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1917
Natchitoches
Fort & Etablissement Francois
Lac a la Clef
Lac a Berbane
Lac Talbot
Riv. Noire
Lac Tacnaas
Riv. des Tacnaas
Riv. des Yatas
Petite Riv. aux Tigres
Petite Pte Coupee Lac
Lac Ste
Petite Pte Coupee
Fort de Tombeche
Village des Tchactas
Tchicachaes
Riv. de la Mobile
Youanis
Pointe du Diable
Nocce
Acchoupa
Bacatane
Tout ce Pays est coupe de Bayoux ou Ruisseaux
Riv. des Pascagoulas
Bayou qui se conduit aux Natchitoches
Riv. aux Canards
Riv. des Tonicas
Bayou Rouge
Bayou
Riv. Rouge
Riv. Blanche
Riv. aux Arcs
Lac
Lac Maurepas
Riv. Manchac
Fort de la Mobile
Baye des Pascagoulas
Riv. aux Iberville
Mississipi
Fleuve
Riv. du Fermont
Riv. Pamanoutabé
Riv. des Chetes
Lac des Chetes
Nle Orleans
Riv. des Natchez
Lac Pontchartrain
Lac
Lake Borgne
Lake Ste Chandeleur
Riv. Mobile
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A Potential Indicator of the Cumulative Impact of Sublethal Stress in Coastal Plant Communities

Final Report to
Louisiana Department of Transportation and Development

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August 1978

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SUMMARY

The increasing influence of man's impact on coastal plant communities magnifies the need for a sensitive and reliable index which reflects the cumulative impact of sublethal environmental stresses. This study was designed to develop such an index and to evaluate its suitability in the major wetland habitats of Louisiana. The index chosen for testing was the energy charge (E.C.) ratio ($\frac{ATP + \frac{1}{2}ADP}{ATP + ADP + AMP}$), a measure of "energy rich" adenylate compounds, in addition to, the general adenylate (ATP, ADP, and AMP) patterns present in an organism.

The adenylates were assayed, after their conversion to ATP, by the ATP-dependent light yielding reaction of the firefly lantern luciferin-luciferase system. The amount of light emitted during this reaction is proportional to the concentration of ATP present.

Several methods for extracting adenylates from leaf tissue were examined. The highest ATP concentrations and recovery rates (96%) of added ATP occurred when frozen, lyophilized, and ground tissue was extracted in boiling 1mM EDTA (ethylenediamine-tetraacetic acid) and 5% PVPP (polyvinylpolypyrrolidone) for 30 s. This method extracted 45% more ATP than when fresh leaf tissue was boiled followed by homogenization with mortar and pestle in the same extractant. The addition of PVPP to the extraction solution alone increased ATP concentrations 48%.

The use of liquid scintillation counting to measure ATP-dependent light production proved extremely satisfactory. The ATP standard curve when plotted on a log-log scale was linear from 5×10^{-10} to 10^{-12} moles ATP ml^{-1}. Coefficients of variation for standard ATP concentrations were generally lower than 3%.

The exposure of Spartina alterniflora to sublethal levels of petroleum hydrocarbons (Louisiana crude) significantly decreased the

E.C. ratio of both leaf and root tissue before obvious visual symptoms appeared.

The apparent sublethal stress associated with subsided-inland Spartina marshes was correctly defined by E.C. ratio methodology. In addition, subtle differences in stress, as defined by Spartina above-ground standing crop, were highly associated ($r^2 = 0.91$) with E.C. ratio along a transect east of Barataria Bay, Louisiana. This relationship, however, was not significant for Spartina sampled along a transect west of Barataria Bay. The reason for this difference has not yet been determined.

Spartina patens and S. alterniflora collected from a newly created dredge-spoil area had a significantly higher energy status than those plants from an adjacent unperturbed marsh. Under specific circumstances, newly deposited dredge material may provide a more suitable site for plant growth than a mature natural marsh.

The E.C. ratios of Sagittaria falcata from an impounded fresh water marsh and Acer rubrum var. drummondii from an impounded cypress swamp were significantly higher than those for the same plant species growing in unimpounded control sites. The higher energy status of plants in the impounded sites suggest that the effect of impoundment on vegetation is confounded by the effect of other environmental factors. For example, the higher E.C. ratio of red maple in the impounded cypress swamp was probably due to greater solar penetration through the open cypress canopy. The small difference in the energy status of Taxodium distichum between the two sites was in agreement with cypress productivity estimates for these two areas.

In general, this study provides preliminary information indicating the suitability of adenylate composition and/or E.C. ratio as a monitor

of environmental stress in coastal plant communities. However, further testing of this method under both field and laboratory conditions is required before a final conclusion concerning its ultimate use can be stated.

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INTRODUCTION

Urban, industrial, and agricultural activities affecting coastal environments have increased at an alarming rate. These activities either directly or indirectly have generated a variety of environmental stresses which, in association with natural stresses, determine the stability of highly productive and ecologically important wetland communities. The intensity of anthropogenic activities in coastal Louisiana is illustrated by the fact that 50% of the permits issued in the United States by the Army Corps of Engineers are issued for projects on the Louisiana coast.

These man-induced stresses are generated primarily from the following activities: 1) oil and gas exploration and drilling which directly affect wetlands by the release of potentially toxic hydrocarbons or indirectly from dredging activities, 2) dredge and fill operations which result in the loss of wetland habitats and smothering of wetland vegetation, in addition to changes in hydrological parameters, 3) industrial and waste discharge which contributes potentially detrimental heavy metals and organic toxins, and 4) agricultural runoff which introduces pesticides and fertilizer nitrogen and phosphorus into wetland environments.

Environmental stresses occurring along the Louisiana coast are of a natural origin, as well as man-induced. Salinity, soil anaerobiosis, low nutrient conditions, fluctuating water regimes, etc. are all natural stresses influencing wetland vegetation. In addition, land subsidence has a great influence on Louisiana's wetlands. Subsidence increases the waterlogging of wetland soils resulting in increased stress due to anoxic soil conditions.

The majority of stresses mentioned above have sublethal effects which, while not killing the plant community, gradually reduce its vigor and

productivity. Although it is obvious that wetlands are under stress, the intensity of stress is extremely difficult to quantify.

As population and industrial pressures increase further, management decisions will have to be made concerning which wetland areas should be developed and which left in their natural state. If an area must be developed, those habitats that are already highly stressed should be sacrificed and developed first while habitats under relatively little stress should be maintained as they are. In order to pursue this approach, it is essential to develop a means of evaluating stress intensity. The need for such an index is also justified from the standpoint of point sources of pollution. If, for example, an oil spill should occur in a Louisiana wetland, the extent of clean-up and containment could be predicated on the degree of stress to which that vegetational community was subjected.

