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Adenine nucleotide levels and adenylate energy charge in Zostera marina (eelgrass): determination and application

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ADENINE NUCLEOTIDE LEVELS AND ADENYLATE ENERGY CHARGE IN ZOSTERA MARINA (EELGRASS): DETERMINATION AND APPLICATION

The College of William and Mary in Virginia PH.D. 1982

ADENINE NUCLEOTIDE LEVELS AND ADENYLATE ENERGY CHARGE IN *ZOSTERA MARINA* (EELGRASS): DETERMINATION AND APPLICATION

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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by

Damon A. Delistraty

1982

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Damon Delistraty

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ABSTRACT

An analytical technique was developed to measure adenine nucleotide levels (ATP, ADP, AMP) and adenylate energy charge (EC) in Zostera marina (eelgrass), a submerged marine angiosperm. A tissue comparison and seasonal survey provide baseline information on natural adenylate variability. The methodology developed can be suitably adapted to other macrophyte species as well. Plants were frozen, lyophilized, scraped free of epiphytes, and homogenized. Adenylates were extracted with boiling 1 mM EDTA + 5% (w/v) PVPP (pH 7.6), and assayed by enzymic conversion of AMP and ADP to ATP, followed by quantitative analysis of ATP via the firefly bioluminescent reaction. ATP, ADP, total adenylates (AT), and EC were highest in leaf tissue (photophosphorylating source), while all adenylates were lowest in root plus rhizome. Monthly time series with aboveground tissue show ATP concentration highest in August and lowest in April, corresponding to periods of senescence (decreased ATP utilization) and growth (increased ATP utilization), respectively.

Response of adenine nucleotides and EC in Z. marina to nutrient enrichment, light reduction, and herbicide (atrazine) exposure was evaluated as a monitor of metabolic state. Nutrient enrichment over 2 weeks increased epiphyte colonization, which in turn, appeared to negatively impact Z. marina adenylate content, net productivity, and growth. Z. marina ATP, AT, and EC were weakly and positively correlated with nutrients and light, but decreased over time. Short-term (6 hr) atrazine stress reduced ATP and AT at both 10 and 100 ppb, but EC remained constant. Net productivity decreased at 100, but not at 10 ppb atrazine over 6 hrs. Long-term (21 day) atrazine stress was evident from growth inhibition and 50% mortality near 100 ppb. EC was reduced at 0.1, 1.0, and 10 ppb atrazine, but ATP and EC increased with physiological adaptation to severe stress (100 ppb) after 21 days. Apparently, ATP and AT decrease over the short-term but rebound over the long-term with severe atrazine stress, increasing beyond control levels before plant death results. Supplementing adenine nucleotide and EC results with more conventional quantitative analyses would afford greater knowledge of physiological response to environmental variation.

ADENINE NUCLEOTIDE LEVELS AND ADENYLATE ENERGY CHARGE IN *ZOSTERA MARINA* (EELGRASS): DETERMINATION AND APPLICATION

GENERAL INTRODUCTION

Adenylate Energy Charge

The adenylate energy charge (EC) was first defined by Atkinson and Walton (1967):

$$EC = \frac{[ATP] + 1/2\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

This ratio was proposed as a fundamental metabolic control parameter. As such, EC represents the metabolic energy state of the cell. The biochemical basis of the EC is presented in Appendix A. Broad applications of EC include the following:

1) disciplines, ranging from cellular biochemistry (Atkinson, 1977) to community ecology (Wiebe and Bancroft, 1975);

2) different cellular and organismic types, prokaryote vs. eukaryote, autotroph vs. heterotroph, and single vs. multicellular organisms (Chapman et al., 1971); and

3) a range of environments, including marine (Karl and Holm-Hansen, 1978), estuarine (Mendelssohn and McKee, 1981), and terrestrial systems (Ching and Kronstad, 1972).

Recent application of EC measurement to higher plants is extensive, primarily involving agriculturally important crop species (e.g. Raymond and Pradet, 1980; Saglio et al., 1980; Bonzon et al., 1981; Quebedeaux, 1981; Hampp et al., 1982). In contrast, adenylate

literature on seagrasses (Knauer and Ayers, 1977) is extremely limited. Plants respond to environmental stress in numerous ways (Levitt, 1972; Cottenie and Camerlynck, 1979; Rabe and Kreeb, 1979). Since the metabolic energy state of an organism is sensitive to environmental variation, both natural and anthropogenic, EC has been advanced as an index of sublethal stress (Ivanovici, 1980).

Ecology of Zostera marina

Zostera marina (eelgrass), a submerged marine angiosperm, functions as a food source, habitat, nutrient pump, and sediment stabilizer. The basic biology (Setchell, 1929; Burkholder and Doheny, 1968; Harrison and Mann, 1975; Orth et al., 1981) and ecological value (McRoy and Helfferich, 1977; Stevenson and Confer, 1978; Phillips and McRoy, 1980; Wetzel et al., 1981) of Z. marina have been well documented.

Historically and more recently, the distribution and abundance of Z. marina have undergone large fluctuations in the Chesapeake Bay (Orth and Moore, 1981). The reduction of eelgrass beds has been attributed to disease (Renn, 1934), temperature increase (Orth, 1976), herbicide input (Stevenson and Confer, 1978), cownose ray disturbance (Orth, 1975), and to a lesser extent, dredging and boating activities (Orth, 1976). A reliable method to assess the metabolic state of eelgrass is, therefore, essential. Application of energy charge measurement to Z. marina is a logical choice.

Objectives

1. A major objective of this study was development of a methodology to quantitatively measure adenine nucleotides and adenylate energy charge (EC) in Zostera marina (eelgrass). The remaining objectives have incorporated these optimized techniques.

2. Adenylates and EC were compared among Z. marina tissues, including leaf, leaf sheath, root plus rhizome, and seed pod. Comparative measurements were made on eelgrass epiphytes, aboveground Ruppia maritima (widgeongrass), and aboveground Spartina alterniflora (saltmarsh cordgrass).

3. Monthly variation of adenylates and EC was assessed in above and belowground Z. marina tissue over a one year period. Associated environmental and morphometric data were collected.

4. Adenylate and EC responses to three nutrient levels and two light levels, over 1 and 2 week periods, were examined in Z. marina leaf tissue and epiphytes. Additionally, morphometric, productivity, and epiphyte colonization data were obtained.

5. Adenylate and EC responses to two atrazine levels over 6 hours, and five atrazine levels over 21 days, were assessed in Z. marina leaf tissue. Hourly production rates were measured during the 6 hour experiment. Weekly morphometric changes and mortality were examined over the 21 day atrazine exposure period.

Overview

This dissertation is divided into two major sections. Section 1 (Objectives 1-3) focuses on analytical determination of adenine nucleotides. Section 2 (Objectives 4 and 5) evaluates adenylate

response to selected environmental variables. Appendix A reviews the metabolic role of the adenine nucleotides. Appendix B outlines assumptions underlying several parametric procedures, used in statistical analyses.

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SECTION 1. DETERMINATION

INTRODUCTION

Adenine nucleotides, expressed as the adenylate energy charge (EC) ratio, regulate cellular energetics (Atkinson, 1977). Problems associated with methodology for the determination of in situ adenine nucleotide levels may limit the utility of the EC concept, in general (Pradet and Raymond, 1978; Karl, 1980; Ivanovici, 1980). Methodology must be tailored to the specific chemical characteristics of a particular biological material in order to accurately determine in situ levels of intracellular adenine nucleotides. In addition, ease of operation and reproducibility are essential to any useful analytical technique.

The most frequently employed methods for determination of adenine nucleotides have involved enzymic conversion of adenosine monophosphate (AMP) and adenosine diphosphate (ADP) to equivalent amounts of adenosine triphosphate (ATP), followed by quantitative analysis of the ATP via the firefly bioluminescent reaction (Karl and Holm-Hansen, 1978). Determination of ATP by the firefly luciferase reaction, reviewed by Leach (1982), has been widely applied (DeLuca, 1978; DeLuca and McElroy, 1981). After reviewing the literature, Sofrova and Leblova (1970) concluded that the firefly reaction is the most rapid, sensitive, and specific method for ATP determination in plant tissue. Several studies, which specifically address methodology

for adenylate determination in higher plants, have utilized the firefly reaction (Pradet, 1967; Guinn and Eidenbock, 1972; DeGreef et al., 1979; Mendelssohn and McKee, 1981).

Employing the firefly assay, this study developed a methodology to optimize determination of adenine nucleotides in Zostera marina (eelgrass), a submerged marine angiosperm. Z. marina is an ecologically important macrophyte species (McRoy and Helfferich, 1977; Stevenson and Confer, 1978; Phillips and McRoy, 1980; Wetzel et. al., 1981; Orth et al., 1981), occurring in temperate and subarctic coastal and estuarine waters in the Northern Hemisphere (den Hartog, 1970). Major analytical procedures were evaluated, including sample collection and preparation, adenylate extraction, conversion of AMP and ADP to ATP, firefly lantern extract preparation, and photometry. Methodology for adenylate determination, presented in the Methods section, represents methodology which has been optimized from experimental data presented in the Results section. Tissue composition and seasonal patterns of adenine nucleotides were also assessed in order to provide baseline information on natural adenylate variability in Z. marina.

METHODS

Sampling sites

Zostera marina was collected at low tide from an extensive grassbed (37°15'40" N, 76°23'50" W) off Sandy Point at the mouth of the York River in the lower Chesapeake Bay estuary. This bed is close to the laboratory and accessible by land. Epiphytes and *Ruppia maritima* were also obtained from Sandy Point. *Spartina alterniflora* was collected from nearby Indian Field Creek (37°16'5" N, 76°33'30" W). Locations of these sites are shown in Figure 1.1.

Adenine Nucleotides

Assay Principles

Adenylate assay reactions have been described by Pradet (1967), Holm-Hansen and Karl (1978), and DeLuca (1976). ATP was assayed with the firefly bioluminescent reaction (Figure 1.2). AMP and ADP were first converted enzymically to ATP (Figure 1.3), which was then analyzed by the firefly reaction. The equilibrium constant for the PK reaction is sufficiently large to convert most ADP, and consequently most AMP, to ATP (Adam, 1965).

Sample Collection and Preparation

Plants were uprooted with a shovel, swirled in river water to remove macro-algae and loose sediment, and stuffed in a 180 or 530 ml plastic bag (Whirl-Pak). Liquid nitrogen was poured into the bag

Figure 1.1. Sampling site locations in the lower Chesapeake Bay.

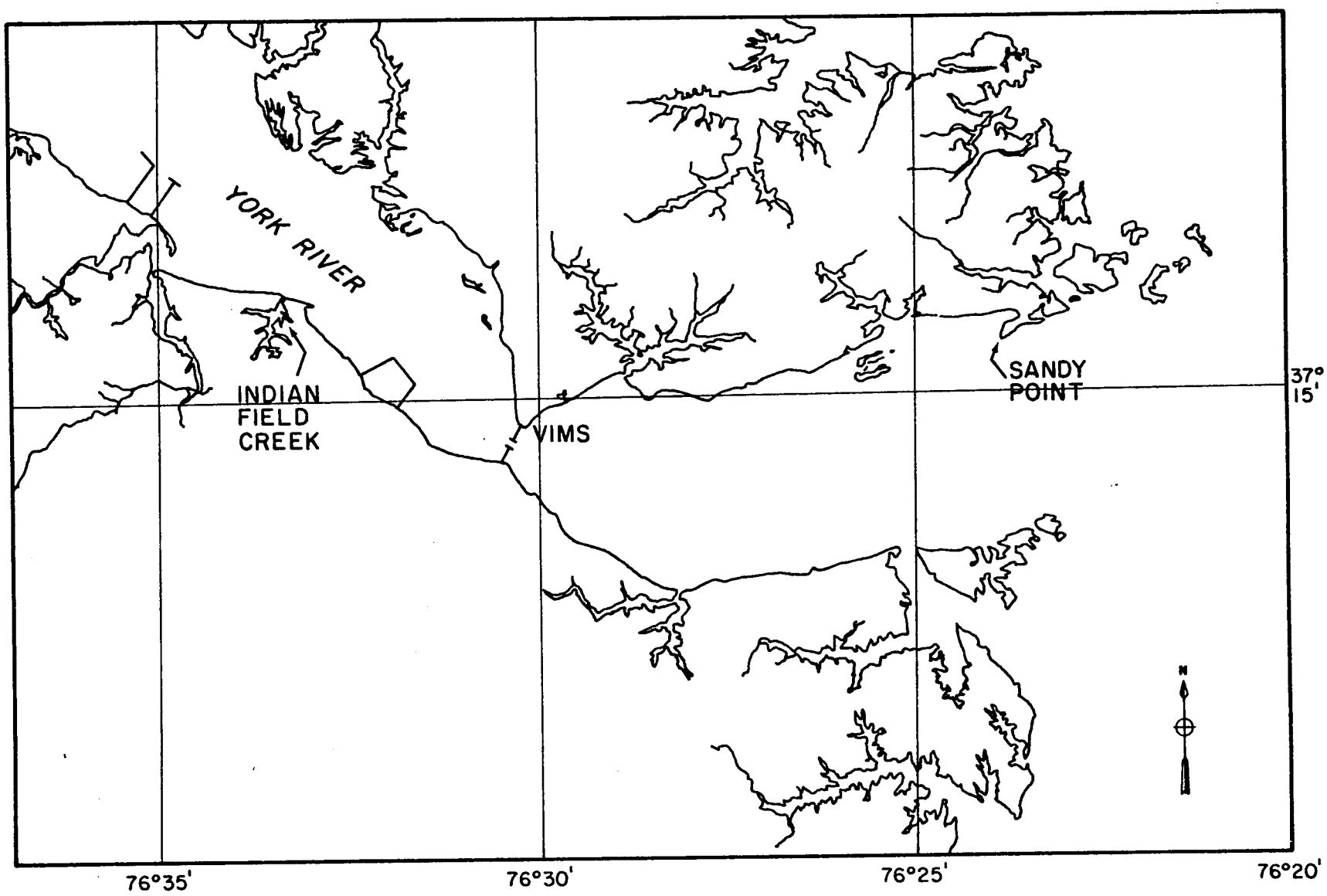
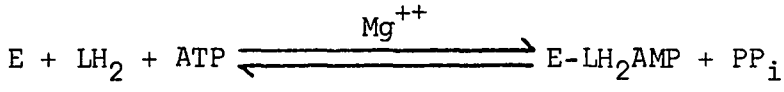
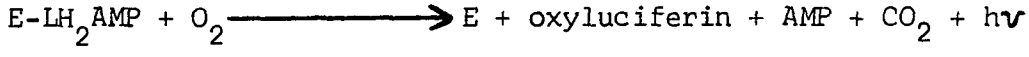

YORK RIVER
INDIAN
FIELD
CREEK
VIMS
SANDY
POINT
37°
15'
N
76°35'
76°30'
76°25'
76°20'

Figure 1.2. Firefly bioluminescent reaction.





E: firefly luciferase (EC 1.13.12.7)

LH_2: luciferin

$E\text{-}LH_2AMP$: enzyme-bound luciferyl-adenylate

Figure 1.3. Enzymic conversion reactions.

ADP conversion

$$ADP + PEP \underset{K^+,\ Mg^{++}}{\overset{PK}{\rightleftharpoons}} ATP + pyruvate \qquad K = 2 \times 10^3 \text{ (Adam, 1965)}$$

Coupled AMP conversion

$$AMP + ATP \underset{Mg^{++}}{\overset{AK}{\rightleftharpoons}} 2\ ADP \qquad K = 1.2 \text{ (Atkinson, 1977)}$$

$$ADP + PEP \underset{K^+,\ Mg^{++}}{\overset{PK}{\rightleftharpoons}} ATP + pyruvate$$

PEP: phosphoenolpyruvate

PK: pyruvate kinase (EC 2.7.1.40)

AK: adenylate kinase (EC 2.7.4.3)

(within 1 min of harvest), and the entire bag was submerged in liquid nitrogen contained in a 4 l polyethelene dewar flask (Nalgene) for return to the laboratory.

Liquid nitrogen was drained from the bag which was kept opened and placed in a lyophilizer. The chamber was sealed, and vacuum was initiated, with condenser temperature allowed to reach -55°C before sample introduction. Chamber shelves, which support the sample, were not heated. Samples were lyophilized for 70-90 hrs.

After lyophilization, plant tissue was handled with forceps to prevent hydration. Brown aboveground tissue was discarded, since this material was considered dead at time of harvest. Leaves were scraped with a flat spatula which removes 70-90% of the epiphytes (Penhale, 1977).

For methodology experiments (excluding freeze delay), plants were pooled to provide a uniform substrate for experimental treatments. For tissue comparison and seasonal survey experiments, plants within a treatment (i.e. tissue type or monthly sample, respectively) were pooled in order to minimize within treatment variation and spotlight between treatment variation. In addition, pooling provides a larger quantity of plant material for analysis. The pooled sample was then divided into specific tissues to be assayed. Leaf tissue was used for methodology experiments. Leaf, leaf sheath, root plus rhizome, and seed pod tissue were examined in the tissue comparison experiment. Aboveground (stem plus leaf) and belowground (root plus rhizome) parts were analyzed in the seasonal survey.

Tissues were ground in a cutting mill to pass a #40 (425 μ) mesh screen. Scrapings (epiphytes) off lyophilized Z. marina leaves were ground by hand with mortar and pestle. Samples were either processed immediately or stored in a vacuum desiccator (Nalgene) in the dark for up to 5 days. Tissue preparation was adapted from the method of Mendelssohn and McKee (1981).

Extraction

Tissue was weighed into 20-80 mg aliquots and held in a desiccator. Extractant solution was 1 mM ethylenediaminetetraacetic acid (EDTA) + 5% (w/v) polyvinylpolypyrrolidone (PVPP) at pH 7.6. Four to eight ml of extractant were heated to 100°C in a 50 ml beaker on a hot plate (Corning). Tissue was added (≤1% w/v), and the beaker swirled for 30 sec at 100°C. The extract was quantitatively transferred to a centrifuge tube. The beaker was rinsed with additional 1 mM EDTA which was poured into the centrifuge tube to bring the final volume up to 5 or 10 ml. These tubes were held on ice and centrifuged at 14000 g for 15 min at 4°C. The supernatant was decanted, held on ice, and processed as soon as possible. Extraction methodology was adapted from Mendelssohn and McKee (1981).

EDTA extractant solution was stored at 4°C in the dark and used for periods up to 1 month. A working aliquot of EDTA solution was vacuum-filtered through a 0.2 μ nitrocellulose membrane (Nalgene) for each day's analyses and discarded immediately after use. Reagents were routinely filtered as a precaution against microbial

contamination. PVPP was added to the filtered EDTA solution approximately 30 min prior to extraction.

Conversion

AMP and ADP were enzymically converted to ATP. Three sets of reaction mixtures (13 x 100 mm disposable glass tubes) were prepared as follows:

Tube A (ATP Reagents):	400 µl blank (extractant), standard (ATP in extractant), or sample extract 400 µl reaction buffer (45 mM TRICINE, 18 mM $MgSO_4$, pH 7.6) 400 µl distilled water (DW)
Tube B (ADP + ATP Reagents):	400 µl blank, standard, or sample extract 400 µl reaction buffer 400 µl PK (30 µg), PEP (1.5 mM)
Tube C (AMP + ADP + ATP Reagents):	400 µl blank, standard, or sample extract 400 µl reaction buffer 400 µl PK, PEP, AK (30 µg).

Activities of PK and AK were 350-500 and 1000-1500 units mg^{-1} protein, respectively. Tubes were incubated (30°C, 30 min), heat deactivated (100°C, 2 min), and allowed to re-equilibrate (on ice, 20 min). Composition of conversion reaction mixtures with ATP standards appears in Table 1.1.

Buffer was stored at 4°C in the dark and used for periods up to 2 weeks. Working aliquots of buffer and DW were filtered (0.2 µ) for each day's analyses and discarded immediately after use. Fresh solutions of [PEP + PK] and [PEP + PK + AK] were prepared in filtered

Table 1.1. Composition of conversion reaction mixtures.

Component	Units	Reaction Mixture		
		[ATP] Reagents	[ATP+ADP] Reagents	[ATP+ADP+AMP] Reagents
Na_2ATP	ng ml^{-1}	13-1333	13-1333	13-1333
TRICINE buffer	mM	15	15	15
$MgSO_4$	mM	6	6	6
Na_2EDTA	µM	333	333	333
PK	µg ml^{-1}	-	25	25
AK	µg ml^{-1}	-	-	25
Na_3PEP	µM	-	500	500
$(NH_4)_2SO_4$[1]	mM	-	15	30

[1] From PK and AK suspensions

DW in glass vials for each day's analyses, held on ice, and discarded immediately after use.

Firefly Lantern Extract Preparation

One vial of lyophilized firefly lantern extract (FLE), commercially prepared from 50 mg dried lanterns, was hydrated with 25 ml filtered (0.2 μ) 45 mM TRICINE-18 mM $MgSO_4$ (pH 7.6) and aged (room temperature, 6-8 hrs) in order to degrade endogenous ATP. After aging, the insoluble residue was removed by centrifugation at 3000 RPM for 15 min. Whenever a large volume of FLE was required, several vials were pooled in order to eliminate variation between individual vials (Holm-Hansen and Karl, 1978).

Photometry

The photometer was allowed to warm up for at least 1 hr prior to assays. A sensitivity setting of 7.00 was utilized, since best instrument stability is achieved by using the lowest setting adequate for analysis (SAIT, 1975). Dark current was nulled by adjusting the zero (4.80 - 4.90 at sensitivity 7.00), just before each standard set was run.

One hundred μl of solution from Tubes A, B, or C were pipetted (Eppendorf) into a 6 x 50 mm disposable glass tube. Fifty μl of FLE were pipetted (Eppendorf) into this tube, while simultaneously initiating the 10 sec delay mode of the photometer timing circuit with the footswitch. During this delay period, the tube was vortexed (Vortex-Genie) to ensure thorough mixing, inserted into the photometer, and the shutter was opened. Counts were recorded for the

following 10 sec integration period. A chart recorder was interfaced with the photometer to follow reaction kinetics in order to detect interferences or instrument malfunction.

For peak height measurements, 100 µl of solution from Tubes A, B, or C were pipetted into a 6 x 50 mm tube. The tube was then placed inside the photometer, the shutter opened, and 50 µl of FLE injected with the electronic pipet system which simultaneously activates the photometer. Peak height counts were recorded. Sensitivity settings from 7.00 - 10.00 were used. As a check on initial reagent mixing for peak height measurements, each tube was read, removed from the photometer, vortexed, and re-inserted into the photometer. If the recorder trace exhibited continuity, the reading was considered valid (Karl and Holm-Hansen, 1978). If not, the tube was discarded, and the process repeated until a continuous trace (i.e. thorough initial mixing) was obtained.

Composition of firefly reaction mixtures with ATP standards appears in Table 1.2. In addition, pH values for reaction components and mixtures are presented in Table 1.3.

Standards and Blanks

A primary standard was prepared with a weighed amount of ATP dissolved in filtered (0.2 µ), distilled, deionized water. This primary standard was divided into 1 ml aliquots and stored frozen (-20°C) in glass vials for a period up to 3 months. A fresh set of working standards was prepared in glass vials for each day's analyses. An aliquot of primary standard was thawed and serially diluted with

Table 1.2. Composition of firefly reaction mixtures.

Component	Units	Reaction Mixture		
		[ATP] Reagents	[ATP+ADP] Reagents	[ATP+ADP+AMP] Reagents
Na_2ATP	ng ml^{-1}	8-888	8-888	8-888
TRICINE buffer	mM	25	25	25
$MgSO_4$[1]	mM	11	11	11
Na_2EDTA	µM	222	222	222
PK	µg ml^{-1}	-	17	17
AK	µg ml^{-1}	-	-	17
Na_3PEP	µM	-	333	333
$(NH_4)_2SO_4$[2]	mM	-	10	20
FLE[3]	µg ml^{-1}	667	667	667
KH_2AsO_4[4]	mM	3	3	3

[1] 1 mM from FLE preparation
[2] From PK and AK suspensions
[3] Expressed as precursor firefly lanterns
[4] From FLE preparation

Table 1.3. Reaction component and mixture pH values.

Label	Component or Mixture		pH[1]
R	Distilled water		7.45
S	75 µg ml^{-1} PK + 1.5 mM PEP		7.05
T	75 µg ml^{-1} PK + 1.5 mM PEP + 75 µg ml^{-1} AK		6.90
	1 mM EDTA		7.58
U	1 mM EDTA + 1 µg ml^{-1} ATP		7.66
V	45 mM TRICINE + 18 mM $MgSO_4$		7.62
W	[2]Tube A = 400 µl R + 400 µl U + 400 µl V	Conversion	7.55
X	[3]Tube B = 400 µl S + 400 µl U + 400 µl V	Reaction	7.55
Y	[4]Tube C = 400 µl T + 400 µl U + 400 µl V	Mixtures	7.53
Z	2 mg ml^{-1} FLE[5] + 45 mM TRICINE + 18 mM $MgSO_4$		7.43
	[2]Tube A = 100 µl W + 50 µl Z	Firefly	7.48
	[3]Tube B = 100 µl X + 50 µl Z	Reaction	7.49
	[4]Tube C = 100 µl Y + 50 µl Z	Mixtures	7.49

1 pH meter calibrated with .05M (KH_2PO_4 - NaOH) buffer to pH 7.00 at 25°C
2 [ATP] Reagents
3 [ATP+ADP] Reagents
4 [ATP+ADP+AMP] Reagents
5 Expressed as precursor firefly lanterns

filtered (0.2 μ) extractant solution (1 mM EDTA) to produce a set of standards which bracket sample ATP levels. Working standards were held on ice and discarded immediately after use. Although Holm-Hansen and Karl (1978) have reported no significant loss of these standard adenylates during an 8 hr period, a standard set was run at least every 2 hrs. Working standards and blanks were carried through enzymic conversion and incubation steps to parallel sample processing. This results in similar ionic composition and ATP reactivity, permitting more accurate adenylate quantification (Holm-Hansen and Karl, 1978). Standards and blanks were each read in duplicate per reaction Tube A, B, or C. In cases where a large discrepancy in duplicate readings occurred, a third reading was taken.

Another primary standard was prepared with weighed amounts of ATP, ADP, and AMP dissolved in filtered (0.2 μ), distilled, deionized water. The resultant standard, containing equal concentrations of ATP, ADP, and AMP, was used to calculate recovery and conversion efficiencies.

Data Reduction

Net light output was computed by subtracting the appropriate blank value from each total light emission value. Log [net light emission], dependent variable, was regressed against log [ATP concentration], independent variable, for three separate series of standards, processed in reaction Tubes A (ATP Reagents), B (ATP + ADP Reagents), and C (ATP + ADP + AMP Reagents).

Each sample extract was similarly processed in reaction Tubes A, B, and C (duplicate reading per tube), and tube concentrations were calculated from corresponding standard regressions. Amounts of adenylates (ATP equivalents ml^{-1}) and EC were computed from tube concentrations as follows:

$$ATP = \text{Tube A}$$

$$ADP = \text{Tube B} - \text{Tube A}$$

$$AMP = \text{Tube C} - \text{Tube B}$$

$$AT = \text{Tube C}$$

$$EC = \frac{\text{Tube A} + \text{Tube B}}{2\ (\text{Tube C})}$$

An ATP equivalent is the amount of AMP, ADP, or AT, given as the weight of an equimolar amount of ATP (Pamatmat and Skjoldal, 1979). The formulation used for EC (Ball and Atkinson, 1975) reduces propagation of errors by using directly measured quantities. Since standards, blanks, and sample extracts all underwent identical dilution:

$$\frac{\mu g \text{ ATP equivalent}}{g \text{ dry wt tissue}} = \frac{\mu g \text{ ATP equivalent}}{ml} \times \frac{ml \text{ extraction volume}}{g \text{ dry wt tissue}}$$

Recovery and Conversion Efficiencies

Efficiency of adenylate recovery after extraction was determined by assaying two aliquots: 1) sample with addition of known amounts of ATP, ADP, and AMP (internal standard) immediately before extraction, and 2) sample without internal standard addition. Recovery was calculated as follows (Mendelssohn and McKee, 1981):

$$\% \text{ Recovery} = \frac{[AN_{\text{Tissue + Internal Standard}} - AN_{\text{Tissue}}] \text{ Determined by Assay}}{[AN_{\text{Internal Standard}}] \text{ Known Addition}} \times 100$$

where AN = ATP, ADP, or AMP.

Strehler (1968) has offered two recommendations: 1) light output of sample plus added adenylate should not be more than 50% greater than the response of sample alone, and 2) maximum ATP concentration (sample plus internal standard) should be well below the K_m value for ATP with respect to luciferase (i.e. maintenance of linearity between light output and ATP concentration). Both of these recommendations were followed.

Efficiency of enzymic conversion (i.e. AMP and ADP to ATP) was evaluated by assaying a standard containing known of amounts of ATP, ADP, and AMP (Mendelssohn and McKee, 1981):

$$\% \text{ Conversion} = \frac{[AN_{\text{Standard}}] \text{ Determined by Assay}}{[AN_{\text{Standard}}] \text{ Known Amount}} \times 100$$

where AN = ADP or AMP.

Reagents and Equipment

The following reagents were obtained from Sigma Chemical Co.: firefly lantern extract (FLE-50), ATP (A 5394), ADP (A 6521), AMP (A 1877), GDP (G 6506), PEP (P 7002), PK (P 1506), AK (M 3003), PVPP (P 6755), TRIS-HCl (T 3253), HEPES (H 3375), and TRICINE (T 0377). Other chemicals used in this study were analytical reagent grade.

Adenylates were measured with an ATP photometer (Model 3000, SAI Technology Co.) and, in the case of peak height measurements, with the Enzyme Kinetics Kit electronic injection pipet (No. 020302, SAI Technology Co.). A chart recorder (Model 250/MM, Linear) was modified to accommodate an input voltage from 0.01-10 V.

Other equipment included a lyophilizer (Model 10-100, VirTis), mechanical analytical balance (Model H31, Mettler), electronic top-loading balance (Model PL 200, Mettler), drying oven (Model SW-17TA, Blue M Electric Co.), refrigerated centrifuge (Model PR-2, International Equipment Co.) with high capacity attachment, high speed angle centrifuge (Model SS-1,Sorvall), Thomas-Wiley intermediate mill (Model 3383-L10, Arthur H. Thomas Co.), water bath (Model MW-1110A-1, Blue M Electric Co.), vacuum pump (No. XX60000000, Millipore), and digital pH meter (Model 610, Fisher Scientific Co.), equipped with a glass-body combination electrode (No. 13-639-90, Fisher Scientific Co.). Disposable tubes, vials, filters, pipets, and pipet tips were routinely used. Reusable glassware was acid-washed, rinsed three times with DW, and oven-dried to minimize contamination.

Tissue Comparisons and Seasonal Survey

Environmental Data

Environmental data included water temperature, salinity, and pH. Water samples, collected in brown bottles (Nalgene), were returned to the laboratory for salinity (induction salinometer, Model RS 7B, Beckman) and pH measurements. Daylength and low tide time and height data were obtained from tide tables (NOAA, 1979, 1980).

Morphometrics

At each harvest, one 0.03 m^2 plug of eelgrass was cored down to a depth of 10 cm with a plexiglass tube (0.10 m radius), placed in a coarse mesh bag, and washed free of sediment. This sample was returned to the laboratory and analyzed for total number of shoots, shoot lengths, and above and belowground biomass, according to Orth (1977).

Statistical Analysis

Procedures

The following procedures in the SPSS software package (Nie et al., 1975; Hull and Nie, 1981) were used: ONEWAY (single factor ANOVA, Hartley F max and Cochran C tests for homoscedasticity, Student-Newman-Keuls multiple range test), NPAR TESTS (Kruskal-Wallis single factor ANOVA by ranks and Kolmogorov-Smirnov one sample test for normality), and NONPAR CORR (Spearman rank correlation).

Other statistical procedures employed included analysis of covariance (test for homogeneity of linear regression slopes and intercepts) with an associated multiple range test (Zar, 1974), nonparametric multiple range testing by rank sums (Zar, 1974), linear regression, and Pearson correlation. Statistical assumptions are discussed in Appendix B.

Standard Curve

In standard curve regressions, ATP net count and concentration data were log-transformed. It was initially determined that log-transformed count data satisfy the assumptions of homoscedasticity

and normality. Pearson correlation coefficients corresponding to these log-log regressions were calculated.

Adenine Nucleotide Methodology Experiments

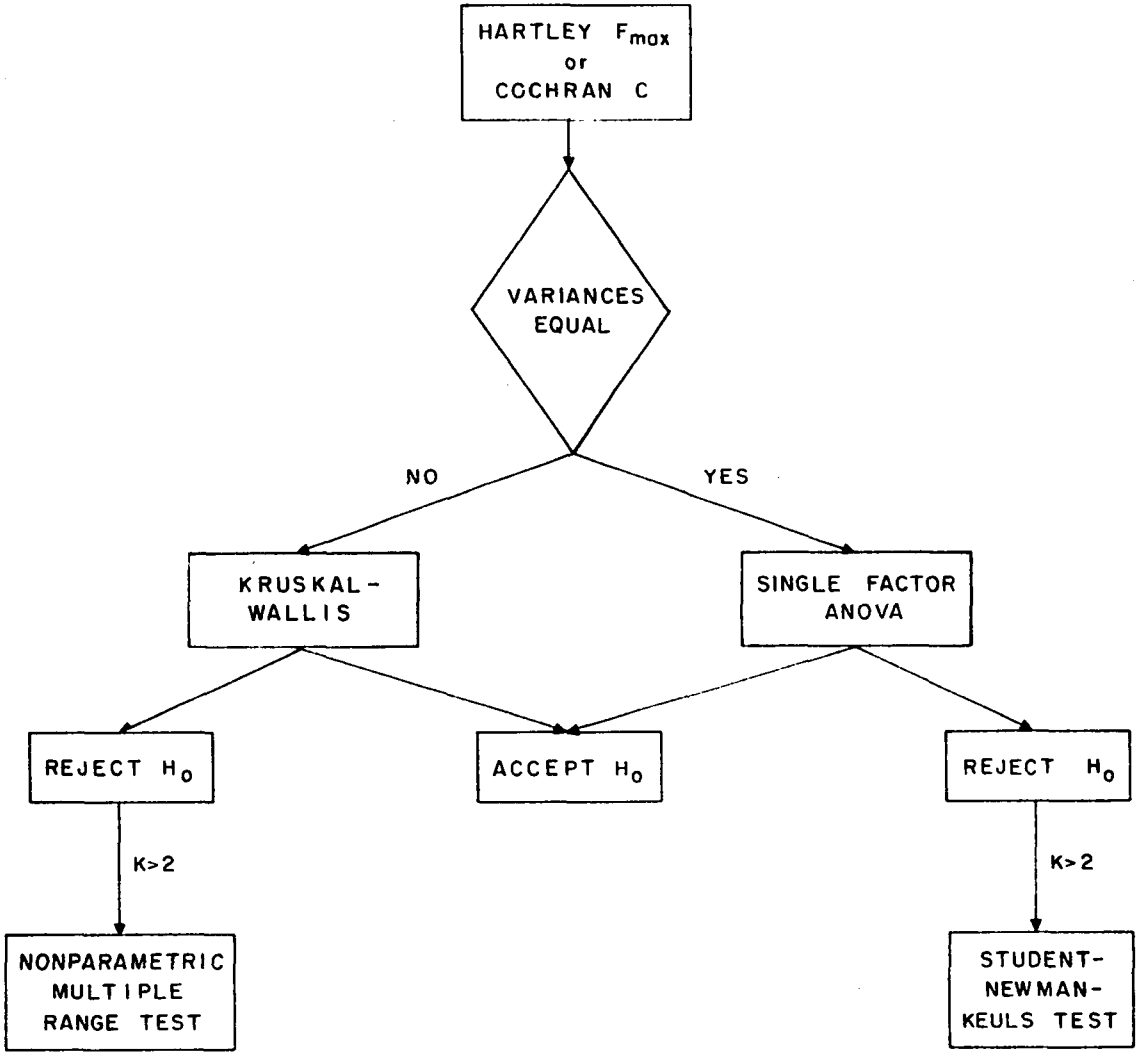
Differences between adenylates, subjected to various analytical treatments, were detected and located by the procedure diagrammed in Figure 1.4. Dependent variables are ATP, ADP, AMP, AT, and EC. Independent variables are treatment levels. The null hypothesis states no difference in adenylates between k treatments (i.e. H_0 : $\mu_1 = \mu_2 = \ldots = \mu_k$).

Standard curves, generated by three different photometer counting modes, were compared. Homogeneity of these linear regression slopes and intercepts was tested by analysis of covariance (ANCOVA). Data were log-transformed and satisfy the assumptions of homoscedasticity and normality. Pearson correlation coefficients for log-log regressions used in ANCOVA were calculated. Null hypotheses state no difference in slopes (H_0 : $\beta_1 = \beta_2 = \beta_3$) or intercepts ($H_0$: $\alpha_1 = \alpha_2 = \alpha_3$) between regressions. Significant differences were located by the Student-Newman-Keuls multiple range test.

Tissue Comparisons

Differences between adenylates in four tissue types were detected and located by the procedure diagrammed in Figure 1.4. Relationships among adenylates were evaluated by Spearman rank correlation.

Figure 1.4. Detection and location of treatment differences.


HARTLEY F_{max}
or
COCHRAN C
VARIANCES EQUAL
NO
YES
KRUSKAL-WALLIS
SINGLE FACTOR ANOVA
REJECT H_0
ACCEPT H_0
REJECT H_0
K>2
K>2
NONPARAMETRIC MULTIPLE RANGE TEST
STUDENT-NEWMAN-KEULS TEST

Seasonal survey

Differences between adenylates at monthly intervals were detected by the procedure diagrammed in Figure 1.4. Relationships among adenylate, environmental, and morphometric data were analyzed by Spearman rank correlation.

RESULTS

Adenine Nucleotide Methodology Experiments

Overview

Table 1.4 summarizes tested factors and their associated treatment levels, grouped under the appropriate analytical procedure. Standards and blanks, used to quantify samples and internal standard recovery and conversion, were processed in parallel with samples and internal standards (i.e. with corresponding treatment levels) for the following factors: extractant, all conversion factors, all FLE preparation factors, and photometer counting mode.

Sample Collection and Preparation

Eight harvest-freeze delay periods were compared (Table 1.5). The delay period represents the time interval between uprooting the plants and freezing them in liquid nitrogen. ATP, AT, and EC generally increased as delay period lengthened. These trends are shown graphically in Figure 1.5. Associated regression statistics are presented in Table 1.6. Results suggest that adenylate concentrations reflect *in situ* levels for a period $\leq$2 min following harvest.

Adenylate levels in plants harvested during the day or at night, each at two delay intervals, appear in Table 1.7. Results suggest that increases in ATP, AT, and EC (as delay period lengthens) are

Table 1.4. Summary of tested factors.

Procedure	Factor	Levels
Sample Collection and Preparation	harvest-freeze delay	.25, .5, 1, 2, 5, 10, 30, 60 min
	day vs night harvest with freeze delay	1200 hrs (30 sec, 10 min) 2400 hrs (30 sec, 10 min)
	tissue state	fresh-chopped (5 mm) vs frozen-lyophilized-ground (425 μ)
	epiphyte removal	scraped vs unscraped leaf
	desiccated-dark storage of frozen-lyophilized-ground tissue	0, 5, 20 days
Extraction	extractant	boiling 1 mM EDTA (pH 7.6), boiling 1 mM EDTA + 5% (w/v) PVPP (pH 7.6), boiling distilled water, 0-4°C .6 N H_2SO_4 + 1 mM EDTA (neutralized to pH 7.6-7.9 after extraction)
	extraction time	5, 30, 120 sec
	extracted tissue	individual vs pooled plants
	frozen extract storage	0, 5, 20 days

Table 1.4. (continued)

Procedure	Factor	Levels
Conversion	buffer	15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6), 15 mM HEPES + 6 mM $MgSO_4$ (pH 7.6), 15 mM TRIS-HCl + 6 mM $MgSO_4$ (pH 7.6)
	enzyme cofactors	15 mM TRICINE (pH 7.6), 15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6), 15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 7.6), 15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 8.1)
	heat deactivation	heated, not heated
FLE Preparation	reconstituent	distilled water, 45 mM TRICINE + 18 mM $MgSO_4$ (pH 7.6), 45 mM TRICINE + 18 mM $MgSO_4$ (pH 8.1)
	aging time and temperature	6 hrs (4, 25°C), 24 hrs (4, 25°C)
	GDP addition	0, 6.85 µg ml^{-1}
Photometry	counting mode	peak height, 10 sec delay + 10 sec integral, 10 sec delay + 30 sec integral
	standard volume/FLE volume (peak height mode)	20, 50, 100 µl standard / 15, 25, 50, 100, 200 µl FLE

Table 1.5. Effect of time delay, between harvest and freezing, on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=2).

