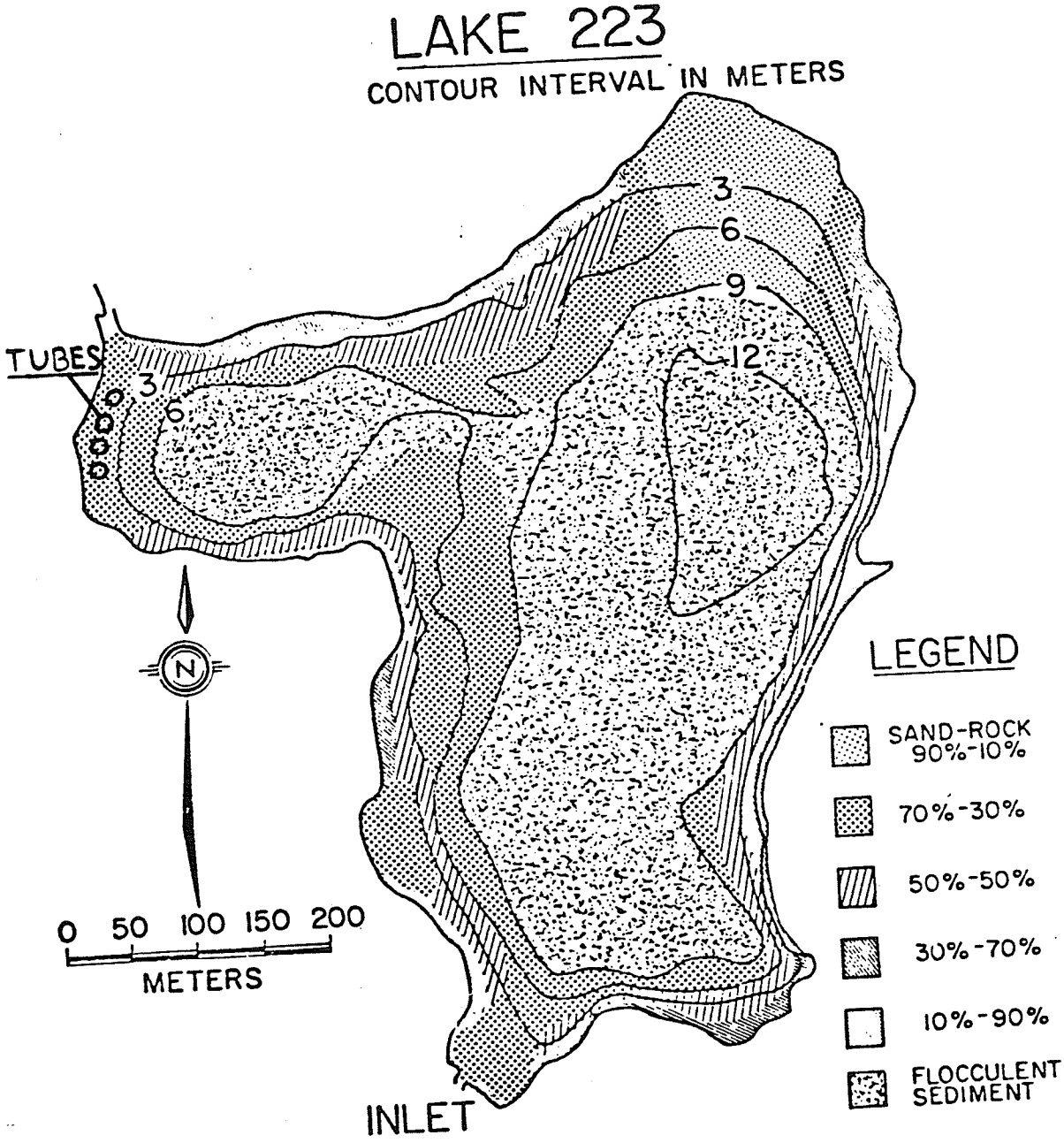
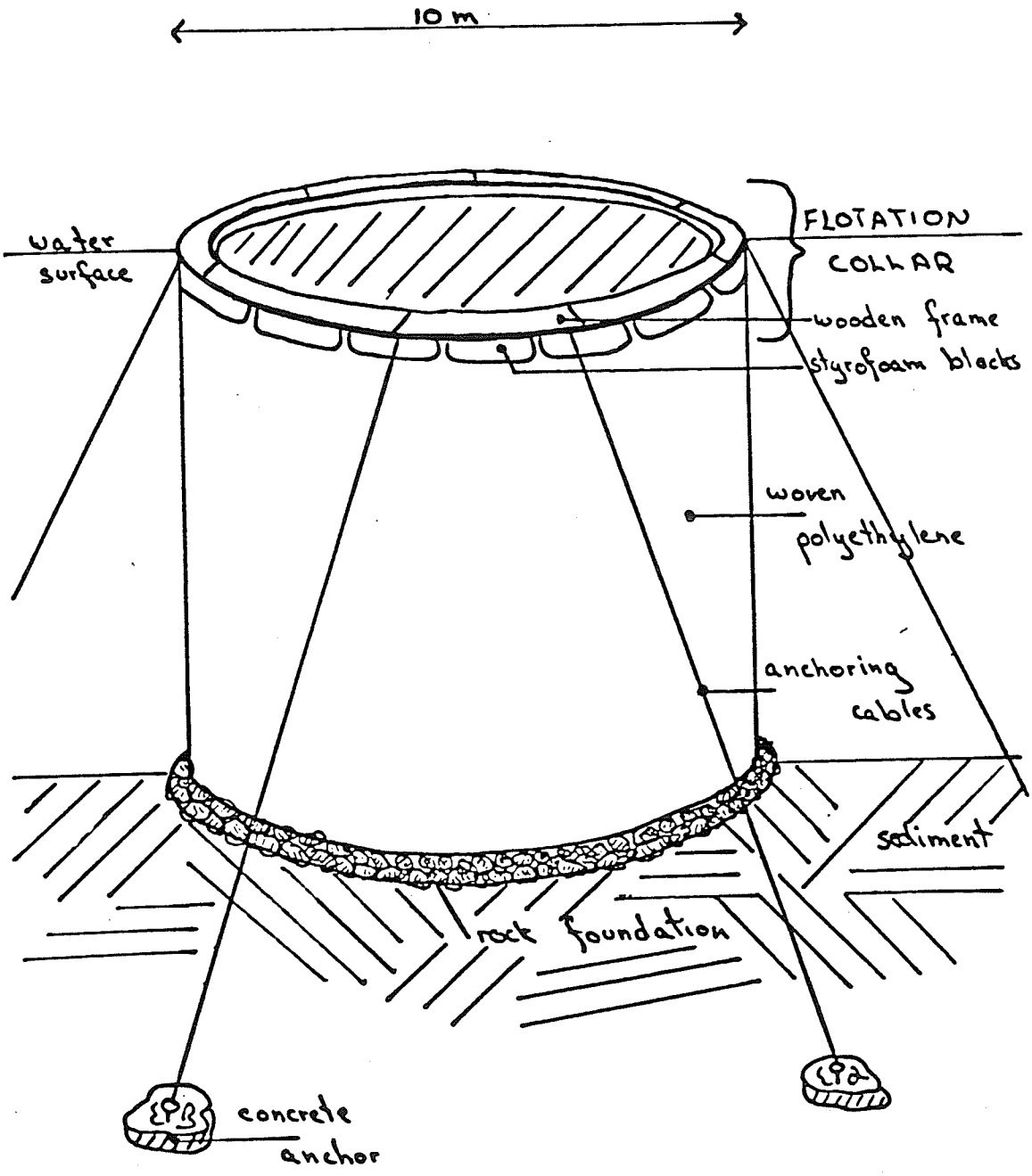

LAKE 223
CONTOUR INTERVAL IN METERS
3
6
9
12
TUBES
3
6
N
0 50 100 150 200
METERS
INLET
LEGEND
SAND-ROCK
90%-10%
70%-30%
50%-50%
30%-70%
10%-90%
FLOCCULENT
SEDIMENT

Figure 2. Details of experimental enclosures designed to mimic later stages of lake acidification. From Schindler *et al.* (1977).


10 m
water surface
FLOTATION COLLAR
wooden frame
styrofoam blocks
woven polyethylene
anchoring cables
sediment
rock foundation
concrete anchor

constructed and titrated with acid to simulate communities at pH 4.0 (tube D), pH 5.0 (tube B), pH 6.0 (tube C), and control pH 6.6 (tube E), no treatment.

It was expected that these tubes would provide an opportunity to observe bacterial heterotrophs in communities at pH conditions similar to those which occur during later stages of lake acidification.

Tube Effects

Comparisons of experimental enclosures with whole lakes have demonstrated significant effects of enclosure (Jones 1973). Increased epiphytic growth and altered rates of nutrient feedback from the sediments make extrapolation to whole lakes difficult. Furthermore, the large acid additions which were at times necessary to achieve and maintain the experimental pH levels would only occur in special circumstances in natural systems (i.e. spring melt water).

The tubes are intended only to represent similar habitats differing only in pH. It is recognized that differences in sediments underlying the tubes may result in some unknown variability due to differential rates of nutrient feedback from the sediments. Ideally, all acid treatments should be performed on replicate tubes to assess the role of sediment variability. However, the resource investment required to construct replicate tubes was beyond the scope of this study.

HISTORY

HISTORY

Bacterial Response to Acidification

The effects of low pH on the growth of pure cultures of bacteria and other heterotrophs have been studied (Buchannan and Fulmer 1930). The results of these pure culture studies, though useful in a general sense, cannot be used to predict bacterial tolerance of acid *in situ* due to the complex interactions in the aquatic ecosystem.

The inhibitory effects of low pH may be more pronounced in the lake environment than in culture due to synergistic interactions between environmental stress and acidification. Competition with non-bacterial heterotrophs, not present in pure culture, can be expected to reduce the pH range over which bacteria successfully compete *in situ*. For example, some heterotrophic fungi are more tolerant of acid conditions than bacteria (Doetsch and Cook 1973). This competitive advantage of the strictly aerobic fungi at low pH is also enhanced by the lack of oxygen demand resulting from reduced bacterial growth and respiration.

Although pure culture studies may estimate a minimum pH tolerance, the inability to culture many forms of aquatic bacteria (Jannasch 1970) requires that we generalize, assuming that the response of aquatic heterotrophs resembles that of other bacteria studied. Pure culture studies show that bacteria generally display optimal growth in neutral to slightly alkaline conditions (Stanier *et al*. 1963). The lower limits of growth of heterotrophic bacteria fall between pH 4 and 5 although

some acidophiles grow at pH 1 (Brock 1969; Doetsch and Cook 1973).

Studies of nutrient enriched acidified lake water demonstrate a variable response dependent on the substrate chosen and a succession to fungi which became dominate after several weeks incubation. In short term studies mixed heterotrophic cultures obtained from acidified environments demonstrated tolerance of acidification to pH 3.5 to 4.5 when grown on soluble labile substrates (Bick and Drews 1973; Hendrey et al. 1976). Exponential growth on glucose or glutamic acid at pH 3.5 was nearly equal to the rate observed in the neutral control although growth at the low pH was preceded by an extended lag period (Hendrey et al. 1976). Cultures incubated at low pH demonstrated a succession from bacterial to fungal decomposers, the latter becoming dominant after several weeks (Bick and Drews 1973; Hendrey et al. 1976). Bacterial glucose uptake in water overlying acidified sediment cores at pH 5 was only 2 percent of the rate observed at pH 6 in these fungal-dominated environments (Hendrey et al. 1976). Artificially prepared cores with equal concentration of substrate also displayed a succession to fungal decomposers and a similar reduction in bacterial glucose uptake at pH 5 relative to pH 6 (Hendrey et al. 1976).

Bacterial utilization of more recalcitrant particulate substrates is more sensitive to acidification than metabolism of soluble substrates. Hydrolysis of cellulose by soil bacteria has a pH optimum of 6.5 to 8.0 with increasingly acidic conditions favoring cellulytic fungi (Doetsch and Cook 1973). Hendrey et al. (1976) found the rate of decomposition

of homogenized birch leaves, inoculated with activated sewage sludge, to be highly dependent upon pH. Decomposition, estimated by respiration, at pH 5.2 was only one-half the rate observed at pH 7.0. Hendrey et al. (1976) found similar reductions in respiration rates of sediment cores as the pH of overlying water was reduced.

Bacteria show a variable reaction to acidification which appears to be dependent on the substrate and competition with fungi. Soluble sugars and amino acids tested support bacterial growth to pH 4 and below, whereas bacterial degradation of cellulose was significantly inhibited below pH 5.5. Extended incubations with all substrates demonstrated a succession to fungal decomposers with a resulting reduction in mineralization rates.

Field observations of acidified lakes confirm the results of laboratory studies. The accumulation of coarse detritus in lake sediments has been attributed to a reduction of decomposer activity (Almer et al. 1974; Grahn et al. 1974; Hendrey et al. 1976; Levistad et al. 1976; Wright et al. 1975; Hendrey and Wright 1976). These studies cite dense fungal mycelia and reduced oxygen consumption in the sediments as evidence of the succession to fungal decomposers. The inability of bacterial decomposers to compete with fungi at low pH is therefore believed to result in reduced nutrient cycling and the loss of productivity observed in acidified lakes.

Methods of Assessing Bacterial Activity

The bacterial populations of aquatic ecosystems are composed of many physiological types, and as a consequence no single technique can

adequately characterize the total bacterial activity. However, a number of methods have been developed, each of which focuses upon a specific aspect of the population. Methods of assessing bacterial activity fall into three general categories: metabolic indicators, enumeration techniques, and detection of cellular constituents. The selection of a specific technique depends upon the nature of the questions to be answered and the character of the population.

The metabolic activity of a population may be estimated by following a key step in metabolism, such as substrate uptake or electron transport. The heterotrophic uptake methods first used by Parsons and Strickland (1962) are best suited to situations where the primary energy source of the population is known. Heterotrophic uptake methods may estimate either the in situ rate of substrate uptake (kinetic analysis), or the rate of substrate uptake at saturation (heterotrophic potential). The primary problems with this approach are the difficulty in selection of a substrate used by all organisms present (Wright 1973) and an unexplained inability to demonstrate a kinetic response in oligotrophic waters (for example: Hamilton and Preslan (1970)).

Bacterial heterotrophs are known to fix small amounts of inorganic carbon during growth (Wood and Strjernholm 1962). Estimates of bacterial production based upon heterotrophic $^{14}CO_2$ uptake assume that the inorganic carbon fixed represents a small and relatively constant portion of total carbon assimilated in aerobic environments (Romanenko 1964). However Tuttle and Jannasch (1977) have demonstrated the

existence of mixotrophic organisms which display increased rates of heterotrophic $^{14}CO_2$ uptake in the presence of thiosulfate in aerobic marine environments. The inability to establish a meaningful conversion factor limits heterotrophic $^{14}CO_2$ production methods to environments where autotrophic and mixotrophic organisms can be assumed to be absent, a tenuous assumption in many cases, or to comparative studies where the conversion to total heterotrophic production is unnecessary.

Methods estimating electron transport, biological oxygen demand (BOD) or electron transport system (ETS) (Packard 1969) activity generally are well suited to productive systems only. The extended incubations required to develop observable changes in oxygen for the BOD or accumulation of reduced dyes in the ETS method produce unacceptable bottle effects in oligotrophic systems (Jannasch 1970).

Enumeration of bacteria may be achieved with cultural methods or microscopic counts. Cultural methods are inadequate for estimates of total numbers because many groups of aquatic bacteria cannot be cultured (Jannasch 1970).

Direct counting methods are confounded by the inability to distinguish viable bacteria from non-viable bacteria and bacteria-sized detritus present in samples from aquatic communities.

Epifluorescent counts of samples treated with vital stains provide a means of distinguishing actively growing bacteria from senescent bacteria and bacteria sized detritus (Hobbie et al. 1977). Epifluorescent counting is a potentially valuable tool although restricted to

facilities which possess the specialized microscopic equipment.