What is needed to accomplish the above is a sensitive index of sublethal stress which at any instant can provide an integrated measure of the physiological "health" of a particular plant community, population, or organism. Growth fails as such an index because it is too slowly affected by subtle alterations in the physical environment and thus, does not provide an early enough warning system of stress intensity. Considerable evidence has accumulated which indicates that the energy charge (E.C.) ratio $\left(\frac{ATP + \frac{1}{2}ADP}{AMP + ADP + ATP}\right)$, a measure of the "energy rich" adenylate compounds present in an organism, may be such an indicator.

The basic premise of this specific indicator is that an organism maintains a particular cellular ratio of ATP (adenosine triphosphate) to ADP (adenosine diphosphate) and AMP (adenosine monophosphate), and that this ratio depends upon the organism's physiological vigor and state of growth. When the E.C. ratio is greater than 0.5, ATP-utilizing systems

increase their activities. At a lower E.C. ratio than 0.5 in cells, ATP regeneration systems are dominant. Growing and multiplying cells maintain a high energy charge around 0.8, but senescent cells have a low energy charge of 0.5 (Chapman et al., 1971).

Although the E.C. ratio has not been utilized to define environmental stress in field studies, except for microbial communities for which it nicely delineated their metabolic state (Weibe and Bancroft, 1975), it has been used as a monitor of stress in a number of laboratory experiments with higher plants. Environmental stresses such as anaerobiosis (Bromsel and Pradet, 1968), salinity (Hasson-Porath and Poljakoff-Mayber, 1971), chilling injury (Stewart and Guinn, 1969), and heat injury (Jones, 1970) have generated a reduction in the E.C. ratio, indicating a reduction in the plant's metabolic health. In addition, Ching and Kronstad (1972) demonstrated that the growth potential of different plant varieties was directly related to their E.C. ratio.

The overall objectives of this study were to develop a technique for measuring the E.C. ratio in wetland plants and to use this index to evaluate the intensity of sublethal stress in a variety of coastal plant communities.

MATERIALS AND METHODS

Energy Charge Ratio Methodology

Tissue Storage

In order to prevent changes in adenylate (ATP, ADP, and AMP) composition between time of sampling and extraction, plant leaf tissue was frozen in dry ice (-57 C) immediately upon harvest. The effect of this storage method on the amount of extractable ATP was thus determined. Changes in ATP concentration due to various extraction techniques were used as indicative of the effects of these methods on all adenylates.

Leaf tissue of Spartina alterniflora, salt marsh cordgrass, grown in in the greenhouse was harvested and separated into three aliquots: 1) extracted immediately, 2) frozen in dry ice, thawed, lyophilized for 24 hrs, ground, and extracted immediately, and 3) frozen in dry ice, lyophilized for 24 hrs, ground, and extracted immediately. The above extractions were made in 10 ml of boiling 1mM EDTA (ethylenediamine-tetraacetic acid).

Extraction of Adenylates

The following extraction procedures were compared to determine which produced the highest ATP concentrations: 1) fresh tissue in boiling 1mM EDTA, 2) fresh tissue in boiling 1mM EDTA and 5% (0.5 g per 10 ml extraction solution) PVPP (polyvinylpolypyrrolidone) (Sigma Chemical Co.), 3) fresh tissue in boiling 1mM EDTA and 5% PVPP and subsequent homogenization with mortar and pestle, 4) frozen-lyophilized tissue in boiling 1mM EDTA and 5% PVPP.

For each of the above extraction procedures, 0.5 g fresh wt of leaf tissue, cut into 5 mm sections, or 0.1 g of frozen-lyophilized tissue, ground in a Wiley mill to pass a #60 mesh screen, was placed in 10 ml of boiling extraction solution for 30 s and 45 s, respectively. These optimum boiling durations were determined experimentally. Extracts of the frozen-lyophilized tissue and the fresh tissue ground with mortar and pestle were centrifuged at 20,000 g and the supernatant used for ATP analysis. For fresh tissue extracts which were not homogenized, the supernatant was decanted without prior centrifugation and assayed for ATP.

Estimation of Percent Recovery of Added ATP

In order to estimate percent recovery during extraction, ATP was added during the extraction procedure. Fresh leaf samples were extracted with boiling 1mM EDTA containing 5% PVPP followed by either no

homogenization or mortar and pestle homogenization. Frozen-lyophilized leaf samples were extracted with boiling 1mM EDTA containing 5% PVPP. Two leaf aliquots were used for each extraction procedure: 1) without ATP and 2) with ATP added 15 s after the addition of the sample. Each extraction was done in triplicate.

Conversion of AMP and ADP to ATP and Calculation of Energy Charge Ratio

Supernatants from the extraction solutions were cooled and 0.2 ml incubated at 30 C for 30 min in each of the following mixtures:

A. For ATP determination, 0.2 ml of a reaction buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, and 50mM magnesium acetate and 0.2 ml of deionized water.

B. For ADP plus ATP determination, 0.2 ml of the reaction buffer and 0.2 ml of a solution containing 40 µg of pyruvate kinase (EC 2.7. 1.40) and 1 µmole of trisodium phosphenolpyruvate (Sigma Chemical Co.).

C. For total adenylate phosphates, 0.2 ml of reaction buffer and 0.2 ml of a solution containing 40 µg of pyruvate kinase, 1 µmole of trisodium phosphoenolpyruvate, and 50 µg of adenylate kinase (EC 2.7.4.3) (Sigma Chemical Co.).

After incubation, the extracts were placed in a boiling water bath for 3 min, cooled, and assayed immediately. Concentrations of ATP, ADP plus ATP, and total adenylate phosphates were calculated from the following equation:

$$\underset{\text{tissue}}{\text{nmoles ATP}}\ g^{-1} = \frac{\text{nmoles ATP/ml x volume extracting solution x dilution factor}}{\text{aliquot volume x wt of tissue (dry or fresh)}}$$

ATP concentration was measured directly, while ADP and AMP concentrations were obtained by subtraction. The energy charge ratio was calculated from the following formula:

$$\frac{[ATP] + 0.5[ADP]}{[AMP + ADP + ATP]}$$

Theory of ATP Analysis

Of the several methods available for quantitatively assaying ATP, the most sensitive and practical method utilizes the bioluminescent reaction occurring in the firefly Photinus pyralis (McElroy, 1947). The amount of light produced in the bioluminescent reaction, which can be monitored with a liquid scintillation counter, is directly proportional to the amount of ATP present.