Variable	Time Delay (min)							
	.25	.5	1	2	5	10	30	60
ATP	$138^{a1} \pm 1^2$	$134^{a} \pm 4$	$157^{b} \pm 2$	$140^{a} \pm 1$	$216^{c} \pm 6$	$196^{d} \pm 1$	$184^{e} \pm 3$	$240^{f} \pm 3$
ADP	$91^{a} \pm 1$	$91^{a} \pm 3$	$103^{a} \pm 1$	$98^{a} \pm 4$	$68^{a} \pm 13$	$91^{a} \pm 5$	$102^{a} \pm 1$	$106^{a} \pm 1$
AMP	$78^{a} \pm 4$	$97^{b} \pm 3$	$84^{a} \pm 1$	$73^{ac} \pm 1$	$53^{d} \pm 5$	$61^{cd} \pm 4$	$82^{a} \pm 5$	$63^{cd} \pm 1$
AT	$306^{a} \pm 2$	$322^{ab} \pm 11$	$344^{bc} \pm 3$	$311^{ab} \pm 3$	$337^{abc} \pm 15$	$347^{bc} \pm 9$	$368^{c} \pm 4$	$408^{d} \pm 5$
EC	$.60^{a} \pm .01$	$.56^{b} \pm <.01$	$.61^{a} \pm <.01$	$.61^{a} \pm <.01$	$.75^{c} \pm .01$	$.70^{d} \pm .01$	$.64^{e} \pm .01$	$.72^{f} \pm <.01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Figure 1.5. Semi-log regressions of ATP, AT, and EC vs. harvest-freeze delay interval (n = 2).

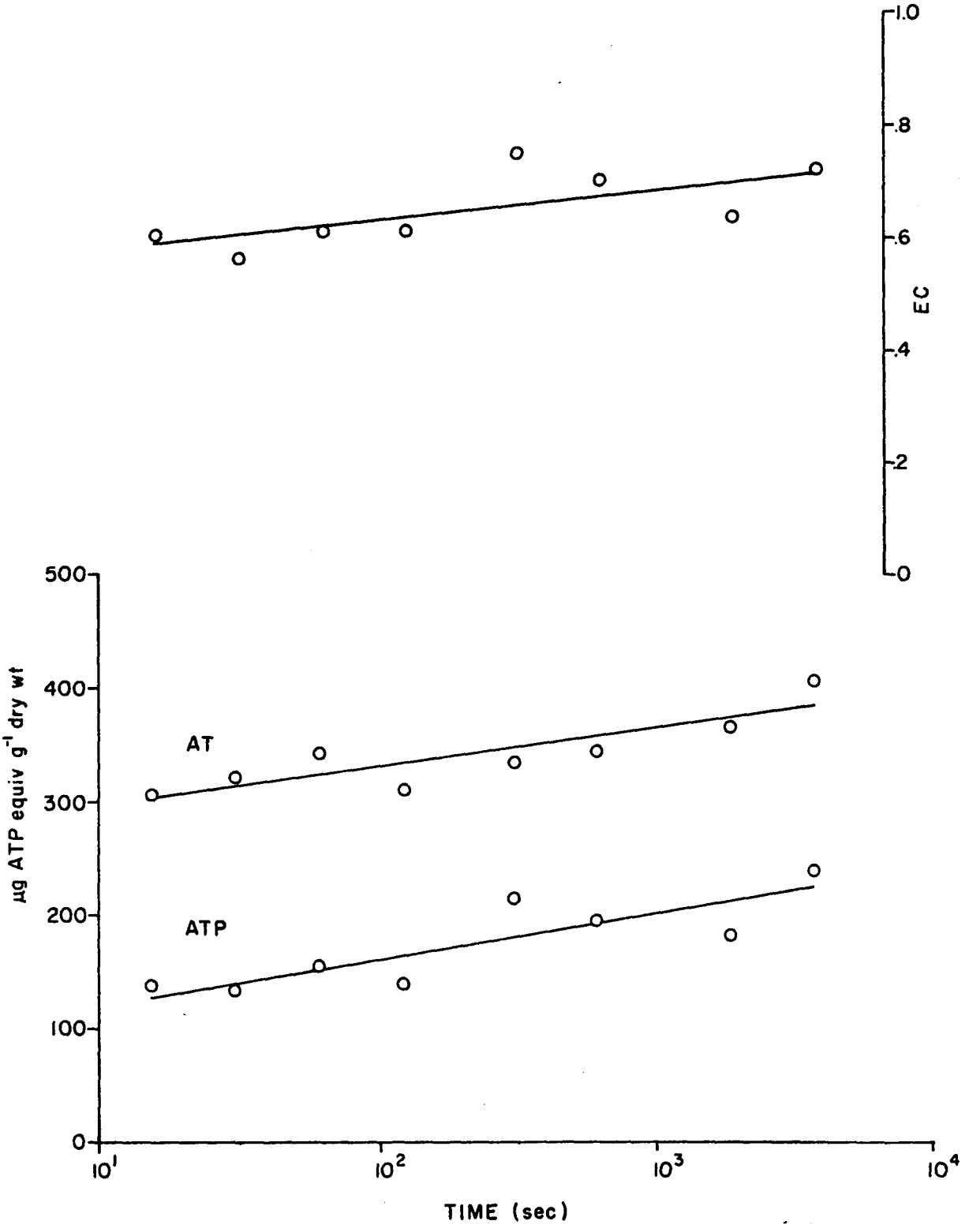

1.0
.8
.6
EC
.4
.2
0
500
400
300
200
100
0
µg ATP equiv g⁻¹ dry wt
AT
ATP
10¹
10²
10³
10⁴
TIME (sec)

Table 1.6. Semi-log regression (n=8) statistics for harvest-freeze delay.

Statistic	ATP	AT	EC
Slope	.3991	.3431	.0559
Intercept	.8294	2.6321	.5188
Pearson Correlation Coefficient	.8507*	.8729*	.7078*

* $P < .05$

Table 1.7. Effect of day vs night harvest, at two freeze delay intervals, on adenine nucleotides (µg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Day (1200 hrs)		Night (2400 hrs)	
	30 sec	10 min	30 sec	10 min
ATP	$173^{a1} \pm 4^2$	$227^{b} \pm 4$	$169^{a} \pm 6$	$167^{a} \pm 3$
ADP	$92^{a} \pm 3$	$84^{a} \pm 4$	$53^{b} \pm 3$	$59^{b} \pm 1$
AMP[3]	$52^{a} \pm 1$	$34^{a} \pm 4$	$33^{a} \pm 1$	$35^{a} \pm 2$
AT	$317^{a} \pm 5$	$344^{b} \pm 8$	$254^{c} \pm 8$	$260^{c} \pm 3$
EC	$.70^{a} \pm <.01$	$.78^{b} \pm .01$	$.77^{bc} \pm .01$	$.76^{c} \pm .01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.
[3] Although the Kruskal-Wallis test shows a signifincant difference, the nonparametric multiple range test failed to detect differences between any pair of means for AMP.

light-related, since corresponding increases are not observed at night. It is of interest to note that EC was higher at night than during the day for the 30 sec delay.

Fresh-chopped vs. frozen-lyophilized-ground tissue was compared (Table 1.8). Although data show no significant difference, variability (i.e. standard error) in the lyophilized tissue was considerably lower, reflecting increased homogeneity of the quick-frozen, lyophilized, and more finely ground tissue. Fresh tissue was held (4 hrs) in river water at at situ temperature and light levels prior to processing.

The effect of epiphytes was evaluated with scraped vs. unscraped lyophilized leaves (Table 1.9). ATP, ADP, AT, and EC were significantly lower in unscraped tissue than in scraped tissue. The decreases were apparently due to low epiphyte adenylate levels.

Two modes of sample storage were evaluated (Table 1.10). Desiccated-dark storge of frozen-lyophilized-ground tissue and frozen extract storage were both suitable over 5 days, but not 20 days. AT significantly decreased in both preparations over a 20 day storage period.

Extraction

Four extractants were compared (Table 1.11). The superiority of boiling 1 mM EDTA + 5% PVPP (pH 7.6) was evident, among those extractants tested. Without addition of PVPP to the EDTA solution, light output was reduced and firefly reaction kinetics did not display

Table 1.8. Effect of tissue state on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Fresh-Chopped (5 mm)	Frozen-Lyophylized-Ground (425 μ)
ATP	226[a1] ± 21[2]	253[a] ± 4
ADP	192[a] ± 36	151[a] ± 15
AMP	112[a] ± 14	129[a] ± 4
AT	530[a] ± 71	533[a] ± 20
EC	.61[a] ± .01	.62[a] ± .01

[1] Values with same letter superscripts (between treatments) do not differ significantly (P > .05).
[2] Standard error.

Table 1.9. Effect of epiphyte removal, by scraping lyophilized leaf tissue, on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Scraped Leaf	Unscraped Leaf	Scrapings[1] (Epiphytes)
ATP	313[a][2] ± 2[3]	253[b] ± 1	43 ± 2
ADP	91[a] ± 1	84[b] ± 1	33 ± 1
AMP	106[a] ± 5	95[a] ± 4	25 ± 1
AT	509[a] ± 7	432[b] ± 3	101 ± 2
EC	.71[a] ± .01	.68[b] ± .01	.59 ± .01

[1] Scrapings excluded from comparison test.
[2] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[3] Standard error.

Table 1.10. Effect of two storage methods at 5 and 20 days on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Initial	5 Days		20 Days	
		Frozen-Lyophilized-Ground and Desiccated-Dark	Frozen Extract (-20°C)	Frozen-Lyophilized-Ground and Desiccated-Dark	Frozen Extract (-20°C)
ATP	278[a1] ± 4[2]	267[a] ± 3	277[a] ± 4	248[b] ± 3	272[a] ± 4
ADP	91[a] ± 5	96[a] ± 4	87[ab] ± 2	88[ab] ± 3	75[b] ± 3
AMP	120[a] ± 3	114[ab] ± 2	112[ab] ± 4	107[b] ± 3	84[c] ± 3
AT	489[a] ± 10	477[a] ± 4	475[a] ± 8	443[b] ± 6	430[b] ± 10
EC	.66[a] ± <.01	.66[a] ± <.01	.67[a] ± .01	.66[a] ± <.01	.72[b] ± <.01

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 1.11. Effect of extractant on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Boiling 1 mM EDTA + 5% PVPP (pH 7.6)	Boiling 1 mM EDTA (pH 7.6)	Boiling Distilled Water	0-4°C .6N H_2SO_4 + 1 mM EDTA (neutralized to pH 7.6-7.9 with NaOH after extraction)
ATP	144[a1] ± 2[2]	27[b] ± <1	31[b] ± 1	38[b] ± 4
ADP	102[a] ± 2	25[b] ± <1	23[b] ± 1	22[b] ± 2
AMP	108[a] ± 4	61[b] ± 1	29[c] ± 1	27[c] ± 5
AT	354[a] ± 6	113[b] ± 1	82[c] ± 1	87[c] ± 11
EC	.55[a] ± <.01	.35[b] ± <.01	.52[c] ± .01	.57[a] ± .01

[1] Values with same letter superscripts (between treatments) do not differ significantly (P > .05).
[2] Standard error.

their characteristic decay pattern (Figure 1.6). Data on recovery of added adenylates (internal standard) appear in Table 1.12. Again, the superiority of boiling 1 mM EDTA + 5% PVPP (pH 7.6) was evident. Standards, prepared in EDTA, quenched light output to a lesser extent than those prepared in either distilled water or neutralized acid (Table 1.13).

Duration of three extraction times was evaluated (Table 1.14). No significant differences were observed for extraction times of 5, 30, or 120 sec.

Extraction of individual plants vs. extraction of multiple aliquots from a pooled sample was compared (Table 1.15). Adenylates show no significant difference, although variability (i.e. standard error) in the pooled plant sample was considerably lower, as would be expected. Pooling masked natural variability between plants but yielded mean adenylate levels, similar to those obtained from individually extracted plants. Standard errors, associated with individually extracted plants, provide information on adenylate variability between plants in the field.

Conversion

Methodology experiments in the conversion procedure were tested by calculating conversion efficiency of AMP and ADP (internal standard) to ATP. Concentrations given for treatment levels refer to the conversion reaction mixture.

Figure 1.6. Reaction kinetics obtained from EDTA extraction of sample with and without PVPP addition. FLE is injected at time zero, the tube is vortexed, inserted into the photometer, and the shutter is opened (indicated by arrow).

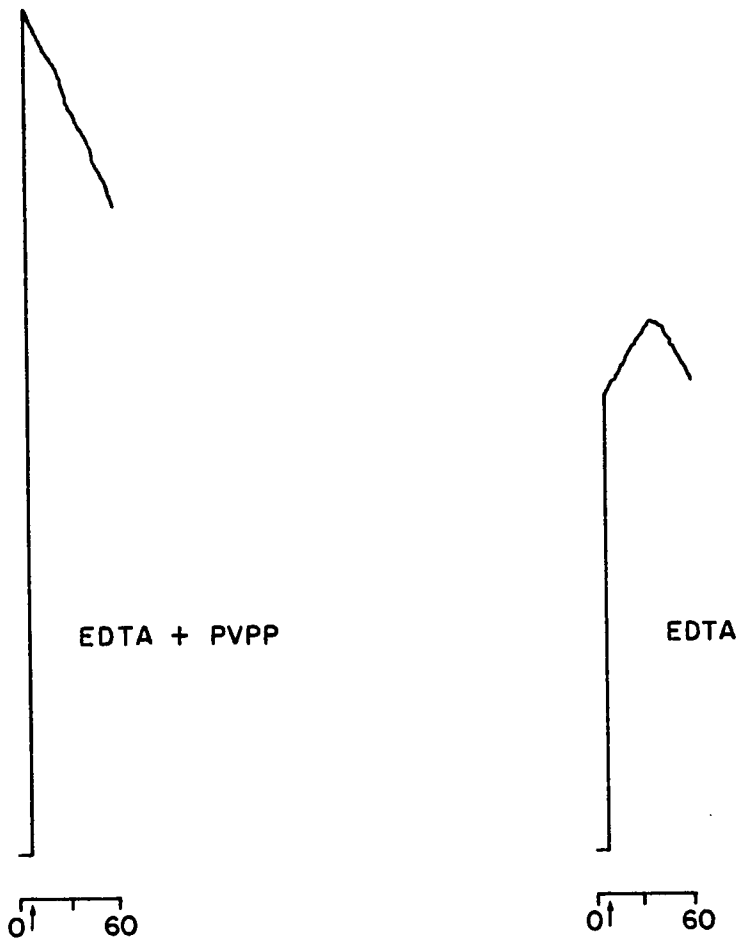

EDTA + PVPP
EDTA
0
60
0
60
TIME (sec)

Table 1.12. Effect of extractant on recovery (%) of 200 ng ml^{-1} ATP, ADP,AMP added immediately prior to extraction (n=4).

Variable	Boiling 1 mM EDTA + 5% PVPP (pH 7.6)	Boiling 1 mM EDTA (pH 7.6)	Boiling Distilled Water
ATP	$82^{a1} \pm 4^{2}$	$17^{b} \pm 1$	$22^{b} \pm 2$
ADP	$83^{a} \pm 22$	$31^{b} \pm 4$	$25_{b} \pm 5$
AMP	$112^{a} \pm 12$	$64^{b} \pm 10$	$51^{b} \pm 7$

1 Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
2 Standard error.

Table 1.13. Effect of extractant on light output (net counts) (n=2).

ATP Standard (ng ml^{-1})	[ATP] Reagents			[ATP+ADP] Reagents			[ATP+ADP+AMP] Reagents		
	X	Y	Z	X	Y	Z	X	Y	Z
4000	75096	47370	13760	66377	38828	12841	53072	32408	12682
40	426	287	86	350	252	83	294	204	77

X = 1 mM EDTA (pH 7.6)
Y = Distilled Water
Z = .6N H_2SO_4 + 1 mM EDTA (neutralized)

Table 1.14. Effect of extraction duration on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Extraction Duration (sec) 5	30	120
ATP	165[a1] ± 4[2]	178[a] ± 4	162[a] ± 8
ADP	142[a] ± 9	133[a] ± 4	141[a] ± 8
AMP	116[a] ± 11	144[a] ± 11	111[a] ± 6
AT	423[a] ± 5	455[a] ± 10	414[a] ± 22
EC	.56[a] ± .01	.54[a] ± .01	.57[a] ± <.01

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 1.15. Effect of pooling plants on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Individual Plants	Pooled Plants
ATP	369[a1] ± 11[2]	372[a] ± 4
ADP	65[a] ± 5	62[a] ± 1
AMP	66[a] ± 4	68[a] ± 3
AT	499[a] ± 16	501[a] ± 6
EC	.80[a] ± .01	.81[a] ± <.01

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Three buffers were evaluated (Table 1.16). AMP and ADP conversion efficiencies show no significant difference among the three buffers. TRICINE yielded the highest light output (Table 1.17).

Conversion enzyme cofactors were compared (Table 1.18). Results indicate that $MgSO_4$ is abolutely required, but that K_2SO_4 is not. Furthermore, K_2SO_4 addition may decrease conversion accuracy. $MgSO_4$, K_2SO_4, and higher pH all quenched light emission (Table 1.19).

The effect of heat deactivation on AMP and ADP conversion was assessed (Table 1.20). It is clear that this procedural step was essential. Without heat deactivation, ATP was produced in the presence of PEP and PK, presumably from ADP contained within the crude firefly lantern extract (Figure 1.7). With heat deactivation, ATP was not produced, and firefly reaction kinetics displayed their characteristic decay pattern.

Firefly Lantern Extract Preparation

Three solutions to reconstitute lyophilized firefly lantern extract (FLE) were compared (Table 1.21). One vial of Sigma FLE-50 was trisected by weight to minimize FLE variability. Specified $MgSO_4$ concentration is exogenous, since Sigma FLE-50 also contains $MgSO_4$. The buffer solution at pH 8.1 resulted in significantly lower AMP and AT than either of the other reconstituents tested.

FLE aging times and temperatures were evaluated (Table 1.22). Sample extract was frozen between 6 and 24 hr assays, in order to minimize adenylate degradation. Although the 24 hr ATP levels were

Table 1.16. Effect of buffer on AMP and ADP conversion efficiency (%), using 80 ng ml^{-1} ATP,ADP,AMP (n=3).

Variable	15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6)	15 mM HEPES + 6 mM $MgSO_4$ (pH 7.6)	15 mM TRIS-HCl + 6 mM $MgSO_4$ (pH 7.6)
ADP	$106^{a1} \pm 2^2$	$103^a \pm 2$	$104^a \pm 3$
AMP	$83^a \pm 5$	$75^a \pm 5$	$84^a \pm 4$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 1.17. Effect of buffer on light output (net counts) (n=1).

ATP Standard (ng ml^{-1})	[ATP] Reagents			[ATP+ADP] Reagents			[ATP+ADP+AMP] Reagents		
	X	Y	Z	X	Y	Z	X	Y	Z
4000	59163	54416	53769	46227	45020	43242	38650	37885	37091
40	367	343	323	303	283	269	294	264	258

X = 15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6)
Y = 15 mM HEPES + 6 mM $MgSO_4$ (pH 7.6)
Z = 15 mM TRIS-HCl + 6 mM $MgSO_4$ (pH 7.6)

Table 1.18. Effect of enzyme cofactors on AMP and ADP conversion efficiency (%), using 80 ng ml^{-1} ATP,ADP,AMP (n=3).

Variable	15 mM TRICINE (pH 7.6)	15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6)	15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 7.6)	15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 8.1)
ADP	-1[a1] ± 1[2]	108[b] ± 4	114[b] ± 2	114[b] ± 1
AMP	-1[a3] ± 1	110[b] ± 3	85[ab] ± <1	82[ab] ± 4

[1] Values with same letter superscripts (between treatments) do not differ significantly (P > .05).
[2] Standard error.
[3] Group 1 shows no difference with groups 3 and 4 for AMP conversion, because the nonparametric multiple range test uses ranks.

Table 1.19. Effect of enzyme cofactors on light output (net counts) (n=2).

ATP Standard (ng ml^{-1})	[ATP] Reagents				[ATP+ADP] Reagents				[ATP+ADP+AMP] Reagents			
	W	X	Y	Z	W	X	Y	Z	W	X	Y	Z
4000	48753	45351	33145	28813	38247	37239	27919	24775	30355	31460	23637	19925
40	283	270	177	152	217	209	147	123	166	172	126	111

W = 15 mM TRICINE (pH 7.6)
X = 15 mM TRICINE + 6 mM $MgSO_4$ (pH 7.6)
Y = 15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 7.6)
Z = 15 mM TRICINE + 6 mM $MgSO_4$ + 7.5 mM K_2SO_4 (pH 8.1)

Table 1.20. Effect of heat deactivation on AMP and ADP conversion efficiency (%), using 80 ng ml^{-1} ATP,ADP,AMP (n=4).

Variable	Heat (2 min, 100°C)	No Heat
ADP	$111^{a1} \pm 4^2$	$55^b \pm 13$
AMP	$102^a \pm 7$	$185^b \pm 14$

[1] Values with same letter superscripts (between treatments) do not differ significantly (P > .05).
[2] Standard error.

Figure 1.7. Reaction kinetics with and without heat deactivation. FLE is injected at time zero, the tube is vortexed, inserted into the photometer, and the shutter is opened (indicated by arrow).

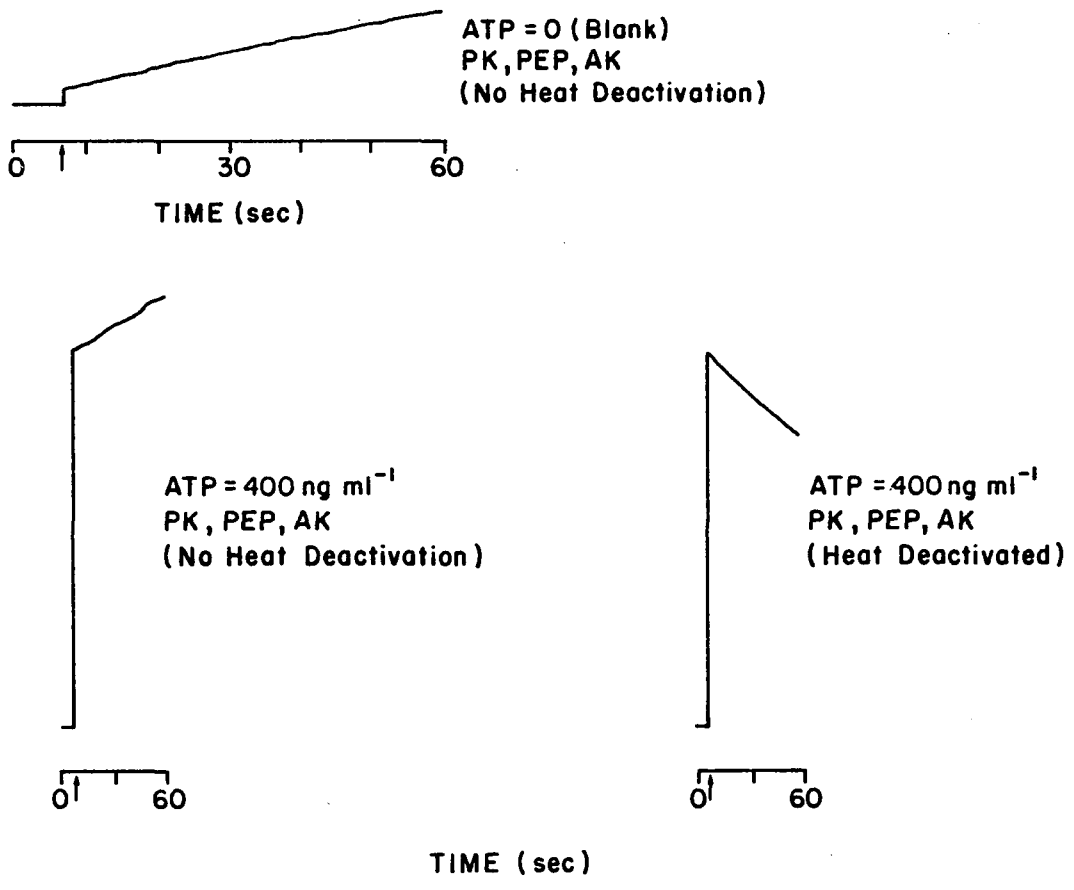

ATP = 0 (Blank)
PK, PEP, AK
(No Heat Deactivation)
0
30
60
TIME (sec)
ATP = 400 ng ml^{-1}
PK, PEP, AK
(No Heat Deactivation)
0
60
ATP = 400 ng ml^{-1}
PK, PEP, AK
(Heat Deactivated)
0
60
TIME (sec)

Table 1.21. Effect of FLE reconstituent on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	Distilled Water	45 mM TRICINE + 18 mM $MgSO_4$ (pH 7.6)	45 mM TRICINE + 18 mM $MgSO_4$ (pH 8.1)
ATP	$92^{a1} \pm 1^2$	$93^a \pm 1$	$92^a \pm 1$
ADP	$65^a \pm 1$	$66^a \pm 2$	$62^a \pm 1$
AMP	$65^a \pm 3$	$67^a \pm 3$	$54^b \pm 1$
AT	$221^a \pm 4$	$226^a \pm 2$	$208^b \pm 1$
EC	$.57^a \pm .01$	$.56^a \pm .01$	$.59^b \pm <.01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 1.22. Effect of FLE aging time and temperature on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	6 hr		24 hr	
	4°C	25°C	4°C	25°C
ATP	88[a1] ± 1[2]	89[a] ± 1	91[b] ± 1	93[b] ± 1
ADP	72[a] ± 1	70[a] ± 2	67[a] ± 3	68[a] ± 2
AMP	48[a] ± 4	60[a] ± 1	56[a] ± 3	55[a] ± 3
AT	208[a] ± 3	218[a] ± 2	214[a] ± 2	215[a] ± 2
EC	.60[a] ± .01	.57[a] ± <.01	.58[a] ± .01	.59[a] ± <.01

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$)
[2] Standard error.

significantly higher than the 6 hr levels, the magnitude of the increase was slight. Significance resulted from the low variability within treatments. No other adenylate differences were observed. As both aging time and temperature increased, light output was reduced (Table 1.23).

The effect of guanosine diphosphate (GDP) addition to FLE was examined. ATP may be produced from ADP in the presence of guanosine triphosphate (GTP), or any other nucleoside triphosphate (NTP), and nucleoside diphosphokinase (NDPK):

$$\text{ADP (FLE)} + \text{NTP (Sample)} \overset{\text{NDPK (FLE)}}{\rightleftharpoons} \text{ATP} + \text{NDP (K=1.0)}.$$

Specified GDP concentration refers to the FLE preparation. Although results show no difference in adenylate levels (Table 1.24), firefly reaction kinetics differ markedly (Figure 1.8). With GDP addition, light output was reduced and decay was more rapid in both standards and samples.

Photometry

Three photometer counting modes were evaluated (Table 1.25). Although ATP levels differed significantly among the three modes, the magnitudes of these differences were not large. No other adenylate differences were observed. Log-log standard regressions, derived from the three counting modes, were compared for ATP Reagents (Table 1.26). Slopes show no difference, but intercepts are significantly higher for the 30 sec integration. Correlation coefficients were highly significant. These regressions are plotted in Figure 1.9.

Table 1.23. Effect of FLE aging time and temperature on light output (net counts) (n=2).

ATP Standard (ng ml^{-1})	[ATP] Reagents				[ATP+ADP] Reagents				[ATP+ADP+AMP] Reagents			
	W	X	Y	Z	W	X	Y	Z	W	X	Y	Z
4000	87610	76269	75334	46857	63902	50851	50782	31777	50775	40664	42445	25405
40	536	388	407	266	384	271	283	187	306	209	224	146

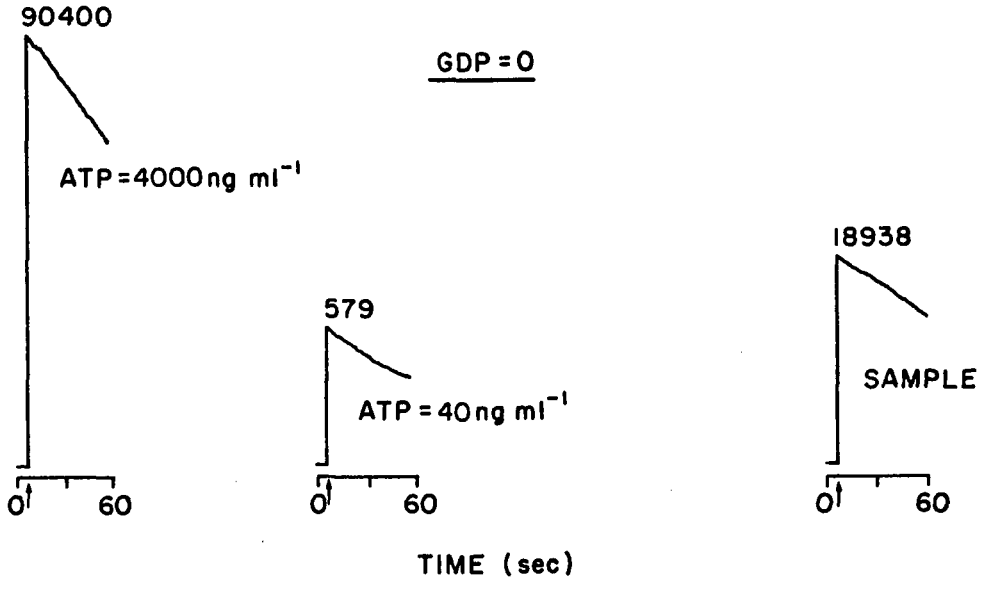
W = 6 hr, 4°C
X = 6 hr, 25°C
Y = 24 hr, 4°C
Z = 24 hr, 25°C

Table 1.24. Effect of GDP addition to FLE on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	No GDP	6.85 μg ml^{-1} GDP
ATP	104[a1] ± 1[2]	104[a] ± 2
ADP	62[a] ± 2	65[a] ± 6
AMP	35[a] ± 2	38[a] ± 1
AT	202[a] ± 2	206[a] ± 1
EC	.67[a] ± .01	.66[a] ± <.01

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Figure 1.8. Reaction kinetics with and without GDP addition. FLE is injected at time zero, the tube is vortexed, inserted into the photometer, and the shutter is opened (indicated by arrow). Counts represent a 10 sec integration period, immediately following a 10 sec delay from time zero.


90400
GDP = 0
ATP = 4000ng ml⁻¹
579
ATP = 40ng ml⁻¹
18938
SAMPLE
0
60
0
60
0
60
TIME (sec)

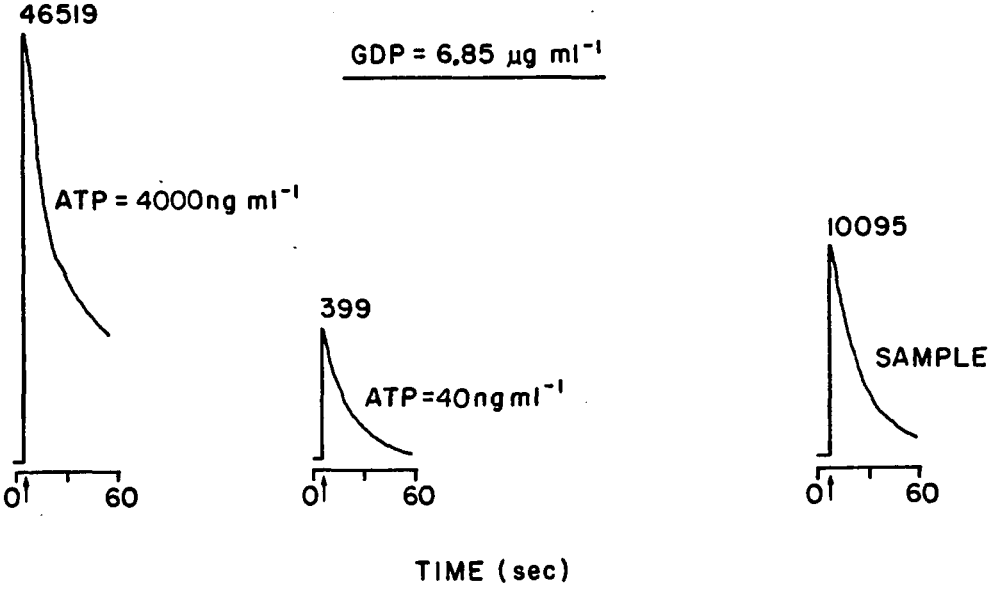

46519
GDP = 6.85 µg ml⁻¹
ATP = 4000ng ml⁻¹
399
ATP = 40ng ml⁻¹
10095
SAMPLE
0
60
0
60
0
60
TIME (sec)

Table 1.25. Effect of photometer counting mode on adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC (n=4).

Variable	10 Sec Delay followed by:		Peak Height
	10 sec Integral	30 sec Integral	
ATP	$162^{a1} \pm 2^2$	$171^{b} \pm 2$	$150^{c} \pm 2$
ADP	$161^{a} \pm 7$	$145^{a} \pm 4$	$141^{a} \pm 6$
AMP	$230^{a} \pm 2$	$235^{a} \pm 17$	$238^{a} \pm 7$
AT	$552^{a} \pm 5$	$551^{a} \pm 14$	$529^{a} \pm 7$
EC	$.44^{a} \pm <.01$	$.44^{a} \pm .01$	$.42^{a} \pm <.01$

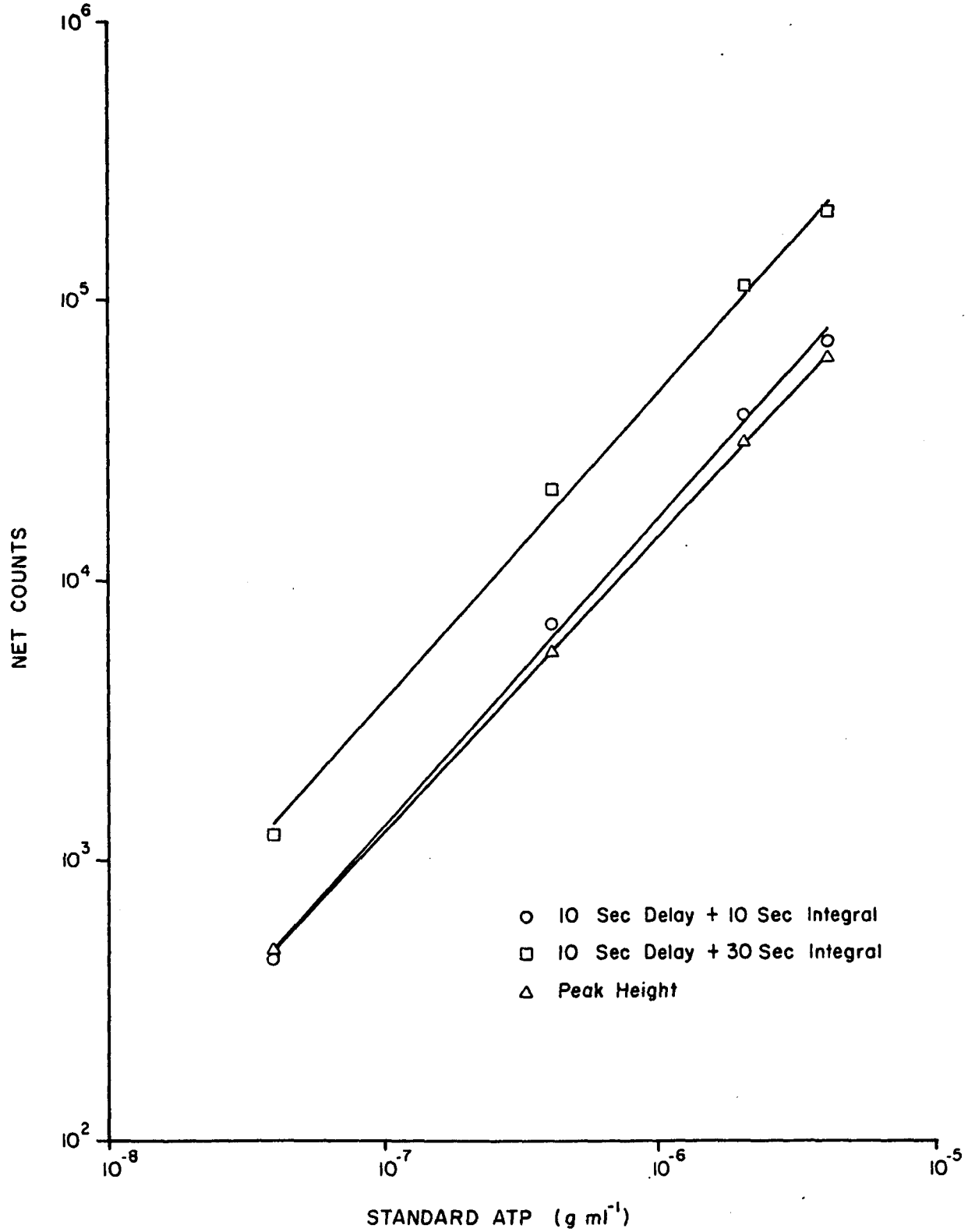
1 Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
2 Standard error.

Table 1.26. Comparison of log-log regression (n=4) statistics, obtained from three photometer counting modes with [ATP] reagents.

Statistic	10 Sec Delay followed by:		Peak Height
	10 Sec Integral	30 Sec Integral	
Slope	1.1150[a1]	1.1289[a]	1.0699[a]
Intercept	10.9392[a]	11.4838[b]	10.6016[a]
Pearson Correlation Coefficient	.9989*	.9985*	.9999*

[1] Values with same letter superscripts (between treatments) do not differ significantly (P > .05).
* P < .001

Figure 1.9. Comparison of photometer counting modes with ATP reagents (n = 2).


10^6
10^5
10^4
10^3
10^2
NET COUNTS
10^{-8}
10^{-7}
10^{-6}
10^{-5}
STANDARD ATP ($g\ ml^{-1}$)
○ 10 Sec Delay + 10 Sec Integral
□ 10 Sec Delay + 30 Sec Integral
△ Peak Height

Mixing kinetics with the peak height mode, using a 6 x 50 mm tube, were examined by varying the ATP standard/FLE volume ratio (Table 1.27). Proper mixing was evaluated, as described in Figure 1.10. Although several standard/FLE volume ratios mixed properly (i.e. 20/15, 50/15, 100/50), samples would not consistently mix well. Therefore, whenever peak height was used, proper mixing was evaluated.

Standard Curve

Six standards and one blank were routinely run for each reaction Tube A, B, and C. Using a 10 sec delay followed by a 10 sec integration, representative standards, net mean counts, and regression-calculated ATP concentrations appear in Table 1.28. Log-log regression plots are shown in Figure 1.11. Associated statistics are presented in Table 1.29. Correlation coefficients were highly significant. Differential quenching of light output was apparent among the three regressions.

Analytical Variability

Optimized recovery and conversion efficiencies were presented in Table 1.30. Since these efficiencies were near 100% with relatively low variability (i.e. small standard error), no correction factors were applied in data reduction.

Photometer variability, expressed as coefficient of variation, appears in Table 1.31. Coefficients were generally <2%, with the exception of blank readings. Higher coefficients for blanks were the mathematical result of division by a small mean rather than

Table 1.27. Examination of reagent mixing in peak height mode (counts) (n=5).

ATP Standard Volume (µl)	Statistic	FLE Volume (µl)				
		15	25	50	100	200
20	$\bar{x}$	4532	8314	13310	8452	10038
	$s/\bar{x}$	.08	.11	.19	.28	.07
	% PM[1]	100	80	60	0	0
50	$\bar{x}$	3625	9776	19896	22097	26449
	$s/\bar{x}$	.04	.11	.06	.22	.15
	% PM	100	80	0	0	0
100	$\bar{x}$	1287	6077	24241	40259	38310
	$s/\bar{x}$	.33	.08	.03	.78	.13
	% PM	0	0	100	20	0

[1] Properly Mixed Tubes.

Figure 1.10. Mixing kinetics in peak height mode. The sample tube is inserted into the photometer, and FLE is injected at time zero with the electronic pipet system which simultaneously activates the photometer. After 15 sec, the tube is removed, vortexed, and re-inserted into the photometer. Continuity in decay kinetics indicates proper initial mixing.