A number of cellular constituents have been used to estimate bacterial biomass. This approach assumes that the constituent is present as a constant percentage of total biomass or is related directly to the activity of the bacterial cell. The constituent should be unique to living organisms and degrade rapidly upon cell death. It would also be desirable to use a constituent unique to bacterial cells to avoid interference from other organisms.

ATP has been used extensively to characterize bacterial populations despite its inability to satisfy completely all of these criteria. The intracellular ATP pools of bacteria fluctuate with the temporary metabolic state of the cell (Forrest 1965) so conversion of ATP concentration to biomass is possible only in situations where the population is in a known and constant growth state (Jannasch 1970).

The ATP method of estimating bacterial activity and/or biomass was originally developed for the detection of bacteria in the deep sea where photosynthetic organisms were absent (Holm-Hansen and Booth 1966). Enumeration of bacteria in the euphotic zone requires physical separation of bacteria and interfering algae.

However, despite the difficulty of comparing ATP concentrations to biomass estimates and the inherent difficulty of separating bacterial and algal ATP, the method has been used extensively in environmental studies.

The strength of the ATP method lies in the simplicity and

sensitivity of the ATP assay based upon the firefly bioluminescent reaction. Inexpensive crude enzyme preparations yield reproducible results at concentrations as low as 0.5 ng per ml extract when assayed with a liquid scintillation spectrophotometer (Rudd and Hamilton 1973). The sensitivity of the technique may be increased by several orders of magnitude by using specialized ATP photometers, aged crude enzyme preparations (Jones and Simon 1977) and luciferin additions (Karl and Holm-Hanson 1976).

Measurement of ATP in oligotrophic waters without specialized equipment requires that the samples be concentrated by filtration. Filtration has been reported to reduce the observed ATP to about one-half that observed with direct extraction without filtration; however, the extraction efficiency of filters is reproducible allowing its use for comparative purposes (Jones and Simon 1977). The low apparent extraction efficiencies reported by Jones may be due in part to dissolved ATP which causes overestimates in the direct injection method (Azam and Hodson 1977). The ATP losses observed during filtration appear to be due to enzymatic degradation of ATP to ADP and AMP (Karl and Holm-Hansen 1977). These investigators found that although ATP concentrations decreased during filtration, the total adenylate (AMP + ADP + ATP) pool remained constant.

Since ATP is not unique to bacteria, interference by non-bacterial ATP is troublesome. Size fractionation may be used to separate bacteria and algae if they are sufficiently different in size. Numerous studies

have used filters with pore sizes from 1 to 10 μm to exclude algae from the filtrate (Rudd and Hamilton 1973; Burnison 1975; Harrison et al. 1977). The selection of pore size is dependent upon the size of the algae present in the sample. Size fractionation assumes that the bacteria or the substrates to which they are attached are smaller than the algae present. In many environments bacteria appear to be free living and partition themselves into the fraction less than 1 μm during size fractionation (Burnison 1975; Harrison et al. 1977). Some investigators (Paerl 1975; Goulder 1977), however, have found bacteria intimately associated with larger particulate material. Thus bacteria appear to be associated with detritus in estuaries and other turbid environments and free-living in waters low in suspended particulate (Wiebe and Smith 1977). Therefore size fractionation is only appropriate in environments low in suspended detritus.

The adenylate charge or energy charge (E.C.) described by Atkinson (1969) provides a means of estimating the growth state of a natural community (Wiebe and Bancroft 1975). The energy charge is calculated from the concentrations of ATP, ADP and AMP by the following equation:

$$E.C.= \frac{ATP + \frac{1}{2} ADP}{ATP + ADP + AMP}$$

The basis for the energy charge concept lies in the role of these adenylates in the regulation of key enzymes in metabolism (Atkinson 1969). Activities associated with growth such as protein synthesis and DNA synthesis are observed during conditions of high energy charge (0.8-0.9),

whereas low energy charge values (0.5-0.7) occur during periods of very slow growth or senescence (Chapman et al. 1971).

Measurement of energy charge presumably could shed light on a long-standing controversy of environmental microbiology. Are the organisms present in natural waters growing or are they just senescent populations? If results showed that the energy charge of samples from natural waters was clearly above or below the lower limit for growth observed by Chapman et al. (1971), it would provide the substantial evidence necessary to resolve the issue.

Wiebe and Bancroft (1975) applied the method to natural marine communities and concluded that natural populations are growing. However, they assumed that filtration stress had caused an underestimate of the energy charge. Without this assumption the results remain controversial, the energy charge values clustered about 0.6 to 0.7 just below the threshold of growth defined by Chapman et al. (1971). More recent work by Karl and Holm-Hansen (1977) employing more sensitive equipment and methods has demonstrated the existence of metabolically-active bacterial populations in some areas, such as the oxic-anoxic interface at 100-125 m in the Black Sea.

At its present state of development the adenylate charge still appears to be useful as an indicator of gross differences in the metabolic state of populations.

METHODS

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Sampling

All water samples for ATP analysis were collected in the early morning, within 15 minutes of sunrise. Samples were collected in 750 ml Nalgene^{R} screw top bottles which had been twice washed with distilled water and inverted to dry. The bottles were again rinsed with a small volume of sample immediately before sample collection. Manually-integrated samples were drawn with a portable peristaltic pump by slowly lowering and raising a weighted intake hose through the water column. Care was taken to avoid sampling both sediment flock and surface films. After collection, samples were packed in an insulated back pack for transport back to the laboratory.

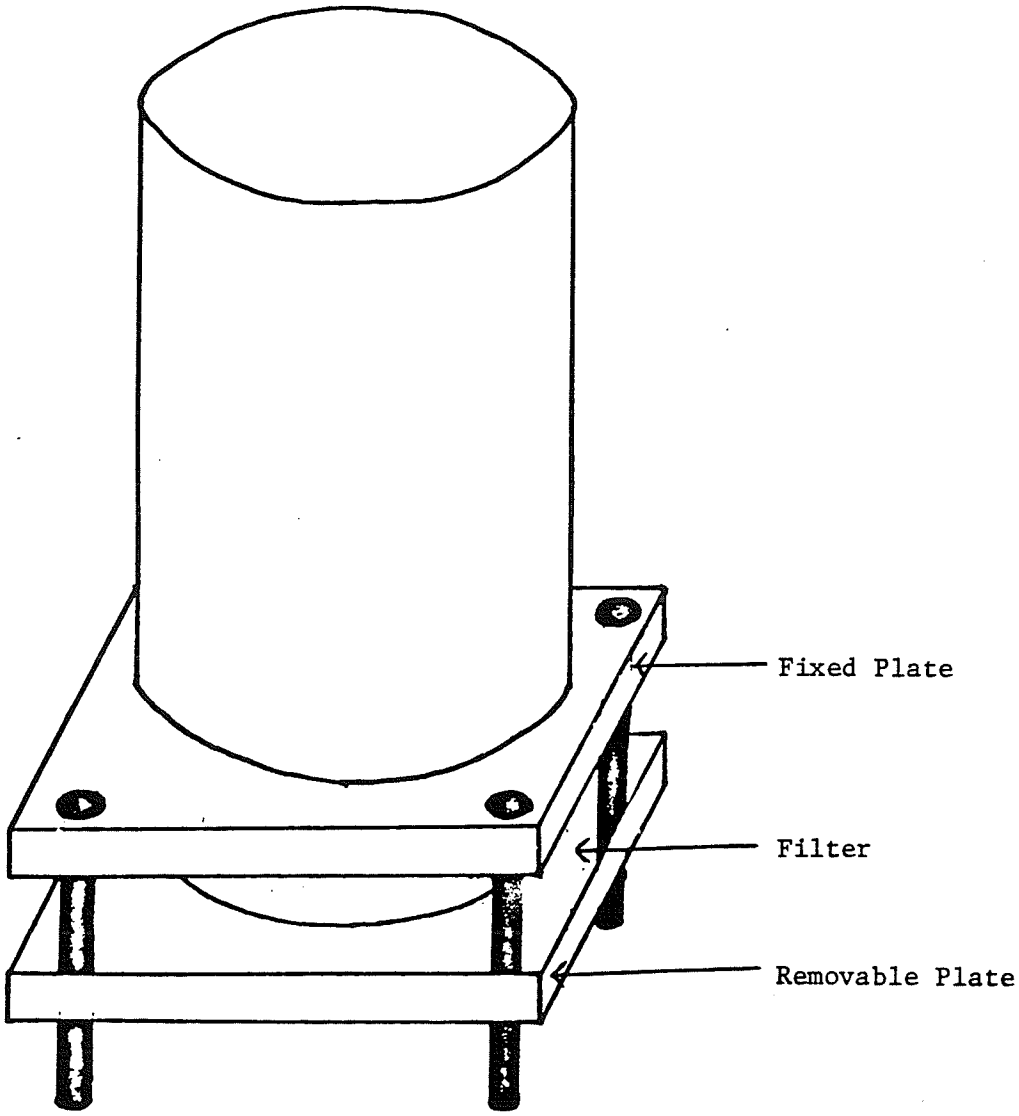
pH

The pH of each sample was determined with an electronic pH meter. The meter was calibrated immediately before use with a buffer near the pH of the sample, pH 4 buffer for tubes B and D and pH 7.0 buffer for tubes C and E.

Size Fractionation

The particulates of each sample were size fractioned using reverse flow filtration (Dodson and Thomas 1964) in a devise similar to that of Wiebe and Smith (1977). The fractionation apparatus was a plexiglass tube, 7.0 cm I.D. and 12 cm in length, closed at one end with a sheet of Nucleopore^{R} filter material (Figure 3), 3 μm pore size. This filter

Figure 3. Reverse flow filtration device. Nucleopore^{R} filters were clamped between fixed and removable plexiglass plates greased to prevent bypass leakage.


Fixed Plate
Filter
Removable Plate

tube was placed, filter down, into a beaker containing the sample to be fractionated. It was necessary to place a small amount of distilled water upon the slightly hydrophobic membrane to initiate flow. This small dilution error was ignored as it represents a volume change of less than 1 percent. Size fractionation of 175-200 ml of sample required from 2-10 minutes dependent upon the burden of particulate material in the sample. Filters were reusable if back washed with distilled water between samples. Filters showing reduced flow rates were discarded to avoid changes in effective pore size.

The portion of the sample within the filter tube contained particles with a nominal size of 3 μm or less. This size fractionated sample was removed and divided into portions of 25 ml and 150 ml for algal counts and ATP analysis respectively.

Algal Counts

A 25 ml portion of the fractionated sample was microscopically screened for algal contaminants as a test of fractionation efficiency. Samples were prepared for phase contract counts by passing 5 ml of the fractionated sample through a 0.22 μm x 25 mm Millipore^{R} filter. The filters were dried and then cleared with immersion oil and observed at 400x. The total number of observed algae in 20 fields was then used to calculate the number of algae per liter of fractionated sample. The amount of ATP contributed by these algal contaminants was estimated by making the following assumptions:

The algae were spherical, 3 μm in diameter, have a specific gravity of 1.1, have a dry weight equal to 20 percent of wet weight (Healey 1975), have a carbon content equal to 40 percent dry weight, and have a carbon to ATP ratio of 250:1 (Hamilton and Holm-Hanson 1967). Although these assumptions are not strictly valid, they served to approximate the contribution of algal ATP.

Controls run on July 19 showed that the algal biomass present in the fractionated samples represented about 0.8 percent of the total algal biomass in the original sample (total algal biomass data from D. Findlay). The estimated ATP contribution of the algae in the 3 μm sample represented 4, 8, 9 and 0.2 ng ATP/l in samples from tubes B, C, D, and E respectively. Quantitative algal counts were not run on any other sampling dates although a 25 ml portion of each fractionated sample was preserved in acid Lugols solution. Samples from periods showing high ATP concentrations were opened, treated with several drops of formalin, and allowed to clear for several days. The colorless samples were then filtered through a darkened (0.20 μm x 25 mm) Nucleopore^{R} filter (Hobbie et al. 1977). When the filters were viewed under near UV, cells containing chlorophyll fluoresced brilliant red against the darkened filter allowing rapid screening for algal contaminants. Algae were observed infrequently in all samples tested.

ATP Extraction

The particulate ATP was captured by passing 150 ml of the fractionated sample through a (0.45 μm x 47 mm) Millipore^{R} filter at a

vacuum of 100 torr. As the filter passed the last of the sample, the filter was quickly transferred into a beaker containing 10 ml of rapidly boiling extraction buffer consisting of 5 ml 0.05 M Tris (pH 7.7) and 5 ml distilled water. The filter remained in the boiling buffer for 5 minutes before it was drawn up the side of the beaker and washed with several ml of distilled water. The washed filter was then removed and discarded. The extraction buffer was allowed to boil down to a volume of about 5 ml. The final volume was recorded and the extract was bottled and frozen at -20°C for storage. The total elapsed time from sample collection to extraction ranged from 45-75 minutes. The bacterial and algal populations were separated for less than 15 minutes prior to extraction.

ATP Analysis

ATP analysis used the ATP dependent light emission of firefly lantern extract (Sigma Fle-50). Emitted light was measured with a liquid scintillation spectrophotometer (Picker-Nuclear Liquimat 220) as described by Rudd and Hamilton (1973).

The low ATP levels in the samples required aging of the rehydrated luciferin-luciferase preparation to reduce blank counts due to endogenous ATP. Aging of 4 to 6 hours at room temperature followed by overnight storage at 4°C resulted in a 20-fold reduction of blank values and an order of magnitude increase in the sensitivity reported by Rudd and Hamilton (1973). Some aged enzyme preparations showed a loss of activity, presumably due to depletion of luciferin and a consequential reduction

in light emission. The suitability of each batch of enzyme was judged by the linearity of the regression line describing the standard curve. All points were in triplicate (Fig. 4). Enzyme preparations were discarded if the regression coefficient (r) was less than 0.98 or if the sample counts were less than double background counts.

Raw counts from samples were converted to ATP concentration, expressed as mg ATP per ml, with the equation expressing the regression line derived from the ATP standard. The ATP concentration in the lake sample was then calculated from the volume of sample filtered (150 ml), the filter extract volume, and the ATP concentration of the extract.