The detailed mechanism and the biochemistry of the firefly bioluminescent reaction have been well described (McElroy et al., 1969). The overall sequence of the firefly reaction can be summarized as follows:

$$\underset{\text{(Luciferin)}}{LH_2} + \underset{\substack{\text{Luficerase}\\ \text{enzyme}}}{E} + \underline{ATP} \rightleftharpoons$$

$$\underset{\substack{\text{(Luciferyl adenylate}\\ \text{complex)}}}{E.LH_2.AMP} + \underset{\text{(Pyrophosphate)}}{PP} \qquad (1)$$

$$E.LH_2.AMP + O_2 \longrightarrow E + \text{Product} + CO_2 + AMP + \underline{\text{Light}} \qquad (2)$$

In the initial activation step (Equation 1) luciferin and ATP, catalyzed by luciferase, react to form a luciferase-luciferin-adenosine monophosphate complex and inorganic pyrophosphate. This complex reacts with molecular oxygen to produce light and a product molecule (oxyluciferin adenylate). The firefly lantern enzyme luciferase has a specific requirement for ATP.

Enzymatic Assay of ATP with Liquid Scintillation Counter

The ATP-dependent luminescence was detected with a Model 230 Beckman liquid scintillation counter. The scintillation vials (Beckman Bio-Vials)

which acted as the reaction vessel contained 2 ml of 50mM glycine buffer, pH 7.4, 50 µl of plant extract, and 1.9 ml of deionized water. Light emission was initiated by adding 0.1 ml of a 20% dilution of reconstituted firefly lantern extract (Sigma FLE-50). It is essential that the time between addition of the firefly lantern extract and the first reading of the liquid scintillation counter be constant for all samples. This was easily accomplished by using the automatic mode of the liquid scintillation counter. Out of coincidence circuitry, a 50-150 discriminator setting, 6 s counting time, and a gain of 100%, produced a linear standard curve over a broad concentration range (5×10^{-10} to 1×10^{-12} moles ATP ml^{-1}). The second 6 s count was used to plot the ATP standard curve.

ATP Standard Curve

The determination of standard curves for each series of ATP analyses was essential since the standard curve may vary with firefly lantern extract batch and fluctuations in the efficiency of the liquid scintillation counter. Standards were prepared to cover a range of concentrations from 5×10^{-10} to 10^{-12} moles ATP ml^{-1}. Triplicates of each standard were run to determine the degree of analytical variation.

Utilization of the Energy Charge Ratio in Greenhouse and Field Trials

Greenhouse Trial

ATP, ADP, AMP, and the E.C. ratio were measured in Spartina alterniflora plants exposed to petroleum hydrocarbons (Louisiana crude). Uniform field transplants were established and allowed to acclimate in the greenhouse. Plants were fertilized with a commercial plant food (RA-PID-GRO, RA-PID-Gro Corp., Dansville, New York) during this period. Four treatment plants, each growing in pots 12 cm in diameter and 12 cm in height, were dosed with 100 ml of Louisiana crude while two untreated plants acted as

controls. The oil was prevented from contacting the plant leaves during the dosing procedure. All plants were harvested after 48 hrs. Fresh leaf and root tissue were extracted in boiling 1mM EDTA and 5% PVPP with subsequent mortar and pestle homogenization. Adenylate analyses were conducted as described above.

Field Trials

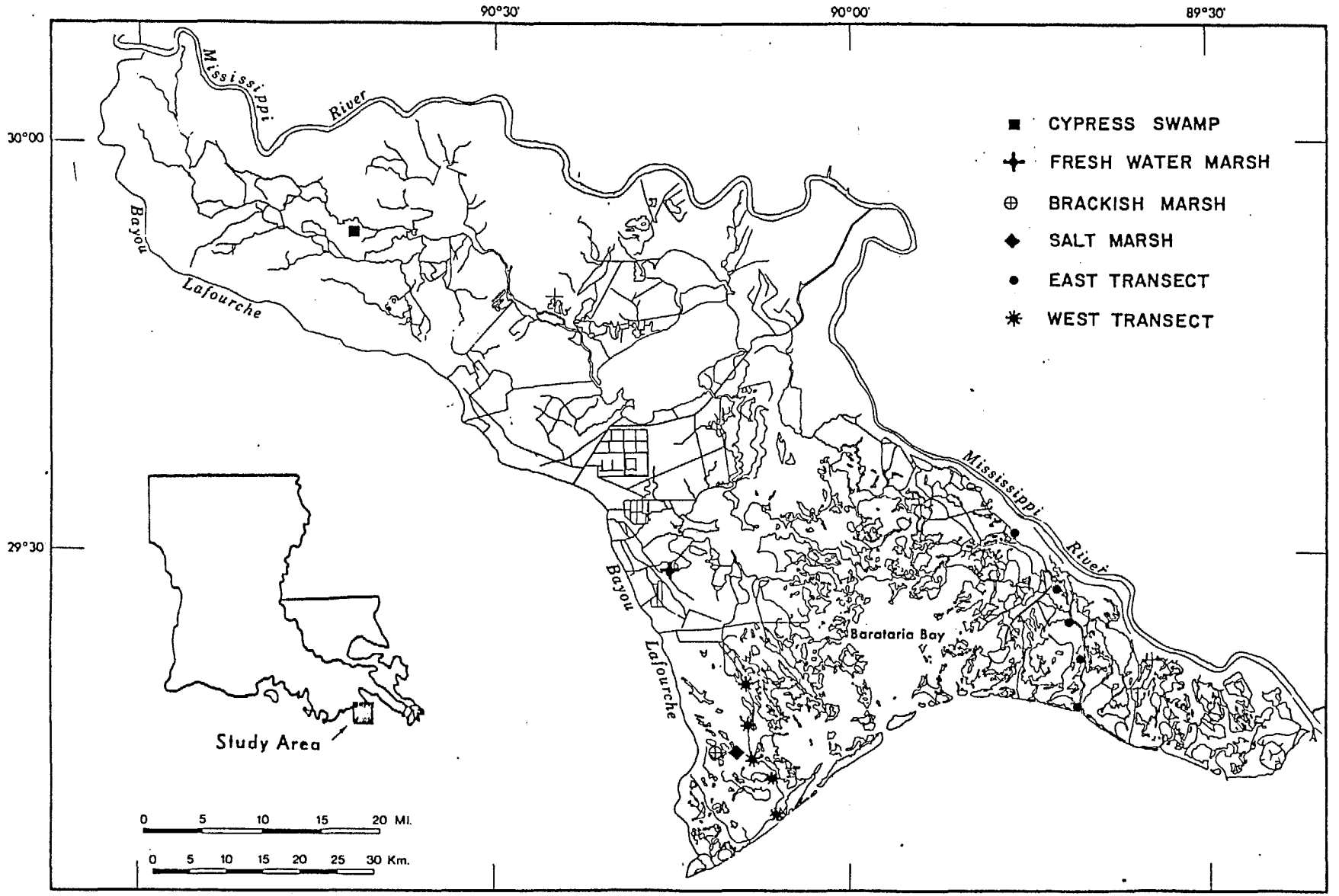
This aspect of the study concentrated on utilizing the E.C. ratio concept to evaluate the cumulative impact of sublethal environmental stress in four wetland habitats of Barataria Basin, Louisiana. We "spot-lighted" Barataria Basin because it contains the gamut of wetland habitats and because considerable ecological data are available for this region (Gosselink et al., 1977). Leaf tissue was collected from the dominant plant species of the major wetland habitats and ATP, ADP, AMP, and the E.C. ratio measured.