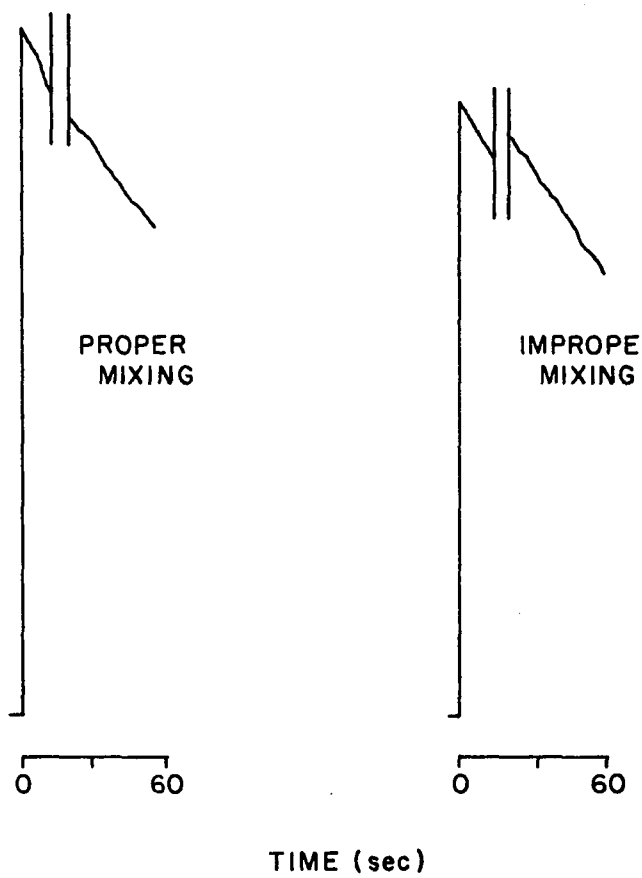

PROPER
MIXING
IMPROPER
MIXING
0
60
0
60
TIME (sec)

Table 1.28. Log-log regression standards, net mean counts (n=3), and predicted ATP concentrations, using a 10 sec delay followed by a 10 sec integral.

Standard ATP (ng ml^{-1})	[ATP] Reagents		[ATP+ADP] Reagents		[ATP+ADP+AMP] Reagents	
	Y	X	Y	X	Y	X
4000	89358	3703	69379	3719	58048	3735
2000	45435	1995	35333	2009	29552	2018
1000	22742	1059	17480	1057	14472	1052
400	8558	433	6477	427	5314	422
100	1719	100	1306	99	1089	99
40	596	38	462	38	385	38

Y = Net mean counts
X = Regression-calculated ATP (ng ml^{-1})

Figure 1.11. Standard curve regressions, using a 10 sec delay followed by a 10 sec integration (n = 3).

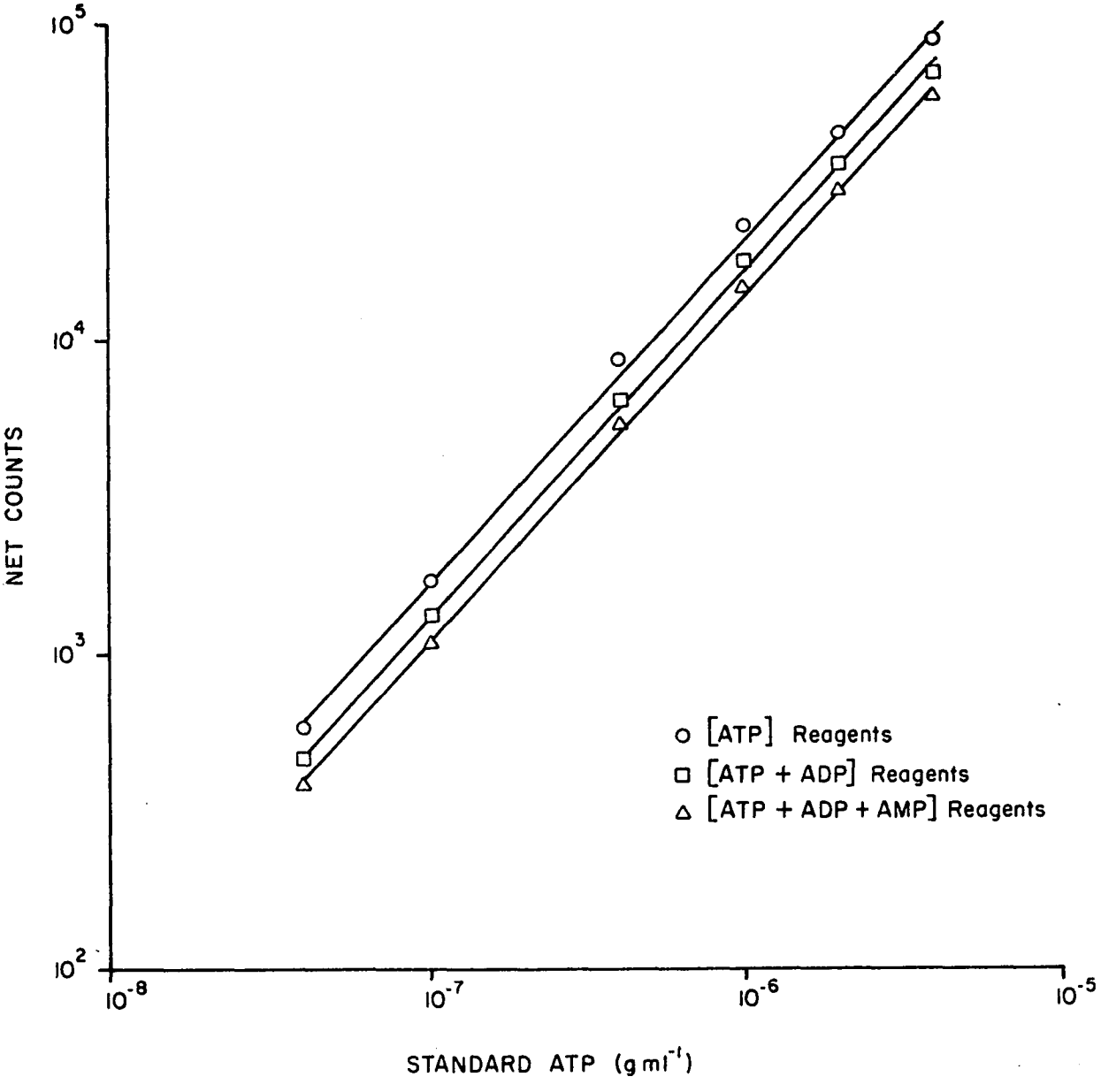

10^5
10^4
10^3
10^2
NET COUNTS
10^{-8}
10^{-7}
10^{-6}
10^{-5}
STANDARD ATP (g ml^{-1})
○ [ATP] Reagents
□ [ATP + ADP] Reagents
△ [ATP + ADP + AMP] Reagents

Table 1.29. Log-log regression (n=6) statistics, using a 10 sec delay followed by a 10 sec integral.

Statistic	[ATP] Reagents	[ATP+ADP] Reagents	[ATP+ADP+AMP] Reagents
Slope	1.0932	1.0957	1.0963
Intercept	10.8890	10.7902	10.7144
Pearson Correlation Coefficient	.9994*	.9995*	.9996*

* P < .001

Table 1.30. Recovery and conversion efficiency (%) with optimized method (n=4).

Variable	Recovery: 200 ng ml^{-1} ATP,ADP,AMP added immediately prior to extraction	Conversion: ATP,ADP,AMP Standard (ng ml^{-1})	
		1000	80
ATP	109 ± 9[1]	-	-
ADP	96 ± 5	102 ± 1	104 ± 1
AMP	97 ± 4	108 ± 2	96 ± 2

[1] Standard error.

Table 1.31. Photometer variability (coefficient of variation) with optimized method (n=5).

Standard ATP (ng ml^{-1})	[ATP] Reagents	[ATP+ADP] Reagents	[ATP+ADP+AMP] Reagents
Blank	.250	.026	.057
4000	.010	.006	.010
2000	.006	.012	.005
1000	.010	.012	.015
400	.007	.008	.019
100	.010	.011	.016
40	.015	.022	.003

multiplication by a large standard deviation. These data were based on a 10 sec delay, followed by a 10 sec integration.

Tissue Comparisons

Zostera marina

Adenylate levels in four types of tissues from Z. marina are presented in Table 1.32. Leaf tissue clearly had the highest level of ATP, ADP, AT, and EC, while root plus rhizome tissue showed the lowest measured levels of ATP, ADP, AMP, and AT. An adenylate correlation matrix was derived by pooling values from all four tissues (Table 1.33). ATP was positively correlated with ADP, AT, and EC, while ADP was positively correlated with AT and EC. Environmental and morphometric data, associated with this eelgrass sample, are presented in Table 1.34.

Other Species

Adenylate levels in Z. marina epiphytes, aboveground Ruppia maritima (widgeongrass), and aboveground Spartina alterniflora (saltmarsh cordgrass) appear in Table 1.35 for comparative purposes. Z. marina leaf tissue and aboveground R. maritima had comparable adenylate concentrations. Both were higher than either the epiphytes or aboveground S. alterniflora tissue. Environmental data, associated with collection of these samples, are presented in Table 1.36.

Seasonal Survey

Monthly mean aboveground adenylates (Figure 1.12), belowground adenylates (Figure 1.13), and resultant EC values (Figure 1.14) in Z. marina are plotted. Each of these time series contained significant

Table 1.32. Adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC in four types of tissue from Z. marina (n=4).

Variable	Leaf	Leaf Sheath	Root + Rhizome	Seed Pod
ATP	$245^{a1} \pm 2^2$	$72^b \pm <1$	$34^c \pm <1$	$129^d \pm 3$
ADP	$95^a \pm 1$	$49^b \pm 2$	$13^c \pm 1$	$63^d \pm 2$
AMP	$47^a \pm 4$	$55^a \pm 3$	$27^b \pm <1$	$108^c \pm 14$
AT	$387^a \pm 5$	$175^b \pm 5$	$74^c \pm 1$	$299^d \pm 13$
EC	$.76^a \pm .01$	$.55^b \pm .01$	$.55^b \pm <.01$	$.54^b \pm .03$

1 Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
2 Standard error.

Table 1.33. Spearman correlation coefficients among adenine nucleotides and EC, obtained by pooling values from four tissue types (n=16).

	ADP	AMP	AT	EC
ATP	.9512*	.4490	.9608*	.6206*
ADP		.4240	.9594*	.5871*
AMP			.5018	-.2724
AT				.4682

* P < .05

Table 1.34. Environmental data and morphometrics for *Z. marina*, used in tissue study.

1981 Harvest (mo)	May
Low Tide	
EST (hr)	1214
Height (m)	.1
Salinity (°/oo)	22.58
pH	8.00
Water Temp. (°C)	23.8
38°N Daylength (hr-min)	14-15
Density (shoots m^{-2})	1333
Shoot Length (cm)	
$\bar{x} \pm SE$ (n)	25.8 ± 1.4 (40)
Live Dry Wt (g m^{-2})	
Aboveground	291
Belowground	109
Total	400

Table 1.35. Adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC in *Z. marina* epiphytes (n=4), aboveground *Ruppia maritima* (n=2), and aboveground *Spartina alterniflora* (n=4).

Variable	Epiphytes	*R. maritima*	*S. alterniflora*
ATP	43 ± 2[1]	215 ± 5	87 ± 1
ADP	33 ± 1	137 ± <1	69 ± 1
AMP	25 ± 1	41 ± 8	33 ± 1
AT	101 ± 2	394 ± 3	189 ± 2
EC	.59 ± .01	.72 ± .02	.64 ± <.01

[1] Standard error.

Table 1.36. Environmental data for collection of epiphytes, R. maritima, and S. alterniflora.

Variable	Epiphytes	R. maritima	S. alterniflora
1981 Harvest (mo)	Jul	Jun	Apr
Low Tide			
EST (hr)	0951	1336	1702
Height (m)	.1	-.1	0
Salinity (°/oo)	20.87	20.42	22.89
pH	7.86	8.12	8.02
Water Temp. (°C)	28.0	27.1	19.5
38°N Daylength (hr-min)	14-39	14-47	13-24

Figure 1.12. Monthly adenine nucleotides from aboveground Z. marina (n = 4). Error bars are 1 standard error.

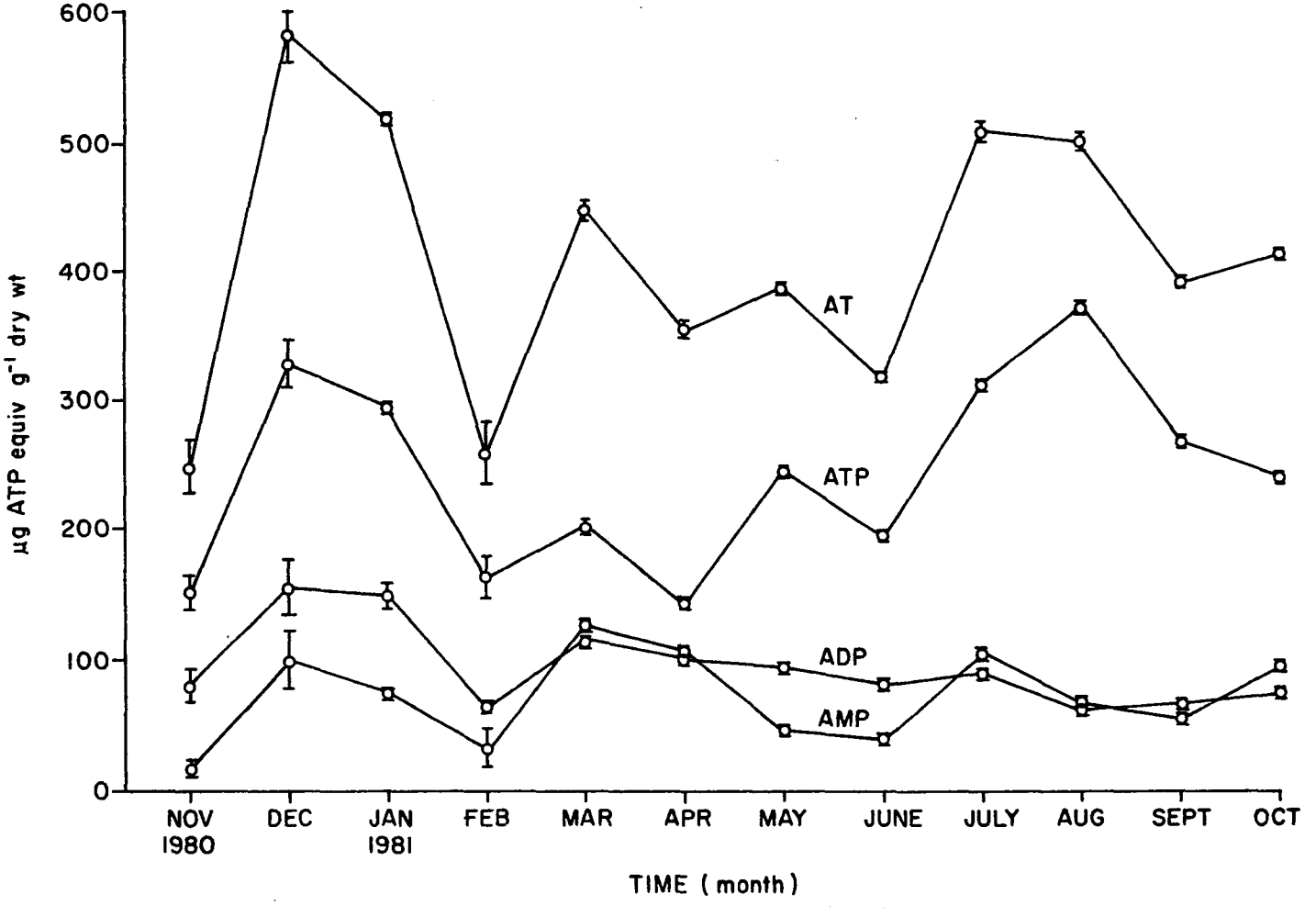

600
500
400
300
200
100
0
µg ATP equiv g⁻¹ dry wt
AT
ATP
ADP
AMP
NOV 1980
DEC
JAN 1981
FEB
MAR
APR
MAY
JUNE
JULY
AUG
SEPT
OCT
TIME (month)

Figure 1.13. Monthly adenine nucleotides from belowground Z. marina (n = 4). Error bars are 1 standard error.

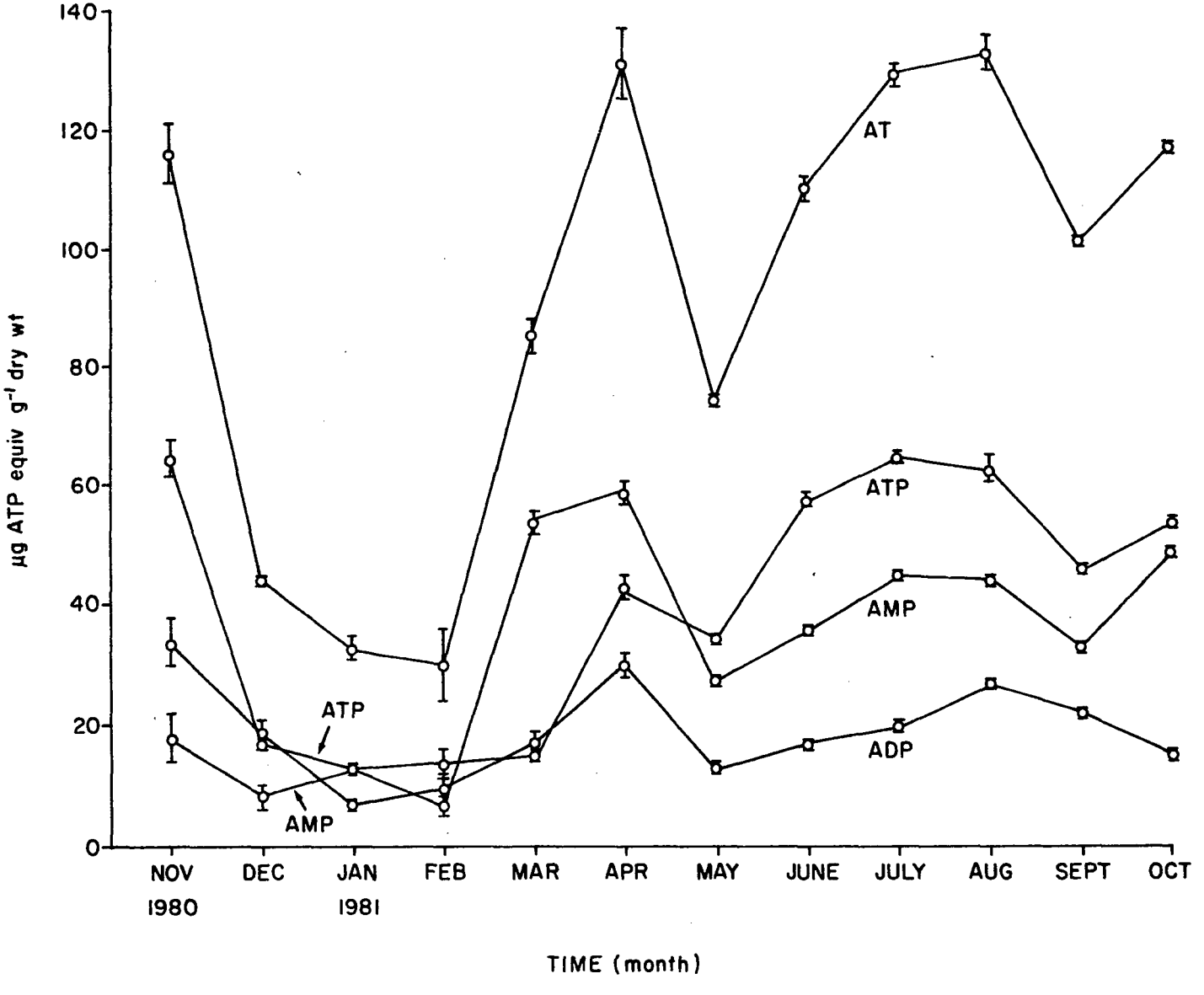
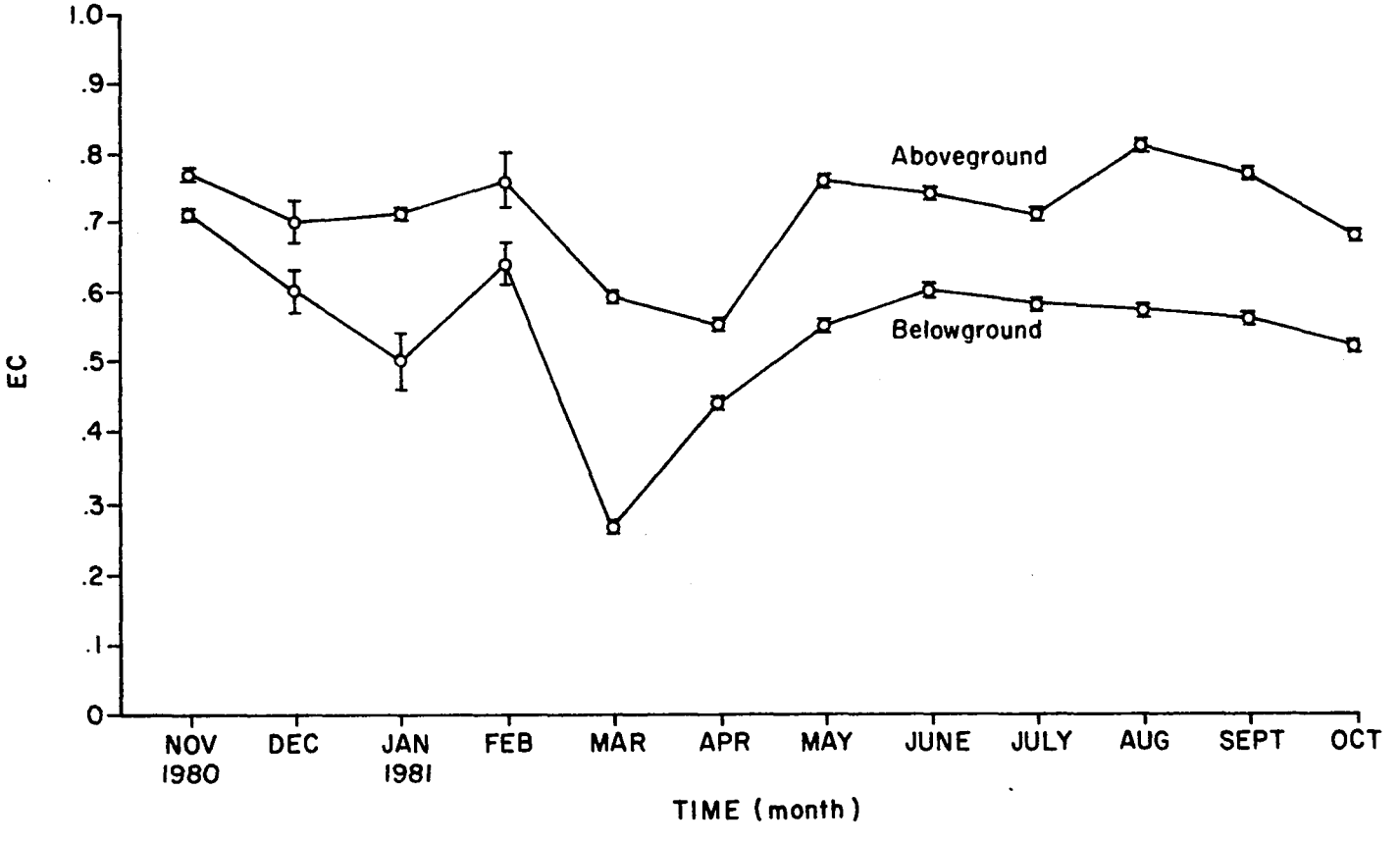

140
120
100
80
60
40
20
0
µg ATP equiv g⁻¹ dry wt
AT
ATP
AMP
ADP
ATP
AMP
NOV
1980
DEC
JAN
1981
FEB
MAR
APR
MAY
JUNE
JULY
AUG
SEPT
OCT
TIME (month)

Figure 1.14. Monthly EC from above and belowground Z. marina (n = 4). Error bars are 1 standard error.


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
EC
Aboveground
Belowground
NOV 1980
DEC
JAN 1981
FEB
MAR
APR
MAY
JUNE
JULY
AUG
SEPT
OCT
TIME (month)

differences ($P < .05$) over the one year period. Adenylates and EC were generally higher in aboveground tissue.

Correlation matrices for aboveground (Table 1.37) and belowground (Table 1.38) adenylates were derived by pooling values from all 12 months. For both above and belowground adenylates, AT was positively correlated with ATP, ADP, and AMP, while EC was negatively correlated with AMP. Correlation coefficients between above and belowground adenylates, using monthly means, are presented in Table 1.39. Above and belowground AMP were positively correlated. Weaker positive correlation ($.05 < P < .10$) existed between above and belowground EC.

Environmental data appear in Table 1.40 and Figure 1.15. Morphometric data are plotted in Figure 1.16. Maxima for biomass, number of shoots, and shoot length occurred in May, June, and July, respectively.

Aboveground and belowground adenylate correlations with environmental and morphometric data, using monthly means, are presented in Tables 1.41 and 1.42, respectively. No significant correlations were observed for aboveground adenylates. Although several belowground adenylate correlations were significant, no clear patterns were evident.

Table 1.37. Spearman correlation coefficients among adenine nucleotides and EC, from aboveground Z. marina used in seasonal survey, obtained by pooling all values (n=48).

	ADP	AMP	AT	EC
ATP	.0943	.2622	.8475*	.2641
ADP		.4282*	.4806*	-.5100*
AMP			.6121*	-.7952*
AT				-.2106

* P < .05

Table 1.38. Spearman correlation coefficients among adenine nucleotides and EC, from belowground *Z. marina* used in seasonal survey, obtained by pooling all values (n=48).

	ADP	AMP	AT	EC
ATP	.6150*	.3280*	.8416*	.3846*
ADP		.3414*	.7160*	.1548
AMP			.7078*	-.6160*
AT				-.0263

* $P < .05$

Table 1.39. Spearman correlation coefficients between above and belowground adenine nucleotides and EC, from Z. marina obtained in seasonal survey, using monthly means (n=12).

Variable	Correlation Coefficient
ATP	.0420
ADP	-.1961
AMP	.6364*
AT	-.0490
EC	.5845

* P < .05

Table 1.40. Monthly environmental data for collection of *Z. marina*, used in seasonal survey.

Harvest (mo)	Low Tide		38°N Daylength (hr-min)
	EST (hr)	Height (m)	
Nov 1980	1022	.1	10-5
Dec	0855	0	9-31
Jan 1981	1730	-.2	9-44
Feb	1706	-.2	10-16
Mar	1301	-.1	11-23
Apr	1359	-.1	12-37
May	1214	.1	14-15
Jun	1336	-.1	14-47
Jul	0951	.1	14-39
Aug	1604	0	14-10
Sept	0603	.1	12-56
Oct	0727	.2	11-44

Figure 1.15. Monthly environmental data for collection of Z. marina, used in seasonal survey.

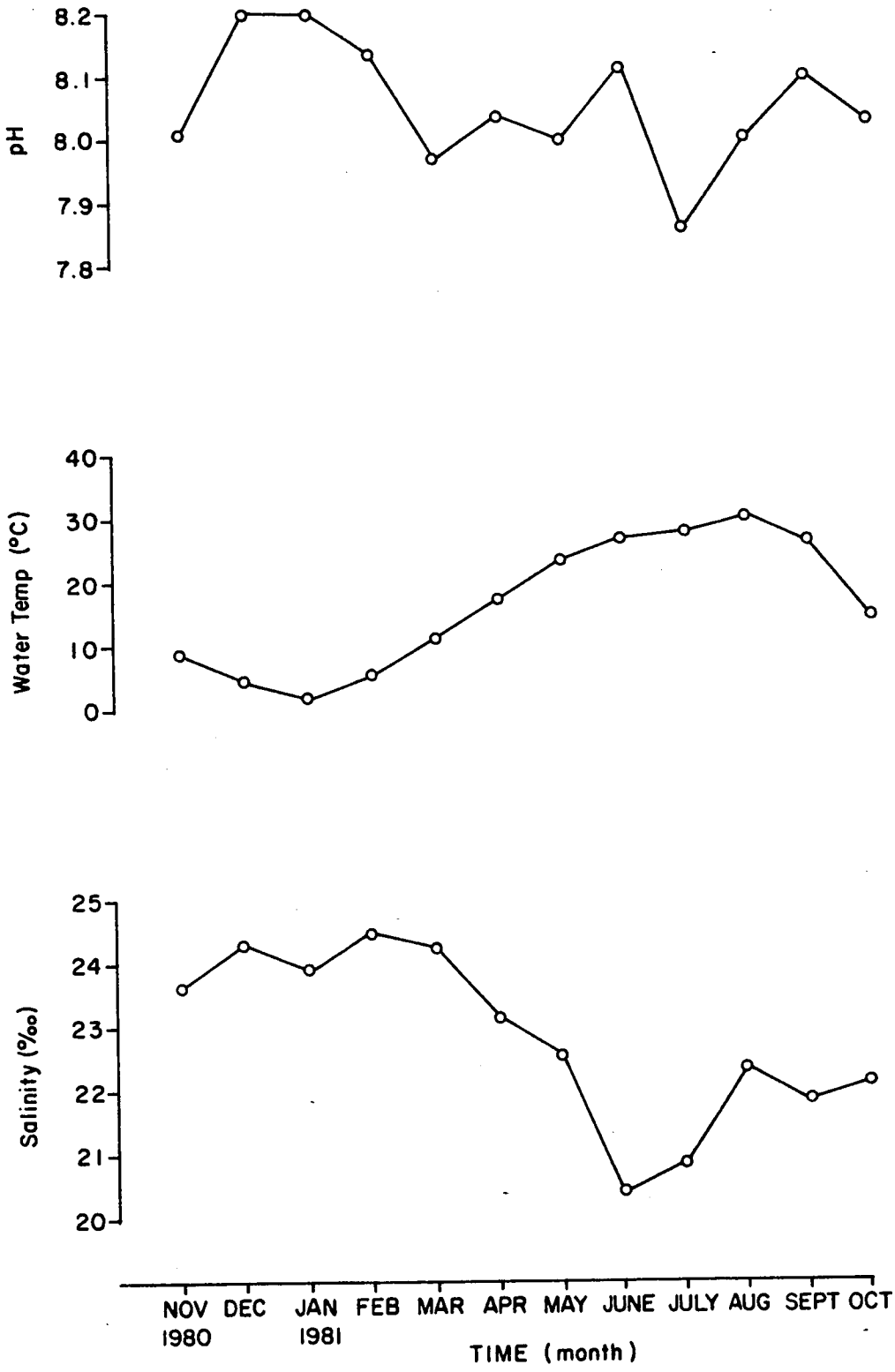

pH
8.2
8.1
8.0
7.9
7.8
Water Temp (°C)
40
30
20
10
0
Salinity (‰)
25
24
23
22
21
20
NOV DEC JAN FEB MAR APR MAY JUNE JULY AUG SEPT OCT
1980
1981
TIME (month)

Figure 1.16. Monthly morphometrics for Z. marina, used in seasonal survey. Shoot length error bars are 1 standard error and numbers in parentheses are n.

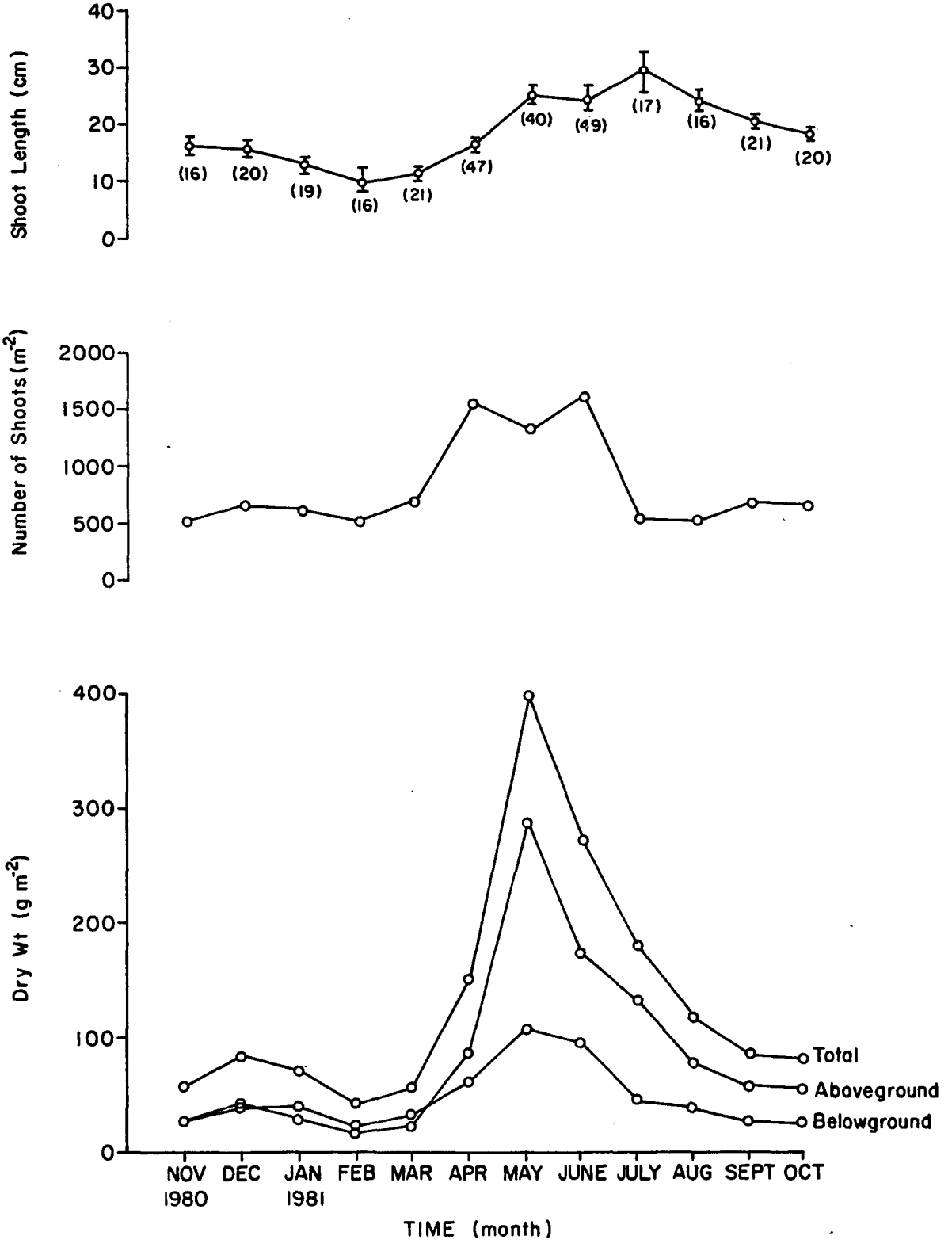

Shoot Length (cm)
40
30
20
10
0
(16)
(20)
(19)
(16)
(21)
(47)
(40)
(49)
(17)
(16)
(21)
(20)
Number of Shoots (m-2)
2000
1500
1000
500
0
Dry Wt (g m-2)
400
300
200
100
0
Total
Aboveground
Belowground
NOV
1980
DEC
JAN
1981
FEB
MAR
APR
MAY
JUNE
JULY
AUG
SEPT
OCT
TIME (month)

Table 1.41. Spearman correlation coefficients of *Z. marina* aboveground adenine nucleotides and EC with environmental and morphometric data, obtained in seasonal survey, using monthly means (n=12).

	Low Tide Height	Salinity	pH	Water Temp.	Daylength	Shoot Density	Shoot Length	Live Dry Wt Aboveground	Live Dry Wt Belowground	Live Dry Wt Total
ATP	.2087	-.1538	-.0526	.2168	.0559	-.2686	.3636	.1958	.1051	.2308
ADP	-.2627	.3427	.1825	-.4825	-.3566	.4064	-.2587	0	.5289	.0559
AMP	-.0432	.0559	-.2597	.0140	-.0559	.2686	-.0420	.0420	.1891	.0490
AT	.0144	.0559	.0175	-.0699	-.1888	-.1095	.0699	.0210	.1436	.0559
EC	.1917	-.1968	-.0494	.3234	.2109	-.4529	.3093	.0668	-.1919	.0738

* $P < .05$

Table 1.42. Spearman correlation coefficients of *Z. marina* belowground adenine nucleotides and EC with environmental and morphometric data, obtained in seasonal survey, using monthly means (n=12).

	Low Tide Height	Salinity	pH	Water Temp.	Daylength	Shoot Density	Shoot Length	Live Dry Wt		
								Aboveground	Belowground	Total
ATP	.6417*	-.7075*	-.5272	.6900*	.5394	-.1558	.6970*	.4518	.0912	.4238
ADP	.3461	-.2557	-.3374	.4098	.1436	-.0726	.2907	.1646	.0281	.1856
AMP	.2303	-.4685	-.6175*	.5385	.4825	.4135	.3427	.3566	.1856	.3007
AT	.4390	-.6154*	-.5719	.7133*	.5175	.0106	.6014*	.4685	.1506	.4266
EC	.0991	.0070	.2127	0	-.0385	-.4726	.0525	-.0981	-.1737	-.0595

* $P < .05$

DISCUSSION

Adenine Nucleotide Methodology Experiments

Sample Collection and Preparation

The logistical problem of sampling a submerged aquatic macrophyte, so that adenylates are maintained at *in situ* levels, was minimized by freezing plants in liquid nitrogen within 2 min after harvest. An increase in light level, associated with the harvest procedure, appears to be responsible for the observed elevation in ATP, AT, and EC. Transitions from dark to light result in rapid chloroplastic ATP increase (Hampp et al., 1982; Cockburn, 1974; Santarius and Heber, 1965), presumably by photophosphorylation. An accompanying elevation in cytoplasmic ATP reflects intracellular adenylate transfer (Sellami, 1976).

Rapid freezing with liquid nitrogen (<3 min) is more effective than slow freezing with dry ice (5-15 min) at preserving *in situ* adenylate levels in *Spartina patens* leaves (Mendelssohn and McKee, 1981). The longer time interval required for dry ice freezing may allow for more transphosphorylase and ATPase activity. Even after plant tissue is frozen, enzymic activity may persist (Bieleski, 1964).

Lyophilization of frozen tissue (e.g. Bomsel and Sellami, 1974; Wilson, 1978) effectively maintained *in situ* adenylate levels, and

homogenization by grinding lowered variability in replicate aliquots. Advantages of lyophilization include adenylate stabilization by enzyme deactivation (dehydration) and direct determination of tissue dry weight (Mendelssohn and McKee, 1981). It is critical that the sample remain frozen below its lowest eutectic point during the time interval required by the lyophilizer to reach sufficient vacuum. Freeze-thaw treatment increases cell permeability to ATP (Rhodes and Stewart, 1974) and may dislodge ATPases from thylakoid membranes (Garber and Steponkus, 1976), reducing ATP content in plant tissue (Mendelssohn and McKee, 1981).

Removal of epiphytes from Z. marina leaf blades was essential in order to quantify adenine nucleotides in eelgrass tissue alone. Epiphytes can be a significant proportion of an aboveground tissue sample. For example, epiphytes comprised an average of 24% of the total eelgrass leaf plus epiphyte biomass (dry wt) in a North Carolina estuary (Penhale, 1977). Low epiphyte adenylate levels, relative to levels in Z. marina leaf tissue, may be attributed to the inclusion of small amounts of sediment, as well as siliceous diatom frustules, in epiphyte preparations.

Storage techniques are aimed at halting enzyme activity, which can alter adenine nucleotide composition. Enzyme activity may be minimized by either dehydration or freezing. Frozen-lyophilized-ground-desiccated tissue (Wilson, 1978) and frozen extract (Holm-Hansen, 1973) consititute two forms of storage. In this study, frozen-lyophilized-ground tissue was stored desiccated-dark for periods up to 5 days.

Extraction

Extraction of adenylates at in situ levels requires rapid nucleotide release and enzyme deactivation by either heating or lowering pH. Destruction of the semipermeable characteristics of cell membranes with boiling extractants causes all soluble constituents (e.g. adenylates) to rapidly diffuse out of the cells, ultimately resulting in a uniform concentration of each constituent throughout the entire suspension (Holm-Hansen, 1973). Hydrolases are released upon disruption of cellular integrity (DeGreef et al., 1979). Deactivation of these enzymes relies on the effectiveness of heat conduction or acid permeation through the tissue. The resultant thermal or $[H^+]$ gradients (Karl et al., 1978) are dependent on tissue chemical and physical properties (e.g. surface to volume ratio, density, chemical composition). Thermal gradients are minimized by homogenization of tissue and by using a low tissue to extractant ratio ($\leq$1% w/v). Holm-Hansen and Karl (1978) recommended a sample to extractant ratio of $\leq$2% (v/v).