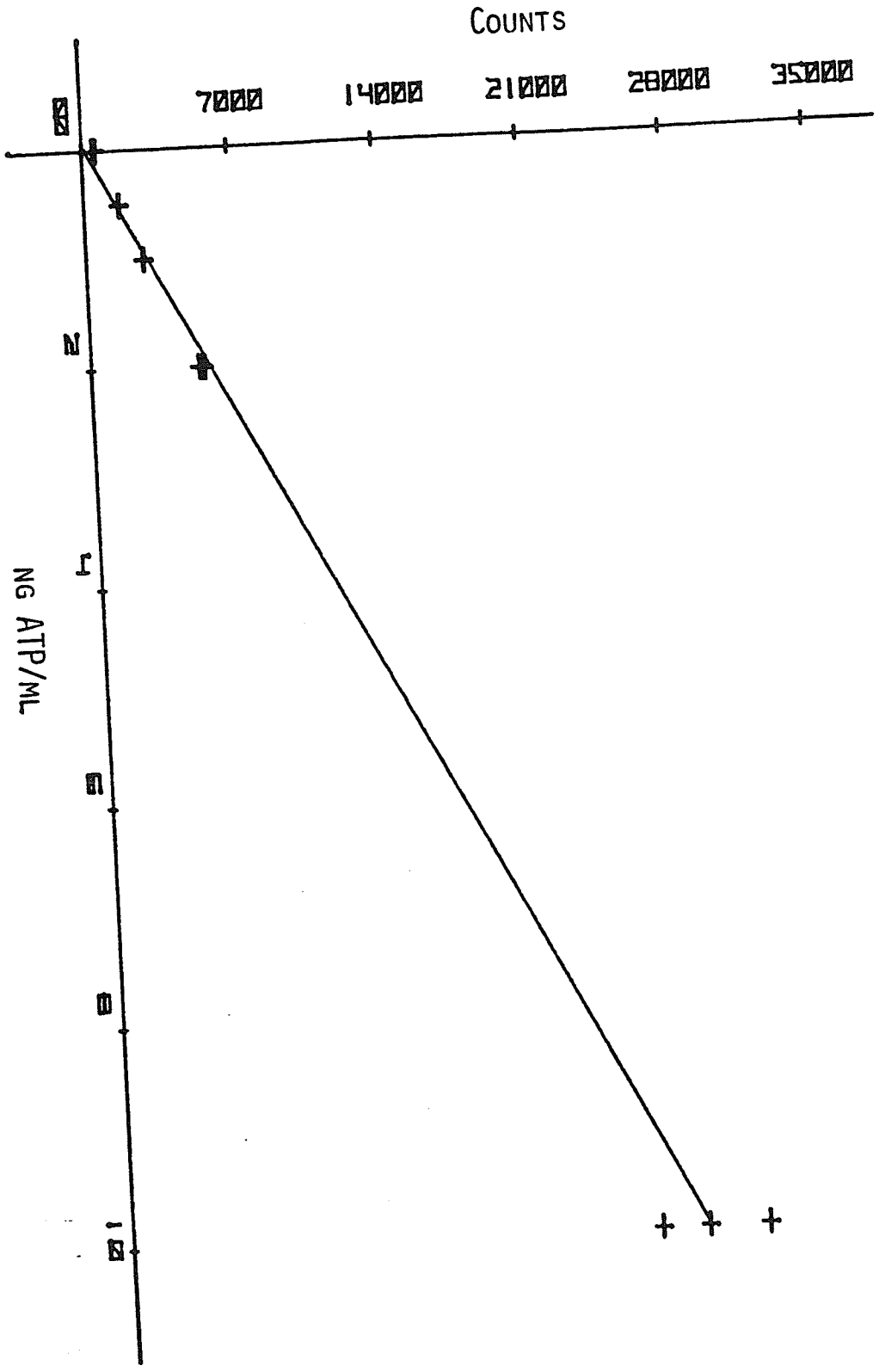
$$\frac{\text{ng ATP}}{\text{Liter Sample}} = \frac{\text{ng ATP}}{\text{ml Sample}} \quad \frac{\text{Extract Volume (ml)}}{\text{Volume Filtered (150 ml)}} \quad \frac{1000 \text{ ml}}{\text{Liter}}$$

Heterotrophic $^{14}CO_2$ Uptake

Integrated water samples were taken from the tubes as previously described except that the collection bottle was allowed to overflow for 2 minutes to purge contaminating atmospheric gases. Replicate water samples for dissolved inorganic carbon (DIC) analysis were taken in 50 ml syringes fitted with plugged needles. These DIC samples were placed on ice and returned to the chemistry laboratory for DIC analysis by conductivity (Stainton et al. 1977).

Four replicate samples from each tube were placed in darkened 125 ml incubation bottles (two of each experimental and control) ten minutes after, controls were killed with 1 ml saturated $HgCl_2$·, all bottles received 2 ml of ^{14}C bicarbonate solution. After four hours incubation

Figure 4. ATP standard regression line, all points in triplicate ($r^2 = 0.991$).


COUNTS
0
7000
14000
21000
28000
35000
0
2
4
6
8
10
NG ATP/ML

at 17°C the experimental bottles received 1 ml of saturated $HgCl_2$ solution. All bottles were then acidified to pH 2.2 with 2.5 ml HCl and bubbled with air for 30 minutes to remove the remaining ^{14}C bicarbonate.

The samples were then concentrated by passing 30 ml through a 0.22 µm x 25 mm Millipore^{R} filter. The filters were then dissolved in 20 ml of dioxane fluor (Schindler and Holmgren 1971) and counted in a liquid scintillation spectrophotometer. Quenched ^{14}C standards were used to determine counting efficiency by channels ratios.

The total DPM added to the sample was calculated from five 10 µl samples of the ^{14}C bicarbonate solution.

The total inorganic carbon fixed was calculated from the fraction of total DPM fixed and the DIC concentration.

Acidification of samples followed by filtration likely caused cell damage and consequently provided an underestimate of uptake. Therefore these results were used as relative indicators of metabolic activity.

Epifluorescent Counts and Energy Charge

Data collected by these methods were for various reasons unsuitable for this study. The methods employed and a discussion of each is provided in the Appendices.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

pH of Tubes

The pH of the acidified tubes proved to be difficult to control during the course of the experiment. As a consequence the pH levels of all tubes were variable and slightly lower than planned (Fig. 5). The effect of minor deviations from the average pH is difficult to assess. The more dramatic decrease of 1.6 pH units in tube B between Day 207 and 221 was accompanied by a decrease in ATP concentration at a time when an increase in ATP was expected based upon the behavior of the other tubes.

Effect of pH on Standing Crop

The ATP concentrations of the fractionated samples for 12 sampling dates appear in Figures 6, 7, 8 and 9. The profiles are remarkably similar in form with ATP concentration averages of 185, 100, 96 and 155 ng ATP/liter for tubes E (pH 6.4), C (pH 5.5), B (pH 4.8) and D (pH 3.9), respectively. The ATP concentrations of the control (E) and lowest pH (D) were significantly greater than the intermediate treatments.

The development of two bacterial blooms is evident from all profiles. ATP maxima appear between Day 150 and 180 and again between Day 200 and 220. In both blooms the control tube (E) responded one week earlier than the treated tubes. In addition to the lag period evident in all treated tubes, tube B and C pH (4.5 and 5.5, respectively) showed only a modest increase in ATP during the second maximum. These tubes (particularly tube B) experienced a significant drop in pH (Fig. 5) due

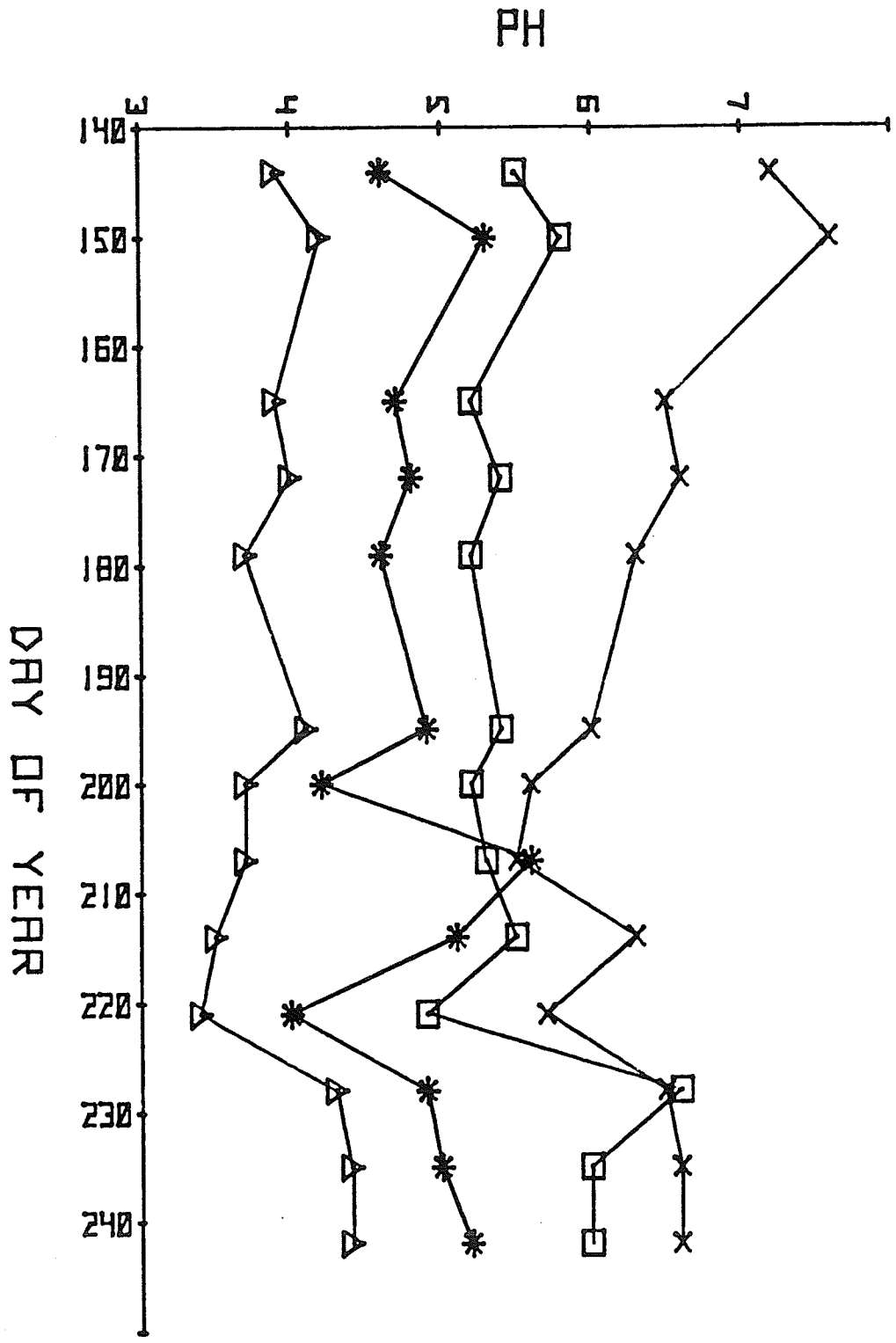

PH
3
4
5
6
7
DAY OF YEAR
140
150
160
170
180
190
200
210
220
230
240

Figure 5. The pH of the experimental enclosures for each sampling date, E X, C□, B*, DΔ. The average pH values were E, control 64; C, 5.5; B, 4.8; D, 3.9.

Figure 6. ATP concentration in ng ATP per liter, tube B.

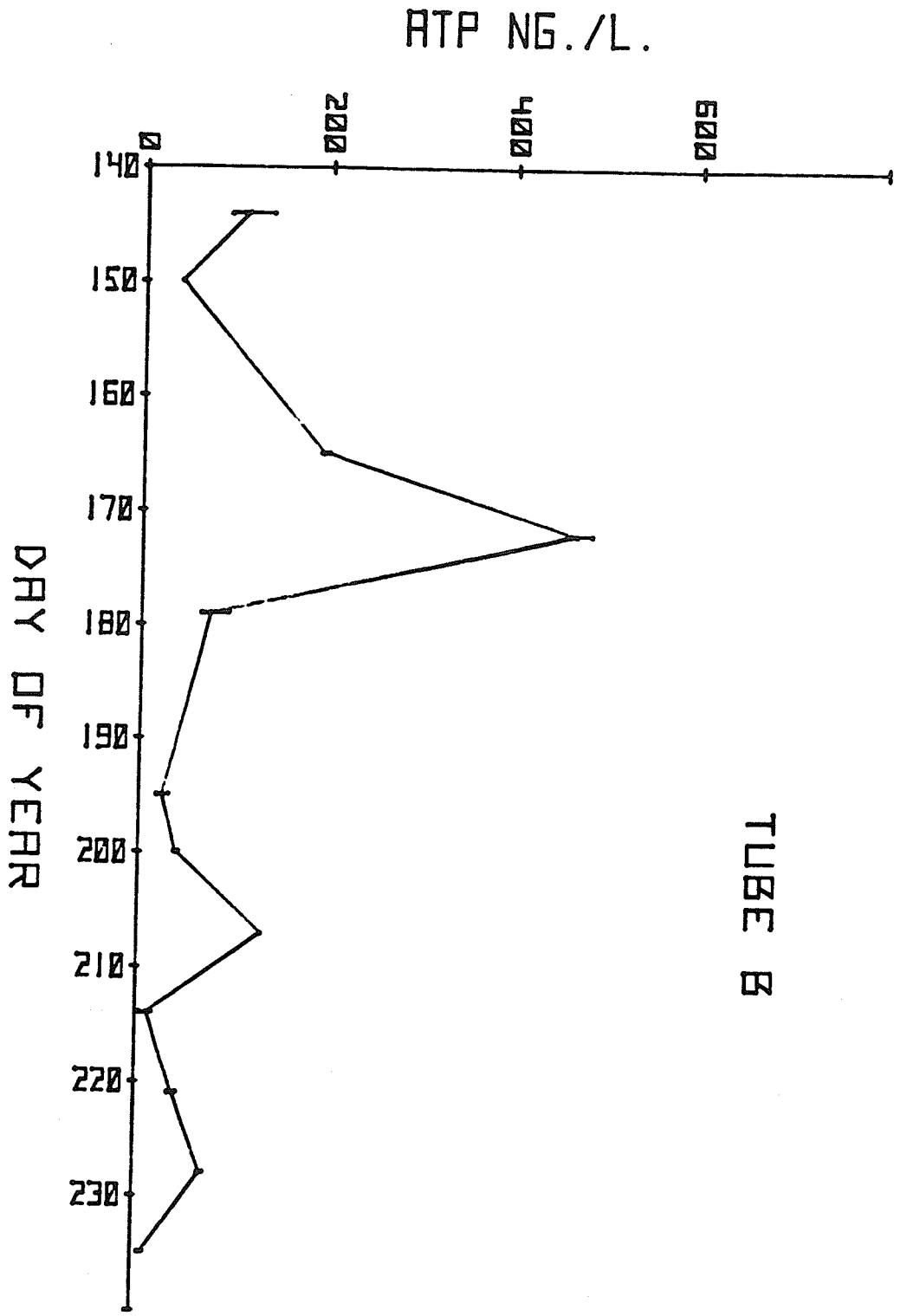

ATP NG./L.
0
200
400
600
140
150
160
170
180
190
200
210
220
230
DAY OF YEAR
TUBE B

Figure 7. ATP concentration in ng ATP per liter, tube C.

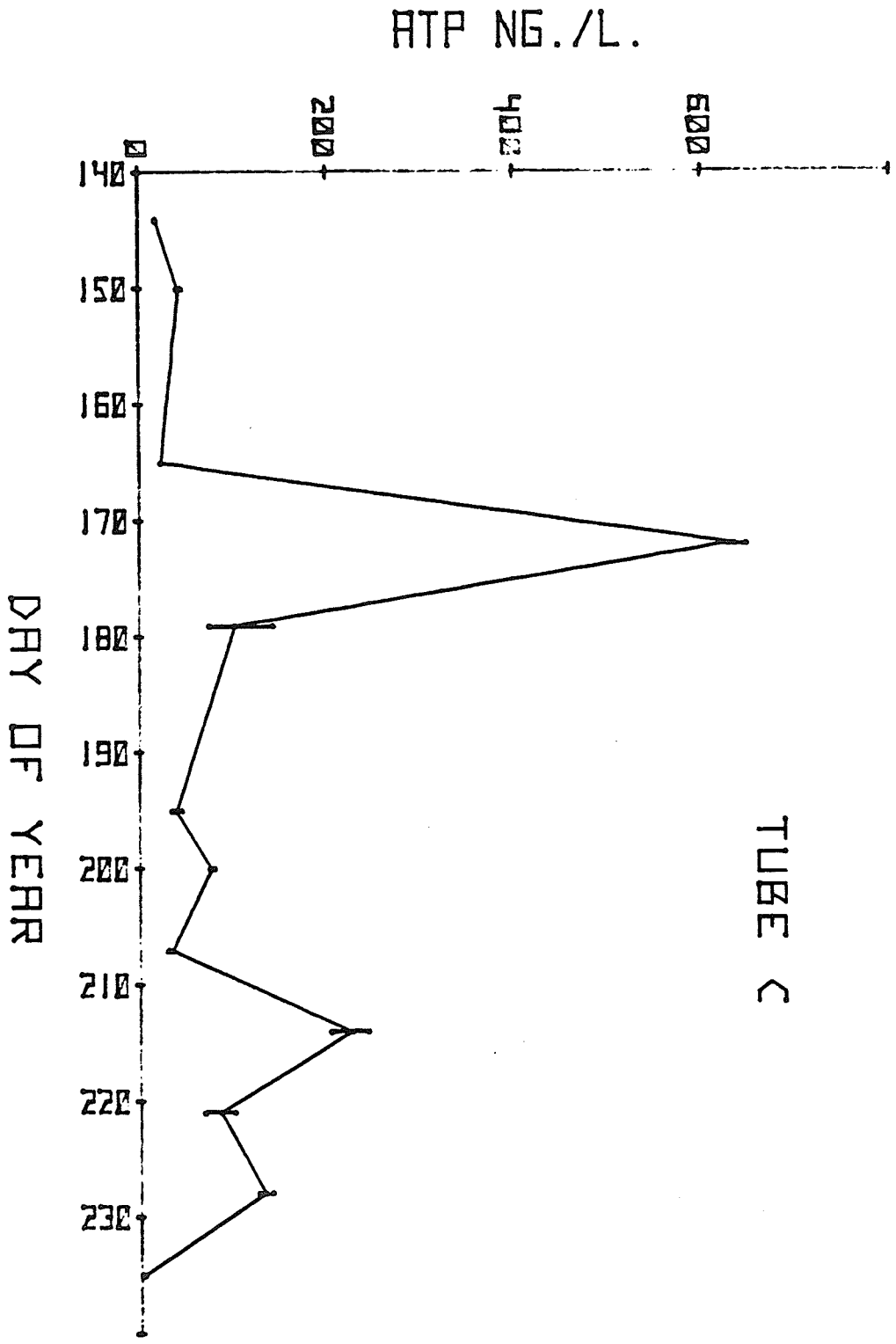

ATP NG./L.
0
002
004
009
140
150
160
170
180
190
200
210
220
230
DAY OF YEAR
TUBE C

Figure 8. ATP concentration in ng ATP per liter, tube D.