Salt marsh habitat

Leaf tissue of Spartina alterniflora, the dominant salt marsh species, was collected from both streamside (area immediately adjacent to a tidal creek) and inland (area 10-20 m from the streamside) locations at five stations along two north-south transects (Fig. 1): 1) A transect following Bayou Grand east of Barataria Bay on July 11, 1978, and 2) A transect following Bayou Ferblanc west of Barataria Bay on June 27, 1978. Three replicate samples, one sample equaling the youngest four leaves from three culms, were taken at each inland and streamside site. At each sampling station, 0.25 m^2 quadrats of Spartina aboveground standing crops were harvested. The amount of living biomass, in itself a relative measure of stress, was compared with the E.C. ratio for that living material.

The salt marshes of Barataria Bay are subsiding at a rate of approximately 1 cm y^{-1} (DeLaune, R. personal communication, Center for Wetland

Fig. 1. Sampling locations and plant species sampled in Barataria Basin: Cypress Swamp - Taxodium distichum and Acer rubrum var. drummondii, impounded swamp versus crayfish farm; Fresh water marsh - Sagittaria falcata, impounded versus non-impounded marshes; Brackish water marsh - Spartina patens and S. alterniflora, dredge spoil site versus natural marsh; Salt marsh - Spartina alterniflora, streamside versus subsided-inland; Transects east and west of Barataria Bay - Spartina alterniflora.


90°30'
90°00'
89°30'
30°00
29°30
Mississippi River
Bayou Lafourche
Bayou Lafourche
Mississippi River
Barataria Bay
CYPRESS SWAMP
FRESH WATER MARSH
BRACKISH MARSH
SALT MARSH
EAST TRANSECT
WEST TRANSECT
Study Area
0 5 10 15 20 MI.
0 5 10 15 20 25 30 Km.

Resources, Louisiana State University, Baton Rouge). This process often results in inland areas of Spartina marsh which are at a lower elevation than adjacent streamside sites. Spartina growing in these areas are apparently highly stressed as suggested by their chlorotic appearance. A site such as described above was sampled near Leeville, Louisiana (Fig. 1) on June 6, 1978. Five replicate samples, each replicate equal to the four youngest leaves were collected from each area.

Brackish marsh habitat

Spartina patens (salt meadow hay), a dominant brackish water species, and Spartina alterniflora were sampled from a dredge-spoil site and from an adjacent natural marsh near Leeville, Louisiana (Fig. 1) on June 20, 1978. Ten replicate samples, each replicate equal to the four youngest leaves from three culms, were collected from each of the two areas.

Fresh water marsh habitat

Sagittaria falcata (arrowhead), a dominant fresh water plant species, was sampled from an impounded and a non-impounded site in a fresh water marsh near Galliano, Louisiana (Fig. 1) on July 20, 1978. Ten replicate leaf samples were collected from each of the two locations. A replicate consisted of squares of tissue cut from three representative leaves from three plants.

Bald cypress swamp habitat

On June 13, 1978, leaf samples from Taxodium distichum (bald cypress) and Acer rubrum var. drummondii (swamp red maple) were collected from a permanently impounded swamp and an adjacent swamp which is managed as a crayfish farm near Vacherie, Louisiana (Fig. 1). Branches from the lower third of the canopy were removed from ten replicate cypress trees in each area by using pruning shears attached to an extensible pole. The

cypress trees sampled had an approximate dbh (diameter at breast height) of 1.6 m. The cypress needles were then removed from each of the branches. The red maples, important understory hardwoods at both sampling sites, had a dbh of approximately 25 cm and were sampled in the upper one third of their canopy. Ten leaves were removed from each of the ten replicate maple trees in each area for analysis.

Sample Handling

All field samples were immediately rinsed with deionized water, placed in plastic bags, and frozen with dry ice. Immediately upon arrival at the laboratory, all samples were lyophilized for 36 hrs. In no instance did the time between sampling and lyophilization exceed 12 hrs. After drying, the samples were ground in a Wiley Mill to pass a #60 mesh screen and analyzed as described in the E.C. Ratio methodology section of the Materials and Methods.

Statistical Analysis

All data were statistically analyzed using analysis of variance and regression analysis, and significant differences and regression effects were determined by the Least Significant Difference (LSD) procedure and F-test, respectively (Steel and Torrie, 1960). Unless otherwise stated, all significant differences are at the 0.05 probability level.

RESULTS AND DISCUSSION

Energy Charge Ratio Methodology

Tissue Storage

Freezing, as a method of storing leaf tissue, resulted in a loss of ATP only when the tissue was allowed to thaw before extraction (Table 1). Rapid freezing of higher plant tissue renders the cells permeable to a wide range of substrate molecules including ATP (Rhodes and Stewart, 1974).

In addition, ATPases (enzymes that hydrolyze ATP to ADP) may become dissociated from their membranes upon freezing. Apparently, the freeze-thaw treatment allows for greater interaction between ATP and ATPases and hence lower ATP concentration.

Since the leaf tissue had to be frozen to prevent changes in adenylate composition between time of sampling and extraction but could not be thawed before extraction, lyophilization of the frozen tissue was tested. Frozen-thawed leaf tissue and fresh leaf tissue resulted in 18% and 52%, respectively, of the ATP extracted from frozen-lyophilized tissue (Table 1). Cheer et al. (1974) found that liquid nitrogen freezing of marine algal samples also increased ATP recovery.

Extraction of Adenylates

Methods for extracting ATP from plant tissue are extremely varied (Sofrová and Leblová, 1970). A number of extraction methods and extractant modifications were tested in this study.