Optimal extraction and recovery were achieved with boiling EDTA plus PVPP (pH 7.6). Boiling EDTA extraction of plant tissue has previously been proven effective (Mendelssohn and McKee, 1981; Guinn and Eidenbock, 1972). As a chelating agent, EDTA binds divalent metal cations which generally inhibit light output in the firefly bioluminescent reaction (Karl and LaRock, 1^75). These authors caution against excessive EDTA addition which complexes Mg^{++} ions (required by luciferase), decreasing light output. Use of PVPP to adsorb phenols serves to increase light output. Higher plants contain

phenolic compounds which bind proteins (Loomis and Battaile, 1966) and apparently inhibit luciferase (Mendelssohn et al., 1978). Z. marina is reported to contain several types of phenolic acids (Zapata and McMillan, 1979). Color quenching was also reduced in extracts treated with PVPP. Guinn and Eidenbock (1972) detected greater amounts of ATP in cotton leaves with polyvinylpyrrolidone (PVP) treatment.

Re-hydration of lyophilized samples during extraction requires that samples, along with standards, be held on ice throughout the assay (excluding the conversion step). This minimizes adenylate changes.

Recovery of internal standards does not assess extractant efficiency per se, since added adenylates are extracellular. However, internal standards are useful in evaluating apparent and/or real nucleotide losses due to hydrolysis, adsorption, coprecipitation, ionic interferences, turbidity, and color quenching (Karl, 1980).

A 30 sec extraction was selected for routine use, although no significant differences in adenylate levels were obtained from 5-120 sec. Similarly, Mendelssohn and McKee (1981) found no significant difference with boiling EDTA plus PVPP extraction over 5-180 sec. However, Karl et al. (1978) caution against prolonged extraction which may hydrolyze nucleoside triphosphates. When using a boiling extractant, it is essential that the temperature be maintained at 100°C in order to deactivate ATPases (Holm-Hansen and Karl, 1978).

Leaf tissue extraction from either individual plants or a pooled plant sample masks adenylate variation on a cellular or organellar level. When multicellular tissue is extracted, mass-weighted mean adenylate values are determined. Cellular compartmentation and tissue heterogeneity may actually sponsor a range of co-existing metabolic states (Pradet and Raymond, 1978; Karl, 1980).

Conversion

TRICINE buffer (25 mM in firefly reaction) was selected for routine use, since this buffer yielded the highest light output. Webster et al. (1980) have also reported maximum light production with 25 mM TRICINE. Apparently, luciferase has the most favorable conformation in TRICINE.

Cofactor requirements have been specified for conversion enzymes, pyruvate kinase (PK) and adenylate kinase (AK), by Kayne (1973) and Noda (1973), respectively. Both PK and AK require a divalent cation (e.g. Mg^{++}). Without $MgSO_4$ addition, essentially no conversion of AMP or ADP occurs. Although the PK reaction also requires a monovalent cation (e.g. K^+), K_2SO_4 addition is not necessary. NH_4^+ (present in commercial PK and AK suspensions) and/or Na^+ (present in commercial EDTA and PEP salts) meet this requirement.

$MgSO_4$, K_2SO_4, and pH 8.1 quenched light output in the firefly reaction. DeLuca et al. (1979) report that $SO_4^=$ inhibits the reaction. Generally, cations and anions reduce light emission (Karl and LaRock, 1975). Apparently, sufficient Mg^{++} is contained in the FLE preparation to meet the luciferase divalent cation requirement

(DeLuca, 1976). Additional $MgSO_4$ inhibits light output, but Mg^{++} is needed in conversion reactions. The pH optimum for the firefly reaction is in the range 7.4 (Strehler, 1968) to 7.8 (Webster and Leach, 1980). pH 7.6 was selected for routine use, since it falls within this range and yielded higher light output than pH 8.1.

The heat deactivation step is essential when using integral measurement. Heating denatures PK, preventing ATP production from reaction of PK and PEP with ADP contained in the crude FLE preparation. Karl and Holm-Hansen (1978) report that heat deactivation is not required when using peak height measurement with *in situ* AT >50 ng ml^{-1}, since PK interference is overwhelmed by the magnitude of the ATP-dependent peak light emission.

When ATP is <30 ng ml^{-1}, AMP conversion to ATP may be incomplete, since ATP is required to initiate the AK reaction (Karl and Holm-Hansen, 1978). An increase in ATP lowers the apparent K_m of AK for AMP. Since all sample extracts in this study contained >50 ng ml^{-1} ATP, addition of ATP was unnecessary.

Firefly Lantern Extract Preparation

Reconstitution of lyophilized firefly lantern extract (FLE) with TRICINE buffer plus $MgSO_4$ (pH 7.6) was selected for routine use in order to stabilize pH. This procedure results in a final buffer concentration of 25 mM (firefly reaction mixture), the optimum prescribed by Webster et al. (1980). $MgSO_4$ addition complies with the recommendation by Karl and Holm-Hansen (1976) to add Mg^{++} when final FLE volume (25 ml) exceeds 5 ml, specified for Sigma FLE-50 by the manufacturer.

A 6-8 hr aging period at room temperature (Mendelssohn and McKee, 1981) was chosen as the routine procedure for FLE preparation. FLE is aged in order to degrade endogenous adenine nucleotides. Prolonged aging and high temperature result in loss of luciferin-luciferase activity. Karl and Holm-Hansen (1976) have demonstrated that loss of Sigma FLE-50 activity over 36 hrs at 25°C is due to luciferin rather than luciferase degradation.

Although firefly luciferase is specific for ATP, transphosphorylases (e.g. NDPK) contained within crude luciferase preparations, regenerate ATP from other NTP's (DeLuca, 1976). Karl and Holm-Hansen (1978) report that GDP addition to the FLE preparation (400 ng ml^{-1}) effectively inhibits ATP production from GTP, uridine triphosphate (UTP), inosine triphosphate (ITP), and cytidine triphosphate (CTP). Christensen and Devol (1980) observed no reduction in light emission with GDP addition.

In the present study, a greater amount of GDP (6.85 µg ml^{-1}) reduced light output in both standards and samples. Since standards contain no NTP (other than ATP), reduced light output with GDP addition reflects ATP consumption by mass-action adjustment via the NDPK reaction. Apparently, NDPK does not compete with luciferase for ATP (10 ng ml^{-1}) with GDP addition under 1 µg ml^{-1} (Karl and Nealson, 1980). Since sample adenylate levels showed no difference with or without GDP, GDP addition to the FLE preparation (6.85 µg ml^{-1}) appears unnecessary.

Photometry

Since the time course of light production resulting from non-adenine NTP's is slower than *in situ* ATP-dependent light emission kinetics, interference is minimized with peak height measurement (Holm-Hansen and Karl, 1978). However, DeLuca et al. (1979) have stated that no single method of measuring light production is adequate for all conditions.

Parallel and linear log-log standard regressions between net light output and ATP (40-4000 ng ml^{-1}) were obtained with peak height (2 sec delay, 1 sec count) and integration (10 sec delay, 10 or 30 sec count). Webster and Leach (1980) have demonstrated parallelism with peak height and integration (15 sec delay, 60 sec count) over 0.2-200 ng ml^{-1} ATP. A 10 sec delay, followed by a 10 sec integration, was selected as the routine counting method for two reasons: 1) mixing problems with peak height are avoided, and 2) after thorough mixing during a 10 sec delay, the shortest machine-available integral (10 sec) minimizes time-dependent interferences.

Standard Curve

Three standard curves, prepared with reagents for determination of [ATP] (Tube A), [ATP+ ADP] (Tube B), and [ATP + ADP + AMP] (Tube C), allow more accurate sample adenylate measurement than single curve determinations (Holm-Hansen and Karl, 1978). Use of multiple standard curves ensured that ionic composition and ATP reactivity were similar in both standards and samples. All three log-log standard regressions between net light output and ATP (40-4000 ng ml^{-1}) were highly linear.

In this study, separate regressions were specifically required, due to $(NH_4)_2SO_4$ addition and heat deactivation. Quenching was lowest in Tube A and highest in Tube C. Commercial preparations of PK (Tubes B and C) and AK (Tube C) contain $(NH_4)_2SO_4$, which reduced light production. The heating step appears to effectively denature PK but not AK. Selective PK deactivation causes the AK reaction to re-equilibrate with backproduction of ADP from ATP in solution (Tube C), reducing light emission (Karl and Holm-Hansen, 1978). Christensen and Devol (1980) have reported a 15% reduction in peak height due to this re-equilibration.

Tissue Comparisons

Zostera marina

Since leaves contained the highest adenylate levels among the four tissues examined, it is suggested that leaf material be routinely sampled as the test tissue for adenylate analyses in Z. marina. Low adenylate levels in Z. marina root plus rhizome tissue are attributed to the presence of structural or metabolically inert material (Pamatmat and Skjoldal, 1979), as well as lowered aerobic respiration in reduced sediments (Mendelssohn et al., 1981). Tissue adenylate distribution in Z. marina contrasts with that observed for Spartina alterniflora (cordgrass), where leaf sheath and roots contained higher levels of ATP than leaves (Mendelssohn and McKee, 1981). This is presumably due to actively dividing meristematic tissue in leaf sheath and roots.

Tissue ATP level reflects ATP generation, utilization, and translocation. Light and oxygen availability permit both photo- and oxidative phosphorylation, respectively (Sellami, 1976), in aboveground tissue. Belowground tissue in reduced sediments must rely on limited oxidative phosphorylation, substrate phosphorylation in glycolysis (Mendelssohn et al., 1981), and possibly translocation (Thigpen, 1981) to maintain an adequate supply of ATP.

Mathematically, EC should be positively correlated with ATP and negatively correlated with AMP. AT should correlate positively with ATP, ADP, and AMP. All of these correlations were observed.

Other Species

Although adenylate analytical techniques were specifically adapted to Z. marina, the methodology was applied to epiphytes of Z. marina, Ruppia maritima (a seagrass), and Spartina alterniflora (a marshgrass) for comparative purposes. As previously suggested, relatively low adenylate levels in epiphytic algae may result from metabolically inert material in epiphyte preparations. Adenylate content of R. maritima aboveground tissue was similar to that of Z. marina leaf tissue.

Differences in methodology and environment preclude strict comparison with the following values reported in the literature. Thalassia testudinum, a tropical seagrass, contained 703 ng ATP per leaf disc dry wt (485 μg ATP g^{-1} dry wt) one day after excision (Knauer and Ayers, 1977). This value represents about twice the amount observed for seagrasses (Z. marina and R. maritima) in the

present study. In a tissue study with S. alterniflora, Mendelssohn and McKee (1981) report a compartively high leaf concentration of 980 nmol ATP g^{-1} dry wt (495 µg ATP g^{-1} dry wt).

Seasonal Survey

Although temperature, light, salinity, and nutrient regimes all exert an influence on growth (Setchell, 1929; Biebl and McRoy, 1971; Backman and Barilotti, 1976; Orth, 1977), temperature appears to be dominant in regulating the seasonal growth pattern of Z. marina in the Chesapeake Bay (Orth et al., 1981). In the present study, maximal shoot density and biomass occurred during spring. At a nearby site (inshore Guinea Marsh), peak shoot density and biomass were observed during June-July for the preceeding two seasons (Orth et al., 1981).

Aboveground tissue ATP levels were highest during winter and summer and lowest during spring and fall. Winter and summer correspond to periods of slow growth and senescence, respectively, with decreased rates of ATP utlization. In contrast, spring and fall correspond to periods of more rapid growth with increased rates of ATP utilization. Seasonal ATP levels in aboveground Z. marina contrast with those reported for Populus gelrica (poplar) twigs, which contained greatest amounts of ATP during active growth and lowest amounts during the no growth season (Sagisaka, 1981).

Sexual reproduction in Z. marina occurs during spring in the Chesapeake Bay (Stevenson and Confer, 1978). This expenditure of energy may reduce ATP content. Low adenylate levels are also observed

in Corbicula fluminea (freshwater clam) during periods of reproductive activity (Giesy and Dickson, 1981).

Belowground tissue ATP levels were highest during summer and fall and lowest during winter and spring. Belowground levels were generally much lower than corresponding aboveground levels. As previously suggested, low belowground adenylate levels may be attributed to metabolically inert material (Pamatmat and Skjoldal, 1979) or lowered aerobic respiration in reduced sediments (Mendelssohn et al., 1981).

Although amounts of adenine nucleotides are routinely reported, there is an important metabolic distinction between amount and turnover rate. The ATP turnover rate or energy flux through the adenine nucleotide pool is actually the more important quantitative assessment of cellular energetics (Weiler and Karl, 1979).

In both above and belowground Z. marina tissue, the following expected correlations were observed: 1) EC positively correlated with ATP and negatively correlated with AMP, and 2) AT correlated positively with ATP, ADP, and AMP. In aboveground tissue over the one year survey, ATP, ADP, and AMP comprised approximately 41-74%, 12-32%, and 7-31%, respectively, of the total adenylate pool. AT fluctuation demonstrates net synthesis and degradation of nucleotides.

Between month variability in EC was damped relative to individual adenylate concentrations. This was also observed in a seasonal study of adenine nucleotides in freshwater clams (Giesy and Dickson, 1981).

Lower EC variability has both biochemical and mathematical rationales. EC is not only regulatory but is also regulated within narrow limits by enzymes, controlling rates of reactions which are coupled to the use and regeneration of ATP (Atkinson, 1977). It has been suggested that AMP removal by adenylate deaminase serves to buffer the cell against a sharp transient decrease in EC (Chapman and Atkinson, 1973). The presence of ATP and ADP in both numerator and denominator of the EC ratio further reduces variability.

Conclusions

Due to the lability of adenine nucleotides, precautions must be taken throughout the analysis in order to quantify adenylates at their *in situ* levels. Freezing plants within 2 min after harvest, prevention of thawing, and lyophilization minimized adenylate change. Prolonged desiccated or frozen storage should be avoided, and hydrated extracts must be held on ice during the assay. High recovery rates of internal standards, added immediately prior to extraction, indicated minimal adenylate loss after extraction during the remainder of the assay. An additional methodological step is unique to aquatic macrophytes. *Z. marina* leaves should be scraped free of epiphytic algae after lyophilization, since substantial epiphytic biomass obscures leaf nucleotide content.

The tissue comparison and seasonal survey provide baseline information on natural adenylate variability in *Z. marina*. Since leaf tissue contained the highest adenylate levels, leaves appear most suitable as a test tissue for routine adenylate analyses. Seasonal

ATP levels in aboveground tissue reflect energy expenditures associated with growth patterns.

The method presented for the determination of adenine nucleotides in Z. marina has several limitations. Tissue adenylate measurement results in a mass-weighted mean value and provides no information on intercellular heterogeneity or intracellular compartmentation. Adenylate levels determined in metabolic or environmental studies with this technique should be interpreted in this context. Direct application of this methodology to other species may be inappropriate. With slight modification, however, the technique should prove suitable to other important macrophyte species.

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SECTION 2. APPLICATION

INTRODUCTION

The role of adenine nucleotides in cellular bioenergetics, including adenylate energy charge (EC) theory, has been presented by Atkinson (1977). Adenine nucleotides are strategically adapted to metabolic regulation, since they are operationally linked with nearly all metabolic sequences. The EC ratio, ([ATP] + 1/2 [ADP])/([ATP]+ [ADP]+[AMP]), represents a linear measure of the metabolic energy stored in the adenylate pool, ranging from 0 (all AMP) to 1.0 (all ATP). EC regulates metabolic sequences by controlling enzymic rates of reactions which are coupled to the use and regeneration of ATP.

Since the metabolic energy state of an organism is sensitive to environmental variation, EC has been proposed as an index of sublethal stress (Ivanovici, 1980) and has been widely applied in this context (e.g. Romano and Daumas, 1981; Giesy et al., 1981; Mendelssohn and McKee, 1981). The present study has evaluated effects of nutrient enrichment, light reduction, and herbicide exposure on adenylate response patterns in Zostera marina (eelgrass), a submerged marine angiosperm. Z. marina is an ecologically important macrophyte species (McRoy and Helfferich, 1977; Stevenson and Confer, 1978; Phillips and McRoy, 1980; Wetzel et al., 1981; Orth et al., 1981), occurring in temperate and subarctic coastal and estuarine waters in the Northern Hemisphere (den Hartog, 1970).

Nutrient-Light Experiment

Since nutrients and light affect rates of photosynthesis and associated photophosphorylation, adenylates are expected to respond to changes in nutrient or light regimes. Several studies have investigated adenylate response in higher plants to nutrient enrichment or light reduction. Ching et al. (1975) have reported increases in ATP, AT, and EC in Triticum aestivum (wheat) leaves with addition of NH_4NO_3 fertilizer over a 2-3 month period. Levels of ATP and AT, but not EC, decreased in Spartina alterniflora (cordgrass) leaves with light reduction (5% of full sunlight) over one week (Mendelssohn and McKee, 1981). Kondo and Nakashima (1979) observed increases in ATP and AT, but not EC, with sucrose enrichment and higher light level over several days in an aquatic macrophyte, Lemna gibba (duckweed). These studies indicate that ATP and AT concentrations are generally more sensitive than EC and consistently increase with nutrient enrichment and higher light intensity. Apparently, EC is stabilized within narrow limits by mechanisms, outlined by Atkinson (1977).

It has been reported that epiphytic growth, stimulated by nutrient enrichment, can adversely affect rooted aquatic macrophytes (Phillips et al., 1978; Sand-Jensen and Sondergaard, 1981). Epiphytes presumably reduce macrophyte growth by shading and by depleting inorganic carbon and nutrients at the leaf surface. In some cases, epiphytic colonization may promote host leaf senescence (Rogers and Breen, 1981). With little or no epiphytic colonization, strong and positive correlations of Z. marina ATP and AT with nutrients and light

are expected, since growth correlates positively with adenylates (Ching, 1975), nutrients (Orth, 1977), and light (Backman and Barilotti, 1976). With heavy epiphytic colonization, these correlations should weaken. Although several studies have addressed adenylate response in algae to nutrient limitation (Holm-Hansen, 1970; Falkowski, 1977; Riemann and Wium-Anderson, 1981) or light limitation (Holm-Hansen, 1970; Weiler and Karl, 1979), few studies have investigated effects of nutrient enrichment (Stephens and Shultz, 1982) or reduced light levels on epiphyte adenylates.

This study has investigated adenylate response patterns in Z. marina and its epiphytes to nutrient enrichment and light reduction. Z. marina morphometric and production data were collected, along with an assessment of epiphytic colonization, in order to facilitate evaluation of physiological response.

Atrazine Experiments

Atrazine, a triazine herbicide, is widely used for selective control of broadleaf and grassy weeds in tolerant crop species, including corn, sorghum, and sugarcane (WSSA, 1974). As an inhibitor of the Hill reaction in photosynthesis (Ebert and Dumford, 1976; Gardner, 1981), atrazine is expected to impair photoevolution of oxygen, net photoreduction, and noncyclic photophosphorylation in the chloroplast and may adversely affect the adenylate pool. Several factors, which may eliminate or offset atrazine toxicity, are reduced herbicide uptake and translocation (Ebert and Dumford, 1976), detoxication (Shimabukuro et al., 1971), or compensatory

phosphorylation potential. Since neither cyclic photophosphorylation (Thompson et al., 1974) nor oxidative phosphorylation (Davis, 1968) are appreciably altered by atrazine, these processes along with substrate phosphorylation may regenerate adequate amounts of ATP.

Several studies have investigated ATP response to atrazine exposure in higher plants. Atrazine, administered through leaves (500 ppm) or through roots (0.5 ppm), generally decreased ATP content in Cucumis sativus (cucumber) leaves and roots over 1-3 days (Decleire and Decat, 1981). In contrast, Gruenhagen and Moreland (1971) have reported slightly elevated levels of ATP in Glycine max (soybean) hypocotyls with atrazine exposure (43 ppm) over 6 hrs. These inconsistent results may reflect differences in exposure time or differences between species in atrazine metabolism or phosphorylation potential.

Nontarget effects of atrazine have been implicated in recent declines of submerged aquatic macrophytes in the Chesapeake Bay (Stevenson and Confer, 1978). Agricultural runoff, leaching, and aerial transport processes introduce atrazine into the Bay (Wu, 1981). Forney and Davis (1981) have reported 3-6 week I_1 values (the concentration inhibiting growth 1%) of a few ppb atrazine for several submerged aquatic macrophyte species. Although atrazine levels in the Chesapeake Bay are generally below 1 ppb (Correll et al., 1978; Wu et al., 1980; Hershner et al., 1981), Hershner et al. (1981) have suggested that Z. marina beds in the lower Bay may experience atrazine

concentrations, ranging from 1-10 ppb, for several days over the growing season.

Assuming Z. marina is susceptible to atrazine toxicity, decreased ATP and EC levels with atrazine exposure are expected. This study has investigated adenylate response patterns in Z. marina over short-term (6 hr) and long-term (21 day) atrazine exposure. Production, morphometric, and mortality data were collected in order to facilitate interpretation of adenylate response to atrazine.

METHODS

Field Collection and Transplanting

Location of the Zostera marina sampling site in the lower Chesapeake Bay was described in Methods of Section 1. Clumps of eelgrass were uprooted with a shovel, swirled in river water to remove macro-algae and loose sediment, transported in a bucket of river water to the laboratory, and acclimated in a flow-through system. Clumps were then divided into "individual" plants (i.e. single shoot with the attached leaf cluster and a 2-5 cm rhizome segment) for transplanting. Transplants, used in the nutrient-light study, were planted in clean white sand in partitioned plastic trays. Transplants, used in atrazine experiments, were planted in natural sediment (obtained from the VIMS beach) in Jiffy Pots. All transplants were submerged in a flow-through system.

Adenine Nucleotides

Samples were processed, as described in Methods of Section 1, with the following specifications:

1) transplants were uprooted by hand,
2) for each treatment, plants were pooled in order to minimize within treatment variation and spotlight between treatment variation,
3) leaf tissue and epiphytes were assayed after 1 and 2 week exposures in the nutrient-light experiment,

4) leaf tissue was assayed at the end of short-term (6 hr) and long-term (21 day) atrazine experiments, and
5) photometry was performed entirely in the integration mode.

Environmental Data

Environmental data included water temperature, salinity, dissolved oxygen (DO), and photosynthetically active radiation (PAR). Minimum and maximum temperatures were recorded with a min-max thermometer (Taylor Instruments). Salinity was measured with a temperature-compensated refractometer (Model 10419, American Optical Corp.) or an induction salinometer (Model RS 7B Beckman). DO was monitored polarographically (Hitchman, 1978) with an oxygen meter (Models 2504 and 2607, Orbisphere Lab). This meter was calibrated in water-saturated air at specified temperature and pressure. Because it was not salinity-corrected, DO values are relative and not absolute. PAR was measured with a light meter (Model LI-185B, Lambda Instruments Corp.), equipped with deck (air) and sea (water) quantum sensors. Underwater readings were taken at a depth of approximately 5 cm (i.e. plant level). PAR includes those wavelengths from 400-720 nm (Parsons et al., 1977).

Nutrient-Light Experiment

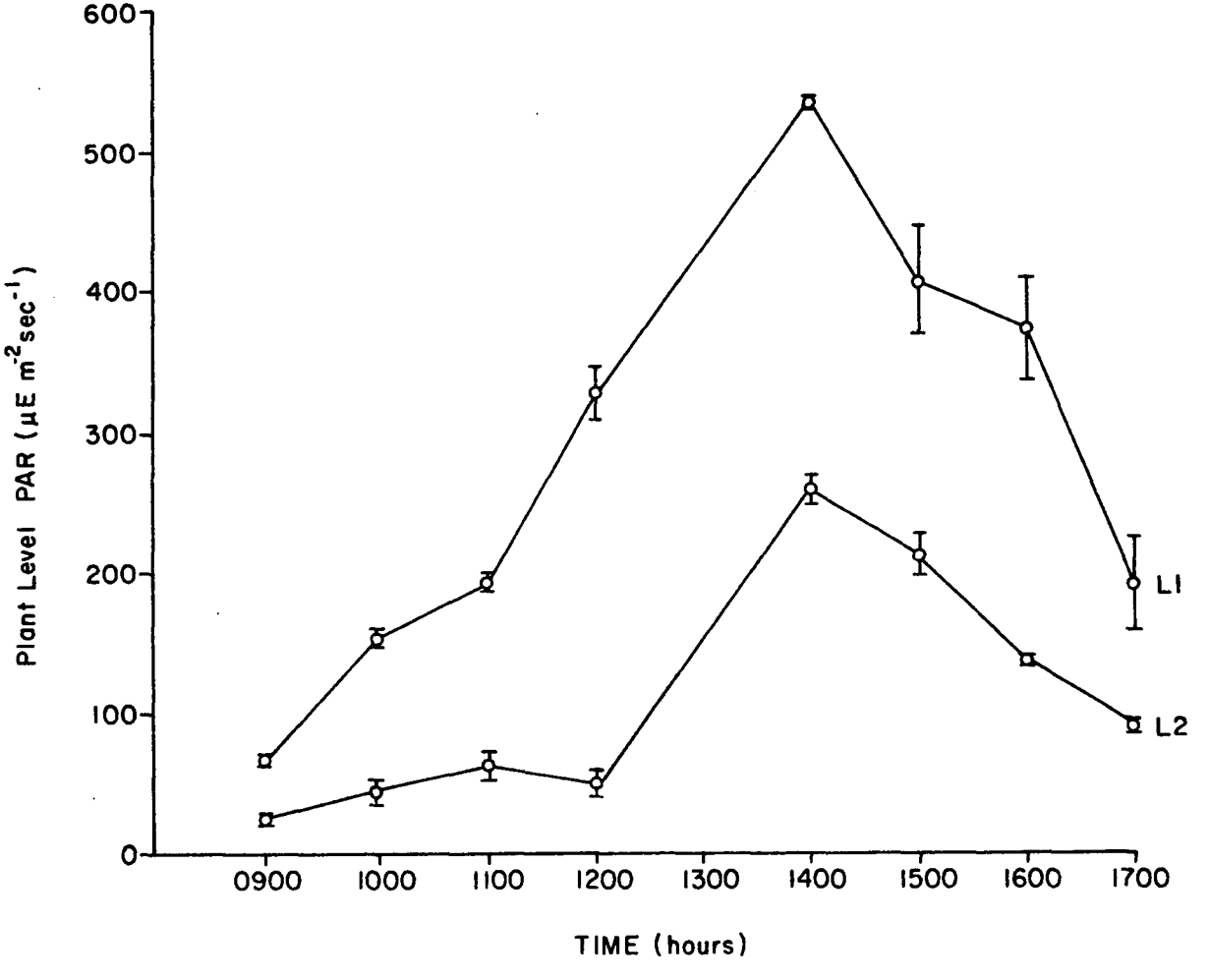
Design

Effects of three nutrient and two light levels over 2 weeks were tested with the resultant six treatments, presented in Table 2.1. L1 and N1 represent ambient levels. Hourly mean light levels over a typical day are plotted in Figure 2.1 Mean nutrient levels and

Table 2.1. Treatments used in nutrient-light experiment.

Nutrient Level	Light Level	
	L1	L2
N1	N1L1	N1L2
N2	N2L1	N2L2
N3	N3L1	N3L2

Figure 2.1 Light levels (L1, L2) in the nutrient-light experiment (n = 3). Error bars are 1 standard error.


600
500
400
300
200
100
0
Plant Level PAR (µE m-2sec-1)
0900
1000
1100
1200
1300
1400
1500
1600
1700
TIME (hours)
L1
L2

resultant N/P ratios were calculated from initial, mid, and final measurements over the course of the experiment (Figure 2.2).

The flow-through system, located on the VIMS pier, is diagrammed schematically in Figure 2.3. Six 38 l glass tanks each held 24 plants. Tank turnover times ranged between 1.7-2.0 hrs.

Nutrient stock solutions were prepared with American Chemical Society (ACS) grade NH_4NO_3 and Na_2HPO_4 (Fisher Scientific Co.), dissolved in deionized water. These solutions were metered in with a peristaltic pump (Model 600-1200, Harvard Apparatus Co., Inc.), so that dilution yielded the desired tank concentrations. Flow rates were monitored daily. Tanks were covered with black polypropylene shade cloths to reach desired light levels. In addition, tanks were equipped with submersible recirculating pumps.

Nutrients

At initial, mid, and final times, water samples from each tank were injected through 0.3 μ glass fiber filters (Type A/E, Gelman) into plastic tubes and stored frozen prior to analysis. Dissolved [orthophosphate]-phosphorus, [ammonia]-nitrogen, and [nitrite plus nitrate]-nitrogen were measured colorimetrically, as described in EPA methods 365.2, 350.1, and 353.2, respectively (U.S. EPA, 1979). Dissolved $[PO_4^{-3}]$-P was measured manually (Model Spectronic 20 with extended spectral range, Bausch and Lomb), while dissolved $[NH_3]$-N and $[NO_2^- + NO_3^-]$-N were determined by an automated procedure (Model AutoAnalyzer II, Technicon Instruments Corp.).

Figure 2.2 Nutrient levels (N1, N2, N3) in the nutrient-light experiment (n = 6). Error bars are 1 standard error.

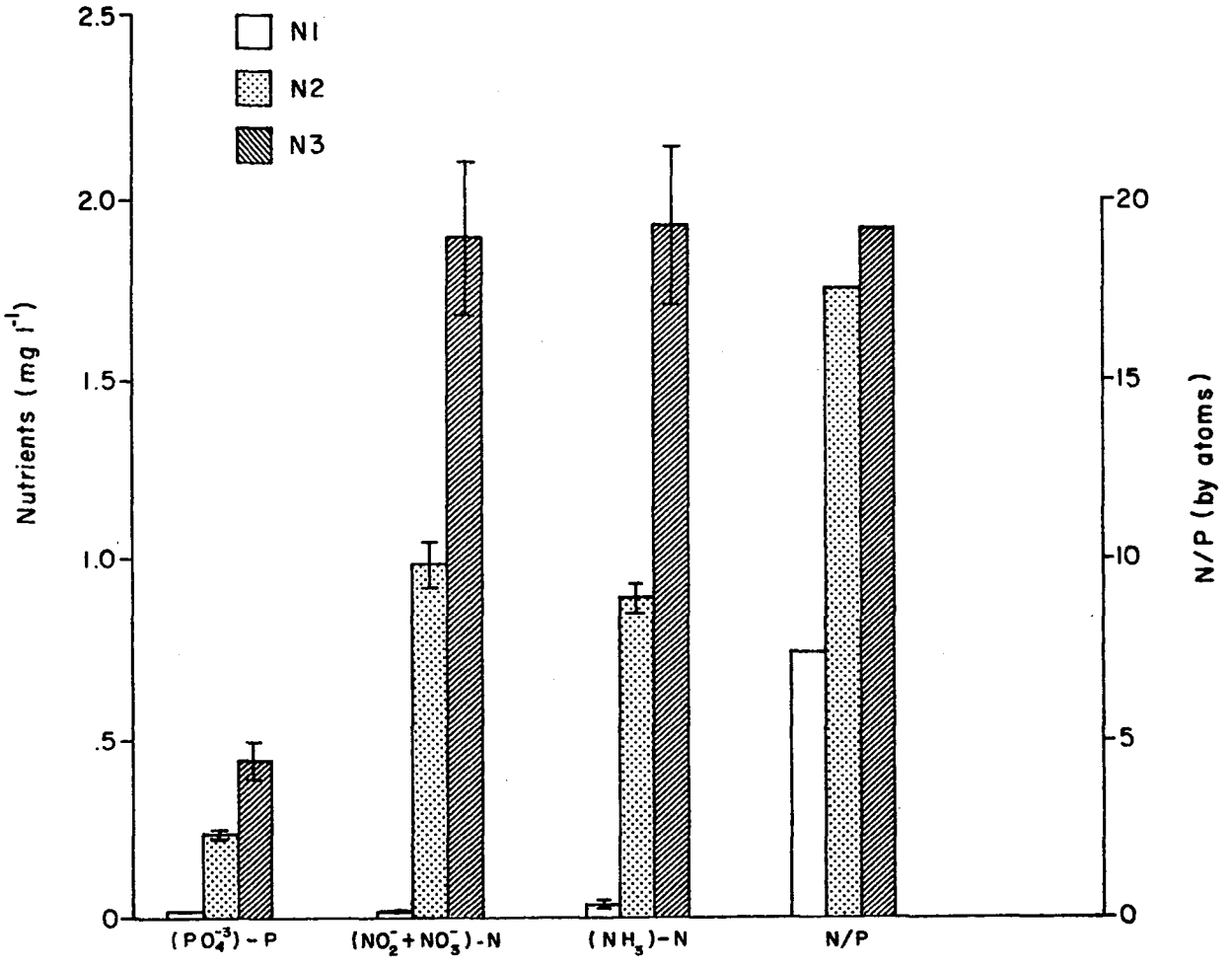
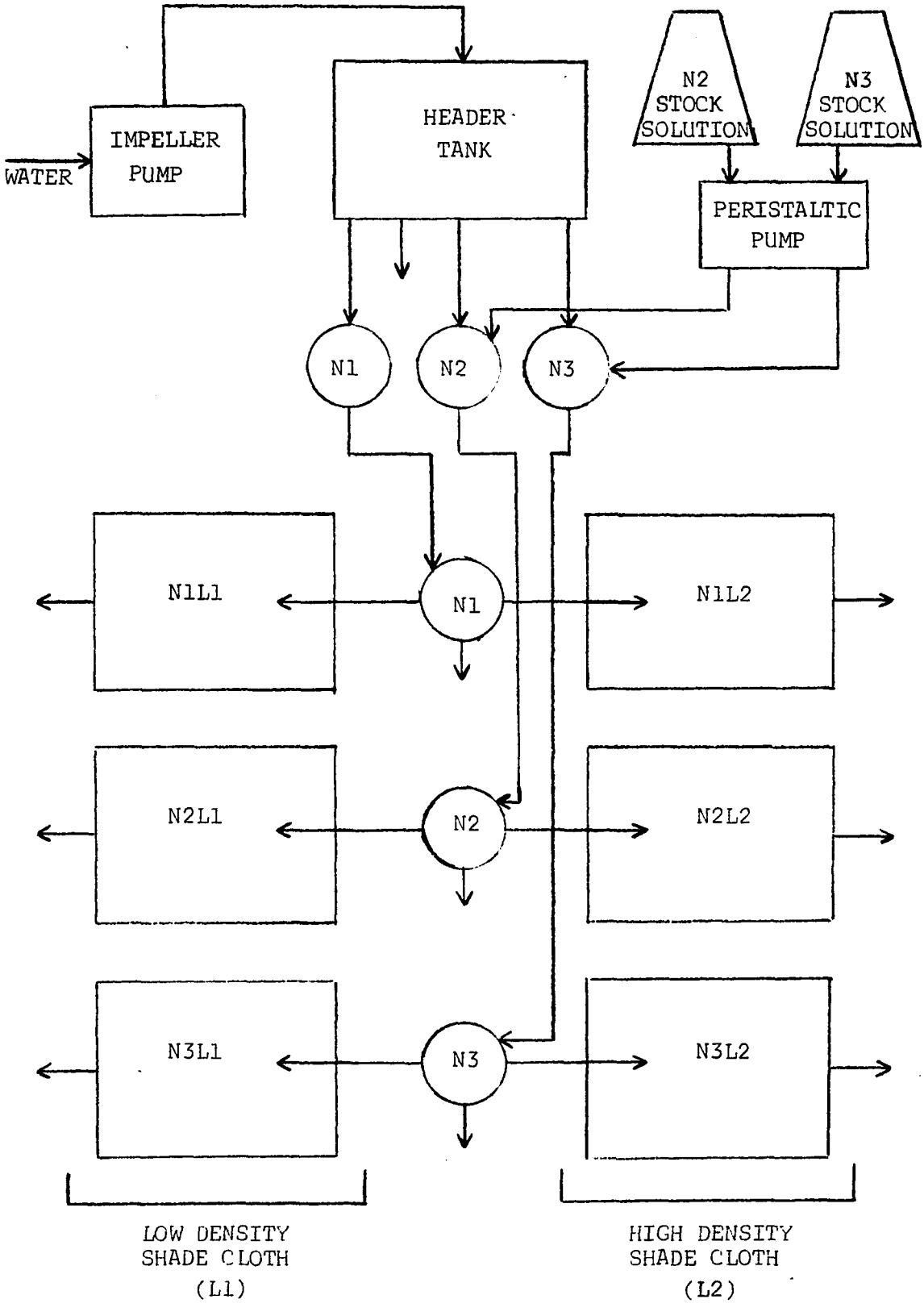

N1
N2
N3
Nutrients (mg l⁻¹)
2.5
2.0
1.5
1.0
.5
0
20
15
10
5
0
N/P (by atoms)
(PO4-3) - P
(NO2- + NO3-) - N
(NH3) - N
N/P

Figure 2.3. Flow-through system used for nutrient-light experiment.


WATER
IMPELLER PUMP
HEADER TANK
N2 STOCK SOLUTION
N3 STOCK SOLUTION
PERISTALTIC PUMP
N1
N2
N3
N1L1
N1
N1L2
N2L1
N2
N2L2
N3L1
N3
N3L2
LOW DENSITY SHADE CLOTH (L1)
HIGH DENSITY SHADE CLOTH (L2)

Morphometrics

The following non-destructive morphometric measurements were obtained on 8 marked plants from each tank at initial and final (2 weeks) times: whole-plant fresh weight, shoot length, and number of leaves. Leaves were scraped free of epiphytes prior to fresh weight determinations. Dead plants were excluded from final mean calculations. Two week morphometric changes were calculated from the following formula:

$$\% \text{ Change} = \frac{\bar{X}_F - \bar{X}_I}{\bar{X}_I} \times 100$$

where $\bar{X}_F$ = final mean

$\bar{X}_I$ = initial mean

Productivity

Z. marina productivity measurements were obtained using a modified light-dark bottle method (e.g. Strickland and Parsons, 1972), following the 2 week nutrient-light exposures. 300 ml BOD glass bottles (Wheaton) were filled with river water (ambient nutrient levels) which had been passed through 0.7 μ glass fiber filters (GF/F, Whatman). Aboveground portions of plants were harvested from specific treatment tanks, scraped free of epiphytes, and placed inside BOD bottles. Bottles were then tightly stoppered and incubated in corresponding source tanks (with associated light levels) from 1030-1430 hrs. Initial and final DO readings were obtained. Dry weights were recorded at the end of the experiment.

Productivity was calculated from the following formula:

$$mg\ O_2\ g^{-1}\ hr^{-1} =$$
$$(\Delta\ mg\ O_2\ \ell^{-1})\ (\ell\ BOD\ bottle)\ (g\ dry\ wt)^{-1}\ (hr\ incubation)^{-1}$$

where Δ = final DO - initial DO

Net production (light bottle), respiration (dark bottle), and gross production (light bottle minus dark bottle) rates were then obtained. Rates, presented in terms of oxygen production, were also converted to carbon fixation by assuming a photosynthetic quotient of 1.25 (Westlake, 1963; McRoy and McMillan, 1977). This results in the conversion: .300 (mg O_2) = mg C. Although these rates were obtained at two light levels but all at ambient nutrient levels, test plants were exposed to the six treatments over the preceeding 2 weeks. As such, these rates represent effects of two light levels on plants with a 2 week history of specific nutrient and light exposure.

Epiphyte Colonization

Two plexiglass plates (each 10.0 x 15.0 x 0.2 cm) were positioned vertically on the bottom of each tank at time zero. At the end of the experiment (2 weeks), PAR readings were taken through the colonized plates. Plates were then scraped free of epiphytes, and PAR readings were obtained again. Shading by epiphyte colonization was calculated from the following formula:

$$\%\ Shade = \left[1 - \frac{PAR_{(P+E)}}{PAR_{(P)}}\right] \times 100$$

where $PAR_{(P+E)}$ = PAR transmitted through plate plus epiphytes

$PAR_{(P)}$ = PAR transmitted through plate alone

Short-Term (6 Hr) Atrazine Experiments

Design

Effects of atrazine exposure over 6 hrs were tested in two sealed 37 l glass tanks, one control and one dosed chamber. The flow-through system inside a greenhouse is diagrammed in Figure 2.4. Nominal atrazine concentrations of 10 and 100 ppb were evaluated in two separate experiments. Design specifications are presented in Table 2.2.

Atrazine stock solution was prepared with technical grade atrazine (97.2%, CIBA-GEIGY Corp.), dissolved in glass-distilled methanol (Burdick and Jackson Labs). This solution was metered in with a peristaltic pump (Model 600-1200, Harvard Apparatus Co., Inc.), so that dilution yielded the desired atrazine concentration (0.07% v/v methanol). Flow rates were checked hourly. Short-term experiments did not incorporate a methanol control.

Atrazine

Water samples were collected, filtered, extracted, and assayed for atrazine by gas chromatography, as described by Hershner et al. (1981). The gas chromatograph (Model 560, Tracor) was equipped with a nitrogen-phosphorus detector (Model 702, Tracor). These samples were collected to spot-check nominal atrazine concentrations.