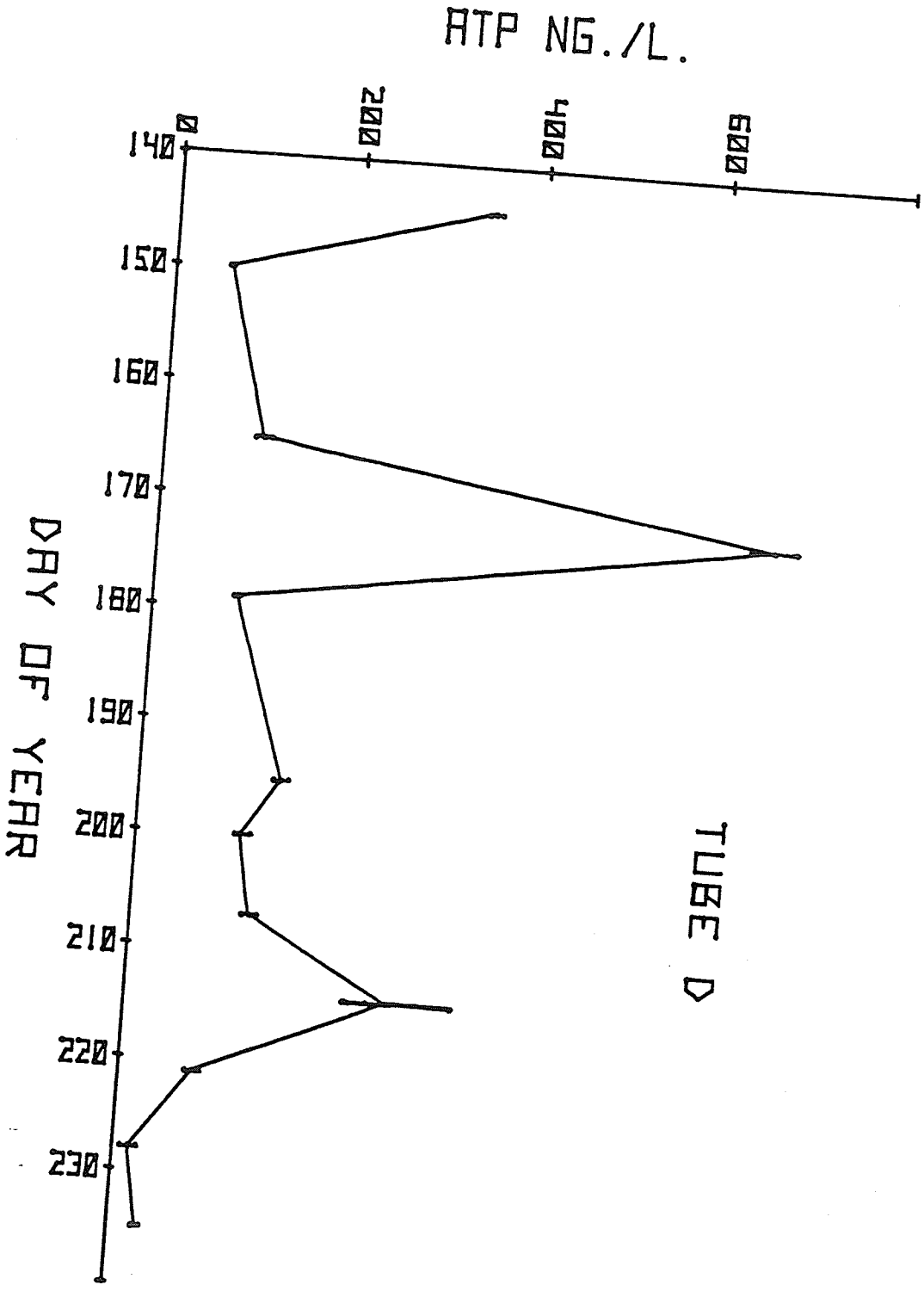
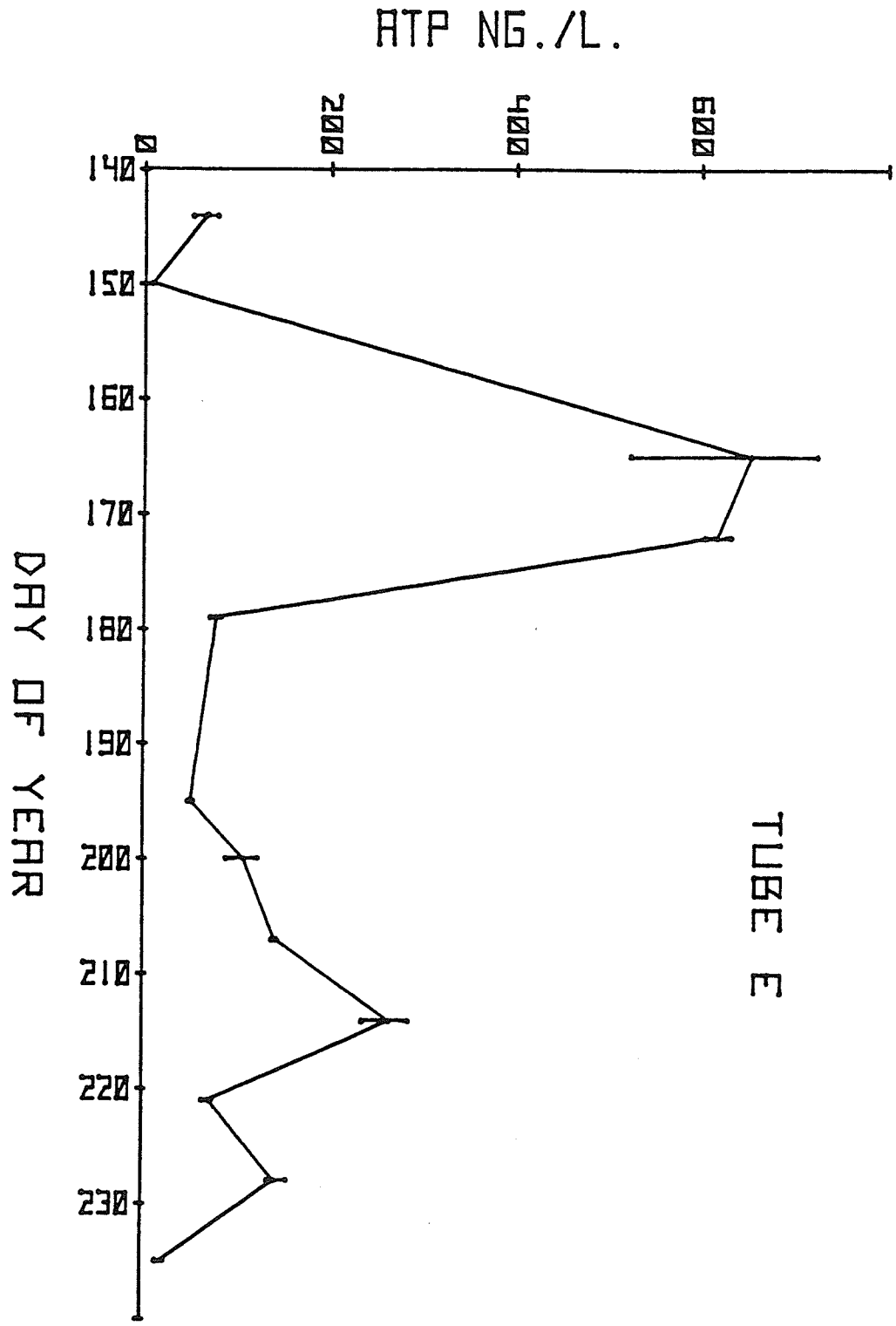

ATP NG./L.
0
002
004
009
140
150
160
170
180
190
200
210
220
230
DAY OF YEAR
TUBE D

Figure 9. ATP concentration in ng ATP per liter, tube E.


ATP NG./L.
0
200
400
600
140
150
160
170
180
190
200
210
220
230
DAY OF YEAR
TUBE E

to an accidental over-addition of acid. All tubes displayed declining pH during this period.

The ATP concentration averages of each of the four different pH environments show that although the bacteria attained the highest standing crop at pH 7, nearly equivalent growth occurred at pH 4. The significant reduction in ATP at intermediate pH values suggests that the bacterial populations are responding to at least two competing regulatory mechanisms. The reduced bacterial standing crop observed at the intermediate pH treatments (tubes B and C) may reflect reduced growth of the bacteria due to acid stress. The increase in standing crop observed at the lowest pH (tube D) may then represent the cumulative effects of increased substrate from primary producers and decreased predation. The consequences of such competing mechanisms would be the observed bimodal response.

The rapid increase in bacterial ATP in tube D (pH 3.9) during the development of both blooms argues that the bacteria are capable of rapid growth even at low pH. The reduced bacterial activity observed in acidified lakes must then be due largely to secondary effects of acidification which result in an unfavorable environment for bacterial growth.

Carbon fixed in primary production may be cycled to the bacterial decomposers in the form of low molecular weight organics released during photosynthesis, or as the lysate of whole algal cells. Both

sources of substrate would be enhanced by increased phytoplankton production. The percentage of fixed carbon released during photosynthesis has been shown to be dependent upon the physiological state of phytoplankton (Fogg 1971). Stress conditions such as nutrient deficiencies and supraoptimal light levels have been shown to increase the rate of extracellular release (Fogg 1971; Hellebust 1965; Pritchard et al. 1962). Analogous increases in extracellular release due to acid stress would enhance bacterial growth by increasing available substrate. Measurements of chlorophyll-a, phytoplankton biomass, and primary production (Table 1) demonstrate the increased algal growth in tubes C and D relative to the control. This increase of algal growth contradicts the evidence of reduced algal production observed in acidified lakes (Grahn et al. 1974). The cause of this increase is unknown but may be a result of reduced predation (Porter 1977; Hurlbert 1975) by acid sensitive zooplankton (Almer et al. 1974), or variability of sediments underlying these tubes.

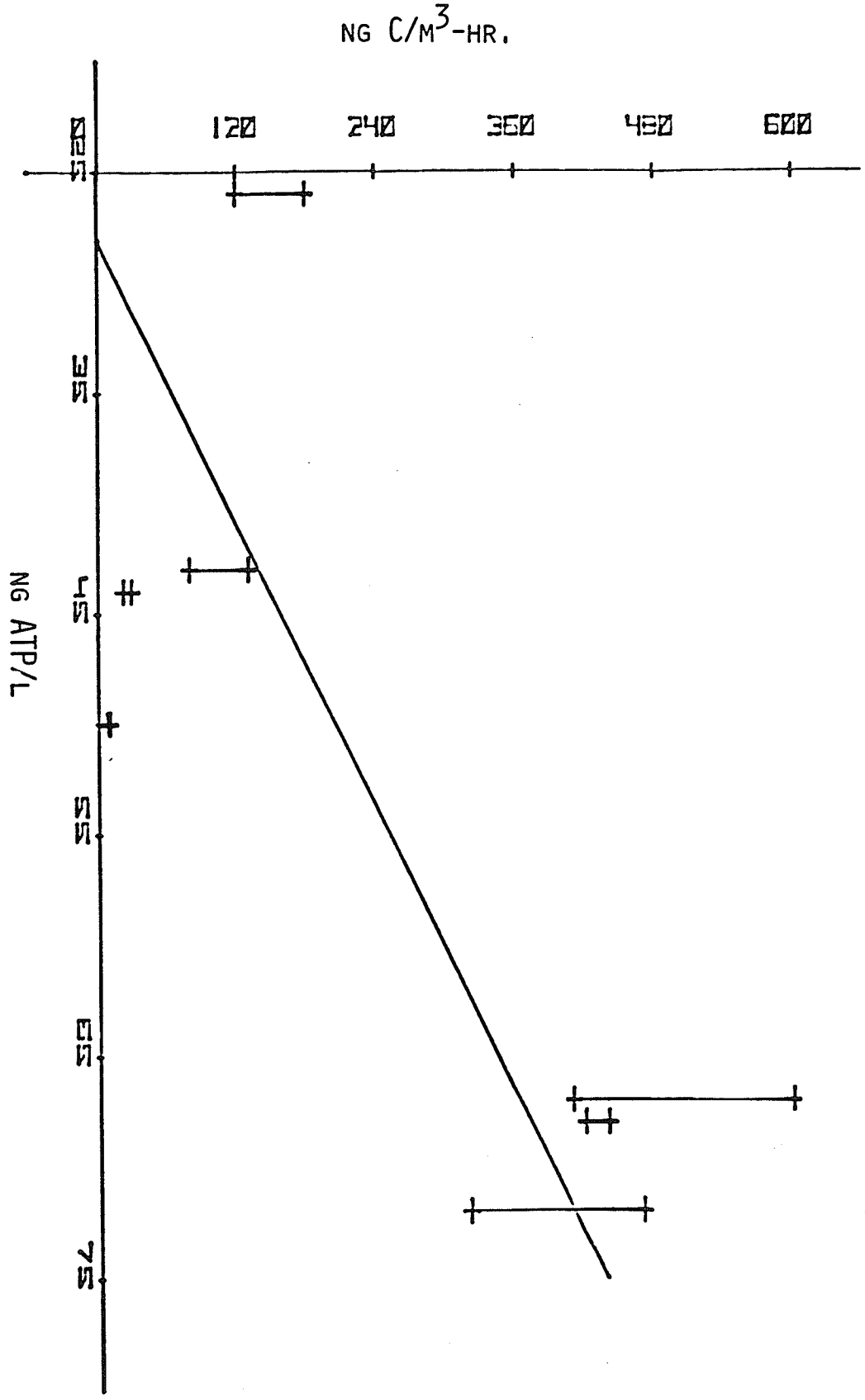
Growth Rate

The reproducibility of replicate dark $^{14}CO_2$ uptake samples was variable. The deviation from the mean of replicate samples ranged from 2.5 to 25 percent, excluding tube C, day 235. The uptake of $^{14}CO_2$ in this sample was probably due largely to chemical adsorption since the killed blanks were very high (16 to 45 times higher than the remaining samples, Table 2). This sample did not contain sufficient ATP to allow detection, which supports the supposition that $^{14}CO_2$ uptake in

Table 1. Phytoplankton chlorophyll-*a*, biomass, and production for each of the tubes. Averaged values for the experimental period.