Higher vascular plants often contain high concentrations of potentially toxic phenolic compounds. The activity of plant enzymes in crude extracts are often reduced by these compounds (Loomis and Battaile, 1966). In this study, PVPP (polyvinylpolypyrrolidone), which complexes phenolic compounds, was added to the extraction medium in an attempt to prevent phenolics from inhibiting the luciferase enzyme of the firefly lantern extract during the ATP assay. Table 2 shows that adding PVPP to the ATP extraction solution resulted in a 48% increase in extractable ATP. Guinn and Eidenbock (1972) also found that purifying cotton leaf extracts with polyvinylpyrrolidone resulted in higher ATP extractions.

Table 2 demonstrates that ATP extraction was not complete when the Spartina leaf tissue was just boiled. Extraction was increased 32% by homogenizing the tissue with mortar and pestle subsequent to boiling.

Table 1

The Effect of Storage Method on the ATP Content of *Spartina alterniflora* Leaf Tissue

Storage Method		
Fresh tissue extracted immediately	Fresh-tissue, frozen, thawed, and extracted after lyophilization	Fresh tissue, frozen, lyophilized, and extracted
(results expressed as the % of the ATP extracted with frozen-lyophilized tissue)		
52	18	100

Table 2

Comparison of Some Methods for Extracting ATP (nmoles g^{-1} dry wt) from Spartina alterniflora Leaves

Replicate	Extraction procedure			
	Fresh tissue in boiling 1mM EDTA	Fresh tissue in boiling 1mM EDTA and 5% PVPP	Fresh tissue in boiling 1mM EDTA and 5% PVPP followed by homogenization	Frozen-lyophilized tissue, ground, in boiling 1mM EDTA and 5% PVPP
1	105	158	206	294
2	99	152	202	290
3	102	152	204	301
$\bar{x} \pm s$	102 ± 3 (A)*	154 ± 4 (B)	204 ± 2 (C)	295 ± 6 (D)

*Values followed by a different letter are significantly different at the 0.05 probability level.

Apparently, boiling does not completely permeabilize cellular membranes so that an equilibrium does not exist between ATP inside and outside the plant tissue. Lin and Hanson (1974) found that homogenization of the plant tissue after boiling was also required for complete extraction of ATP.

Boiling EDTA extraction of frozen-lyophilized tissue resulted in 45% more ATP extracted than boiling extraction of fresh tissue followed by homogenization (Table 2). The use of frozen-lyophilized tissue for adenylate analysis has been proven successful by a number of investigators (Santarius and Heber, 1965; Stewart and Guinn, 1969; Guinn and Eidenbock, 1972).

Several extractants have been used for ATP analysis of plant tissue including 95% ethanol, trichloroacetic acid, perchloric acid, glycine, tris and glycylglycine buffers, and EDTA. A survey of the literature demonstrated that boiling EDTA extracted some of the highest concentrations of ATP (Stewart and Guinn, 1969; Guinn and Eidenbock, 1972) and was used in this study.

Estimation of Percent Recovery

The highest recovery rate (96%) of added ATP occurred when plant tissue was frozen, lyophilized, ground, and extracted in boiling EDTA and PVPP (Table 3). Since this procedure not only produced the highest percent recovery, but also the greatest ATP concentrations (Table 2), it was used to analyze field samples in this study.

ATP Standard Curve

The liquid scintillation counter readings for triplicate samples of different concentrations of standard ATP solutions are given in Table 4, and the ATP standard curve is shown in Fig. 2. It is apparent from the coefficients of variation in Table 4 that after proper calibration and careful operation relatively constant results can be obtained when ATP

Table 3

The Effect of Extraction Method on the Recovery of Added ATP to *Spartina alterniflora* Leaf Tissue

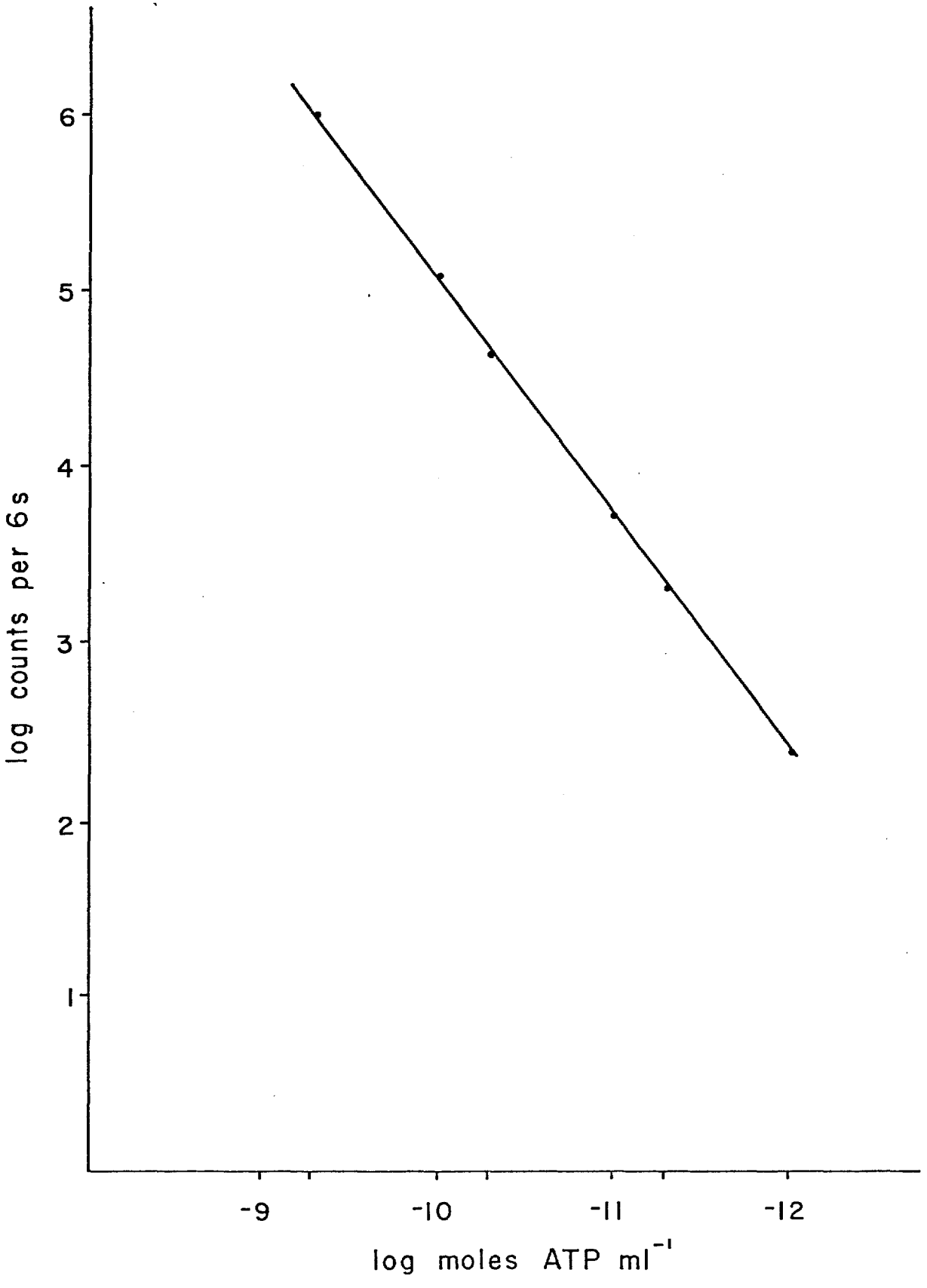
Extraction Method		
Fresh tissue in boiling 1mM EDTA and 5% PVPP	Fresh tissue in boiling 1mM EDTA and 5% PVPP with subsequent homogenization	Frozen-lyophilized tissue, ground, in boiling 1mM EDTA and 5% PVPP
(Percent recovery of added ATP)		
74	78	96