Figure 2.4. Flow-through system used for short-term (6 hour) atrazine experiments.

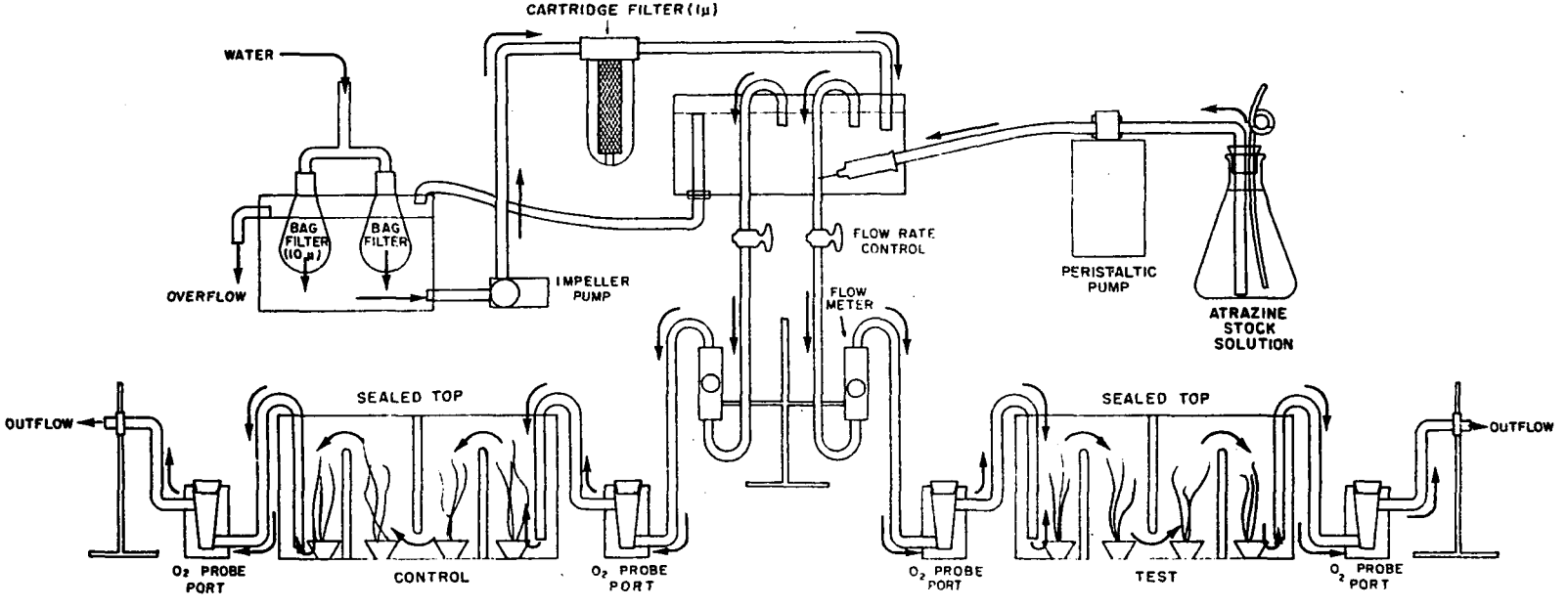

CARTRIDGE FILTER (1μ)
WATER
BAG FILTER (10 μ)
BAG FILTER
OVERFLOW
IMPELLER PUMP
FLOW RATE CONTROL
FLOW METER
PERISTALTIC PUMP
ATRAZINE STOCK SOLUTION
OUTFLOW
SEALED TOP
SEALED TOP
OUTFLOW
O2 PROBE PORT
CONTROL
O2 PROBE PORT
O2 PROBE PORT
TEST
O2 PROBE PORT

Table 2.2. Design specifications for short-term (6 hour) atrazine experiments.

Specification		Nominal Atrazine (ppb) 10	100
Measured atrazine (ppb):	Initial	15.77	97.86
	Final	9.39	91.33
Exposure period (hrs)		1000-1630	1030-1700
Tank turnover time (hrs)		1.74	1.74
Aboveground dry wt (g):	Control	15.04	9.23
	Test	12.48	9.51

Productivity

Z. marina productivity measurements were obtained, using the flow-through system. Water was pumped through a 1 μ cartridge filter, as shown in Figure 2.4. Potted plants were placed in tanks, which were then tightly sealed with glass tops, leaving no air space. After the tank water had turned over one time, DO was monitored hourly at both inflow and outflow ports. Dry weight of aboveground biomass in each tank was obtained at the end of the experiment.

Productivity was calculated from the following formula:

$$\text{mg } O_2\ g^{-1}\ hr^{-1} =$$
$$(\Delta \text{ mg } O_2\ \ell^{-1})\ (\ell \text{ tank})\ (\text{g dry wt})^{-1}\ (\text{hr turnover})^{-1}$$

where Δ = outflow DO - inflow DO

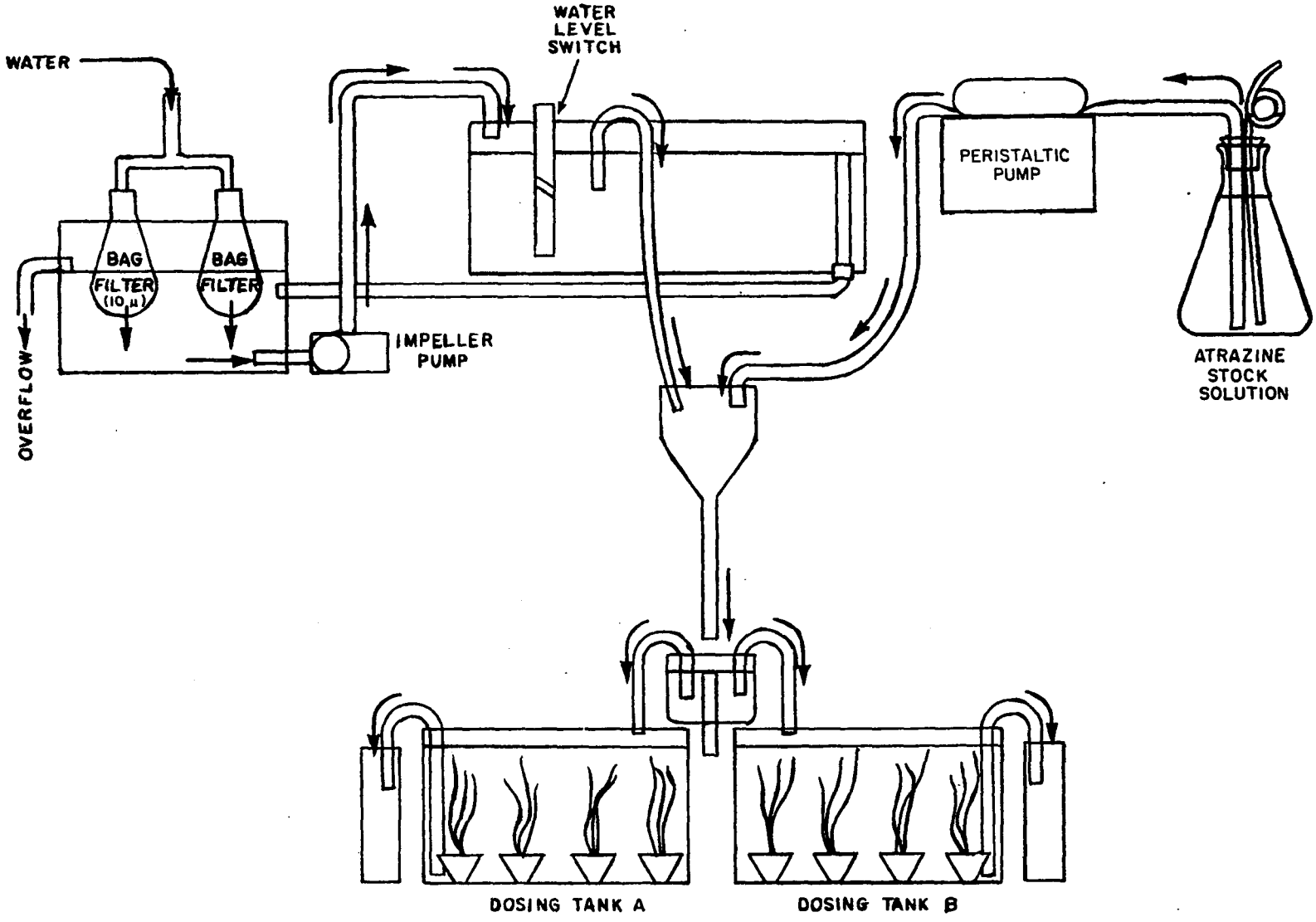
The ratio, tank volume/turnover time, is simply the flow rate. These production rates represent net productivity, since photosynthesis and respiration operate simultaneously during daylight hours.

Long-Term (21 Day) Atrazine Experiments

Design

Effects of atrazine exposure over 21 days were tested in six pairs (each pair consists of A and B replicates) of 38 1 glass tanks, corresponding to the following nominal atrazine concentrations: 0, 0.1, 1.0, 10, 100, 1000 ppb. Each tank initially held 15 potted plants. The flow-through system inside a greenhouse is diagrammed in Figure 2.5. Mean tank turnover times ranged from 7.3-13.5 hrs. This experiment was replicated four times. Replicate Experiments 1-4 were

Figure 2.5. Flow-through system used for long-term (21 day) atrazine experiments. The diagram depicts only one of the six pairs of tanks in the system.


WATER
WATER LEVEL SWITCH
PERISTALTIC PUMP
BAG FILTER (10 μ)
BAG FILTER
IMPELLER PUMP
OVERFLOW
ATRAZINE STOCK SOLUTION
DOSING TANK A
DOSING TANK B

analyzed separately, as well as together, in some cases. Spot-check atrazine measurements are listed in Table 2.3.

Atrazine stock solutions were metered in with a peristaltic pump, so that dilution yielded the desired atrazine concentrations (0.07% v/v methanol). Flow rates were monitored daily. Long-term experiments incorporated a methanol control.

Atrazine

Atrazine measurement was performed according to the procedure described for short-term experiments.

Morphometrics

Shoot length and number of leaves were obtained on all living plants from each tank at 0, 7, 14, and 21 days. Measurements from replicate tanks A and B were combined to calculate means. Weekly morphometric changes were calculated from the following formula:

$$\% \text{ Change} = \frac{\bar{X}_t - \bar{X}_0}{\bar{X}_0} \times 100$$

where $\bar{X}_t$ = mean at time t

$\bar{X}_0$ = mean at time zero

Mortality

Mortality was recorded in each tank at 7, 14, and 21 days. Dead plants were removed from the system. Visual criteria for plant death were loss of green pigmentation (i.e. chlorophyll degradation) and

Table 2.3. Spot-check atrazine measurements in long-term (21 day) atrazine experiments.

Experiment	Exposure Time (days)	Atrazine (ppb)	
		Nominal	Measured
1	21	100	108.60
2	7	1	2.91
		10	22.49
		100	113.53
		1000	1051.08
	14	1	1.06
		1000	1038.69
	21	1	1.26
		1000	1072.86
3	21	.1	.70
		10	11.12
4	21	1	1.27
		100	116.09

loss of structural integrity. Mortality observations from replicate tanks A and B were combined in tabulations.

Statistical Analysis

Procedures

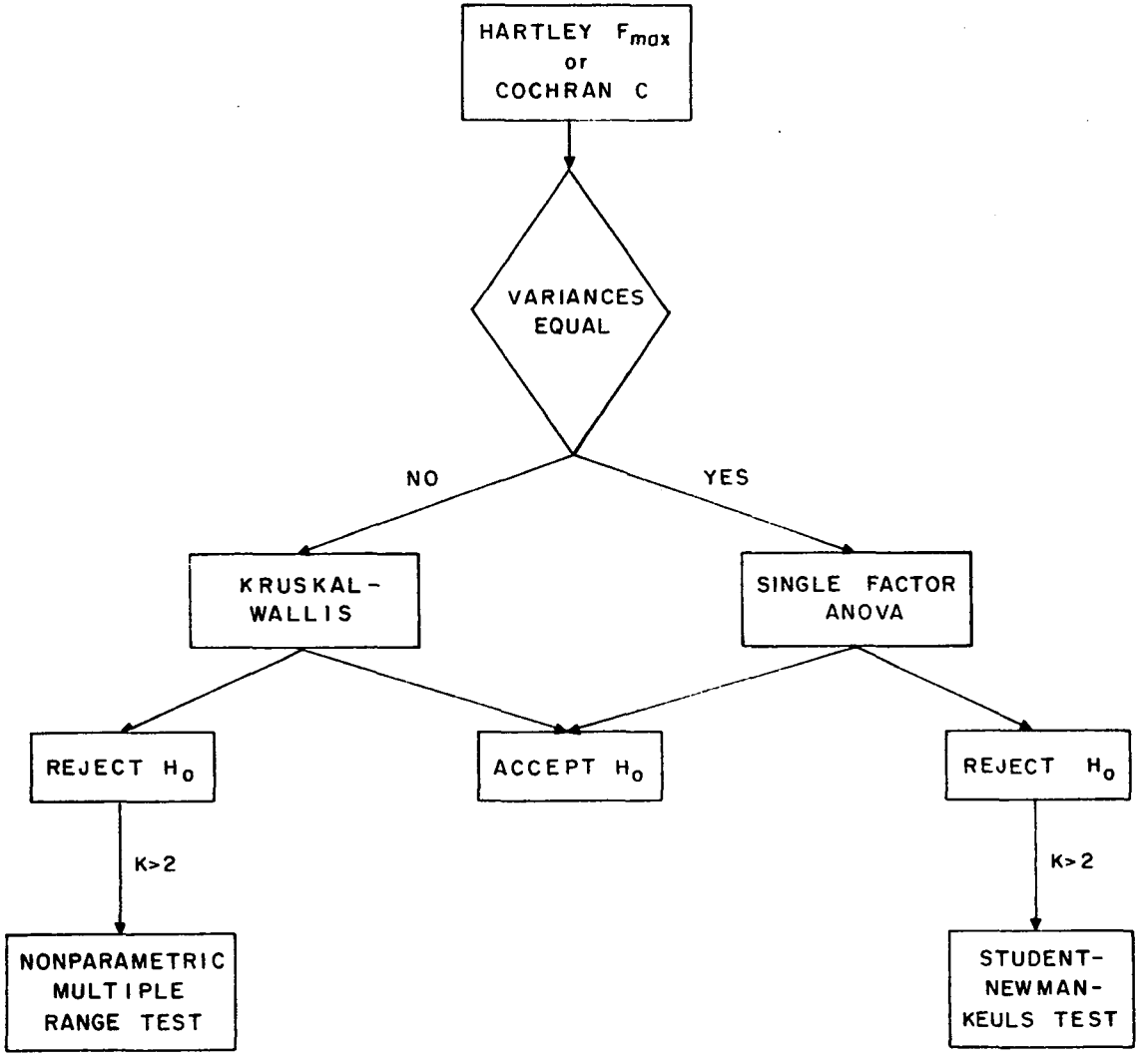
The following procedures in the SPSS software package (Nie et al., 1975; Hull and Nie, 1981) were used: ONEWAY (single factor ANOVA, Hartley Fmax test for homoscedasticity, Student-Newman-Keuls multiple range test), SCATTERGRAM (linear regression and Pearson correlation), NPAR TESTS (Kruskal-Wallis single factor ANOVA by ranks and Kolmogorov-Smirnov one sample test for normality), and NONPAR CORR (Spearman rank correlation).

Other statistical procedures employed included nonparametric multiple range testing by rank sums (Zar, 1974) and dose-effect analysis with log-probit transformation (Litchfield and Wilcoxon, 1949). Statistical assumptions are discussed in Appendix B.

Nutrient-Light Experiment

Differences between adenylates in response to three nutrient levels and two light levels over time were examined. Data were organized into single factor designs with six treatments after 1 and 2 week periods. Differences between adenylates, subjected to these treatments, were detected and located by the procedure diagrammed in Figure 2.6. Dependent variables are ATP, ADP, AMP, AT, and EC. Independent variables are nutrient-light treatments. The null hypothesis states no difference in adenylates between k treatments (i.e. H_0: $\mu_1 = \mu_2 = \ldots = \mu_k$). Relationships among adenylate,

Figure 2.6. Detection and location of treatment differences.


HARTLEY F_{max}
or
COCHRAN C
VARIANCES EQUAL
NO
YES
KRUSKAL-WALLIS
SINGLE FACTOR ANOVA
REJECT H_0
ACCEPT H_0
REJECT H_0
K>2
K>2
NONPARAMETRIC MULTIPLE RANGE TEST
STUDENT-NEWMAN-KEULS TEST

environmental, morphometric, productivity, and epiphyte data were evaluated by Spearman rank correlation.

Short-Term (6 Hr) Atrazine Experiments

Differences between adenylates, resulting from exposure to atrazine, were detected by the procedure diagrammed in Figure 2.6.

Long-Term (21 Day) Atrazine Experiments

Differences between adenylates, resulting from exposure to atrazine, were detected and located by the procedure diagrammed in Figure 2.6. Morphometric change was regressed against time for a control and five atrazine concentrations. Relationships between adenylate and atrazine data were evaluated by Spearman rank correlation. Median and 1% lethal atrazine concentrations (LC 50 and LC 1, respectively) and slope function (S), together with their 95% confidence limits, were estimated by log-probit analysis. Differences between these mortality statistics from replicate experiments were evaluated.

RESULTS

Nutrient-Light Experiment

Flow-Through System Data

Mean temperature and salinity, over the course of the experiment, are presented in Table 2.4. Relatively small standard errors indicate stability in these variables.

Hourly DO concentrations in each treatment, over a typical morning, are plotted in Figure 2.7. These values represent a saturation range from 119-303%. By 1230 hrs, DO's had diverged considerably. At this time, DO was consistently higher with both increased light levels (at a given nutrient level) and nutrient levels (at a given light level). PAR (surface and plant level), DO, and temperature were all positively and significantly correlated (Table 2.5).

Morphometrics

Mean fresh weight, shoot length, and number of leaves for *Z. marina*, obtained at the start of the experiment, are presented in Table 2.6. Most 2 week morphometric changes were negative (Figure 2.8). Change in leaf number was consistently lower with increased nutrient levels (at a given light level). This observation is reflected in the significant negative correlation between leaf number change and nutrients (Table 2.7).

Table 2.4. Temperature and salinity during nutrient-light experiment.

Variable	Statistic		
	n	$\bar{x}$	SE
Minimum temp. (°C)	8	25.1	.4
Maximum temp. (°C)	8	28.6	.5
Salinity (°/oo)	9	20.88	.13

Figure 2.7. Dissolved oxygen concentrations among the six treatments in the nutrient-light experiment.

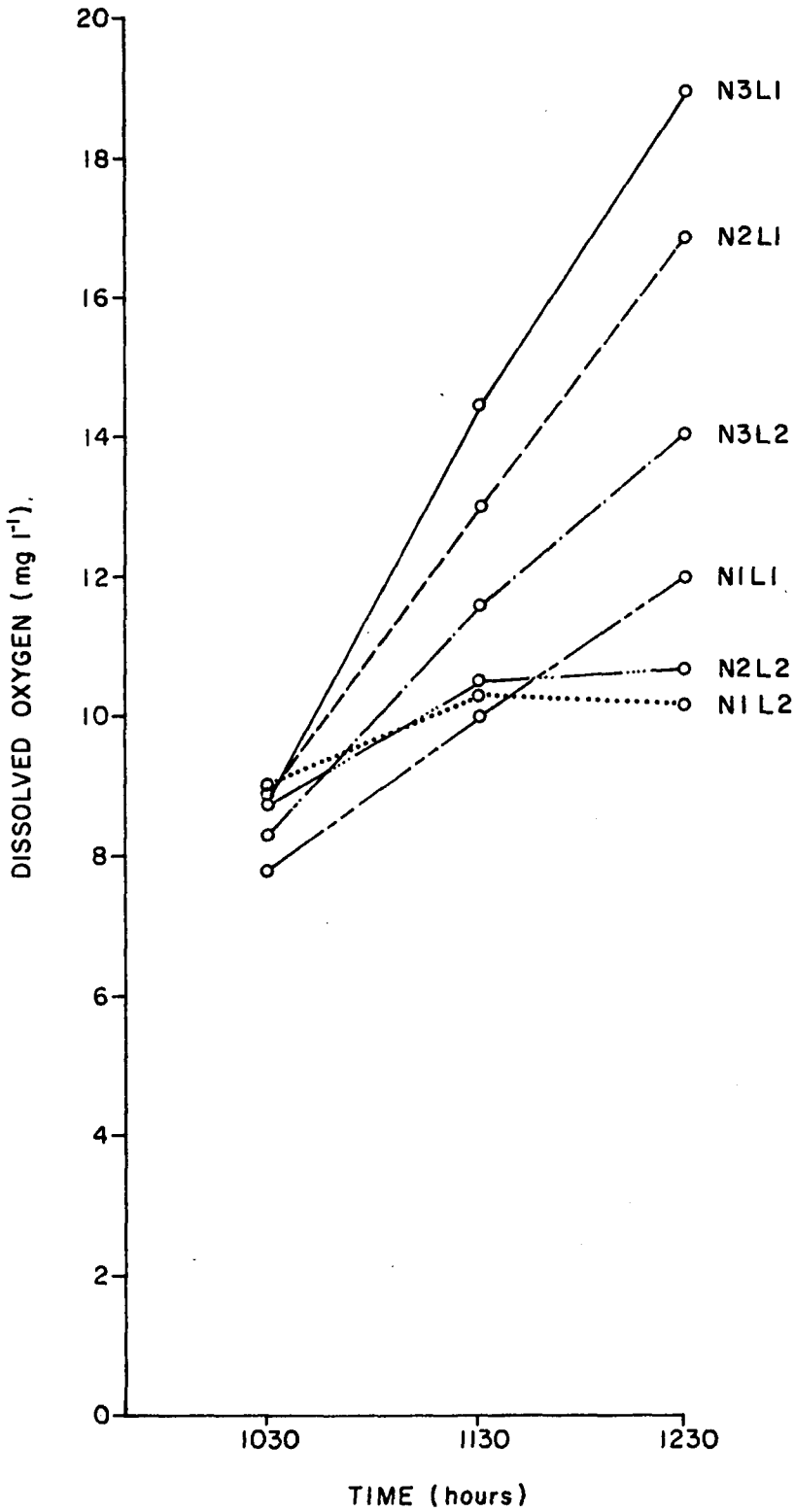

20
18
16
14
12
10
8
6
4
2
0
DISSOLVED OXYGEN (mg l⁻¹)
1030
1130
1230
TIME (hours)
N3L1
N2L1
N3L2
N1L1
N2L2
N1L2

Table 2.5. Spearman correlation coefficients between PAR, DO, and temperature from 1030-1230 hrs in nutrient-light experiment (n=18).

	Plant level PAR	DO	Temp.
Surface PAR	.5284*	.7392*	.9142*
Plant level PAR		.5444*	.5719*
DO			.8450*

* $P < .05$

Table 2.6. Initial *Z. marina* morphometrics in nutrient-light experiment (n=48).

Variable	Statistic	
	$\bar{x}$	SE
Whole-plant fresh wt (g)	1.76	.13
Shoot length (cm)	38.4	1.6
Number leaves	4.6	1.4

Figure 2.8. Two week morphometric changes for Z. marina among the six treatments in the nutrient-light experiment. Numbers in parentheses are n.

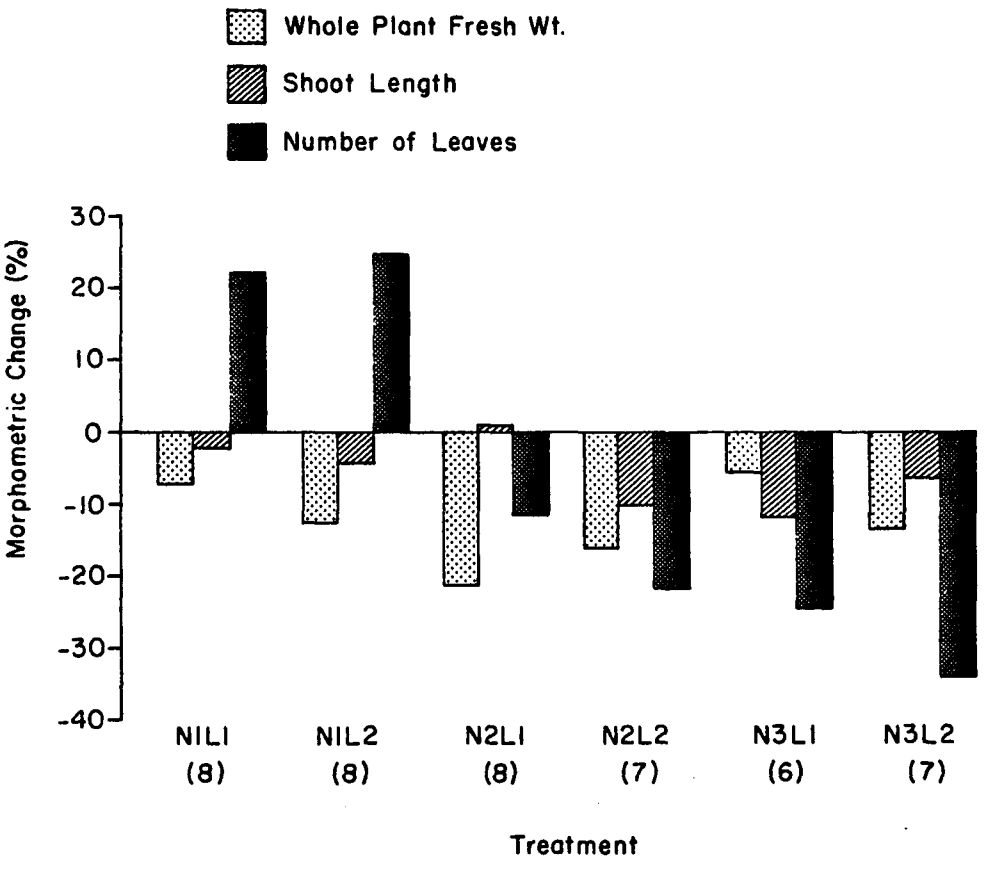

Whole Plant Fresh Wt.
Shoot Length
Number of Leaves
Morphometric Change (%)
30
20
10
0
-10
-20
-30
-40
N1L1
(8)
N1L2
(8)
N2L1
(8)
N2L2
(7)
N3L1
(6)
N3L2
(7)
Treatment

Table 2.7. Spearman correlation coefficients of morphometric change, production, epiphyte shading, and adenylate variables with nutrients and light, paired by treatment, in the nutrient-light experiment (n=6).

Variable		Nutrients		Plant level PAR	
		Wk 1	Wk 2	Wk 1	Wk 2
Fresh wt change			0		.2928
Shoot length change			-.5976		.2928
Leaf number change			-.9562*		.0976
Net production			-.7171		-.0976
Respiration			-.5976[1]		.0976
Gross production			.3586		-.4880
Epiphyte shading			.8367		.2928
Z. marina	ATP	.4781	.5457	.2928	.1980
	ADP	-.3586	-.6670	-.2928	0
	AMP	-.4781	0	-.0976	.8783
	AT	.2390	.5976	.0976	.4880
	EC	.7882	.2425	.2970	0
Epiphyte	ATP	.5976	.9562*	-.0976	.0976
	ADP	.8367	.9562*	.0976	-.2928
	AMP	.8367	.7171	.0976	-.6831
	AT	.7171	.9562*	-.2928	-.0976
	EC	.5457	.4781	-.3961	.8783

* $P < .05$

[1] Because a higher respiration rate is indicated by a more negative value, a negative correlation coefficient indicates higher respiration with higher nutrients.

Productivity

Net production, respiration, and gross production rates for Z. marina appear in Figure 2.9. Net productivity was positive for all treatments, as would be expected during daylight hours. Net productivity generally decreased and respiration rate generally increased (i.e. values became more negative) with higher nutrient levels (at a given light level), administered over the preceeding 2 weeks. Although these correlations were not significant, they were relatively strong (Table 2.7).

Epiphyte Colonization

Two week epiphyte colonization, expressed as a shading index, is shown in Figure 2.10. Shading was consistently higher with higher nutrient levels (at a given light level). This observation is reflected in the relatively strong and positive correlation ($.05 < P < .10$) between epiphyte shading and nutrients (Table 2.7).

Z. marina Adenine Nucleotides

Adenylate and EC values in Z. marina, after 1 and 2 week exposure periods, are presented in Tables 2.8 and 2.9, respectively. These same results are displayed in Figure 2.11. ATP and AT were highest at nutrient level N2 (at a given light level) after both 1 and 2 week periods. Z. marina ATP, AT, and EC were positively but weakly correlated with nutrients and PAR (Table 2.7). Considering all treatments, temporal trends from Week 1 to Week 2 were the following: 1) ATP, AT, and EC generally decreased, and 2) AMP generally increased.

Figure 2.9. Production and respiration rates for Z. marina among the six treatments in the nutrient-light experiment.

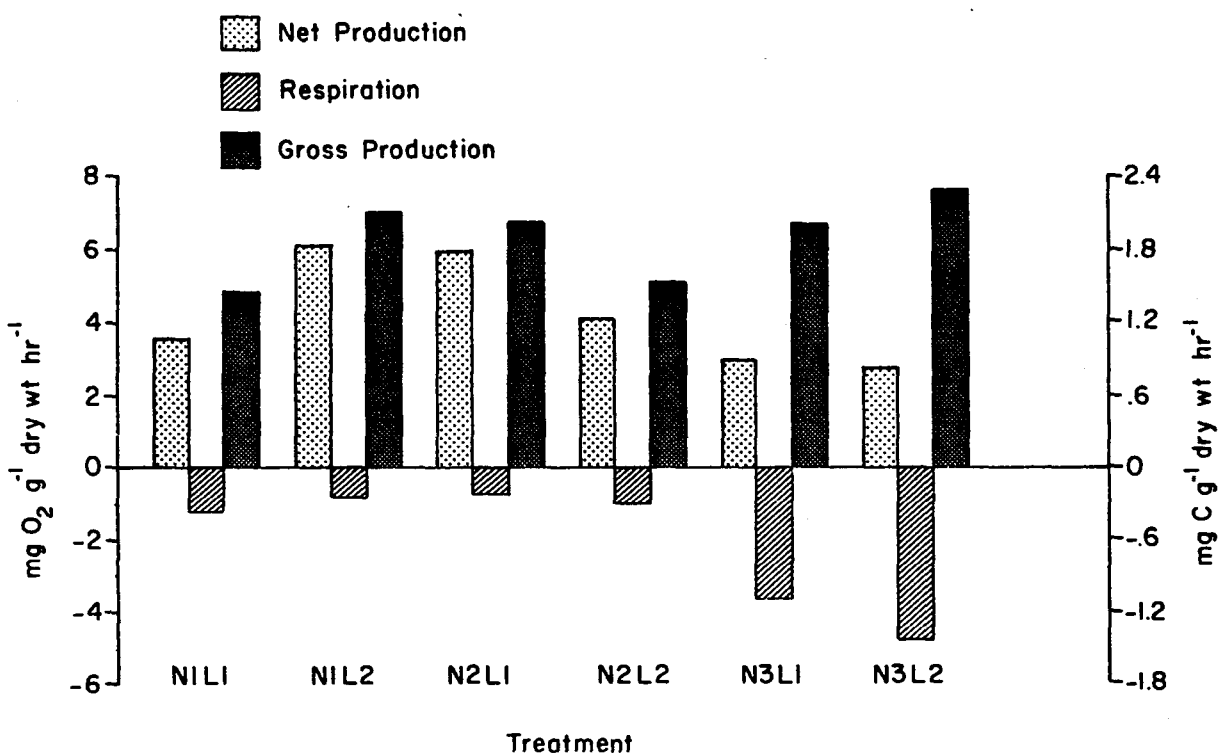

Net Production
Respiration
Gross Production
8
6
4
2
0
-2
-4
-6
mg O_2 g^{-1} dry wt hr^{-1}
2.4
1.8
1.2
.6
0
-.6
-1.2
-1.8
mg C g^{-1} dry wt hr^{-1}
N1L1
N1L2
N2L1
N2L2
N3L1
N3L2
Treatment

Figure 2.10. Two week epiphyte shading among the six treatments in the nutrient-light experiment (n = 2). Error bars are 1 standard error.

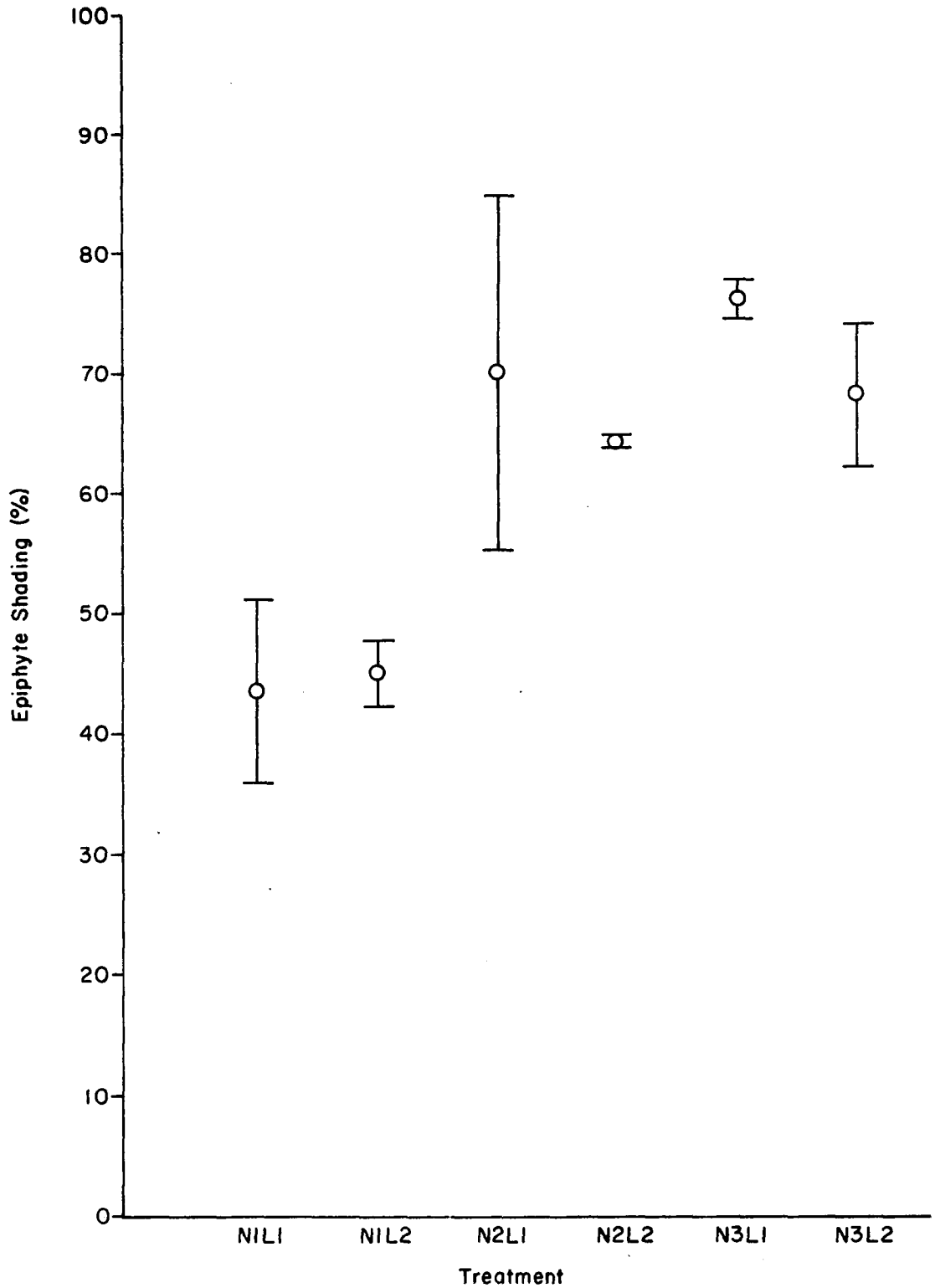

100
90
80
70
60
50
40
30
20
10
0
Epiphyte Shading (%)
N1L1
N1L2
N2L1
N2L2
N3L1
N3L2
Treatment

Table 2.8. Effect of nutrients and light on Z. marina adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 1 week (n=3).

Variable	N1		N2		N3	
	L1	L2	L1	L2	L1	L2
ATP	$253^{a1} \pm 11^2$	$223^{b} \pm 4$	$370^{c} \pm 4$	$306^{d} \pm 7$	$290^{d} \pm 4$	$282^{d} \pm 6$
ADP	$53^{a} \pm 4$	$69^{a} \pm 4$	$63^{a} \pm 4$	$71^{a} \pm 4$	$61^{a} \pm 9$	$51^{a} \pm 4$
AMP	$36^{ab} \pm 2$	$76^{c} \pm 2$	$55^{bc} \pm 6$	$54^{bc} \pm 4$	$37^{ab} \pm 11$	$26^{a} \pm 5$
AT	$342^{a} \pm 12$	$369^{ab} \pm 6$	$488^{c} \pm 11$	$431^{d} \pm 5$	$388^{b} \pm 7$	$359^{ab} \pm 5$
EC	$.82^{ab} \pm .01$	$.70^{c} \pm .01$	$.82^{ab} \pm .01$	$.79^{a} \pm .01$	$.83^{ab} \pm .01$	$.86^{b} \pm .01$

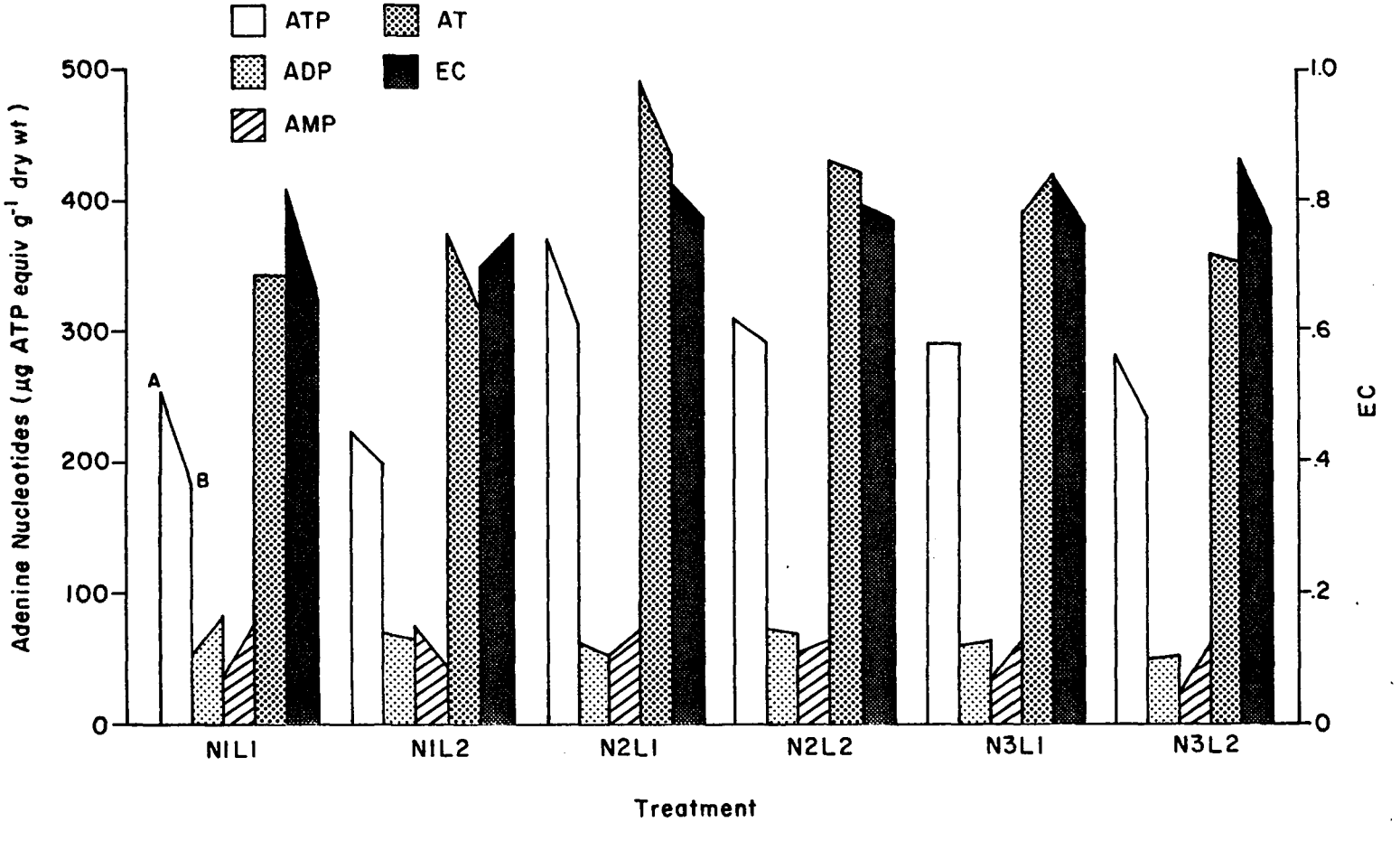
[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 2.9. Effect of nutrients and light on Z. marina adenine nucleotides (µg ATP equiv g^{-1} dry wt) and EC after 2 weeks (n=3).