Treatment pH	Tubes B 4.8	C 5.5	D 3.9	E 6.4 (control)
Chlorophyll-*a* µg/l	2.2	4.0	4.7	2.8
Phytoplankton Biomass gms/m^3	1.76	2.83	2.89	1.70
Phytoplankton Production mgc/m^3- hr	16.7	294	237	57

Figure 10. A comparison of heterotrophic $^{14}CO_2$ uptake and ATP, on seven duplicate samples from days 235 and 242, demonstrated a significant correlation ($F = 14.49$; DF 1-12). Tube C day 235 was omitted (ATP = 0; $\mu gC/m^3$-hr = 1.7 and 22) because of its high and variable control counts.


NG C/M3-HR.
120
240
360
480
600
NG ATP/L

this sample was largely chemical.

A comparison of results obtained with the ATP and $^{14}CO_2$ uptake methods (Fig. 10) demonstrates a significant correlation (F = 14.49; DF 1.12) between bacterial standing crop and dark DIC uptake. This indicates that the heterotrophic activity per unit biomass (specific activity) of the populations was similar.

The ATP and DIC uptake values may be converted to biomass and total heterotrophic uptake by applying standard correction factors. Assuming a carbon to ATP ratio of 250 to 1 (Hamilton and Holm-Hansen 1967), and that DIC uptake equals 3% of total carbon uptake (Romanenko 1964), crude estimate of doubling time may be calculated (Table 2).

The estimated doubling times, all greater than one month, suggest that the bacterial populations were senescent on days 235 and 242. Although $^{14}CO_2$ uptake measurements were not made during bacterial blooms, a minimum estimate of growth rate may be obtained by calculating the doubling time of the ATP pool during the development of bacterial blooms. This is a minimum estimate of growth because the average measured growth rate is probably less than the maximum instantaneous growth rate. The rate is also a net growth rate which ignores losses such as predation.

The most rapid growth rate observed, doubling time in 13.9 hours (tube C between day 165 and 172), appears to be similar to the rates which occurred during the bacterial blooms in all tubes (Figs. 6-9). Thus the bacteria appeared to grow rapidly during brief periods of favorable conditions and remain nearly inactive during unfavorable conditions.

Table 2. Calculated bacterial growth rates for days 235 and 242 (assume carbon to ATP = 250 to 1 and heterotrophic DIC uptake = 3% of total heterotrophic uptake).

Day	Tube	ATP ng/l	Bacterial Biomass $\mu g/m^3$ (ATP x 250)	DIC $\mu g/m^3$	Control DPM per 30 ml Average	Fraction Fixed/hour	DIC fixed ng C/m^3-hr	Bacterial Production $\mu g/m^3$-hr (DIC fixed x 33.33)	Bacterial Doubling Time in Days
235	B	26	6.5×10^3	1910	64	9.41×10^{-5} 6.48×10^{-5}	180 120	6.0 4.0	45 68
235	C	0	-	8455	1410	5.63×10^{-5} 4.23×10^{-6}	480 36	- -	- -
235	D	72	1.8×10^4	5727	56	8.12×10^{-5} 5.55×10^{-5}	470 320	16 11	47 68
235	E	50	1.2×10^4	1227	43	7.08×10^{-6} 8.00×10^{-6}	8.7 9.8	.29 .33	1724 1515
242	B	43	1.1×10^4	2455	49	5.18×10^{-5} 3.23×10^{-5}	130 79	4.3 2.6	107 176
242	C	68	1.7×10^4	7091	83	5.86×10^{-5} 6.19×10^{-5}	420 440	14 15	51 47
242	D	67	1.7×10^4	4909	31	1.23×10^{-4} 8.40×10^{-5}	600 410	20 14	35 51
242	E	44	1.1×10^4	1200	49	1.86×10^{-5} 2.39×10^{-5}	22 29	.73 .97	628 473

Temporal Changes in Standing Crop

The similarity in form among ATP seasonal patterns (Figs. 7, 8, 9 and 10) suggests that the bacterial populations in the acidified tubes shared a common external regulatory mechanism. The coincidence of the June ATP maximum with an extended period of cloudy weather suggested a functional relationship between solar irradiance and bacterial standing crop. As a test of this relationship the log of the ATP concentration was plotted against the average number of hours of bright sun (Fig. 11), measured by a pyroheliometer, for 6 days previous to the sampling date. Linear regression analysis showed the strongest correlation in the control tube and the poorest fit at the lowest pH (Figs. 12, 13, 14, and 15). If the ATP concentrations of the four treatments are averaged, the fit improves demonstrating a negative correlation between solar irradiance and ATP (Fig. 16).

Data from other investigators indicates that the reduction of bacterial ATP at high light intensities may be due to the direct inhibition of bacterial metabolism by visible light. Bacteria are known to be killed by ultraviolet (UV) radiation and susceptible to a lesser degree to photo-inhibition by visible light (Harrison 1967). However, due to the rapid quenching of UV by the organic compounds in water (Armstrong and Boalch 1961) UV inhibition of bacteria has generally been ignored.

The clear water of the shallow (2 m) tubes may have allowed

Figure 11. The average hours of sun between 9:00 and 16:00 hours for 3-day intervals.

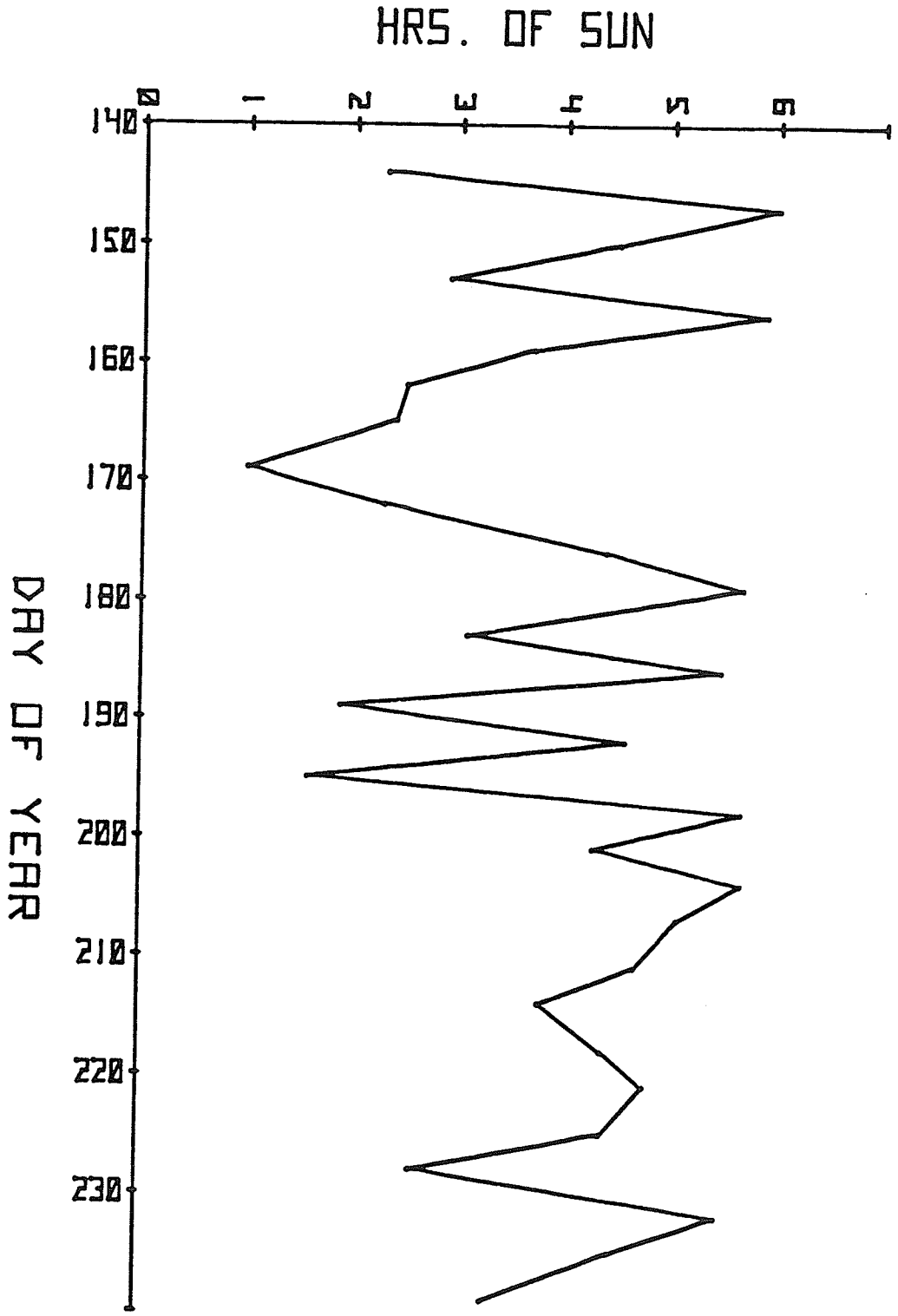

HRS. OF SUN
0
1
2
3
4
5
6
140
150
160
170
180
190
200
210
220
230
DAY OF YEAR

Figure 12. Regression correlating ATP to the amount of bright mid-day sun for tube E, control (average pH 6.4; $r = -0.61$).

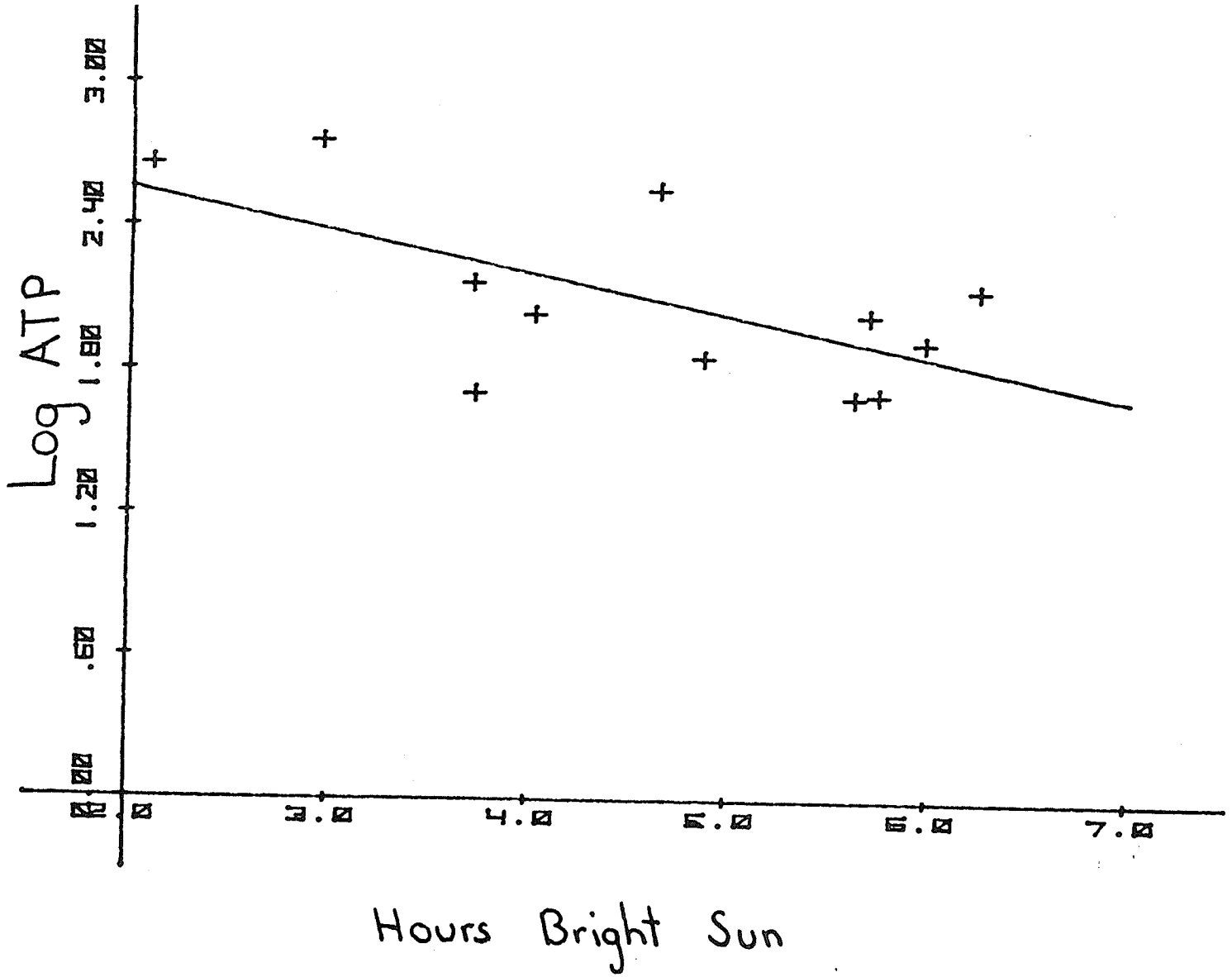

Log ATP
3.00
2.40
1.80
1.20
.60
.00
0
3.0
4.0
5.0
6.0
7.0
Hours Bright Sun

Figure 13. Regression correlating ATP to the amount of bright mid-day sun for tube C (average pH 5.8; r = -0.45).

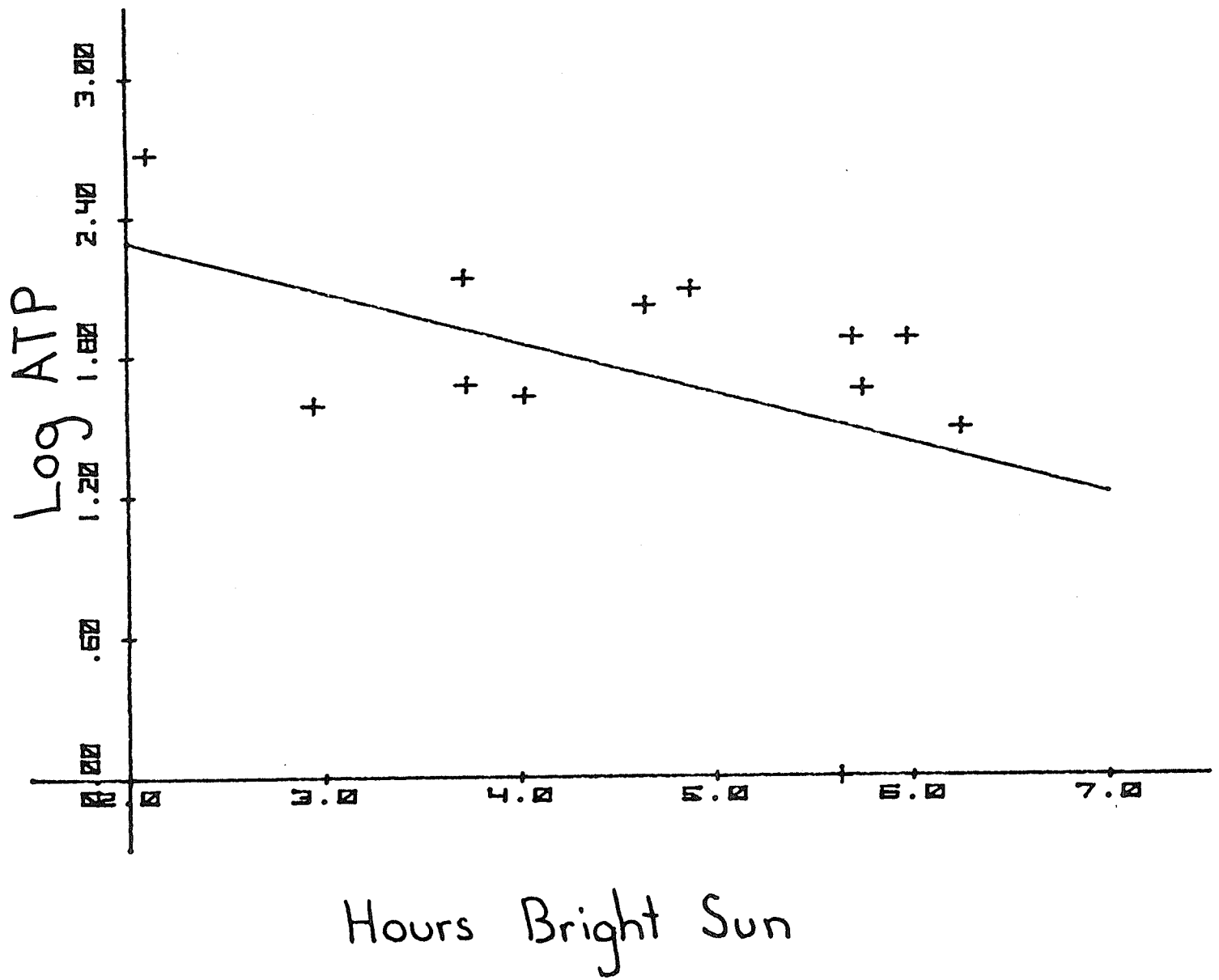

Log ATP
3.00
2.40
1.80
1.20
.60
.00
0.0
3.0
4.0
5.0
6.0
7.0
Hours Bright Sun

Figure 14. Regression correlating ATP to the amount of bright mid-day sun for tube B (average pH 4.8; r = -0.46).