Table 4

Analysis of Standard ATP Solutions Via Beckman Model 230 Liquid Scintillation Counter

Standard ATP (moles ml^{-1})	Liquid scintillation counter reading (log counts $6s^{-1}$)	Mean	C.V. (%)	Calculated ATP (moles ml^{-1})
5×10^{-10}	6.00 6.00 5.97	5.99	0.3	5×10^{-10}
1×10^{-10}	5.10 5.09 5.07	5.09	0.4	1.1×10^{-10}
5×10^{-11}	4.56 4.64 4.55	4.58	1.0	4.84×10^{-10}
1×10^{-11}	3.81 3.62 3.77	3.73	2.7	1×10^{-11}
5×10^{-12}	3.37 3.25 3.28	3.30	1.5	4.84×10^{-12}
1×10^{-12}	2.49 2.46 2.12	2.36	8.9	9.33×10^{-13}

Fig. 2. Standard curve for ATP analysis.


log counts per 6s
6
5
4
3
2
1
-9
-10
-11
-12
log moles ATP ml^{-1}

was assayed via liquid scintillation counting. The linearity of the ATP standard curve in Fig. 2 was representative. When background counts are high, the assay was sensitive to 5×10^{-12} moles ATP ml^{-1}.

Utilization of the Energy Charge Ratio in Greenhouse and Field Trials

Greenhouse Trial

Although the exposure of Spartina alterniflora to petroleum hydrocarbons produced no obvious visual effects to root tissue and only a slight curling of leaf tissue, significant changes in adenlyate composition did result (Table 5). The concentration of all adenylates in the leaf tissue of the treatment plants was significantly lower than in the controls (Table 5). Although the effect of petroleum hydrocarbon exposure was not as intense in the root tissue, significant decreases in adenylate concentrations did occur in hydrocarbon treated plants (Table 5).

Both leaf and root E.C. ratios acceptably delineated the lower energy status, and thus the reduced physiological health, of the treated plants. The E.C. ratio of leaf and root tissue was significantly lower in the hydrocarbon exposed plants. Although petroleum hydrocarbon sublethal stress can be detected by this index, the sensitivity of the E.C. ratio to small changes in hydrocarbon concentration is still to be determined. However, this initial study suggests that changes in adenylate composition occur quickly after petroleum hydrocarbon stress is applied. Drastic decreases in adenylate pattern occurred within 48 hrs after dosing even though there were no obvious visual symptoms.

Field trials

Salt marsh habitat - Transect east of Barataria Bay

Although ATP and ADP concentrations and E.C. ratio were higher in Spartina alterniflora from the streamside location than the inland when

Table 5

ATP, ADP, AMP, Total Adenylate Concentrations (nmoles g^{-1} fresh wt), and Energy Charge (E.C.) Ratio in *Spartina alterniflora* Tissue 48 hrs after Petroleum Hydrocarbon (Louisiana Crude) Exposure

Treatment by plant tissue	n	ATP	ADP	AMP	Total adenylates	E.C. ratio
Leaf						
Control	4	267* (30)	179* (21)	139* (10)	585* (41)	0.61* (0.03)
Treatment	4	38 (10)	60 (24)	63 (9)	161 (26)	0.37 (0.00)
Root						
Control	4	147* (13)	94* (28)	61* (2)	302* (14)	0.65* (0.03)
Treatment	4	51 (9)	25 (5)	34 (5)	110 (18)	0.57 (0.00)

*Asterisk indicates significant difference ($P < 0.05$) between control and hydrocarbon treatments. Value in parentheses is the $s_{\bar{x}}$.

all stations were combined (Table 6), indicating a higher energy status in the streamside plants, these differences were not significant. Field variation in adenylate composition was very high in the salt marsh habitat as evidenced by the large standard errors shown in Tables 6 and 7. Whether this source of variation is natural for field samples or due to the type of plant tissue in this habitat, has not yet been determined.

Regression analysis, however, revealed a significant relationship between aboveground standing crop and E.C. ratio of streamside and inland S. alterniflora (Fig. 3). The E.C. ratio accounted for 91% of the variation in aboveground standing crop. This data indicated that the E.C. ratio correctly reflected the net primary productivity and thus the degree of stress to which these plants were subjected. ADP content (μmoles m^{-2}) was also significantly related to biomass and accounted for 97% of the variation in aboveground standing crop (Fig. 4). No trend in standing crop, adenylate composition, or E.C. ratio was discernible along this north-south transect.

Salt marsh habitat - Transect west of Barataria Bay

Streamside Spartina alterniflora tended significantly to be of a higher energy status than inland S. alterniflora. With data from all stations combined, ATP, AMP, and total adenylate concentrations were significantly higher for streamside than inland S. alterniflora (Table 7). Although the E.C. ratio and ADP concentration were both higher in streamside Spartina, this difference was not significant (Table 7). As was found for the east transect, there were no significant differences in either adenylate concentration or E.C. ratio among stations, and there were no significant trends in either aboveground standing crop or E.C. ratio along this transect. Gosselink et al. (1977) found, along these same transects, a trend of increasing Spartina productivity from south to north. This

Table 6

ATP, ADP, AMP, Total Adenylate Concentrations (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio in *Spartina alterniflora* Leaf Tissue from Streamside and Inland Areas Along a North-South Transect East of Barataria Bay, Louisiana