Variable	N1		N2		N3	
	L1	L2	L1	L2	L1	L2
ATP	$179^{a1} \pm 2^2$	$199^{b} \pm <1$	$303^{c} \pm 5$	$291^{c} \pm 3$	$291^{c} \pm 8$	$235^{d} \pm 6$
ADP	$84^{a} \pm 1$	$67^{b} \pm 4$	$54^{b} \pm 7$	$68^{b} \pm 4$	$64^{b} \pm 2$	$54^{b} \pm 3$
AMP	$80^{a} \pm 5$	$46^{b} \pm 3$	$72^{a} \pm 3$	$63^{ab} \pm 2$	$68^{a} \pm 9$	$64^{ab} \pm 3$
AT	$342^{a} \pm 7$	$312^{b} \pm 2$	$430^{c} \pm 2$	$422^{c} \pm 4$	$423^{c} \pm 4$	$352^{a} \pm 6$
EC	$.65^{a} \pm .01$	$.75^{b} \pm <.01$	$.77^{b} \pm .01$	$.77^{b} \pm .01$	$.76^{b} \pm .02$	$.74^{b} \pm <.01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Figure 2.11. *Z. marina* adenine nucleotides and EC, among the six treatments after 1 (A) and 2 (B) week periods, in the nutrient-light experiment (n = 3).


ATP
ADP
AMP
AT
EC
500
400
300
200
100
0
1.0
.8
.6
.4
.2
0
A
B
Adenine Nucleotides (µg ATP equiv g⁻¹ dry wt)
EC
N1L1
N1L2
N2L1
N2L2
N3L1
N3L2
Treatment

Epiphyte Adenine Nucleotides

Adenylate and EC values in epiphytes after 1 and 2 week exposure periods are presented in Tables 2.10 and 2.11, respectively. These same results are displayed in Figure 2.12. ATP, ADP, AMP, and AT were generally higher with increasing nutrient levels (at a given light level) after both 1 and 2 week periods. This is reflected in strong and positive correlation between epiphyte adenylates and nutrients (Table 2.7). Considering all treatments, temporal trends from Week 1 to Week 2 included the following: 1) ADP, AMP, and AT generally increased, and 2) EC consistently decreased.

Z. marina-Epiphyte Adenine Nucleotide Relationship

Correlations between corresponding adenylates and EC values in Z. marina and its epiphytes, for Weeks 1 and 2, are presented in Table 2.12. Comparatively strong correlation ($.10 < P < .20$) for Week 1 ATP indicates that relative ATP levels among the treatments were somewhat similar in Z. marina and its epiphytes. By week 2, this correlation had weakened. The overall lack of strong correlation indicates that treatment response patterns in Z. marina and epiphyte adenylates were not related.

Short-Term (6 Hr) Atrazine Experiments

Flow-Through System Data

Mean temperature and salinity are presented in Table 2.13. Similar and stable temperature and salinity prevailed over the course of the two experiments.

Table 2.10. Effect of nutrients and light on epiphyte adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 1 week (n=3).

Variable	N1		N2		N3	
	L1	L2	L1	L2	L1	L2
ATP	$48^{a1} \pm 1^2$	$56^{a} \pm 2$	$127^{b} \pm 5$	$197^{c} \pm 7$	$151^{d} \pm 4$	$125^{b} \pm 5$
ADP	$47^{a} \pm 1$	$42^{a} \pm 2$	$77^{b} \pm 3$	$91^{bc} \pm 8$	$94^{c} \pm 1$	$82^{bc} \pm 4$
AMP	$32^{a} \pm 2$	$30^{a} \pm <1$	$45^{b} \pm 1$	$62^{c} \pm 1$	$77^{d} \pm 3$	$46^{b} \pm <1$
AT	$127^{a} \pm 2$	$128^{a} \pm 4$	$249^{b} \pm 7$	$350^{c} \pm 14$	$322^{d} \pm 6$	$253^{b} \pm 9$
EC	$.56^{a} \pm .01$	$.60^{b} \pm <.01$	$.66^{c} \pm .01$	$.70^{d} \pm <.01$	$.61^{b} \pm <.01$	$.66^{c} \pm <.01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 2.11. Effect of nutrients and light on epiphyte adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 2 weeks (n=3).

Variable	N1		N2		N3	
	L1	L2	L1	L2	L1	L2
ATP	$74^{a1} \pm 1^2$	$35^{b} \pm 1$	$87^{c} \pm 6$	$77^{ac} \pm 1$	$141^{d} \pm 1$	$144^{d} \pm 5$
ADP	$88^{a} \pm 4$	$91^{a} \pm 2$	$97^{a} \pm 6$	$124^{b} \pm 5$	$133^{b} \pm 3$	$201^{c} \pm 10$
AMP	$76^{a} \pm 2$	$111^{b} \pm 3$	$80^{a} \pm 4$	$146^{c} \pm 3$	$124^{d} \pm 3$	$175^{e} \pm 5$
AT	$238^{a} \pm 6$	$236^{a} \pm 3$	$265^{a} \pm 16$	$346^{b} \pm 6$	$398^{c} \pm 1$	$520^{d} \pm 19$
EC	$.50^{a} \pm <.01$	$.34^{b} \pm .01$	$.51^{ac} \pm <.01$	$.40^{d} \pm .01$	$.52^{c} \pm .01$	$.47^{e} \pm <.01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Figure 2.12. Epiphyte adenine nucleotides and EC, among the six treatments after 1 (A) and 2 (B) week periods, in the nutrient-light experiment (n = 3).

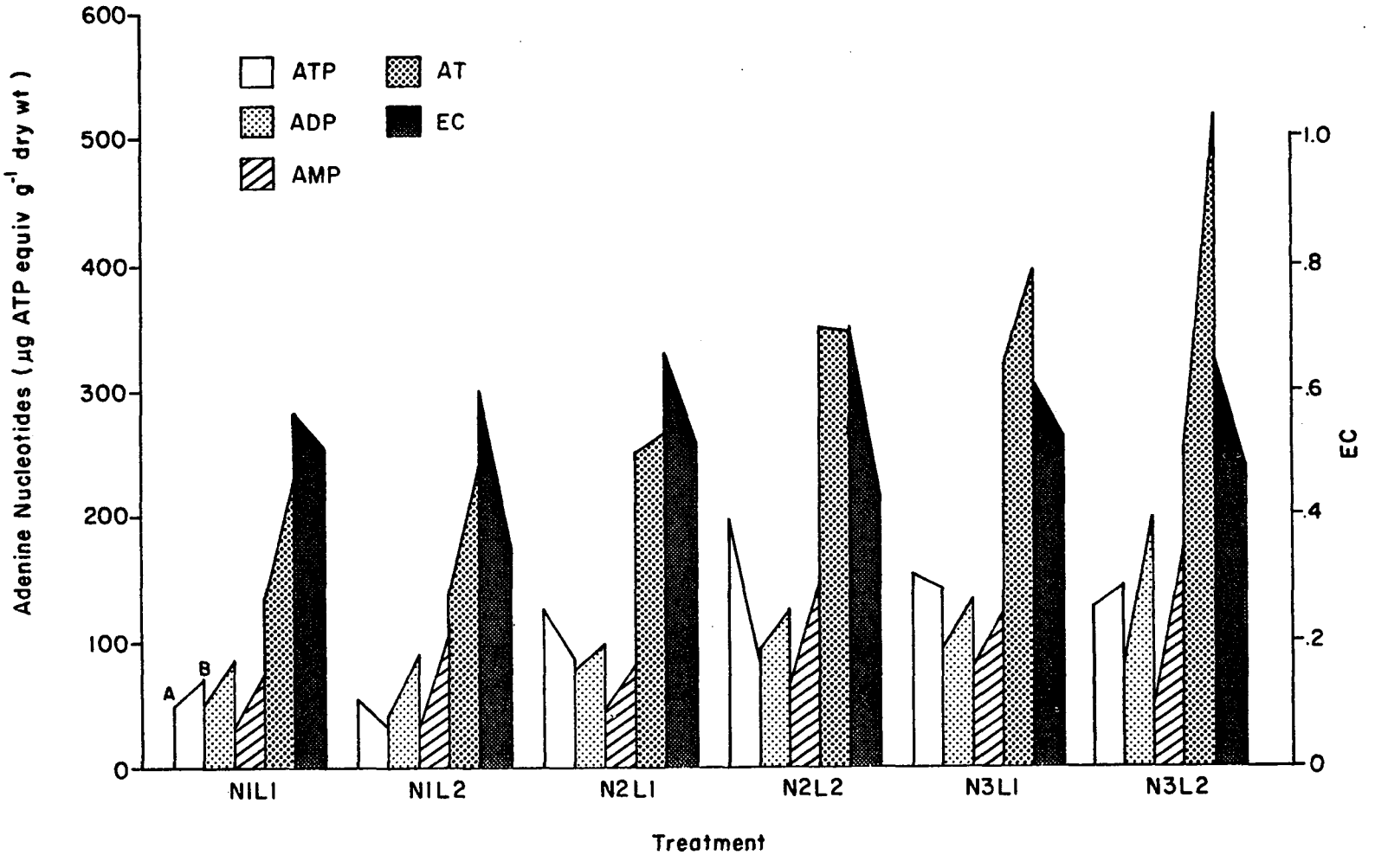

ATP
ADP
AMP
AT
EC
Adenine Nucleotides (µg ATP equiv g⁻¹ dry wt)
600
500
400
300
200
100
0
1.0
.8
.6
.4
.2
0
EC
A
B
N1L1
N1L2
N2L1
N2L2
N3L1
N3L2
Treatment

Table 2.12. Spearman correlation coefficients between Z. marina and epiphyte adenine nucleotides and EC, paired by treatment, in the nutrient-light experiment (n=6).

Variable	Wk 1	Wk 2
ATP	.7714	.4928
ADP	-.0286	-.6377
AMP	-.3714	-.6000
AT	.5429	.4857
EC	.1029	.1160

* P < .05

Table 2.13. Temperature and salinity during short-term (6 hour) atrazine experiments.

Nominal Atrazine (ppb)	Temperature (°C) (n=52)		Salinity (°/oo) (n=1)
	$\bar{x}$	SE	
10	20.5	.1	21.97
100	22.5	.2	22.56

Productivity

Hourly net production rates in both control and test tanks, along with surface PAR readings, are plotted in Figures 2.13 and 2.14 for 10 and 100 ppb atrazine experiments, respectively. At 10 ppb atrazine, net productivity was positive and relatively similar in both control and test tanks. At 100 ppb atrazine, net productivity was positive in the control but generally negative in the test tank. These results indicate that 10 ppb atrazine had little effect on net productivity over 6 hrs, whereas 100 ppb exerted a marked negative effect.

Adenine Nucleotides

Adenylate and EC values in both control and test tanks are shown in Figures 2.15 and 2.16 for 10 and 100 ppb atrazine experiments, respectively. Results at both 10 and 100 ppb were the same. EC values in control and test tanks show no significant difference, whereas ATP, ADP, AMP and AT in test tanks were all significantly lower than their controls at both 10 and 100 ppb atrazine over 6 hrs.

Long-Term (21 Day) Atrazine Experiments

Flow-Through System Data

Mean temperature and salinity, in four replicate experiments, are presented in Table 2.14. Mean minimum and maximum temperatures in Experiment 1 were considerably lower than corresponding temperatures in Experiments 2-4. Salinity was similar in all replicate experiments.

Morphometrics

Mean shoot length and number of leaves, obtained at the start of each experiment, appear in Table 2.15. Mean changes in shoot length

Figure 2.13. Surface PAR and net production rates during short-term (6 hour) 10 ppb atrazine experiment. Tank sealed at 1000 hrs.

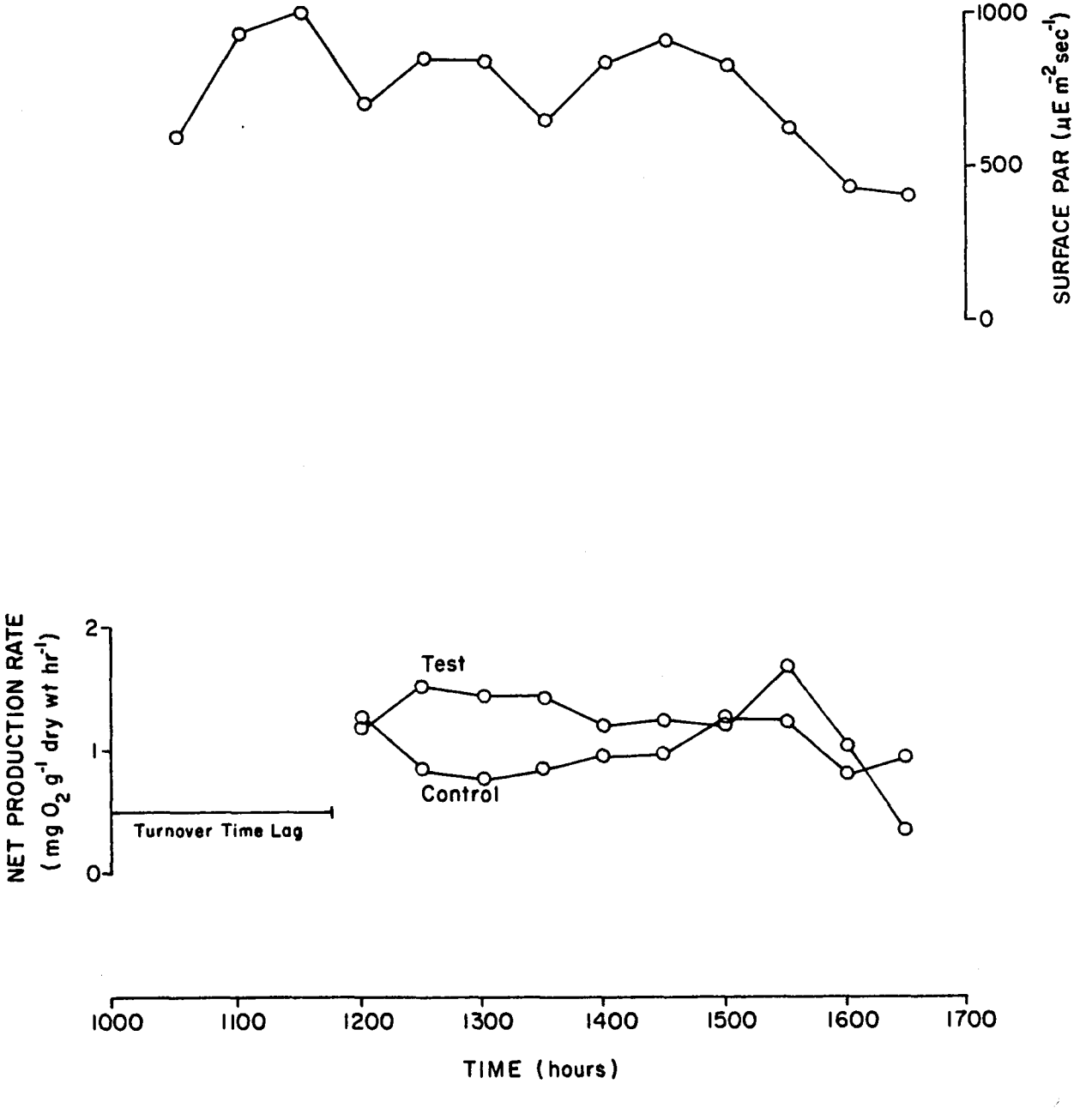

1000
500
0
SURFACE PAR (μE m-2 sec-1)
NET PRODUCTION RATE
(mg O2 g-1 dry wt hr-1)
2
1
0
Test
Control
Turnover Time Lag
1000
1100
1200
1300
1400
1500
1600
1700
TIME (hours)

Figure 2.14. Surface PAR and net production rates during short-term (6 hour) 100 ppb atrazine experiment. Tank sealed at 1030 hrs.

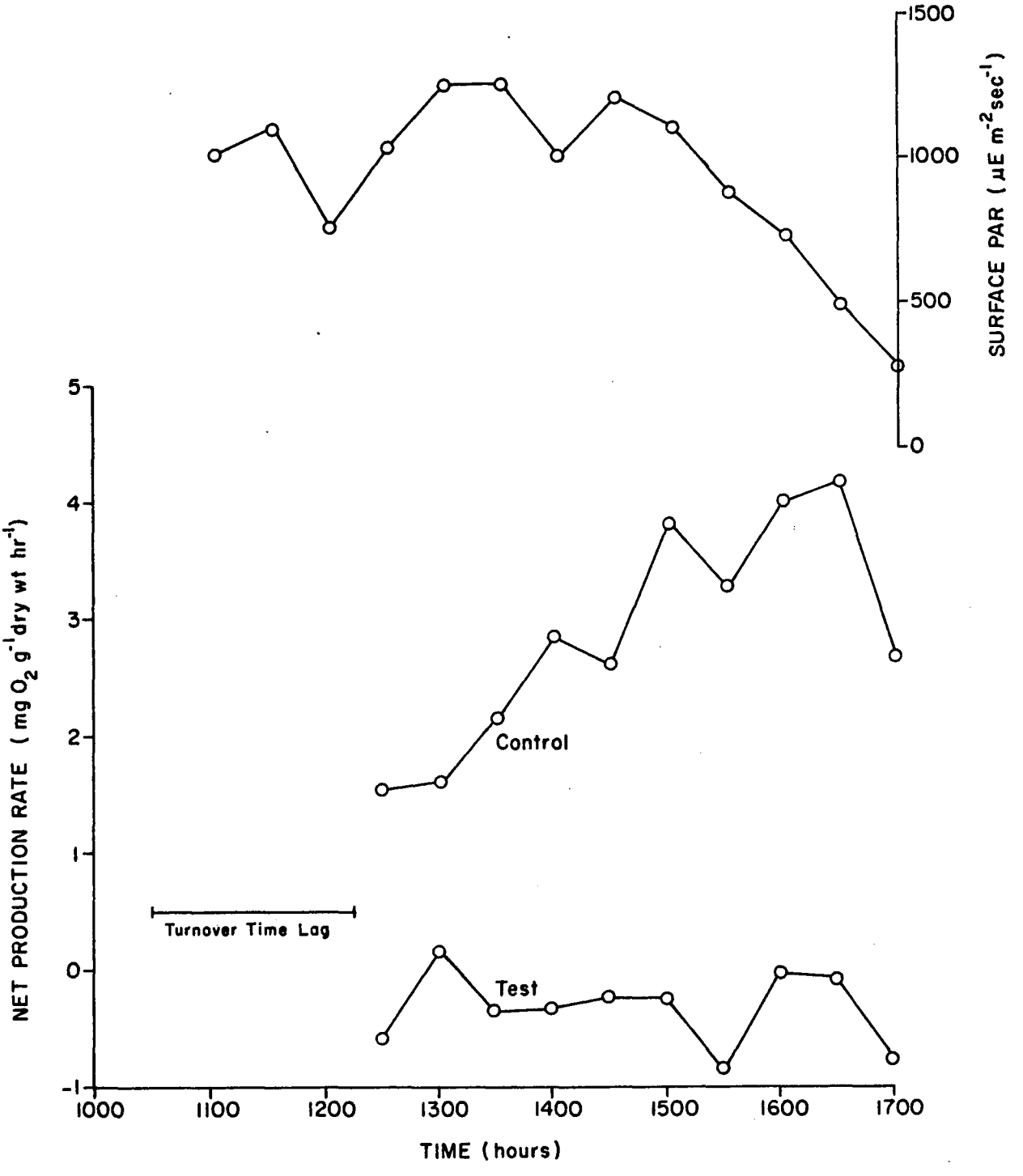

1500
1000
500
0
SURFACE PAR (μE m-2sec-1)
5
4
3
2
1
0
-1
NET PRODUCTION RATE (mg O2 g-1dry wt hr-1)
Control
Test
Turnover Time Lag
1000
1100
1200
1300
1400
1500
1600
1700
TIME (hours)

Figure 2.15. Adenine nucleotides and EC after 6 hours in the short-term 10 ppb atrazine experiment (n = 4). Control-test pairs with same letters do not differ significantly (P >.05). Error bars are 1 standard error.

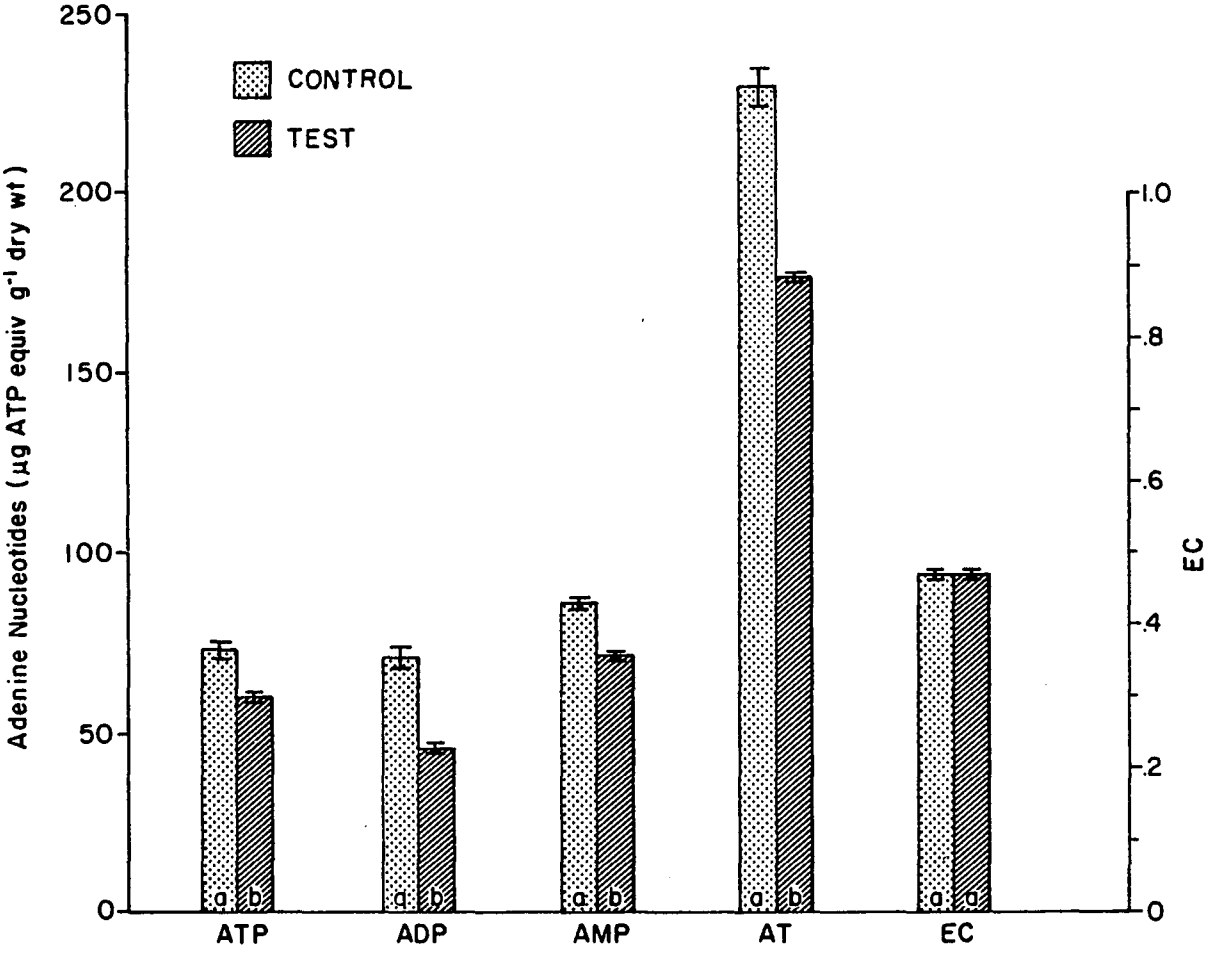

CONTROL
TEST
Adenine Nucleotides (µg ATP equiv g⁻¹ dry wt)
250
200
150
100
50
0
1.0
.8
.6
.4
.2
0
EC
a
b
a
b
a
b
a
b
a
a
ATP
ADP
AMP
AT
EC

Figure 2.16. Adenine nucleotides and EC after 6 hours in the short-term 100 ppb atrazine experiment (n = 4). Control-test pairs with same letters do not differ significantly ($P > .05$). Error bars are 1 standard error.

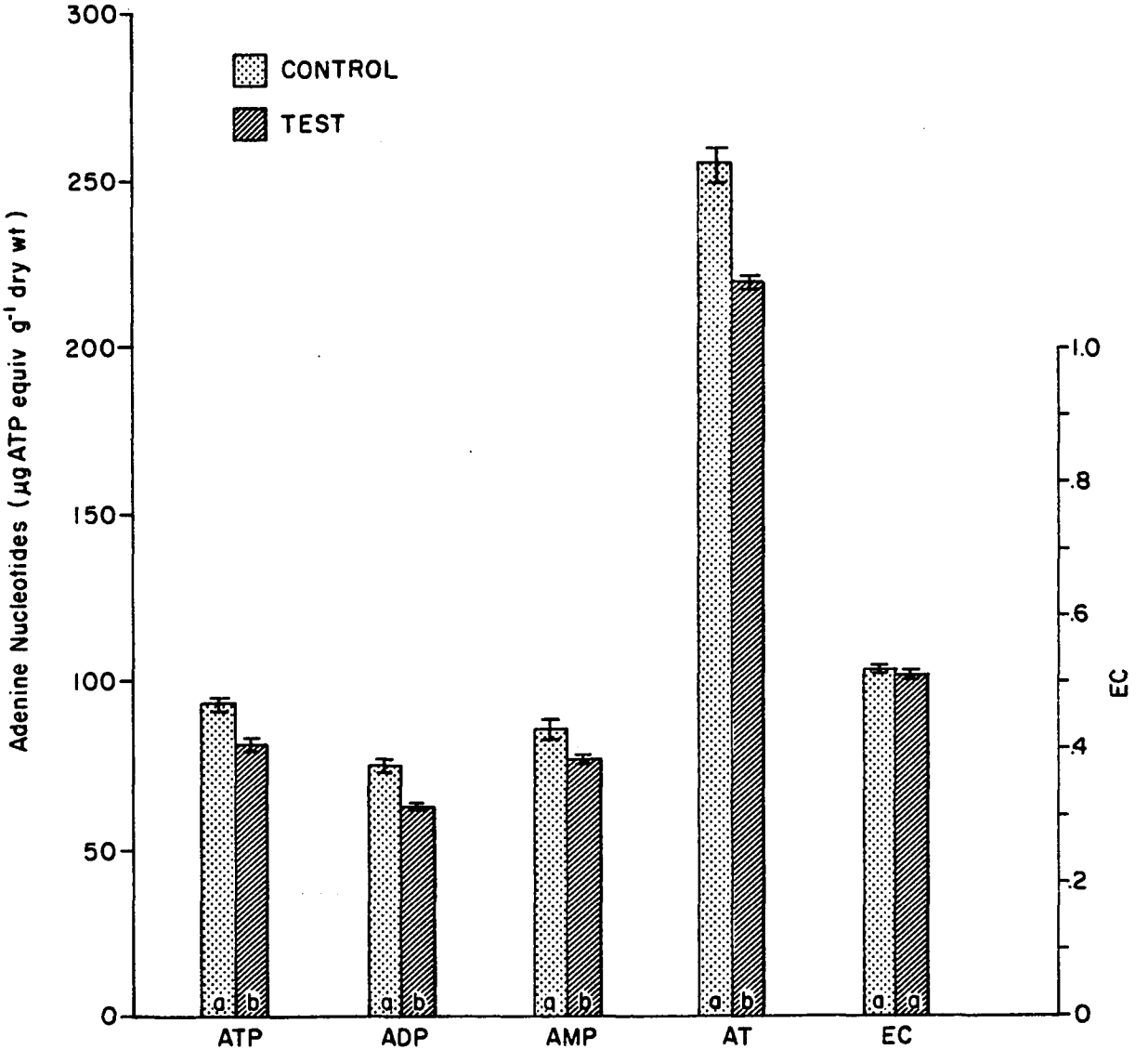

CONTROL
TEST
Adenine Nucleotides (µg ATP equiv g⁻¹ dry wt)
300
250
200
150
100
50
0
1.0
.8
.6
.4
.2
0
EC
a
b
a
b
a
b
a
b
a
a
ATP
ADP
AMP
AT
EC

Table 2.14. Temperature and salinity during long-term (21 day) atrazine experiments.

Experiment	Temperature (°C)					Salinity (°/oo) (n=1)
	n	Minimum		Maximum		
		$\bar{x}$	SE	$\bar{x}$	SE	
1	9	6.3	.5	16.0	.9	21.96
2	9	13.4	.9	25.3	.5	20.14
3	14	15.2	.6	24.6	.6	20.14
4	9	18.7	.7	28.4	1.3	19.13

Table 2.15. Initial Z. marina morphometrics in long-term (21 day) atrazine experiments.

Experiment	n	Shoot Length (cm)		Number Leaves	
		$\bar{x}$	SE	$\bar{x}$	SE
1	180	12.9	.3	3.4	.1
2	165	13.2	.3	4.2	.1
3	180	20.0	.4	4.5	.1
4	180	31.0	.8	5.3	.2

and leaf number at 7, 14, and 21 days, for each atrazine concentration, were pooled from replicate experiments and regressed against time (Figures 2.17 and 2.18, respectively). Statistics associated with these regressions are presented in Table 2.16. Negative slopes and correlation coefficients for shoot length change at 1000 ppb atrazine and for leaf number change at both 100 and 1000 ppb have clearly demonstrated a negative effect of atrazine on growth over 21 days.

Mortality

Twenty-one day mortality, expressed as percent dead, is presented in Table 2.17 for replicate Experiments 1-4. Mortality in controls was <7%, which is acceptable in acute bioassays (Sprague, 1973). Mortality was 100% at 1000 ppb atrazine over 21 days in all replicates, with the exception of Experiment 1.

Results, derived from log-probit analysis of 21 day mortality data, appear in Table 2.18. Estimates of mortality statistics in Experiments 3 and 4 were very similar. The relatively large slope function (S) in Experiment 1, due to incomplete mortality at 1000 ppb, was significantly higher than that obtained in either Experiment 3 or 4 and was reflected in the wide confidence limits, associated with LC 1 and LC 50 values in Experiment 1. The LC 50 estimate for Experiment 2 was significantly lower than those obtained for other experiments, due to higher mortality at 100 ppb in Experiment 2. Overall, results conservatively estimate the 21 day LC 1 and LC 50 at 1 and 100 ppb atrazine, respectively.

Figure 2.17. Regressions of shoot length change vs. time for control and five atrazine concentrations in the long-term (21 day) atrazine experiments. Data from replicate experiments are pooled.

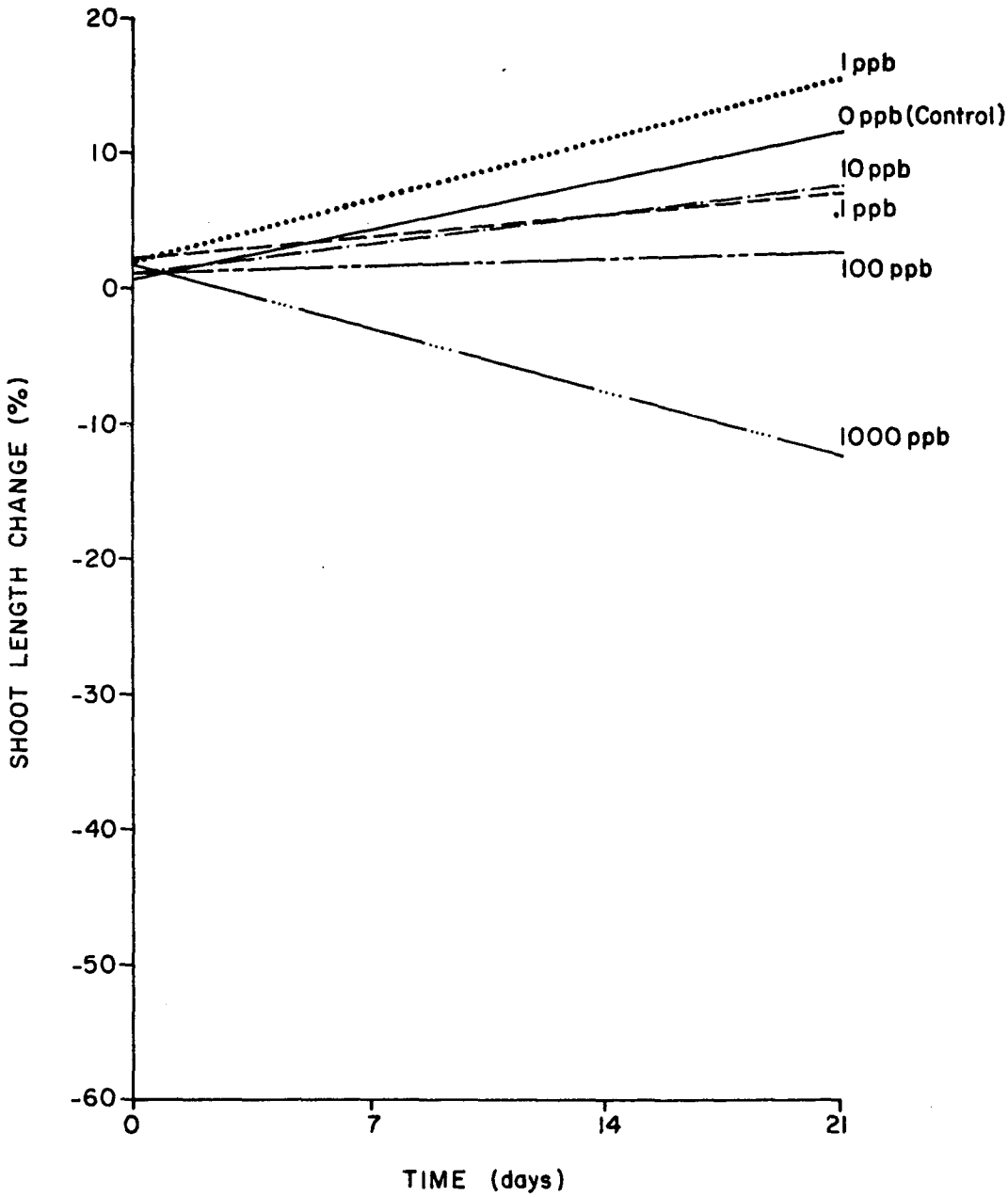

20
10
0
-10
-20
-30
-40
-50
-60
SHOOT LENGTH CHANGE (%)
0
7
14
21
TIME (days)
1 ppb
0 ppb (Control)
10 ppb
.1 ppb
100 ppb
1000 ppb

Figure 2.18. Regressions of leaf number change vs. time for control and five atrazine concentrations in the long-term (21 day) atrazine experiments. Data from replicate experiments are pooled.

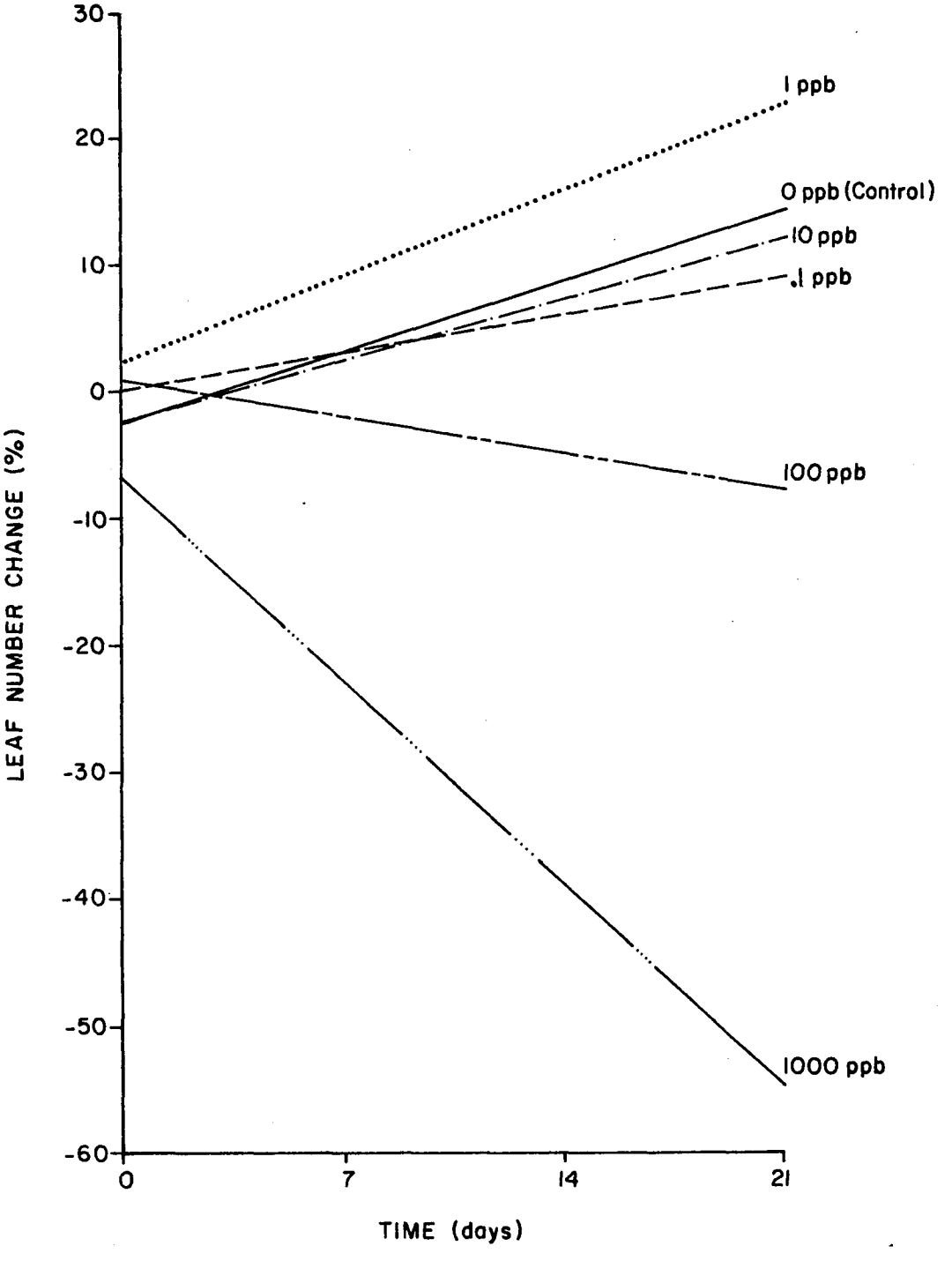

30
20
10
0
-10
-20
-30
-40
-50
-60
LEAF NUMBER CHANGE (%)
0
7
14
21
TIME (days)
1 ppb
0 ppb (Control)
10 ppb
.1 ppb
100 ppb
1000 ppb

Table 2.16. Shoot length and leaf number change regression statistics in the long-term (21 day) atrazine experiments. Data from replicate experiments are pooled.

Nominal Atrazine (ppb)	n	Shoot Length			Number Leaves		
		Slope	Intercept	Correlation Coefficient	Slope	Intercept	Correlation Coefficient
0	16	.5354	.5600	.7100*	.8250	-2.7750	.4017
.1	16	.2318	2.1600	.2403	.4325	.0900	.4142
1	16	.6396	2.0900	.6181*	.9757	2.4675	.5574*
10	16	.3121	1.1225	.3947	.6954	-2.2450	.4886
100	16	.0718	1.2775	.1385	-.4161	.9500	-.2673
1000	11	-.6671	1.7700	-.4811	-2.2671	-7.1119	-.6047*

* $P < .05$

Table 2.17. Mortality (%) after 21 days in the long-term atrazine experiments (n_o=30).

Experiment	Nominal Atrazine (ppb)					
	0	.1	1.0	10	100	1000
1	6.7	13.3	10.0	10.0	20.0	50.0
2	3.3	0[1]	10.0	10.0	46.7	100.0
3	3.3	0	0	0	3.3	100.0
4	6.7	3.3	3.3	0	10.0	100.0

[1] n_o = 15

Table 2.18. Twenty-one day 1% (LC 1) and 50% (LC 50) lethal concentration, along with slope function (S), in the long-term atrazine experiments. LC 1, LC 50, and associated confidence limits (CL) are expressed as ppb atrazine. S and associated CL are unitless.