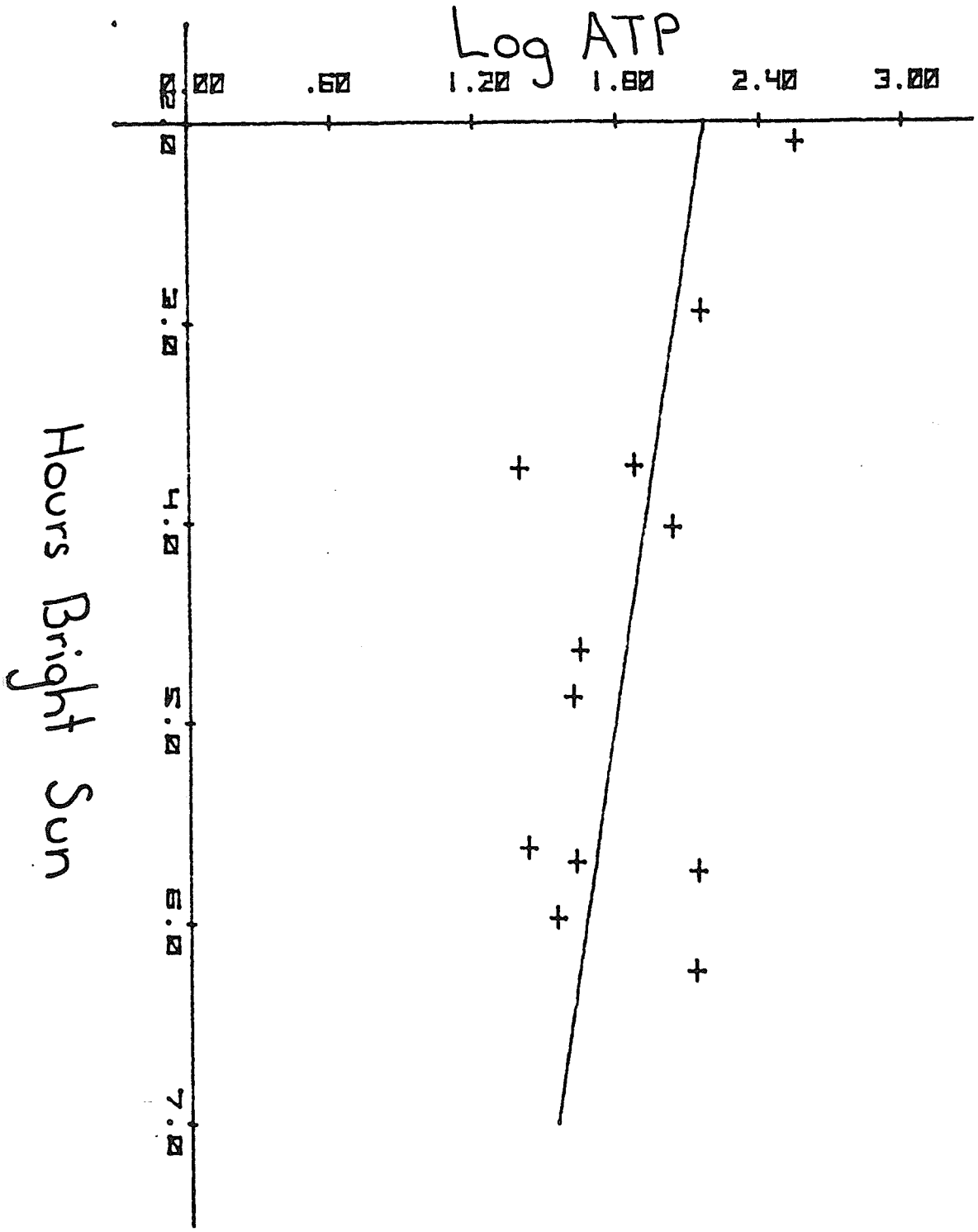

Log ATP
.00
.60
1.20
1.80
2.40
3.00
Hours Bright Sun

Figure 15. Regression correlating ATP to the amount of bright mid-day sun for tube D (average pH 3.9; $r = -0.39$).

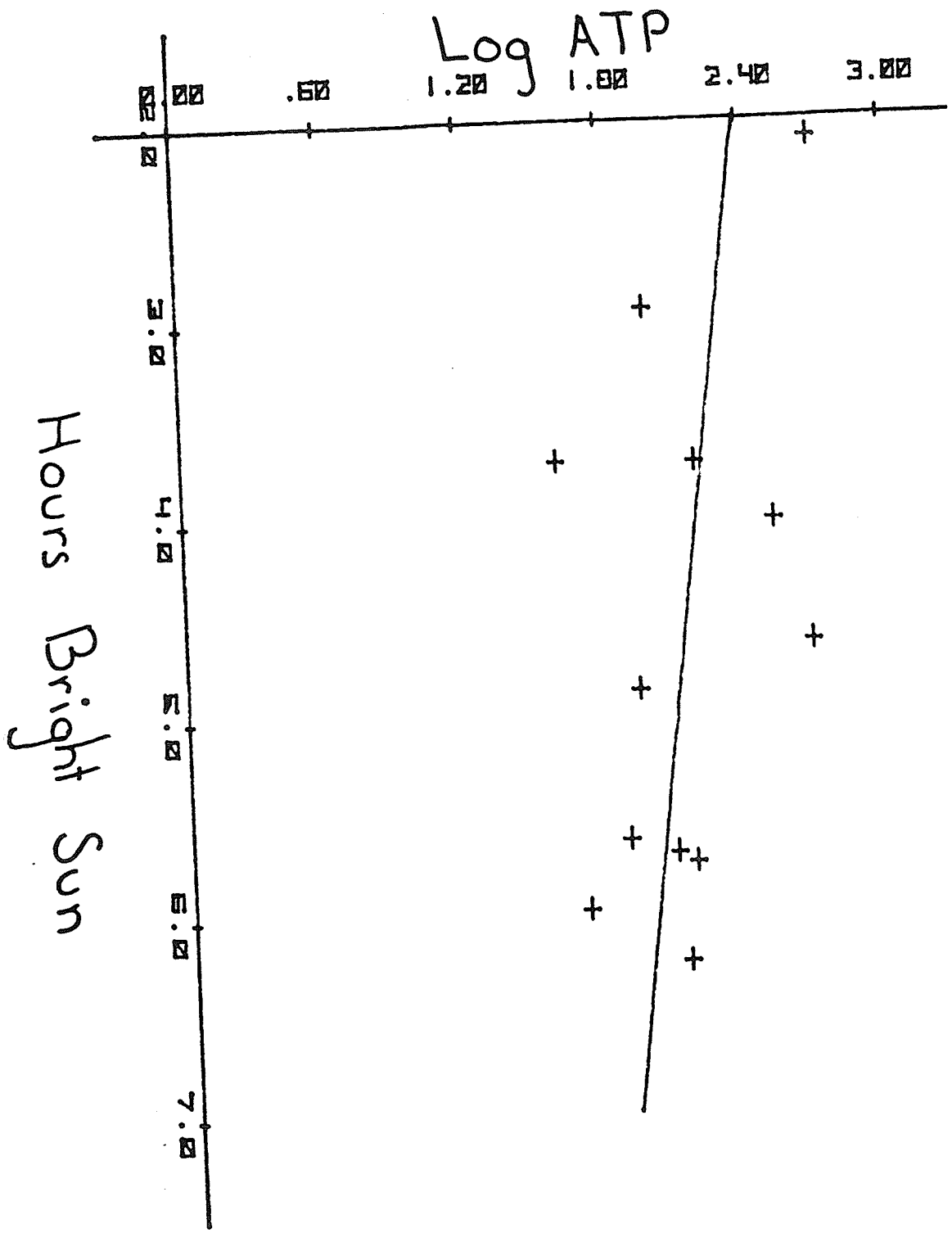
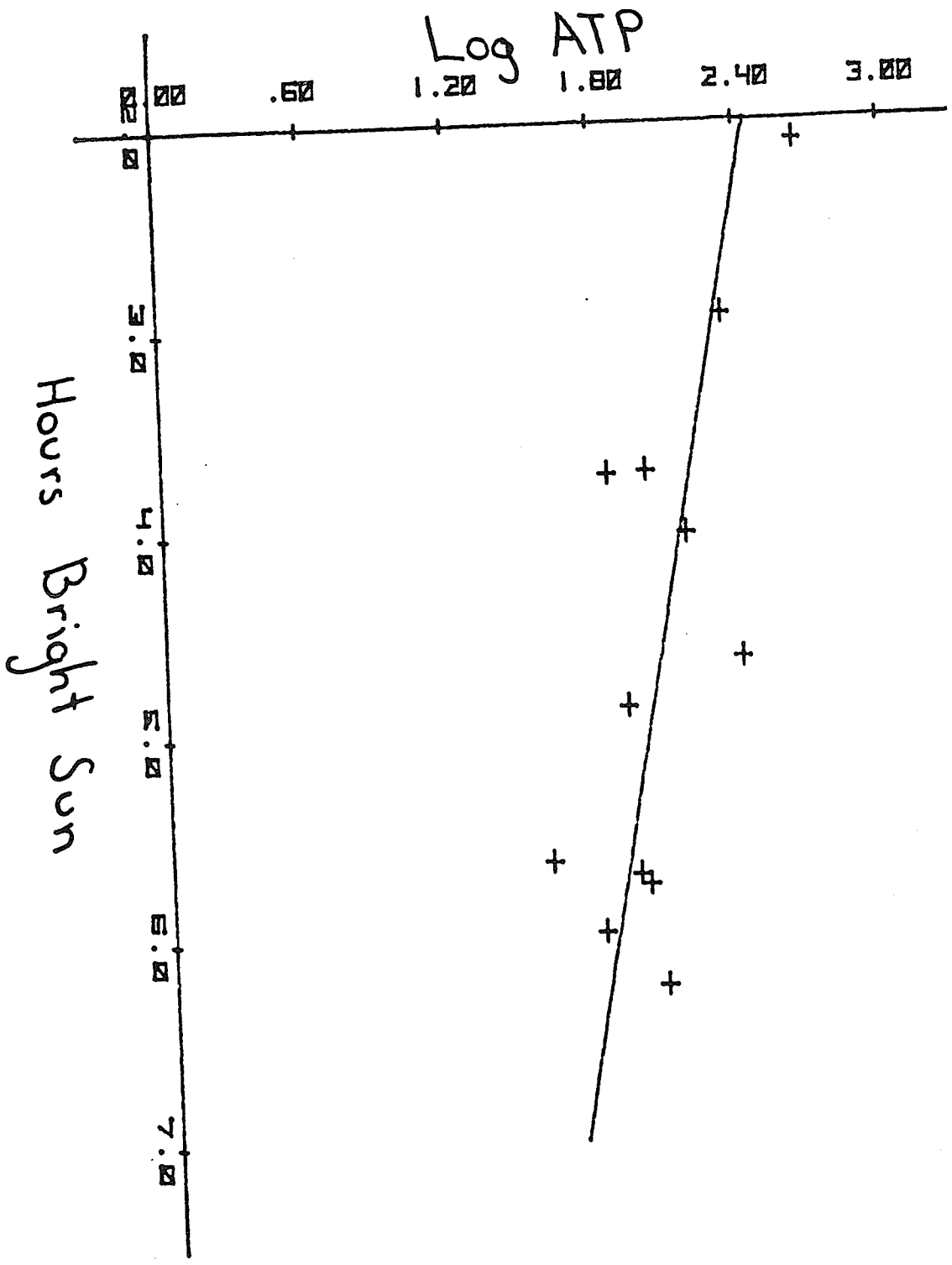

Log ATP
.00
.60
1.20
1.80
2.40
3.00
Hours Bright Sun
0
2.0
4.0
5.0
6.0
7.0

Figure 16. Regression correlating ATP to the amount of mid-day sun for daily average of all tubes ($r = -0.69$).



considerable penetration of UV as evidenced by the damage to the plastic tube material which became photo-oxidized and perforated to a depth of one-half meter forcing an early termination of the experiment. The inhibitory effect of visible light is evident in data obtained from primary production studies.

Measurements of primary production with the ^{14}C tracer method (Steemann-Nielsen 1952) employ dark bottle blanks which correct for non-photosynthetic ^{14}C fixation. Dark uptake includes several heterotrophically mediated components, the Wood-Werkman reaction (Wood and Stjernholm 1962) and mixotrophic CO_2 fixation (Tuttle and Jannasch 1977). The bacterial CO_2 uptake should be greater in the dark than in the light if the bacteria present are inhibited by the light intensities chosen for incubation. This overestimate of respiration would cause underestimates of primary production in samples containing high bacterial activity.

Negative algal production is occasionally observed with the ^{14}C tracer technique in samples containing large numbers of bacteria (E.J. Fee; B. Hecky, personal communications). Other investigators have shown that the addition of bacteria to primary production samples causes an increase in dark uptake which is inhibited by light incubation (Qasim et al. 1972). Using autoradiography, Fee demonstrated that the high dark uptake was associated with detrital material, and that this uptake was sensitive to incubation under tungsten lamps (E.J. Fee, personal communication). The detrital uptake was presumably due to

bacteria associated with the detritus which were fixing carbon in the dark. The light from the tungsten lamps penetrating the glass incubation bottles would not contain significant amounts of UV light so the observed bacterial inhibition must be due primarily to visible wavelengths.

Primary production studies provide evidence of temporary light inhibition of bacterial activity. Although this supports the supposition that light inhibition regulates bacterial standing crop, the connection between temporary activity and standing crop is tenuous.

Studies of bacteria population dynamics have centered upon algae as the source of substrate for substrate-limited bacterial populations (Hickman and Penn 1977; Jones 1971). The failure of such studies to demonstrate a consistent relationship between algal production and bacterial biomass is likely due to other parameters, including light inhibition, which exerts a strong influence upon bacterial growth.

The consequences of light inhibition of bacterial metabolism may be significant in primary production studies which attempt to correct for respiration with a dark control. The increased bacterial activity, in the dark, may be sufficient to invalidate studies of primary production in waters with high bacterial populations (Qasim et al. 1972). Methods of assessing heterotrophic activity which assume the bacteria to be substrate limited would also be invalid if the bacteria are limited by light inhibition rather than substrate.