Sampling station		ATP		ADP		AMP		Total adenylates		E.C. ratio	
		S[a]	I[b]	S	I	S	I	S	I	S	I
North ↓ South	1	418[c] (47)	141 (26)	219 (7)	173 (11)	23 (15)	8 (7)	661 (35)	320 (33)	0.80 (0.05)	0.75 0.01
	2	79 (33)	67 (25)	165 (35)	191 (38)	49 (10)	13 (11)	292 (73)	271 (74)	0.53 (0.05)	0.60 (0.00)
	3	129 (47)	53 (32)	199 (30)	125 (31)	58 (25)	41 (15)	386 (87)	218 (71)	0.59 (0.02)	0.51 (0.01)
	4	155 (104)	165 (103)	174 (36)	196 (39)	13 (10)	86 (73)	343 (122)	446 (181)	0.64 (0.10)	0.57 (0.04)
	5	151 (45)	160 (103)	235 (29)	175 (40)	0 (0)	44 (14)	376 (73)	378 (134)	0.69 (0.02)	0.60 (0.08)
	$\bar{x}$	187 (40)	117 (17)	198 (14)	172 (16)	29 (8)	38 (17)	411 (46)	327 (53)	0.65 (0.03)	0.61 (0.03)

[a] Streamside

[b] Inland

[c] Each value is the mean of three observations. Value in parentheses is the $s_{\bar{x}}$.

Fig. 3. Relationship between aboveground standing crop (g m^{-2}) and Energy Charge Ratio for Spartina alterniflora sampled along a north-south transect east of Barataria Bay, Louisiana.

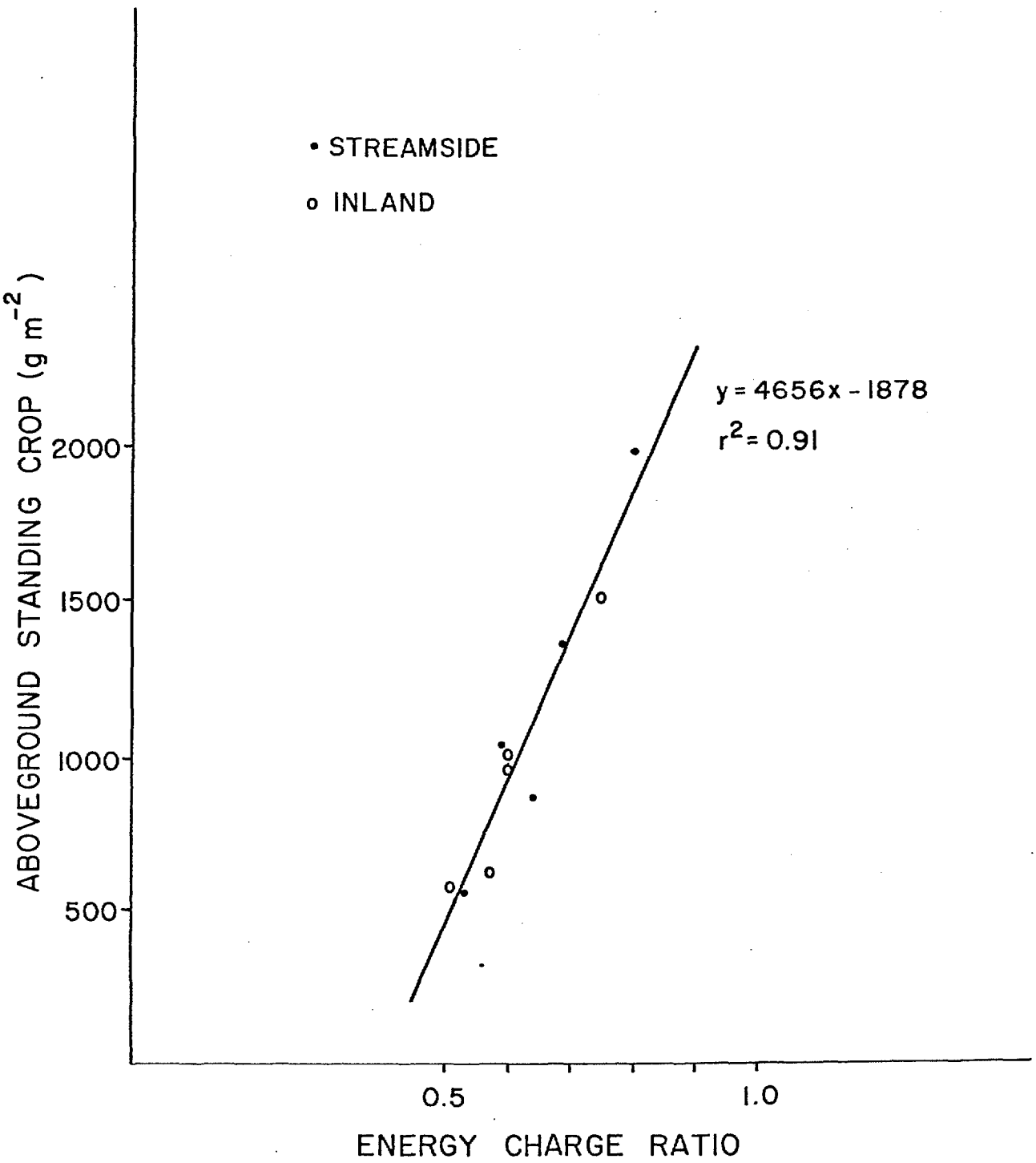

• STREAMSIDE
o INLAND
y = 4656x - 1878
r² = 0.91
ABOVEGROUND STANDING CROP (g m⁻²)
2000
1500
1000
500
0.5
1.0
ENERGY CHARGE RATIO

Fig. 4. Relationship between aboveground standing crop (g m^{-2}) and ADP content (μ moles m^{-2}) for streamside Spartina alterniflora sampled along a north-south transect east of Barataria Bay, Louisiana.