Experiment	LC 1	LC 1 95% CL	LC 50	LC 50 95% CL	S[1]	S 95% CL
1	1.9[ab2]	.1-35.0	540[a]	229-1274	11.02[a]	3.37-36.03
2	2.6[b]	.4-16.4	100[b]	45-221	4.78[ab]	2.35-9.70
3	38.7[a]	16.5-90.9	365[a]	220-606	2.74[b]	2.04-3.67
4	35.5[a]	16.8-74.9	367[a]	221-609	2.71[b]	2.02-3.63

[1] Slope function = .5(LC 84/LC 50 + LC 50/LC 16)
[2] Values with same letter superscripts (between experiments) do not differ significantly ($P > .05$).

Adenine Nucleotides

Adenylate and EC values, in replicate Experiments 1-4, are presented in Tables 2.19-2.22, respectively. These data were pooled, and mean values are displayed in Figure 2.19. In this figure, each experiment was weighted equally and adenylates at 1000 ppb atrazine were excluded, since data at this concentration were obtained in Experiment 1 only.

In this pooled analysis, EC was reduced at 0.1, 1.0, and 10 ppb atrazine over 21 days, but higher ATP at 100 ppb elevated EC to the control level. ADP and AT generally increased with higher atrazine levels. These observations were reflected in relatively strong and positive correlation of ATP, ADP, and AT with atrazine (Table 2.23).

Table 2.19. Adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 21 day atrazine exposure in Experiment 1 (n=6).

Variable	Nominal Atrazine (ppb)					
	Control	.1	1.0	10	100	1000
ATP	$135^{a1} \pm 5^{2}$	$92^{b} \pm 9$	$160^{c} \pm 2$	$170^{d} \pm 1$	$220^{e} \pm 6$	$219^{e} \pm 5$
ADP	$56^{a} \pm 4$	$64^{a} \pm 4$	$106^{bc} \pm 3$	$119^{b} \pm 5$	$94^{c} \pm 3$	$106^{bc} \pm 5$
AMP	$86^{a} \pm 3$	$106^{b} \pm 3$	$188^{c} \pm 4$	$178^{c} \pm 7$	$150^{c} \pm 10$	$161^{c} \pm 23$
AT	$277^{a} \pm 11$	$262^{a} \pm 15$	$454^{b} \pm 6$	$467^{b} \pm 8$	$465^{b} \pm 20$	$487^{b} \pm 26$
EC	$.59^{a} \pm <.01$	$.47^{b} \pm .01$	$.47^{b} \pm .01$	$.49^{b} \pm .01$	$.58^{a} \pm .01$	$.57^{a} \pm .03$

1 Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
2 Standard error.

Table 2.20. Adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 21 day atrazine exposure in Experiment 2 (n=4).

Variable	Nominal Atrazine (ppb)				
	Control	.1	1.0	10	100
ATP	$141^{a1} \pm 6^2$	$90^{b} \pm <1$	$101^{c} \pm 5$	$78^{b} \pm 8$	$166^{d} \pm 5$
ADP	$83^{a} \pm 5$	$74^{a} \pm 3$	$75^{a} \pm 6$	$68^{a} \pm 5$	$108^{b} \pm 10$
AMP	$90^{a} \pm 3$	$131^{b} \pm <1$	$105^{a} \pm 4$	$89^{a} \pm 9$	$76^{a} \pm 8$
AT	$314^{a} \pm 6$	$294^{ab} \pm 4$	$280^{b} \pm 10$	$235^{c} \pm 9$	$350^{d} \pm 7$
EC	$.58^{a} \pm .01$	$.43^{b} \pm <.01$	$.50^{b} \pm .01$	$.48^{b} \pm .03$	$.63^{a} \pm .01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Table 2.21. Adenine nucleotides (µg ATP equiv g^{-1} dry wt) and EC after 21 day atrazine exposure in Experiment 3 (n=6).

Variable	Nominal Atrazine (ppb)				
	Control	.1	1.0	10	100
ATP	$98^{a1} \pm 2^2$	$111^{ab} \pm 4$	$106^{ab} \pm 3$	$110^{ab} \pm 2$	$121^{b} \pm 8$
ADP	$45^{a} \pm 3$	$43^{a} \pm 2$	$52^{a} \pm 5$	$49^{a} \pm 1$	$63^{b} \pm 3$
AMP	$33^{a} \pm 1$	$34^{a} \pm 1$	$42^{ab} \pm 5$	$45^{b} \pm 2$	$47^{b} \pm 4$
AT	$175^{a} \pm 3$	$187^{a} \pm 7$	$199^{b} \pm 12$	$204^{b} \pm 3$	$231^{c} \pm 11$
EC	$.69^{a} \pm .01$	$.71^{b} \pm <.01$	$.66^{a} \pm .02$	$.66^{a} \pm .01$	$.66^{a} \pm .01$

1 Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
2 Standard error.

Table 2.22. Adenine nucleotides (μg ATP equiv g^{-1} dry wt) and EC after 21 day atrazine exposure in Experiment 4 (n=6).

Variable	Nominal Atrazine (ppb)				
	Control	.1	1.0	10	100
ATP	$164^{a1} \pm 4^2$	$164^{a} \pm 10$	$157^{a} \pm 4$	$169^{a} \pm 5$	$202^{b} \pm 2$
ADP	$60^{a} \pm 4$	$78^{b} \pm 4$	$76^{b} \pm 3$	$87^{bc} \pm 2$	$93^{c} \pm 3$
AMP	$26^{a} \pm 3$	$40^{bc} \pm 3$	$51^{d} \pm 4$	$48^{cd} \pm 2$	$37^{b} \pm 4$
AT	$250^{a} \pm 7$	$282^{b} \pm 12$	$284^{b} \pm 3$	$304^{b} \pm 7$	$332^{c} \pm 6$
EC	$.78^{a} \pm .01$	$.72^{b} \pm .01$	$.69^{b} \pm .01$	$.70^{b} \pm <.01$	$.75^{c} \pm .01$

[1] Values with same letter superscripts (between treatments) do not differ significantly ($P > .05$).
[2] Standard error.

Figure 2.19. Adenine nucleotides and EC after 21 days in the long-term atrazine experiments. Data from replicate experiments are pooled (n = 22).

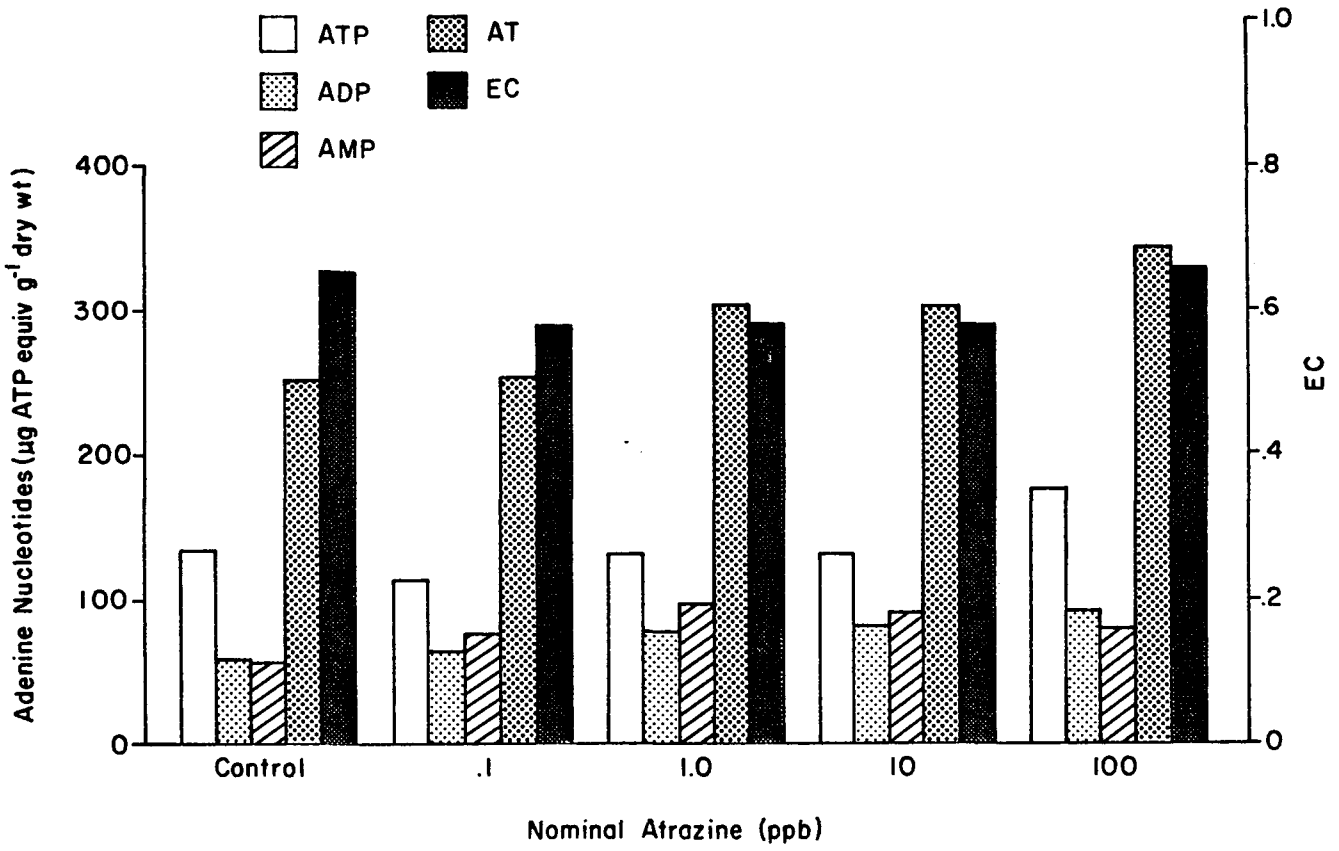

ATP
ADP
AMP
AT
EC
Adenine Nucleotides (µg ATP equiv g⁻¹ dry wt)
400
300
200
100
0
1.0
.8
.6
.4
.2
0
EC
Control
.1
1.0
10
100
Nominal Atrazine (ppb)

Table 2.23. Spearman correlation coefficients of 21 day adenine nucleotides and EC with nominal atrazine concentration in long-term atrazine experiments. Means from replicate experiments are pooled (n=20).

Variable	Correlation Coefficient
ATP	.3956
ADP	.4844*
AMP	.1901
AT	.3679
EC	-.0400

* $P < .05$

DISCUSSION

Nutrient-Light Experiment

Nutrient and Light Levels

It is unlikely that natural eelgrass beds in the Chesapeake Bay encounter nutrient levels as high as the enriched levels used in this study. Since the main stem of the Chesapeake Bay has a high flushing rate and good vertical mixing, it is relatively resilient to fertilizer and sewage loadings (Stevenson and Confer, 1978). Nevertheless, selected nutrient levels function as a diagnostic agent to elicit a measurable response in Z. marina and its epiphytes.

Natural eelgrass beds in the Chesapeake Bay receive light levels comparable to those used in this study. Drew (1979) has estimated the compensation light intensity (I_c) and the saturation light intensity (I_k) as 0.6 and 5.0 mW cm^{-2}, respectively, for Z. marina at 15°C. Employing 52 kcal as the average amount of energy per einstein over PAR wavelengths, I_c and I_k are 28 and 230 $\mu E\ m^{-2}\ sec^{-1}$, respectively, at 15°C. With temperatures of 25-30°C during the present study, Z. marina continues to light-saturate between 200-300 $\mu E\ m^{-2}\ sec^{-1}$ (Wetzel, pers. comm.).

Temperature and Dissolved Oxygen

Although Setchell (1929) proposed that above 20°C Z. marina undergoes heat rigor and growth ceases, Orth et al. (1981) have

observed pronounced vegetative growth in Chesapeake Bay eelgrass seedlings with nutrient enrichment at mean temperatures ranging from 24-27°C. Apparently, Z. marina in the Chesapeake Bay region is relatively eurythermal. Furthermore, Biebl and McRoy (1971) have reported that gross photosynthesis in Z. marina increases up to 30-35°C. Thus, temperatures of 25-30°C during the present study should support growth in Z. marina.

Divergence of DO concentrations, among treatments over morning hours, reflects differential rates of community photosynthesis. Higher nutrient and light levels apparently increase community photosynthesis. Since DO concentrations were relatively high, community photorespiration (Tolbert, 1980) may have reduced photosynthetic efficiency (Warburg effect). Positive correlation among PAR, temperature, and DO was expected. Increased PAR raises water temperature and elevates DO, presumably through stimulation of community photosynthetic activity.

Morphometrics

Increased growth of Z. marina with nutrient enrichment has been observed in field (Orth, 1977; Harlin and Thorne-Miller, 1981) and laboratory studies (Orth et al., 1981), conducted over 2-3 months. Decreased shoot density (Backman and Barilotti, 1976) and shoot length (Burkholder and Doheny, 1968) in Z. marina have been reported with light reduction in field studies. Epiphytic growth, in the present study, may have obscured these effects of nutrient enrichment and light reduction. Two week changes in fresh weight and shoot length were negative in all treatments.

Productivity

Higher respiration rates, a result of nutrient enrichment over the preceeding 2 weeks, decreased net productivity. Increased respiration may reflect residual stress in response to increased epiphytic colonization, associated with higher nutrient levels. During afternoon hours, the higher light level predominantly exceeded saturation for Z. marina, while the lower light level was predominantly subsaturating. Decreased net production is, therefore, expected with the lower light level (at a given nutrient level). This was observed only at the intermediate nutrient level.

Internal cycling of gases within lacunar spaces of leaves may have introduced error into production measurements, which were based on changes in dissolved oxygen. Nevertheless, observed net production rates fell within the range reported for Z. marina in the literature (McRoy and McMillan, 1977).

Epiphyte Colonization

Epiphytic growth has generally increased with nutrient loading (Phillips et al., 1978; Sand-Jensen and Sondergaard, 1981). In a field study, Harlin and Thorne-Miller (1981) observed only small increases in epiphytic algae with nutrient enrichment. Dense epiphyte colonization, in the present study, may have been attributed to higher levels of nutrient supplementation and effects of tank containment.

In general, algae are extremely efficient at exploiting available nutrients. Recirculating pumps in experimental tanks generate currents, which decrease the boundary layer around leaves (Wheeler,

1980; Conover, 1968), facilitating nutrient uptake in epiphytes via the water column (Harlin, 1975). It has also been demonstrated that epiphytes obtain nutrients directly from host macrophytes (McRoy and Goering, 1974; Harlin, 1973). Finally, it should be noted that the use of plexiglass plates to quantify epiphytic growth excludes the influence of leaf surface chemistry, including exudation of antifouling metabolites (Harrison and Chan, 1980; Harrison, 1982).

Adenine Nucleotides

Positive but weak correlations of Z. marina ATP, AT, and EC with nutrients and light, along with decreased ATP, AT, and EC levels over time, are in line with observed Z. marina morphometric and production data. Weiler and Karl (1979) have suggested that a net decrease in ATP and AT may represent either of the following: 1) uniform ATP production, but net removal (i.e. utilization > de novo biosynthesis), or 2) an imbalance in cellular energetics (i.e. ATP hydrolysis > ADP phosphorylation). Adenylate response patterns, observed in Z. marina, may have resulted from epiphyte intervention.

Epiphyte adenylates were correlated strongly and positively with nutrients, and AT increased over time. These observations parallel positive correlation between epiphyte growth and nutrients. Overall lack of correlation between corresponding adenylates in Z. marina and epiphytes is compatible with their generally negative and positive growth, respectively, over the course of the experiment. Low epiphyte ATP, AT, and EC, relative to Z. marina leaf tissue levels, may have been attributed to the presence of metabolically inert material in

epiphyte preparations, including small amounts of sediment, as well as siliceous diatom frustules.

Impact of Epiphytes on Z. marina

Morphometric, production, and adenylate data suggest that epiphytes negatively impact Z. marina. Epiphyte growth increased with nutrient enrichment, whereas Z. marina leaf growth and net productivity decreased with higher nutrients. Furthermore, two week morphometric changes in Z. marina were generally negative. Finally, epiphyte AT increased, while Z. marina AT decreased over time. The size of the AT pool may reflect metabolic activity and associated growth potential. Mechanisms by which epiphytes can reduce the photosynthetic rate of eelgrass leaves include shading and interfering with carbon uptake (Sand-Jensen, 1977).

Short-Term (6 Hr) Atrazine Experiments

Productivity

Z. marina net productivity was inhibited at 100, but not 10 ppb atrazine, over 6 hrs. Net productivity of the Z. marina community, isolated under large plexiglass domes in the field, was similarly depressed at 100 ppb atrazine during daylight hrs (Hershner et al., 1981). Using laboratory microcosms, Correll et al. (1978) have reported a reduction of net productivity with 100 ppb atrazine in another submerged aquatic macrophyte, Zanichellia palustris (horned pondweed), after 1 and 2 week exposures.

Depression of oxygen evolution is expected, since atrazine inhibits the Hill reaction in photosynthesis (Ebert and Dumford,

1976). Although internal cycling of gases within lacunar spaces of leaves may have introduced error into production measurements, based on changes in dissolved oxygen (McRoy and McMillan, 1977), both control and test measurements should have contained the same error.

Adenine Nucleotides

Adenylate levels in Z. marina decreased at both 10 and 100 ppb atrazine over 6 hrs. Since ATP, ADP, and AMP were reduced proportionately, EC ratios remained constant. Apparently, EC was stabilized by removal of AMP with adenylate deaminase (Chapman and Atkinson, 1973). It appears that ATP or AT serves as a more sensitive index of short-term herbicide stress than EC in Z. marina.

Z. marina adenylates, but not net productivity, were reduced at 10 ppb atrazine. This indicates that adenylate determinations were a more sensitive monitor of short-term herbicide stress than net productivity measurements. Noncyclic photophosphorylation may have been impaired with lower amounts of atrazine than photosynthetic oxygen evolution.

Long-Term (21 Day) Atrazine Experiments

Morphometrics

Growth of Z. marina, as measured by shoot length and number of leaves, was clearly inhibited at 100 ppb atrazine over 21 days. It appears that 10 ppb was also inhibitory, but to a lesser extent. Apparent stimulation of Z. marina growth at 1 ppb may have been an indirect result of selective atrazine toxicity toward epiphytic algae, since epiphytes can inhibit Z. marina photosynthesis by interfering

with carbon uptake and by reducing light intensity (Sand-Jensen, 1977). Other studies have demonstrated inhibitory effects of atrazine on algal photosynthesis (Plumley and Davis, 1980) and growth (Veber et al., 1981), although at higher concentrations.

Hershner et al. (1981) have reported 21 day EC 50 values (equivalent to I_{50}, the concentration inhibiting growth 50%) of 410 and 60 ppb atrazine for shoot length and number of leaves, respectively, with Z. marina in laboratory bioassays. Forney and Davis (1981) have calculated 3-6 week I_{50} values of 80-1104 ppb atrazine, based on leaf length measurements with other submerged aquatic macrophyte species under various laboratory conditions. These results are in general agreement with those of the present study.

Mortality

Conservative estimates of the 21 day LC 1 and LC 50 (concentrations lethal to 1 and 50% of the test organisms, respectiively) for Z. marina are 1 and 100 ppb atrazine, respectively. Although a mortality of 1% might not be detectable, an LC 1 serves as a least effect level. A similar 21 day LC 50 value of 72 ppb atrazine for Z. marina has been reported by Hershner et al. (1981). Forney and Davis (1981) have calculated LC 1 and LC 50 values of 11 and 53 ppb atrazine, respectively, for Potamogeton perfoliatus (redheadgrass pondweed). I_{50} values (80-1104 ppb atrazine) for several aquatic macrophyte species were eventually lethal (Forney and Davis, 1981). A comparison of growth inhibitory and lethal concentrations of atrazine, in the present study, supports this observation.

Tolerance of plants toward triazine herbicides may be influenced by temperature (Ebert and Dumford, 1976). Incomplete mortality at 1000 ppb atrazine, over 21 days in replicate Experiment 1, may have been related to cooler prevailing temperatures. Atrazine toxicity appears to increase with warmer temperature, perhaps due to accelerated rates of uptake and translocation.

Adenine Nucleotides

Inability to remove AMP from the adenylate pool contributed to a reduction in Z. marina EC at 0.1, 1.0, and 10 ppb atrazine over 21 days. At 100 ppb, corresponding to the estimated LC 50, ATP and EC unexpectedly rebounded before plant death resulted. Apparently, severe stress (100 ppb) elicits an adaptation response. For example, increased rates of respiration and associated oxidative phosphorylation may have supplied ATP in sufficient amounts to maintain metabolic homeostasis. Continued stress at 100 ppb atrazine, however, became lethal.

ATP and AT response patterns at 100 ppb atrazine appear to follow the triphasic general adaptation syndrome, outlined by Selye (1976). Over the short-term (6 hrs), ATP and AT were reduced (alarm reaction). Over the long-term (21 days), ATP and AT increased beyond control levels (stage of resistance) until death resulted (stage of exhaustion). Giesy et al. (1981) have reported a similar response pattern for ATP, AT, as well as EC, in Palaeomonetes paludosis (glass shrimp) with 30 $\mu g\ l^{-1}$ cadmium exposure.

Morphometric and mortality data facilitate interpretation of adenylate response. EC indicated stress as low as 0.1 ppb atrazine,

but failed to reflect visually apparent stress at 100 ppb. It appears, then, that EC is a sensitive monitor of long-term, sublethal herbicide stress. When Z. marina was confronted with more severe stress, however, physiological adaptation increased EC before death resulted. The utility of EC as an index of long-term herbicide stress in Z. marina may, therefore, be limited.

Conclusions

Adenylate and EC response in Z. marina to selected environmental variables are useful measures of metabolic state under certain conditions. The response is integrative, representing the interaction of genetic disposition with the environmental matrix, both stressful and beneficial. This may be advantageous in an ecological context, but can pose difficulties when attempting to evaluate effects of a single variable. Confoundment of nutrient and light effects on Z. marina adenylates, by epiphytic colonization, represents such a case. Adenylate and EC response may also change in accordance with physiological adaptation over time. Chronic and severe herbicide stress was observed to elicit this adaptive response in Z. marina.

ATP or AT response may be more appropriate than EC in certain cases, as a monitor of environmental stress. ATP and AT decreased in Z. marina with short-term herbicide stress, but EC remained constant. In contrast, EC was reduced with long-term, sublethal herbicide stress. Limitations of adenylate and EC utility must be recognized in order to allow sound interpretation of results. It is suggested that more conventional quantitative analyses accompany adenine nucleotide

measurements in any effort to evaluate physiological response to environmental variation.

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APPENDIX A

METABOLIC REGULATION

Comment

This appendix presents the biochemical framework underlying the use of the adenylate energy charge in environmental studies. The metabolic role of the adenine nucleotides is reviewed. Most of the information was summarized from Atkinson (1977a) and Lehninger (1971, 1975).

Metabolite Concentrations

Many cellular metabolites are held at low concentrations (10^{-6} to 10^{-9} M) in order to accommodate several thousand compounds and to minimize nonenzymic side reactions (Atkinson, 1977a). This permits integration and regulation by enzyme-controlled reactions. Low concentrations are realized with kinetic (enzymes) and thermodynamic (activation and coupling) adaptations.

Enzymes which accelerate corresponding nonenzymic reaction rates by a factor of 10^{10} to 10^{16} enable much lower metabolite concentrations to function effectively. Enzymes lower activation energy barriers and direct metabolic sequences along routes that avoid thermodynamically stable intermediates.

Activation of intermediates by cofactors (carriers) provides a thermodynamically favorable pathway, so that metabolic sequences can proceed smoothly to completion. To generate a useful concentration of product, by way of a metabolic sequence with a relatively small equilibrium constant (K), would require a much higher concentration of reactants than a sequence with a large overall K. By avoiding thermodynamically unfavorable pathways, activating groups hold down metabolite concentrations.

Activated intermediates are held by weak bonds with a large and negative free energy of hydrolysis (Lipmann, 1941). Considered as an activated phosphate and coupling agent, ATP serves to hold down metabolite concentrations. Phosphorylation of simple sugars by ATP provides an example. Free sugar levels are held down, since the equilibria of reactions catalyzed by hexokinase and phosphofructokinase lie far toward the sugar phosphates. An additional selective advantage to this phosphorylation is that sugar phosphates are not permeable to the cell membrane and are thus trapped in the cell, facilitating the glycolytic sequence.

Cellular substrate concentrations are generally near their enzymic K_m (hyperbolic curve) or $S_{.5}$ (sigmoidal curve) values with two notable exceptions: adenine and pyridine nucleotides far exceed their K_m or $S_{.5}$ values, so that corresponding enzymes (e.g. kinases, dehydrogenases) are substrate-saturated. In these cases, reaction velocity is determined, not by the concentration of one single nucleotide, but by nucleotide ratios (i.e. ATP/ADP or ATP/AMP, NAD/NADH or NADP/NADPH).

Undirectional Paired Pathways

Metabolic sequences are organized into paired, oppositely-directed pathways which are essentially unidirectional (large K) and largely independent. If an interconversion proceeded by the same reversible reaction sequence (K = 1) under simple thermodynamic (mass-action or equilibrium) control, response to metabolic need would be slow and inefficient. Clearly, the evolution of paired, unidirectional pathways is more functional to metabolic demand. Both direction and rates can be determined by the concentration of regulatory compounds that need not be directly involved.

Atkinson (1977a) provides an example of the most important paired pathway: respiration ($K = 10^{170}$) and photosynthesis ($K = 10^{60}$). These K's represent physiological concentrations of ATP, ADP, and P_i with other reactants and products at their standard states (1 M glucose, 1 atm partial pressure O_2 and CO_2) and pH 7. The magnitude of these constants ensures thermodynamic unidirectionality and facilitates conversion of very low substrate concentrations to product. The term, "ATP equivalent," has been introduced by Atkinson (1977a) to standardize energy conversions involving coupling agents, including ATP, NADH, NADPH, and $FADH_2$. Using this system, respiration regenerates 38 ATP equivalents, whereas photosynthesis consumes 66 ATP equivalents. Thermodynamically, these two major sequences differ by 104 (=38-[-66]) ATP equivalents, a situation which allows them to be simultaneously poised at all times in their opposite physiological directions. Oneway, oppositely-directed, paired pathways are thus attainable through differences in evolved coupling of ATP equivalents.

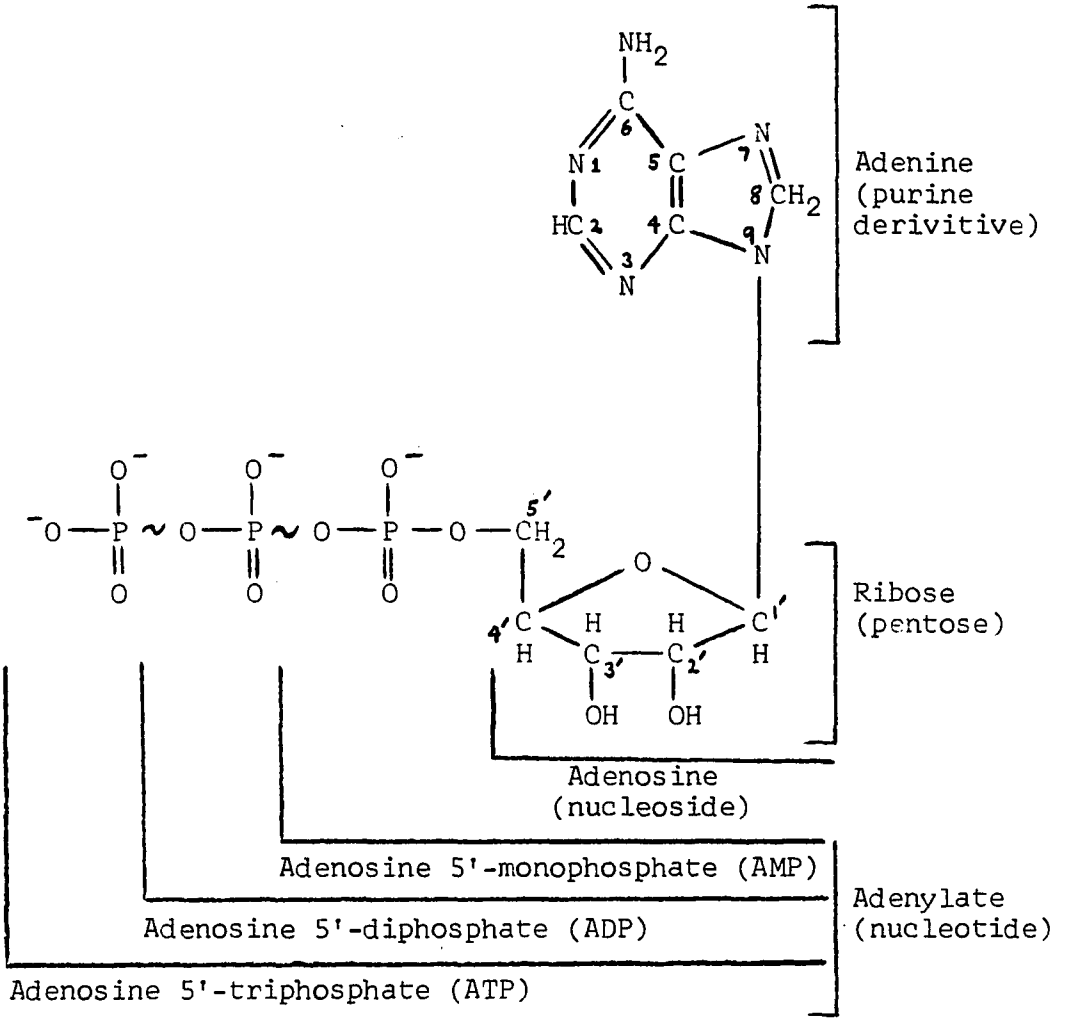
ATP/ADP Nonequilibrium

Living cells are open, steady-state systems, far from thermodynamic equilibrium. Cellular homeostasis reflects this dynamic steady-state, where average rates of component formation and degradation are equal but resultant concentrations are not in true thermodynamic equilibrium. Although the steady-state increases entropy (irreversible process), the rate of entropy production is minimized (Lehninger, 1975). Nonequilibrium enables the cell to perform useful work and establish an efficient regulatory system.

The term, "high energy bond," denotes a large and negative free energy change between products and reactants (Lipmann, 1941). That is, product formation is heavily favored. Activated intermediates, held by these relatively weak bonds, are thermodynamically highly available. Lehninger (1971) outlines two features which are responsible for the large and negative value for the standard free energy of hydrolysis of ATP: 1) strong electrical repulsion within the polyphosphate portion of the ATP molecule, which at pH 7.0, has four negative charges very near to each other (Figure A1), and 2) resonance stabilization between ADP and P_i upon formation.

The observed standard (i.e. equilibrium) free energy of hydrolysis of ATP under approximate physiological conditions (pH 7.0, ionic strength .25, 38°C, water at unit activity, 1 mM Mg^{++}) is -7.60 kcal $mole^{-1}$ (Guynn and Veech, 1973). Intracellular Mg^{++} concentration must be specified, since much of the ATP and ADP is complexed with Mg^{++} at physiological pH (Phillips et al., 1969). The nonstandard

Figure A1. Adenylate and component structures at pH 7.0. ~ denotes high energy bond. Adapted from Lehninger (1971).


Adenine
(purine
derivitive)
Ribose
(pentose)
Adenosine
(nucleoside)
Adenosine 5'-monophosphate (AMP)
Adenosine 5'-diphosphate (ADP)
Adenosine 5'-triphosphate (ATP)
Adenylate
(nucleotide)

(i.e. non-equilibrium) free energy of hydrolysis of ATP, in vivo, is near -11.5 kcal $mole^{-1}$ (Atkinson, 1977a), reflecting the nonequilibrium ATP/ADP ratio. The ATP/ADP ratio under equilibrium and nonequilibrium conditions can be compared, using these free energy change values. Tables A1 and A2 summarize free energy relationships and relevant calculations, respectively.

It is clear that the ATP/ADP ratio, in vivo, is maintained at about 10^8 times greater than the equilibrium ratio by efficient kinetic controls (Atkinson, 1977a). This large nonequilibrium ATP/ADP ratio provides the driving force for nearly all metabolic reactions through ATP coupling. Energy conserved in ATP (by ADP phosphorylation) functions to maintain the ATP/ADP ratio far from equilibrium.

Coupling and Evolutionary Significance

The large and negative free energy of ATP hydrolysis can be coupled to unfavorable thermodynamic reactions to effect a useful conversion. The overall K is the product of individual reaction K values. Equivalently, the overall standard free energy change (ΔG^o) is the sum of individual reaction ΔG^o values. Whenever 1 mole of ATP is coupled to another reaction, the effective equilibrium ratio is increased (ATP hydrolyzed) or decreased (ADP phosphorylated) by a factor of about 10^8 (Atkinson, 1977a). Coupled ATP reactions promote unidirectionality of metabolic pathways, avoid thermodynamically unfavorable pathways, and exploit low substrate levels.

As an effective coupling agent, the ATP/ADP system must be thermodynamically unstable but kinetically stable. Both of these

Table A1. Free energy change (ΔG).

Generalized Reaction

$$A + B \rightleftharpoons C + D \qquad Q = \frac{(C)\ (D)}{(A)\ (B)}$$

At equilibrium: $Q = K$

Relationship of ΔG, Q, K

$$\Delta G = RT \ln Q/K$$

$$= -RT \ln K + RT \ln Q$$

$$= \Delta G^\circ + RT \ln Q$$

where ΔG = free energy change (kcal $mole^{-1}$)
R = gas constant = .001987 kcal $mole^{-1}$ $degree^{-1}$
T = absolute temperature (degrees Kelvin)

ΔG	Q	Reaction
0	K	equilibrium
<0	<K	exergonic
>0	>K	endergonic

Equilibrium

ΔG° = -RT ln K = standard free energy change (kcal $mole^{-1}$)

P = 1 atm, T = 25°C, pH = 0
solute concentrations = 1 M = a/γ
(where a = activity
γ = activity coefficient)

$\Delta G^{\circ\prime}$ = standard free energy change (kcal $mole^{-1}$)

P = 1 atm, T = 25°C, pH = 7.0
solute concentrations = 1 M = a/γ

ΔG°_{obs} = standard free energy change observed at specified conditions (kcal $mole^{-1}$)

Table A2. Calculation of ATP/ADP ratio: Equilibrium and *in vivo*.

ATP Hydrolysis

$$ATP + H_2O \rightleftharpoons ADP + P_i$$

Equilibrium (Guynn and Veech, 1973)

$$\Delta G^\circ_{obs} = -7.6 \text{ kcal mole}^{-1} = -RT \ln K$$

$$K = 2.19 \times 10^5 = (ADP)(P_i)/(ATP)$$

In vivo (Atkinson, 1977a)

$$\Delta G = -11.5 \text{ kcal mole}^{-1} = \Delta G^\circ_{obs} + RT \ln Q$$

$$-11.5 = -7.6 + RT \ln Q$$

$$Q = 1.54 \times 10^{-3} = (ADP)(P_i)/(ATP)$$

ATP/ADP Ratio

$(P_i) = .010$ M, *in vivo* (Atkinson, 1977a)

Equilibrium: $ATP/ADP = (P_i)/K = 4.56 \times 10^{-8}$

In vivo: $ATP/ADP = (P_i)/Q = 6.49$

requirements are met. The ATP/ADP ratio is far from equilibrium (thermodynamic instability), and enzymic catalysis is required to hydrolyze ATP or phosphorylate ADP (kinetic stability).

If $\Delta G^{o'}$ for the terminal phosphate group of ATP is taken as -7.3 kcal $mole^{-1}$ (Lehninger, 1971), efficiency of free energy recovery can be calculated:

$$\% \text{ Efficiency} = \frac{(\text{Number of ATP's formed}) \quad (\Delta G^{o'} \text{ ATP})}{(\Delta G^{o'} \text{ Reaction})} \times 100$$

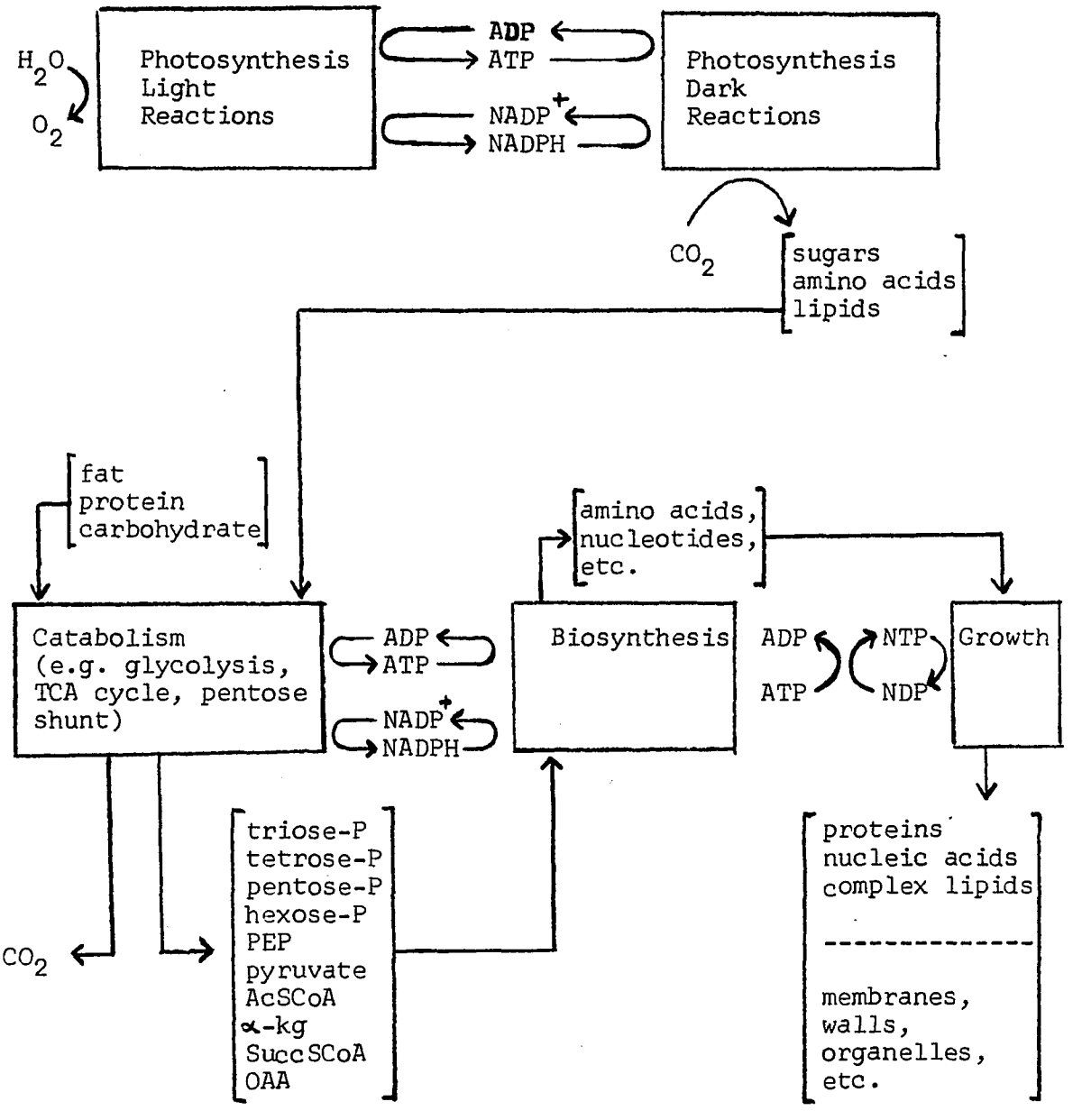
Efficiencies of glycolysis, respiratory chain phosphorylation, and the complete oxidation of glucose are listed in Table A3. The in vivo efficiency of glucose oxidation may approach 60% (Lehninger, 1971).

The functional design of metabolic regulation is a result of evolutionary selection. This design (Figure A2) incorporates coupling and correlation between catabolism and anabolism. Catabolic sequences regenerate ATP and other energized couplers (e.g. NADPH, NADH, $FADH_2$), whereas anabolic processes require these couplers. Catabolic sequences which supply the starting materials for biosynthesis (e.g. glycolysis, TCA cycle, pentose shunt) are sometimes termed, "amphibolic," to indicate their dual catabolic and anabolic functions. Evolved coupling stoichiometries, like all phenotypic characters, reflect compromise. This compromise is apparent from examination of catabolic pathways. Glycolysis, for example, would proceed with an even larger overall K, without ATP coupling. Since glycolysis does involve ATP coupling, the evolutionary significance (i.e. functional value of ATP regeneration) is evident. Evolutionary design is sensitive to overall metabolic need.