CONCLUSIONS

CONCLUSIONS

The results of ATP standing crop estimates suggest that the reported reduction of bacterial activity in acidified lakes is not due to the inhibitory effects of pH alone. Rather, bacterial standing crop and growth rates were enhanced in some of the acidified tubes relative to the control. The enhanced bacterial growth in tubes C and D (pH 5.5 and 3.9 respectively) was accompanied by increased algal standing crop and production. Thus the effects of acidification upon heterotrophs may be mediated by effects upon primary production.

The increased production in tube D (pH 3.9) is in contradiction to reports of decreased primary production in acidified lakes (Grahn et al. 1974). Grahn et al. (1974) attribute this loss of production to decreased mineralization by heterotrophs. My results suggest that the bacterial biomass was primarily dependent upon primary production. The general loss of productivity of acidified lakes is likely due to other factors which may disrupt primary production or mineralization processes. Possible factors include the toxic effects of heavy metals mobilized from watersheds (Schofield 1965) or lake sediments (Schindler et al. 1977) by cation exchange, and competition from acid tolerant fungal decomposers (Grahn et al. 1974).

Estimates of bacterial growth rates from changes in ATP concentration and heterotrophic CO_2 uptake suggest that growth during periods

of low ATP concentration was very slow with doubling times measured in months. However during bacterial blooms conservative estimates of doubling times were as low as 13.9 hours. Thus it appears that a large portion of the bacterial growth and presumably mineralization occurs during relatively brief periods of favorable conditions.

The development of simultaneous bacterial blooms in the tubes coincident with periods of low solar irradiance suggests a functional relationship between bacterial standing crop and light. Although no mechanism may be determined from my data, the results of other investigators show that bacterial metabolism is inhibited by moderate intensities of light of visible wavelengths. The shallow and highly transparent waters of the tubes made these bacterial populations particularly susceptible to light inhibition. It would be of interest to investigate the possible role of light inhibition as a regulator or bacterial populations in situ.

APPENDICES

APPENDIX I

Effect of Filtration Upon ATP Extraction Efficiency

Methods employing filtration and subsequent extraction in boiling buffer have been reported to result in significantly lower estimates of ATP than direct injection methods (Jones and Simon 1977). The loss of ATP has been assumed to be due to the physical stress of filtration or the consumption of ATP by intracellular enzymes during extraction. Extraction by direct injection not only avoids filtration stress but also provides more rapid heating and denaturation of intracellular enzymes.

Several experiments were designed to assess the contribution of these factors. Using steam extraction from filters attempts were made to determine if the methods could be modified to increase the efficiency of ATP extraction. Sample volume from 50 to 500 ml were filtered and extracted to determine if the observed concentration of ATP was dependent upon the volume filtered.

Replicate samples of fractionated lake water were prepared for extraction as described in Methods. One set of samples was extracted as described in Methods. A second set of duplicate filters was then extracted with a blast of steam (112^{o}C) for about 20 seconds before being dropped into boiling buffer to continue the extraction by the standard method. ATP analysis showed essentially equivalent extraction efficiencies with these methods. Due to the hazards associated with

pressurized steam the experiment was discontinued. However, the results suggested that ATP losses were occurring before extraction. To test the effect of filtration stress sample volumes of 50, 100, 250 and 500 ml were filtered and extracted as described in the Methods. The filtration rate was observed to be constant to 250 ml, after which filters became plugged (Fig. 17).

The ATP analysis method employed was not sensitive enough to allow extraction by direct injection, therefore, the absolute extraction efficiency could not be estimated. Filtration of volumes greater than 50 ml resulted in significant loss of ATP (Fig. 18).

The ATP pool appears to have two components, one which is very sensitive to filtration stress and a second more stable pool which is unaffected by increased filtration volumes. The filtration sensitive and insensitive fractions may be intracellular components or separate populations displaying different susceptibilities to filtration stress.

The constant extraction efficiency at volumes greater than 100 ml allows the use of the filtration extraction procedure for comparative studies as suggested by Jones and Simon (1977).

Figure 17. Plot of time required to pass volumes of fractionate lake water through a Millipore^{R} (0.45 μm x 47 mm) filter at 100 torr.

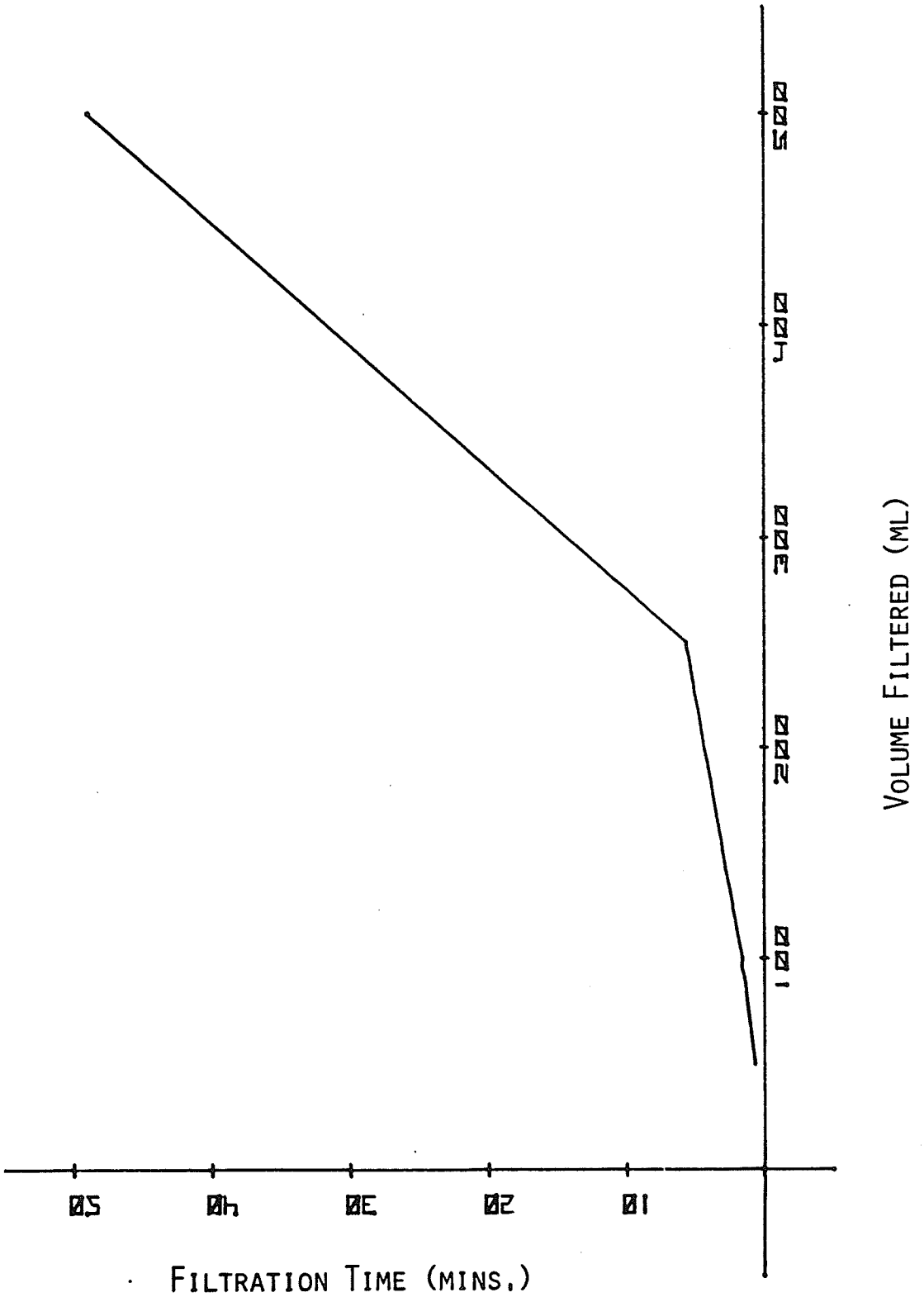
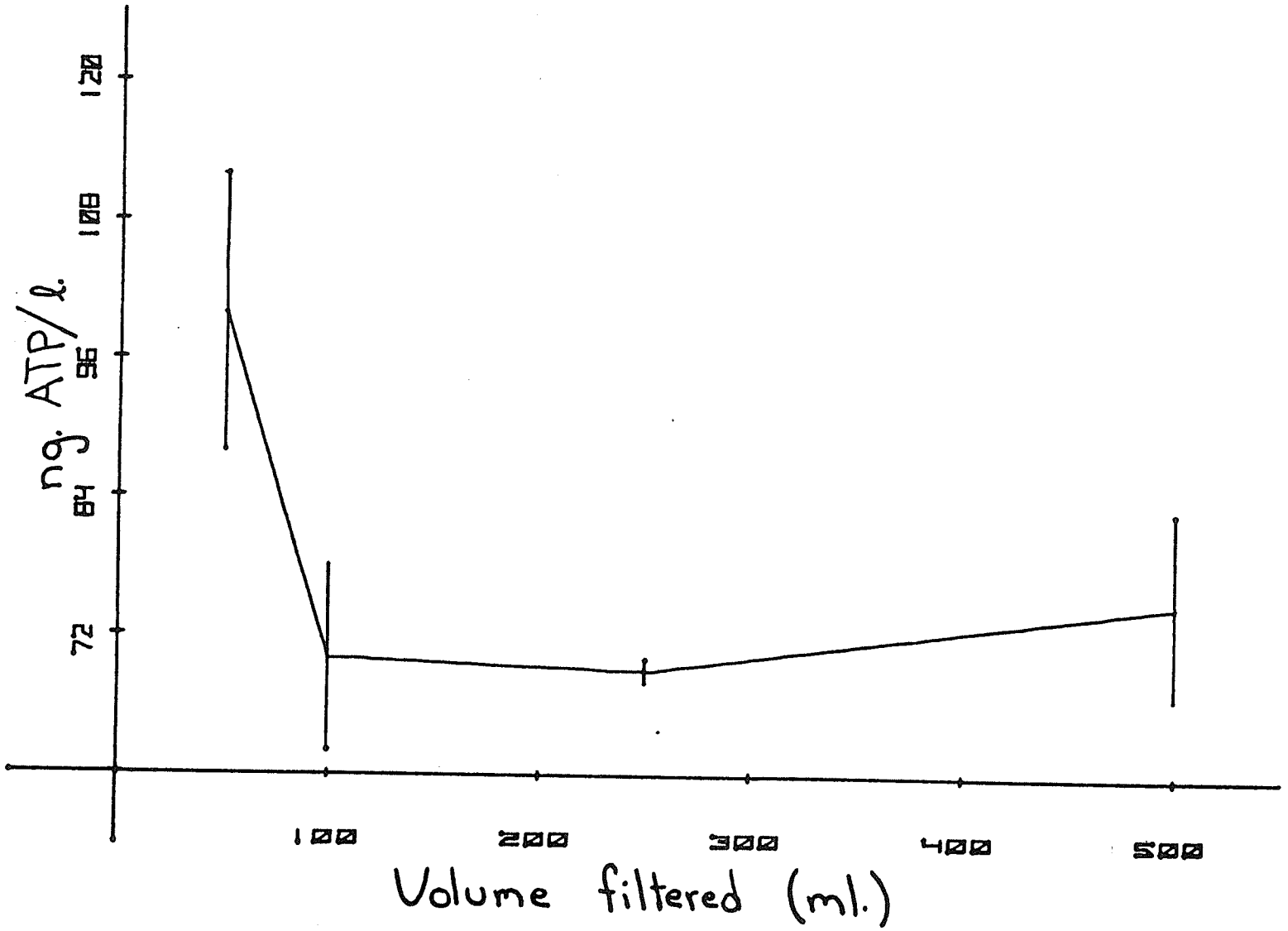

VOLUME FILTERED (ML)
005
004
003
002
001
05
04
03
02
01
FILTRATION TIME (MINS.)

Figure 18. Plot of apparent ATP concentration as a function of volume filtered.


ng. ATP/l.
72
84
96
108
120
100
200
300
400
500
Volume filtered (ml.)

APPENDIX II

Adenylate Analysis

Analysis of adenylates utilized enzymatic conversion of AMP and ADP to ATP followed by ATP analysis as described in Methods. The samples for adenylate analysis were those used in the ATP determinations. The enzymatic conversions of adenylates employed methods adapted from Chapman et al. (1971) (Table 3).

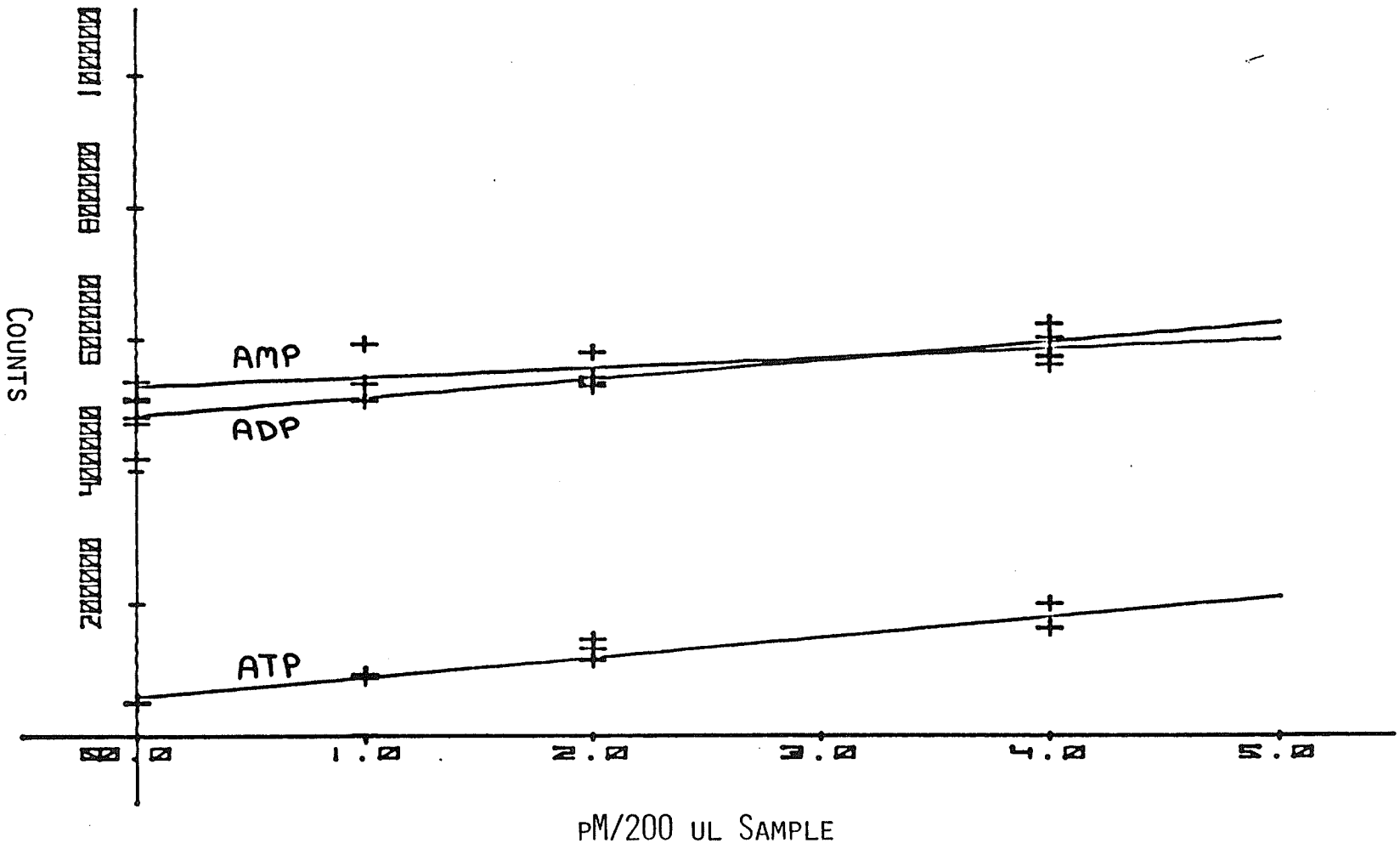
The enzymatic conversion of ADP to ATP was essentially quantitative as evidenced by the nearly parallel standard curves of equal molar concentrations of adenylates (Fig. 19).

The high blank values of the ADP standard were nearly an order of magnitude greater than the blanks of the ATP standard. The high blank values resulted in increased variability among the points on the ADP standard curve and a reduction of the regression correlation coefficient for the standard curve (ADP R = 0.75, ATP R = 0.95).

The AMP blank values were only slightly greater than the ADP blanks suggesting that the blank counts were due to ATP present only in the pyruvate kinase. The non-quantitative conversion of AMP to ATP apparent in the non-parallel ATP and AMP standards is characteristic of this assay (Karl and Holm-Hansen 1977).

The estimation of sample ADP by the difference between ATP and ATP + ADP preparations resulted in high background counts during the ADP assay. This procedural difficulty coupled to the high blank

Figure 19. Typical standard curves for ATP, ADP and AMP analysis.


COUNTS
AMP
ADP
ATP
0.0
1.0
2.0
3.0
4.0
5.0
PM/200 UL SAMPLE

values due to ATP present in the pyruvate kinase preparations required that the firefly lantern preparations used for the ATP analysis maintain a linear light emission over an ATP concentration range of two orders of magnitude. Aging of the firefly lantern preparation to reduce blanks and improve ATP analysis sensitivity was not possible without additions of synthetic luciferin (Karl and Holm-Hansen 1976) since aging depletes the luciferin in the preparation, reducing the maximum ATP concentration which yields a linear light emission. As a consequence of these difficulties ADP analyses yielded reproducible results only in samples containing nearly equivalent amounts of ATP and ADP (i.e. in samples with low energy charge).

The AMP assay was confounded by the cumulative effects of enzyme blank, ATP, ADP, low AMP to ATP conversion efficiency, and the low concentration of AMP present in growing cells. As a consequence of these complications AMP measurements were not successful in any of the samples.

In the absence of AMP data energy charge could not be calculated. However estimations of the energy charge from ATP to ADP ratios were made by developing an expression relating energy charge to ATP to ADP ratios from literature values. Energy charge and ATP/ADP appear to be linearly related between energy charge values of 0.62 and 0.90 (Table 4), although ATP/ADP increased more rapidly above 0.90. A linear regression of energy charge and ATP/ADP (Fig. 20) allows a rough estimation of energy charge from ATP and ADP

Table 3. Flow chart showing reagents and conditions for enzymatic conversions of ADP and AMP to ATP.

SAMPLE EXTRACT (See ATP Methods)		
600 µl extract	600 µl extract	600 µl extract
+	+	+
50 µl PEP STOCK (0.1 M PEP in 0.5 M TRIS pH 7.7)	50 µl PEP STOCK	50 µl PEP STOCK
+	+	+
50 µl Rx Buffer (0.1 M $MgCl_2$ in 0.5 M TRIS pH 7.7)	50 µl Rx Buffer	50 µl Rx Buffer
+	+	+
50 µl TRIS (0.5 M TRIS pH 7.7)	50 µl PK (10µg)	50 µl PK (10 µg)
+	+	+
50 µl TRIS (0.5 M TRIS pH 7.7)	50 µl TRIS	50 µl MK (10 µg)

All samples mixed well and incubated at 30°C for 20 minutes, then placed on ice until ATP analysis (see Methods).

Calculation of Adenylates

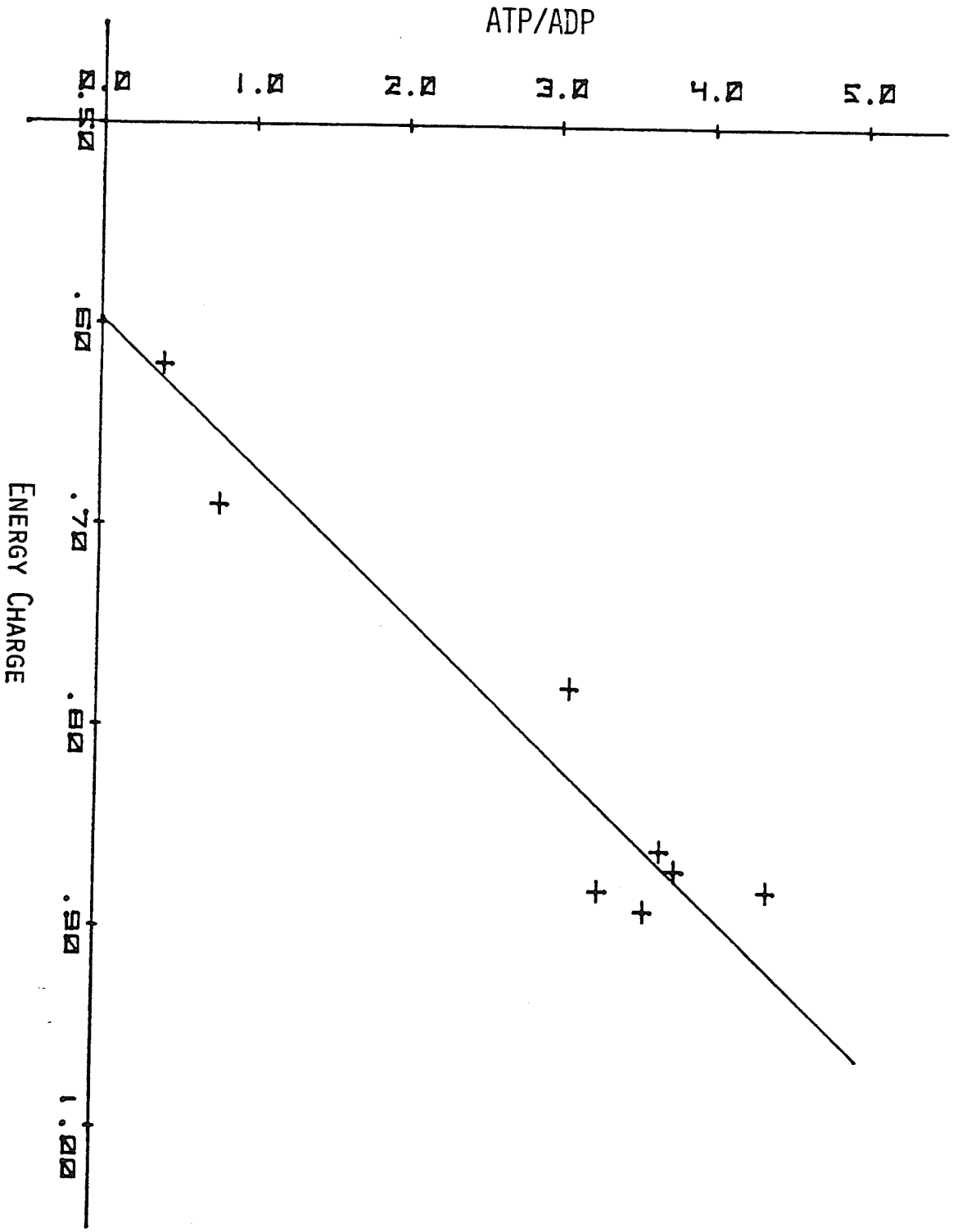
ATP ADP = [ATP + ADP]-[ATP] AMP = [ATP + ADP + AMP]-[ATP + ADP].

PEP - Phospho enol pyruvate; PK - Pyruvate kinase; MK - Myokinase

Table 4. Energy charge and corresponding ATP/ADP ratios calculate from literature.

Energy Charge	$\frac{ATP}{ADP}$	Source	Growth Conditions
.97	35	Dolezac and Kaprálek 1976	N - Limited Chemostat
.94	3.9	"	" "
.92	6.8	"	" "
.91	5.7, 5.1, 6.2	"	C - Limited Chemostat
.91	4.5	"	N - Limited Chemostat
.89	3.6	"	" "
.88	3.3	"	" "
.88	4.4	"	Batch Culture
.87	3.8, 3.8	"	C - Limited Chemostat
.86	3.7	"	" "
.86	3.7	"	Batch Culture
.78	3.1	Karl and Holm-Hansen 1977	Bacteria in Beach Sand
.69	.78	Reece et al. 1976	Starved Batch Culture
.62	.41	"	" " "

Figure 20. Regression of energy charge and ATP/ADP ratios, data from Table 4 ($y = 13.69x - 8.17$; $r = 0.95$).


ATP/ADP
0.0
1.0
2.0
3.0
4.0
5.0
.50
.60
.70
.80
.90
1.00
Energy Charge

values alone. Energy charge values greater than 0.90 can not be estimated from this regression. However, cells with energy charge values of 0.90 and greater contain insignificant amounts of AMP, so energy charge can be calculated directly, assuming AMP equals zero.

The energy charge of the samples, estimated both from ATP to ADP ratios and by assuming AMP equals zero, appear in Table 5. Samples from days 150 and 165 contained appreciable amounts of ATP. The inability to measure ADP is likely due to a high energy charge. Samples from other dates which had undetectable ADP had little ATP, so it is not possible to make a meaningful estimate of the energy charge.

Table 5. Estimates of energy charge: Column I estimated from regression line (Figure 20), Column II assumes AMP equals 0.

DAY	Tube B		C		D		E	
	Estimate Method I	II	I	II	I	II	I	II
144	.91	.93	.74	.83	.88	.90	*	
150	.70	.79	.87	.90	*		.60	.52
165	*		.61	.55	*		*	
172	.67	.74	.71	.80	.76	.85	.76	.85
179	*		*		.65	.71	.83	.88
195	*		.62	.62	.70	.79	.61	.59
200	*		*		.65	.71	.68	.76
207	.65	.72	.68	.77	.65	.72	.64	.68
214	.61	.56	.90	.92	.64	.68	.62	.63
221	.67	.74	*		.63	.64	.65	.70

*These samples contained insufficient adenylates to make a meaningful estimate of energy charge.

APPENDIX III

Epifluorescent Counts

Methods

Fractionated samples fixed with acid Lugol's solution were prepared for epifluorescent counts on Nucleopore^{R} filters (Hobbie et al. 1977). One ml of preserved sample (see Methods) was diluted with 4 ml distilled water and filtered through a 0.2 µm x 25 mm Nucleopore^{R} filter which had been stained with ingralan black. One ml of 0.1 percent acridine orange was then placed in the filtration candle for 15 seconds and removed by filtration. Excess stain was then removed by passing 2 ml of distilled water through the filter. The acridine orange and distilled water were previously filtered through a 0.22 µm Millipore^{R} filter to prevent contamination.

Stained samples were viewed at 1600x with a Zeiss universal research microscope with epifluorescent attachment. The sample was illuminated with a mercury lamp fitted with an exciter bypass filter which transmits wavelengths between 350 mm and 450 mm. The fluorescing cells were then viewed through a barrier filter transmitting wavelengths greater than 520 mm. The average number of bacteria per field from 20 fields was multiplied by 5.34×10^{4} fields per filter. Since 1 ml was filtered this was equivalent to the number of bacteria per ml.

Results

The bacteria numbers from epifluorescent counts did not compare well with ATP concentrations (Table 3). The poor agreement may indicate that the bacteria are not multiplying during growth, but rather increasing in volume and ATP content. Determination of cell volume is very difficult due to the extremely small size of the bacteria, less than 1 μm. Very small, faintly fluorescing cells were obscured by larger neighbouring cells displaying stronger fluorescence. This may account for the apparent decrease in bacterial numbers at high ATP concentrations.

The low counts in samples containing high ATP levels, such as tube D day 221, lead me to believe that acid Lugol's solution was a poor preservative. Samples preserved in formalin have been found to be suitable for epifluorescent counts one year after collection (Akira Furutani, personal communication), indicating that formalin may have been a better preservative.

Table 6. A comparison of epifluorescent counts and ATP concentrations ng/l of four sampling dates.

Day	B		C		D		E	
	Counts[1]	ATP	Counts[1]	ATP	Counts[1]	ATP	Counts[1]	ATP
221	11	40	12	105	6.9	450	21	360
228	32	75	25	135	18	40	20	150
235	15	25	17	0	13	70	25	50
242	26	43	46	68	34	67	40	44

[1]Bacterial cells per ml sample x 10^{-4}.

Table 7. ATP of the size fractionated samples in ng ATP/liter. Note ATP was below detection limits in Tube C on Day 235.

Day of Year	Tube B	Tube C	Tube D	Tube E
144	103	53.2	319	95.4
	109	42.8	322	101
	105	33.2	329	134
150	31.5	67.2	54.3	86.1
	35.2	92.5	42.0	86.0
	38.1	69.6	46.7	84.8
165	143	38.5	81.1	567
	138	38.9	103	583
	140	40.3	104	564
172	347	467	501	459
	365	469	483	472
	349	480	517	451
179	140	39.7	132	47.3
	143	34.0	137	54.7
	123	65.0	142	51.3
195	22.5	48.0	145	53
	31.8	46.7	152	49
	19.2	49.0	162	50
200	41	74	110	90.0
	39	79	110	113
	44	74	126	124
207	132.0	27.5	126	137
	132.3	32.3	120	141
	132.3	32.0	135	141
214	42.0	106	480	366
	48.3	109	440	360
	39.7	101	436	372
221	40.3	119	81.7	67.3
	44.2	132	91.7	75.3
	36.7	119	76.0	76.7
228	76.3	138	47.0	143
	73.7	146	40.7	162
	71.9	131	49.1	141
235	24.3	-	80.0	54.8
	25.1	-	69.9	55.2
	29.3	-	65.7	40.8
242	43	63	78	47
	43	65	62	36
	44	72	62	49

Table 8. pH of the treated tubes on each sampling date.

Day of the Year	Tube			
	B	C	D	E
144	4.6	5.5	3.9	7.2
150	5.3	5.8	4.2	7.6
165	4.7	5.2	3.9	6.5
172	4.8	5.4	4.0	6.6
179	4.6	5.2	3.7	6.3
195	4.9	5.4	4.1	6.0
200	4.2	5.2	3.7	5.6
207	5.6	5.3	3.7	5.5
214	5.1	5.5	3.5	6.3
221	4.0	4.9	3.4	5.7
228	4.9	6.6	4.3	6.5
235	5.0	6.0	4.4	6.6
Average pH	4.8	5.5	3.9	6.4

Table 9. Average hours of bright sun between 9:00-16:00 h for 6 days previous to sampling date. (Data provided by Ken Beaty.)

Sampling Day	Average Hours Bright Sun for 6 Days Previous to Sampling Date
144	4.03
150	5.98
165	2.95
172	2.10
179	5.75
195	3.73
200	5.70
207	6.25
214	4.65
221	4.88
228	2.72
235	5.63

Table 10. Chlorophyll-a µg per liter. (Data provided by Jim Prokopowich.)

Day of Year	Tube			
	B	C	D	E
150	2.5	4.0	2.2	3.5
164	1.3	4.2	7.1	3.4
178	1.1	2.7	1.9	2.4
192	1.9	6.2	1.2	2.6
206	2.6	3.9	8.7	2.7
220	4.5	3.3	7.6	2.9
234	1.5	3.6	4.5	2.2
Average	2.2	4.0	4.7	2.8

Table 11. Phytoplankton biomass g/m^3. (Data provided by Dave Findlay.)

Day of Year	Tube			
	B	C	D	E
122	0.83	1.07	1.80	1.56
153	0.54	2.19	1.76	2.89
165	0.77	4.09	4.22	1.72
174	0.55	2.24	6.03	1.85
192	1.02	5.97	0.31	1.44
201	0.39	4.65	2.53	1.73
222	4.05	1.07	5.37	1.14
229	5.95	1.34	1.12	1.25
Average	1.76	2.83	2.89	1.70

Table 12. Integral primary production in mgc/m^3 -day (data provided by E.J. Fee). Average daily production estimated by Simpson's Rule.

Day of Year	Tubes			
	B	C	D	E
153	54	133	166	103
174	295	632	268	50
209	138	135	553	44
223	84	100	.50	50
229	-	-	88	-
Average Daily Production	167	194	237	57

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