Table A3. Free energy release, recovery (ATP formation), and resultant efficiency of glycolysis, respiratory chain phosphorylation, and complete glucose oxidation (Lehninger, 1971).

Reaction	$\Delta G^{\circ\prime}$ (kcal $mole^{-1}$)	ATP Yield	Efficiency (%)
glucose $\longrightarrow$ 2 lactate	-47	2	31
$\frac{1}{2}$ O_2 + H^+ + NADH $\rightarrow$ NAD^+ + H_2O	-52	3	42
6 O_2 + glucose $\longrightarrow$ 6 CO_2 + 6 H_2O	-686	36	38

Figure A2. Metabolic design of a photoautotrophic and heterotrophic cell. Adapted from Atkinson (1977a).


H_2O
O_2
Photosynthesis Light Reactions
ADP
ATP
$NADP^+$
NADPH
Photosynthesis Dark Reactions
CO_2
sugars
amino acids
lipids
fat
protein
carbohydrate
amino acids, nucleotides, etc.
Catabolism (e.g. glycolysis, TCA cycle, pentose shunt)
ADP
ATP
$NADP^+$
NADPH
Biosynthesis
ADP
ATP
NTP
NDP
Growth
CO_2
triose-P
tetrose-P
pentose-P
hexose-P
PEP
pyruvate
AcSCoA
α-kg
SuccSCoA
OAA
proteins
nucleic acids
complex lipids
membranes,
walls,
organelles,
etc.

ATP Use and Distribution

The adenine nucleotides participate directly or indirectly in nearly all metabolic energy transductions. Some of these conversions include catabolism (e.g. glycolysis, TCA cycle, pentose shunt), anabolism (chemical and photosynthesis), mechanical work (e.g. muscle contraction, cell division), osmotic work (e.g. active transport of Na^+, K^+), and several specialized reactions (e.g. bioluminescence). The adenylate pool is necessarily distributed throughout the cell. Depending on cell type (i.e. prokaryote vs. eukaryote, autotroph vs. heterotroph) and its associated structures, the adenine nucleotides are located in the cytosol (e.g. glycolysis, pentose shunt), mitochondria (TCA cycle, electron transport chain), chloroplast grana (light reactions of photosynthesis) and stroma (dark reactions of photosynthesis), nucleus (nucleic acid synthesis), and associated with membranes (active transport) and ribosomes (protein synthesis).

ATP Regeneration

The half-time of turnover of ATP is on the order of seconds for rapidly respiring bacteria and 1-2 minutes for slowly respiring eukaryotic cells (Lehninger, 1975). That is, the amount of ATP regenerated (or consumed) per second by bacteria is about equal to the steady-state amount of ATP in the cell (Atkinson, 1977a), which ranges from .001-.01 M or about .5-5.0 mg ml^{-1} (Lehninger, 1971). Erecinska and Wilson (1978) have calculated that a resting human consumes about 40 kg ATP/24 hrs. During strenuous exercise, the ATP utilization rate is approximately .6 kg ATP min^{-1}.

Enzymic phosphorylation of ADP to regenerate ATP proceeds by substrate, oxidative, and/or photophosphorylation. These three modes of phosphorylation are briefly reviewed.

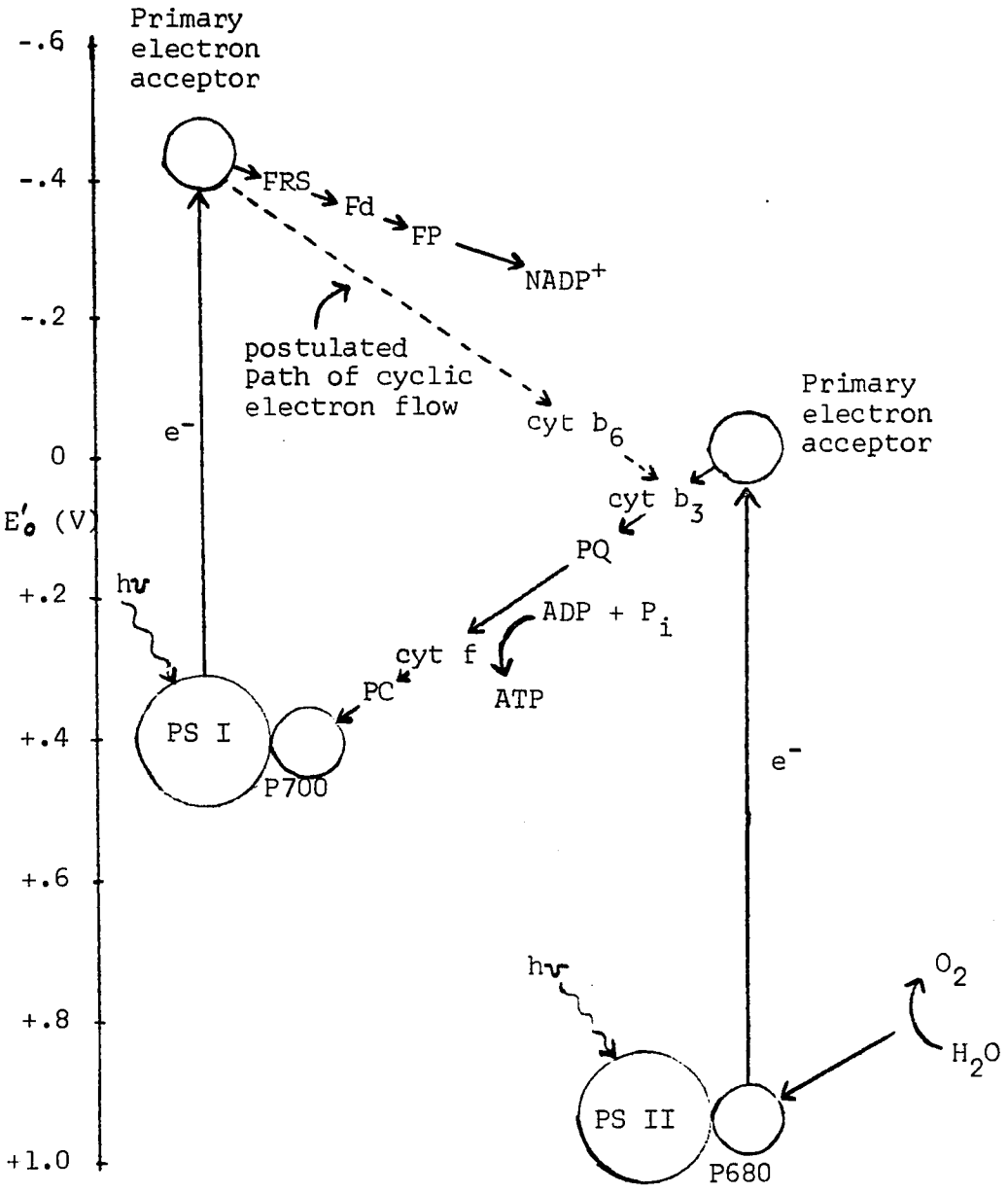
Phosphorylation by organic substrates occurs mainly in the cytosol. Phosphate compounds with a large and negative free energy of hydrolysis (e.g. phosphoenolpyruvate, 1-3 diphosphoglycerate, phosphocreatine, acetyl phosphate) donate phosphate to ADP. Both phosphoenolpyruvate and 1-3-diphosphoglycerate participate in glycolysis. Phosphocreatine functions as a "high energy" phosphate reservoir, particularly in muscle and nerve cells (Lehninger, 1971). Nucleoside triphosphates (NTP), including GTP, CTP, UTP, dATP, dTTP, dGTP, and dCTP, can phosphorylate ADP in reversible reactions. All NTP's have the same standard free energy of hydrolysis as ATP.

Oxidative phosphorylation (respiratory chain phosphorylation) occurs within the cristae of the inner membrane of the mitochondrion under aerobic conditions. In a highly exergonic reaction, ATP is formed as electrons flow from NADH or $FADH_2$ to O_2 along a series of cytochromes (Stryer, 1975). Three ATP's are regenerated per NADH oxidized (P/O = 3), whereas two are regenerated per $FADH_2$ oxidized (P/O = 2). Normally, "state 3" respiration occurs in which electron transport (oxidation of NADH and $FADH_2$) is tightly coupled to ADP phosphorylation. In contrast, "state 4" respiration is not coupled to ATP generation. The respiratory control rate (state 3/state 4) characterizes the tightness of coupling between substrate oxidation and phosphorylation.

Photophosphorylation, cyclic and noncyclic, occurs in the chloroplast grana during the light reactions of photosynthesis. Theoretical electron flow patterns (Z scheme) and the formation of ATP, NADPH, and O_2 are diagrammed in Figure A3. In cyclic electron flow (involving Photosystem I), light energy excites chlorophyll electrons, causing them to leave the molecule. As the electrons pass along a circular chain of carriers back to chlorophyll, they lose energy, some of which is coupled to ADP phosphorylation. In noncyclic electron flow (involving Photosystems I and II), light-excited electrons flow from chlorophyll along a series of carriers to $NADP^+$, resulting in NADPH and ATP formation. Noncyclic photophosphorylation requires both an external electron donor (H_2O) and acceptor ($NADP^+$), whereas cyclic photophosphorylation requires neither of these. The function of cyclic electron flow may be to generate ATP, without the necessity of concomitant generation of NADPH and O_2 (Lehninger, 1971). Recently, Arnon et al. (1981) have proposed an alternative to the Z scheme, viewing the two photosystems as basically autonomous.

Three hypotheses for the mechanism of oxidative and photophosphorylation differ with respect to the intermediate which transfers energy from the redox reactions of electron transport to ATP synthesis: 1) chemical coupling involves a high energy, covalently bonded intermediate, 2) conformational coupling suggests a high energy conformational state, and 3) chemiosmotic coupling proposes an electrochemical trans-membrane pH gradient, as the coupling vehicle. The chemiosmotic hypothesis (Mitchell, 1976) has received the most support. In this hypothesis, electron transport pumps protons outward across the inner membrane of the mitochondrion (oxidative

Figure A3. Electron transport in oxygen-evolving photosynthesis in plants, diagrammed in terms of the standard redox potentials (E'_o) of the interacting redox couples. Adapted from Lehninger (1975).


-.6
-.4
-.2
0
E'o (V)
+.2
+.4
+.6
+.8
+1.0
Primary electron acceptor
FRS
Fd
FP
NADP+
postulated path of cyclic electron flow
e-
cyt b6
Primary electron acceptor
cyt b3
PQ
hν
ADP + Pi
cyt f
PC
ATP
PS I
P700
e-
hν
O2
H2O
PS II
P680

phosphorylation) or inward across the thylakoid membrane of the chloroplast (photophosphorylation). The resultant proton motive force (pmf) is comprised of two components: a membrane potential and a proton gradient (Jensen, 1980). Collapse of the pmf provides the driving force for ATP synthesis.

Energy Charge

The adenine nucleotide pool (AMP + ADP + ATP) can be considered as a chemical energy storage system with the following interconversions (Atkinson, 1977a):

$$2\ ADP + 2\ P_i \rightleftharpoons 2\ ATP + H_2O$$

$$AMP + ATP \xrightleftharpoons{\text{adenylate kinase}} 2\ ADP$$

The system is fully charged when all adenylate is converted to ATP and fully discharged when only AMP is present. From the adenylate kinase reaction, 2 moles of ADP are required to produce 1 mole of ATP. On a molar basis, ADP contributes half the amount of energy as ATP, since ATP contains two phosphoanhydride ("high energy") bonds and ADP contains only one.

The adenylate energy charge (EC) has been defined by Atkinson and Walton (1967):

$$EC = \frac{[ATP] + 1/2\ [ADP]}{[ATP] + [ADP] + [AMP]}$$

As the "effective" mole fraction of ATP (i.e. the actual mole fraction of ATP plus half the mole fraction of ADP), EC represents a linear

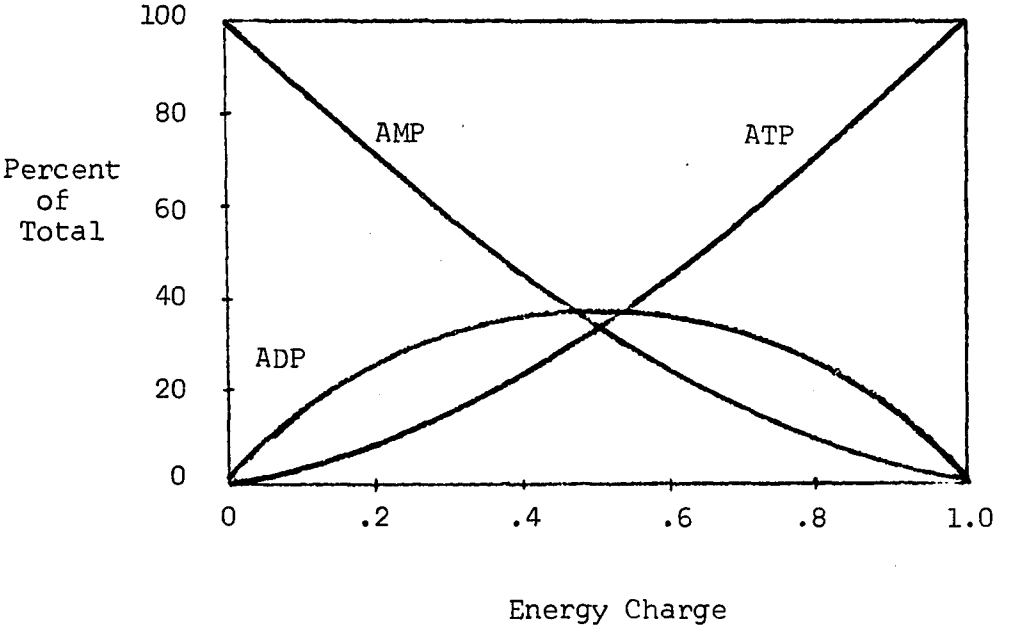
measure of the metabolic energy stored in the adenylate pool. Numerically, the charge ranges from 0 (all AMP) to 1 (all ATP) and is equal to half the average number of anhydride-bound phosphate groups per adenosine moiety.

If the adenylate kinase reaction is assumed to be at equilibrium, the relative amounts of AMP, ADP, and ATP are fixed as a function of EC (Kremen, 1976). The apparent K for the adenylate kinase reaction varies with Mg^{++} concentration, since ATP binds Mg^{++} to a greater extent than either ADP or AMP. The value of K, in vivo, is uncertain, although it is probably near 1.0 (Atkinson, 1977a). The relative concentrations of AMP, ADP, and ATP, as a function of EC, are graphed in Figure A4. The adenylate kinase reaction is assumed to be at equilibrium, and a value of 1.2 is used as the effective K, in the direction shown.

Regulatory Mechanisms

Metabolic regulatory mechanisms are reviewed by Pradet and Bomsel (1978). Regulation can be partitioned into coarse (e.g. enzyme amount) and fine (e.g. enzyme activity) control mechanisms. EC, phosphorylation state ($[ATP]/[ADP][P_i]$), redox charge ([NADH + NADPH]/[NAD + NADH + NADP + NADPH], i.e. degree of saturation in electrons of pyridine nucleotides), pH, mono- and divalent cations, and inorganic phosphate (P_i) exert regulatory influence. In addition, cellular compartmentation (eukaryotes) and distinct paired reaction pathways for biosynthesis and degradation facilitate regulation (Stryer, 1975).

Figure A4. Relative adenine nucleotide concentrations, as a function of energy charge. Adapted from Atkinson (1977a).


100
80
60
40
20
0
Percent of Total
AMP
ATP
ADP
0
.2
.4
.6
.8
1.0
Energy Charge

Since nearly all cellular reactions are enzyme-catalyzed, metabolic regulation is achieved by controlling type and rate of enzyme function (Hochachka and Somero, 1973). Rates of catalysis are regulated by amount and/or activity of enzymes. Regulation by changing enzyme amount results in slower, less sensitive, but more versatile response, relative to a change in enzyme activity. The amount or concentration of enzyme can be controlled at four levels: 1) transcription (operon model), 2) translation (primary protein structure), 3) protein assembly (secondary through quintinary protein structure), or 4) degradation. Enzyme activity is dependent on substrate concentration and physical variables (e.g. temperature, pressure). Other factors which may influence activity are cofactor availability, covalent modification, proteolytic activation, hormonal levels, and enzyme modulators. Modulators (both positive and negative) provide rapid, accurate, and sensitive regulation.

Regulatory or allosteric enzymes are strategically positioned in multi-enzyme sequences at metabolic branch points and have both a catalytic (i.e. substrate) and regulatory (i.e. modulator) site. In most cases, modulators exert their effect by altering the enzyme-substrate (E-S) affinity, and not the V_{max} potential of the enzyme (Hochachka and Somero, 1973). Since physiological substrate concentrations usually sub-saturate enzymes (near the region of the K_m or $S_{.5}$ value), enzymic rates are highly sensitive to changes in this E-S affinity, and thus highly sensitive to modulation.

Modulators include adenine and pyridine nucleotides, as well as specific metabolites (e.g. pathway end product inhibitors). Hochachka

and Somero (1973) suggest that EC modulation is a relatively coarse metabolic control which is finely tuned by specific product modulation. For example, a high EC stimulates ATP-utilizing pathways in general, and a specific metabolite designates which energy requiring process is needed in particular.

Energy Charge Regulation

Since they are operationally linked with nearly all metabolic sequences, it is apparent that adenine nucleotides are strategically adapted to metabolic regulation (Atkinson, 1977a). This statement is supported by the following two observations.

First, nearly all enzymic phosphate transfers involve ATP and ADP (i.e. common intermediate). The ATP/ADP couple occupies an intermediate position among activated phosphate compounds (Figure A5). Phosphate transfer from high energy donors (e.g. PEP) to low energy acceptors (e.g. G-6-P) must funnel through the ATP/ADP system. This facilitates regulation.

Secondly, four ribonucleoside triphosphates (ATP, GTP, CTP, UTP) and four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) channel ATP energy into different biosynthetic pathways (Figure A6). Among nucleoside diphosphates (NDP), only ADP accepts phosphate groups during glycolysis, and oxidative and photophosphorylation. Thus, ATP is required to phosphorylate all NDP's by reversible reactions, catalyzed by nucleoside diphosphokinase (NDPK):

$$ATP + NDP \overset{NDPK}{\rightleftharpoons} ADP + NTP \quad (K = 1.0).$$

Figure A5. Phosphate transfer from high energy donors to low energy acceptors. Adapted from Lehninger (1971).

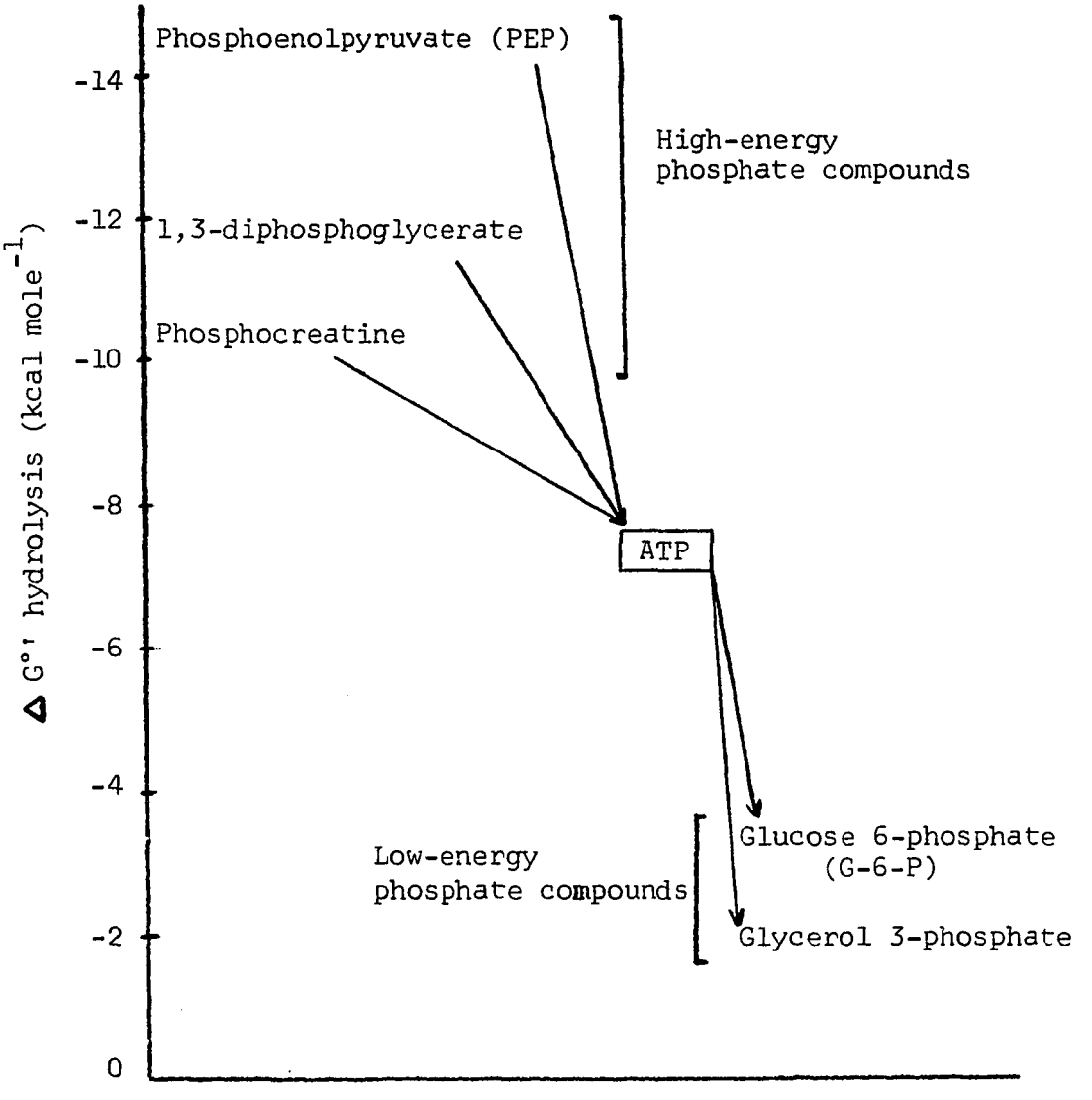
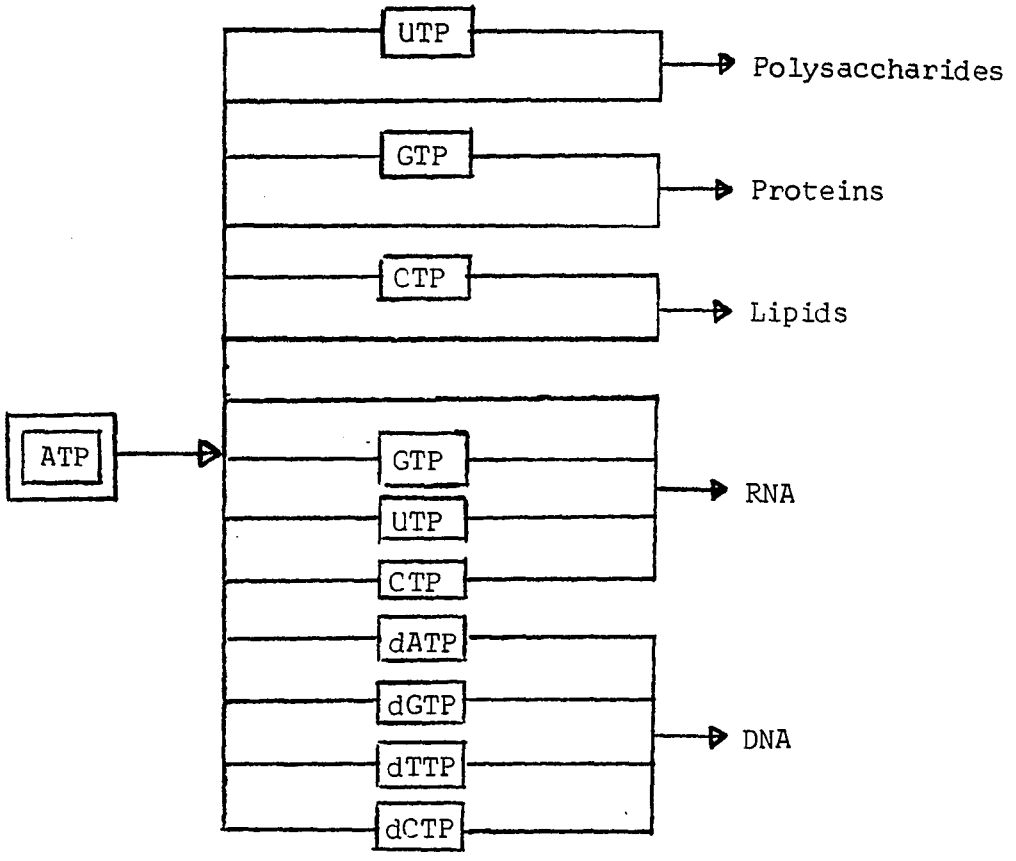

Phosphoenolpyruvate (PEP)
-14
High-energy
phosphate compounds
-12
1,3-diphosphoglycerate
Phosphocreatine
-10
-8
ATP
-6
-4
Glucose 6-phosphate
(G-6-P)
Low-energy
phosphate compounds
-2
Glycerol 3-phosphate
0
Δ G°' hydrolysis (kcal mole⁻¹)

Figure A6. Channeling of ATP phosphate bond energy into specific biosynthetic pathways. Adapted from Lehninger (1971).


UTP
Polysaccharides
GTP
Proteins
CTP
Lipids
ATP
GTP
UTP
CTP
RNA
dATP
dGTP
dTTP
dCTP
DNA

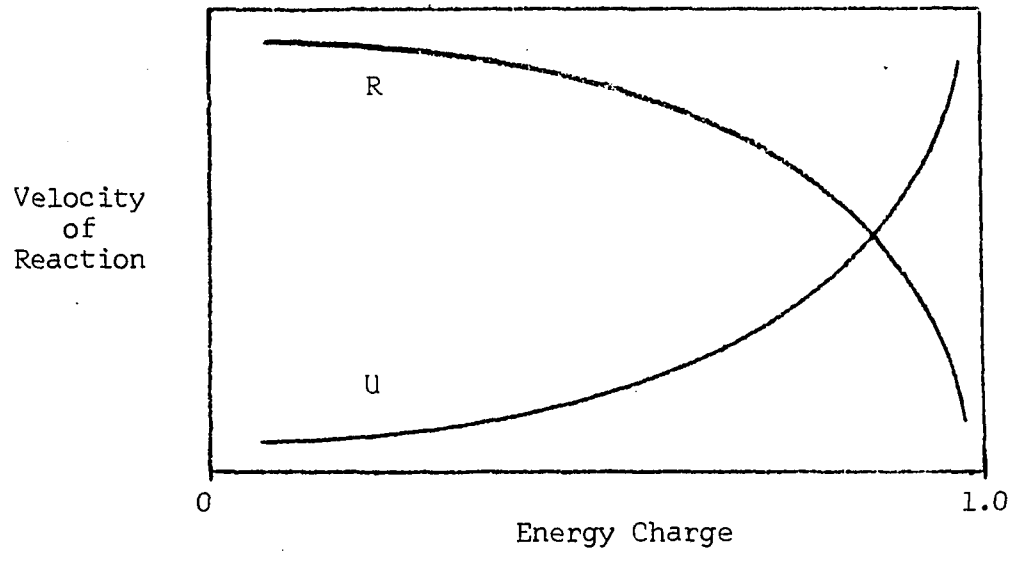
This adaptation may have evolved, so that macromolecular syntheses could be regulated by the adenylate system. As a logical extension, Atkinson (1977a) suggests that NDPK is a key control enzyme which partitions the use of ATP between macromolecular syntheses and other ATP-utilizing processes.

EC regulates metabolic sequences by controlling enzymic rates of reactions which are coupled to the use (U) and regeneration (R) of ATP, as shown in Figure A7. Only key enzymes at metabolic branch points need be controlled, since these enzymes catalyze the "first committed reaction" in a sequence (Atkinson, 1977b). Reciprocally, EC is stabilized by corresponding regulatory enzymes, in the range where the curves intersect in Figure A7, near a value of 0.85 (e.g. Chapman et al., 1971). This general pattern (Figure A7), considered a weighted average of R and U sequences, is derived from <u>in vitro</u> studies but lends strong support to the contention that EC functions as a regulatory parameter <u>in vivo</u>.

Regulation of glycolysis, the TCA cycle, and the electron transport chain (Lehninger, 1971) provides an example of enzyme response to EC or more specifically, the ATP/ADP ratio. In glycolysis, phosphofructokinase (PFK) activity is increased with high ADP concentration (positive modulation) and decreased with high ATP concentration (negative modulation):

$$\underset{\text{(inactive)}}{\text{PFK}} \underset{\text{ATP, citrate}}{\overset{\text{ADP, AMP}}{\rightleftharpoons}} \underset{\text{(active)}}{\text{PFK}}$$

Figure A7. Velocity of reactions which regenerate (R) and utilize (U) ATP, as a function of energy charge. Adapted from Atkinson (1977a).


Velocity
of
Reaction
R
U
0
1.0
Energy Charge

If ATP concentration is greater than is required for saturation of PFK, catalytic activity is depressed. As an end product of respiration, ATP serves as a feedback inhibitor. In the TCA cycle, isocitrate dehydrogenase (IDH) activity is modulated in a similar manner:

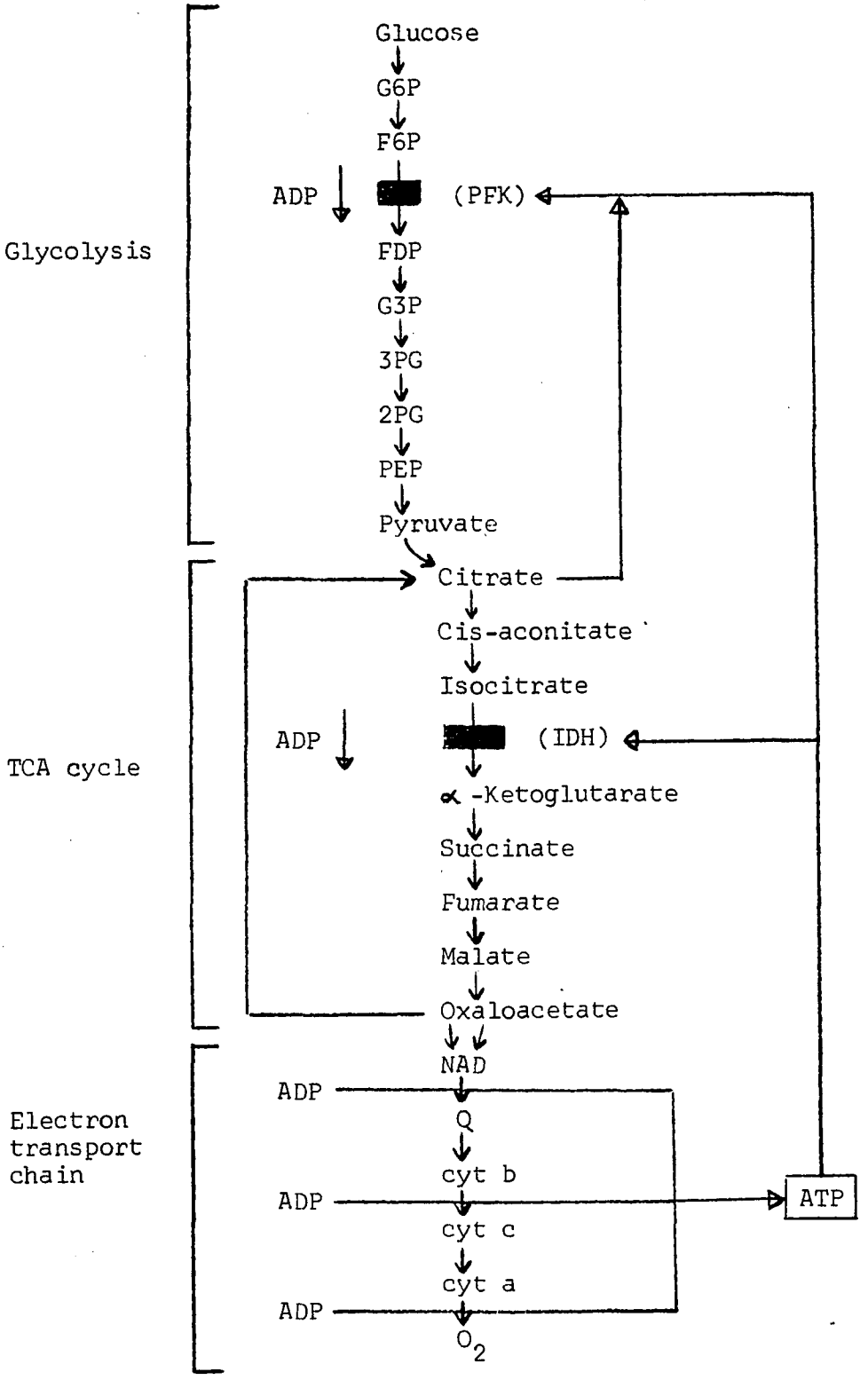
$$\underset{\text{(inactive)}}{\text{IDH}} \underset{\text{ATP, NADH}}{\overset{\text{ADP, NAD}^+}{\rightleftharpoons}} \underset{\text{(active)}}{\text{IDH}}$$

It follows that whenever the ATP/ADP ratio is larger than its steady-state value, both the rates of glycolysis and the TCA cycle are decreased. The rates of glycolysis and the TCA cycle are integrated through negative modulation of PFK by citrate (i.e. if citrate is being overproduced, PFK activity is throttled down). Within the respiratory chain, the rate of electron transport decreases and the carriers become more reduced when the ATP/ADP ratio is high. Regulation of glycolysis and respiration is diagrammed in Figure A8.

Glycolysis and respiration have self-regulatory features, involving the adenine nucleotides. Glycolytic and respiratory rates are adjusted to stabilize the large nonequilibrium ATP/ADP ratio. In this way, glycolysis and respiration are geared to metabolic demand for ATP.

The central thermodynamic feature of metabolic organization (i.e. unidirectional paired pathways with evolved ATP coupling stoichiometries) and the kinetic feature (i.e. correlated control between these pathways) are functionally interdependent. Kinetic

Figure A8. Regulation of glycolysis, TCA cycle, and electron transport chain by positive (ADP) and negative (ATP, citrate) modulation. Adapted from Lehninger (1971).


Glycolysis
TCA cycle
Electron transport chain
Glucose
G6P
F6P
ADP
(PFK)
FDP
G3P
3PG
2PG
PEP
Pyruvate
Citrate
Cis-aconitate
Isocitrate
ADP
(IDH)
∝ -Ketoglutarate
Succinate
Fumarate
Malate
Oxaloacetate
NAD
ADP
Q
cyt b
ADP
ATP
cyt c
cyt a
ADP
O_2

control is mandatory, in vivo, where cellular component concentrations are far from equilibrium. The adenine nucleotides, most conveniently expressd in terms of EC, regulate the rates of ATP-utilizing and ATP-regenerating sequences (kinetic feature) to maintain an ATP/ADP ratio far from equilibrium (thermodynamic feature). It is, therefore, evident that thermodynamic and kinetic roles of the adenylates are functionally interdependent.

Energy Charge Controversy and Limitation

Cellular regulation by EC has not received universal acceptance. Atkinson (1977a) has refuted criticisms, presented by Banks and Vernon (1970) on the role of ATP, in vivo. Atkinson (1977b) and Fromm (1977) have recently debated the theory of EC regulation. Using computer simulations, Purich and Fromm (1972, 1973) have shown that total adenylate pool size, pH, free Mg^{++}, kinetic reaction mechanisms, and adenylate kinase mass-action ratio alter enzyme response to EC. These simulations suggest that EC does not regulate enzyme response effectively.

Some experimental evidence is inconsistent with EC theory. Two examples are cited which involve higher plants. First, Pradet (1969) and Raymond and Pradet (1980) have demonstrated stabilization of very low EC values (near 0.3), using lettuce seeds under reduced oxygen tension. Since modification of EC at low values is not accompanied by large changes in the ATP/ADP or ATP/AMP ratios, Pradet and Raymond (1978) suggest that low EC values are regulated by mechanisms other than those described by Atkinson's theory. Second, the regulatory

enzyme, phosphofructokinase (PFK), catalyzes an early step in the glycolytic sequence. Animal PFK, but not plant PFK, appears to respond to EC (Pradet and Bomsel, 1978). Because both enzymes are inhibited by ATP, but only animal PFK is activited by AMP, Turner and Turner (1980) suggest that EC theory may require modification before it can be effectively applied to some plant enzyme systems.

Problems associated with methodology for the determination of in situ adenylate levels limit the utility of the EC concept, in general (Karl, 1980). Even when measurement error is eliminated, results may be obscured by cell compartmentation, tissue heterogeneity, or community variability (Pradet and Raymond, 1978; Karl, 1980). When multicellular tissues or populations of organisms (e.g. microbial assemblage) are extracted, a mass-weighted mean EC is determined. Because this measurement reflects a range of metabolic states, subsequent interpretation is often difficult.

As a unitless quantity, EC does not specify information on nucleotide concentrations or turnover rates. These can vary significantly at a fixed EC (Karl, 1980). Lowry et al. (1971) have agrued that EC is an insensitive metabolic indicator, since relatively small changes in EC obscure much larger changes in ATP/ADP or ATP/AMP ratios, to which enzymes are actually responding.

As an index of sublethal stress, application of EC to some organisms may be inappropriate (Ivanovici, 1980). For example, yeast cells remained viable at EC values below 0.1 during starvation (Ball and Atkinson, 1975).

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APPENDIX B

STATISTICAL ASSUMPTIONS

Introduction

Nonparametric and parametric statistical tests have been contrasted by Siegel (1956). Every statistical test is associated with a model and measurement requirement. The parametric model specifies conditions about population parameters from which the sample is drawn. Interval or ratio level measurement is required. The nonparametric model does not specify conditions about population parameters. Nominal, ordinal, interval, or ratio level measurement may apply. Assumptions are listed for the following parametric procedures, used herein: single factor Model 1 Analysis of Variance (ANOVA), Model 1 Linear Regression, and single factor Analysis of Covariance (ANCOVA).

Model 1 ANOVA

Assumptions for Model 1 ANOVA have been outlined by Steel and Torrie (1960), mathematically presented by Guenther (1964), and discussed in detail by Sokal and Rohlf (1981):

1) treatment (i.e. factor level) and environmental (i.e. block) effects are additive,

2) for each treatment, variate error components (and hence, variate values, assuming an additive relationship between variates and their associated errors) are random, independently and normally distributed with mean zero (or equal to the treatment mean, in the case of variate values) and a common variance which estimates the population variance, and

3) treatments are fixed (i.e. Model 1).

These assumptions are referred to as additivity (or linearity), randomness, independence, normality, homoscedasticity, and fixed effects, respectively.

Model 1 Linear Regression

Assumptions underlying Model 1 Linear Regression have been outlined by Zar (1974) and Sokal and Rohlf (1981):

1) for any value of X (independent variable), the Y values (dependent variable) are random, independently and normally distributed with a common variance which estimates the population variance, accounting for the dependence of Y on X,

2) expected Y values are a linear function of X and lie on a straight line, and

3) X values are fixed and measured without error (i.e. Model 1).

ANCOVA

The assumptions for ANCOVA are a combination of those for ANOVA and linear regression (Steel and Torrie, 1960; Guenther, 1964):

1) for each X within a given treatment, Y value error components (and hence, Y values, assuming an additive relationship between Y values and their associated errors) are random, independently and normally distributed with mean zero (or equal to the treatment mean for a particular X, in the case of Y values) and a common variance which estimates the population variance,
2) for each treatment, expected Y values are a linear function of X and lie on a straight line,
3) slopes of these lines are equal among treatments, and
4) X values are fixed and measured without error.

Treatment of Assumptions

Assumptions for these parametric tests were either presumed or evaluated. Additivity was not directly evaluated, although homoscedasticity implies a certain degree of additivity (Schefler, 1969). Homoscedasticity was tested for in ANOVA, linear regression, and ANCOVA. Random assignment of experimental units to treatment levels (i.e. completely randomized design) satisfied the randomness assumption and may have overcome any lack of independence. In most cases, normality was not evaluated due to small sample sizes. Treatment sample sizes were equal in experiments subjected to ANOVA testing. Consequences of nonnormality and heteroscedasticity are not severe, as long as these sample sizes are equal (Snedecor and Cochran, 1967; Zar, 1974). Because treatments were specifically chosen, Model 1 ANOVA was appropriate. Linear correlation between X and Y values

was tested for in linear regression and ANCOVA. Homogeneity of slopes was evaluated in ANCOVA.

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