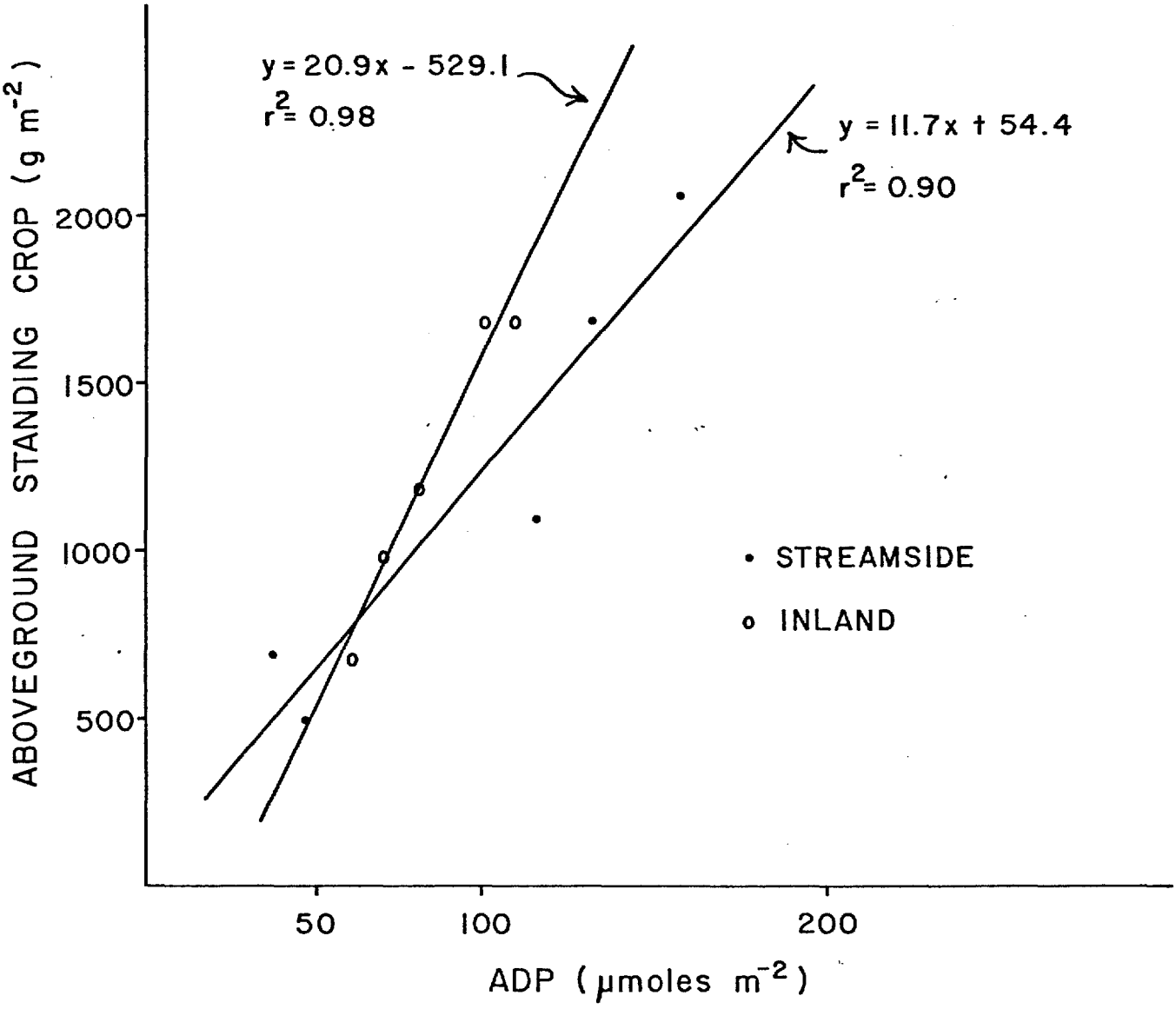

y = 20.9x - 529.1
r² = 0.98
y = 11.7x + 54.4
r² = 0.90
• STREAMSIDE
o INLAND
ABOVEGROUND STANDING CROP (g m⁻²)
2000
1500
1000
500
50
100
200
ADP (μmoles m⁻²)

Table 7

ATP, ADP, AMP, Total Adenylate Concentrations (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio in *Spartina alterniflora* Leaf Tissue from Streamside and Inland Areas Along a North-South Transect West of Barataria Bay, Louisiana

Sampling station		ATP		ADP		AMP		Total adenylates		E.C. ratio	
		S[a]	I[b]	S	I	S	I	S	I	S	I
North ↓ South	1	54[c] (25)	54 (42)	174 (34)	98 (34)	23 (9)	27 (7)	252 (68)	178 (79)	0.55 (0.01)	0.51 (0.00)
	2	104 (33)	30 (12)	147 (21)	97 (12)	45 (23)	27 (5)	284 (35)	154 (29)	0.61 (0.10)	0.51 (0.01)
	3	80 (65)	22 (7)	110 (35)	90 (18)	22 (5)	26 (13)	213 (104)	138 (26)	0.56 (0.02)	0.48 (0.01)
	4	85 (40)	78 (20)	165 (63)	180 (38)	52 (2)	48 (10)	302 (103)	306 (50)	0.50 (0.09)	0.54 (0.02)
	5	135 (35)	35 (7)	167 (41)	103 (11)	71 (7)	8 (8)	373 (70)	146 (16)	0.57 (0.05)	0.60 (0.00)
	$\bar{x}$	92* (18)	44 (6)	153 (17)	114 (13)	43* (7)	27 (4)	285* (35)	185 (24)	0.56 (0.03)	0.53 (0.02)

[a] Streamside

[b] Inland

[c] Each value is the mean of three observations. Value in parentheses is the $s_{\bar{x}}$.

* Asterisk indicates significant difference ($P < 0.05$) between streamside and inland values.

trend was not apparent from the adenylate concentrations, E.C. ratios, or standing crop data (not shown) determined in the present study.

Regression analysis revealed a significant relationship between aboveground standing crop and leaf ADP content (μmoles m^{-2}) (Fig. 4), but no significant relationship with ATP or E.C. ratio (Fig. 6). Leaf ADP accounted for 90% and 98% of the variation in aboveground standing crop in the streamside and inland areas, respectively (Fig. 5).

The results obtained from transects east and west of Barataria Bay are somewhat contradictory, e.g., a significant relationship between standing crop and E.C. ratio in the east transect, but a non-significant relationship in the west transect. However, the strong association ($r^2 = 0.91$) found for the east transect suggests that adenylate patterns and E.C. ratio may be a sensitive way of determining plant growth potential. A much more intensive sampling design must be initiated at different times during the growing season, in order to satisfactorily test this hypothesis.

Salt marsh habitat - Streamside versus subsided-inland

The energy charge ratio of streamside Spartina alterniflora was significantly greater than for subsided-inland S. alterniflora (Table 8). Spartina plants in subsided-inland sites are depauperate and of a chlorotic appearance compared to streamside Spartina. This apparent stress was correctly delineated by the E.C. ratio.

Although there was no significant difference in leaf ATP concentration between the two sites, AMP concentration, interestingly, was significantly greater for subsided-inland Spartina than streamside Spartina. This observation indicated significant hydrolysis of high energy carrying ATP and ADP. The high concentration of the lowest energy carrying adenosine phosphate, AMP, relative to the ATP concentration for

Fig. 5. Relationship between aboveground standing crop (g m^{-2}) and ADP content (μ moles m^{-2}) for streamside and inland Spartina alterniflora sampled along a north-south transect west of Barataria Bay, Louisiana.

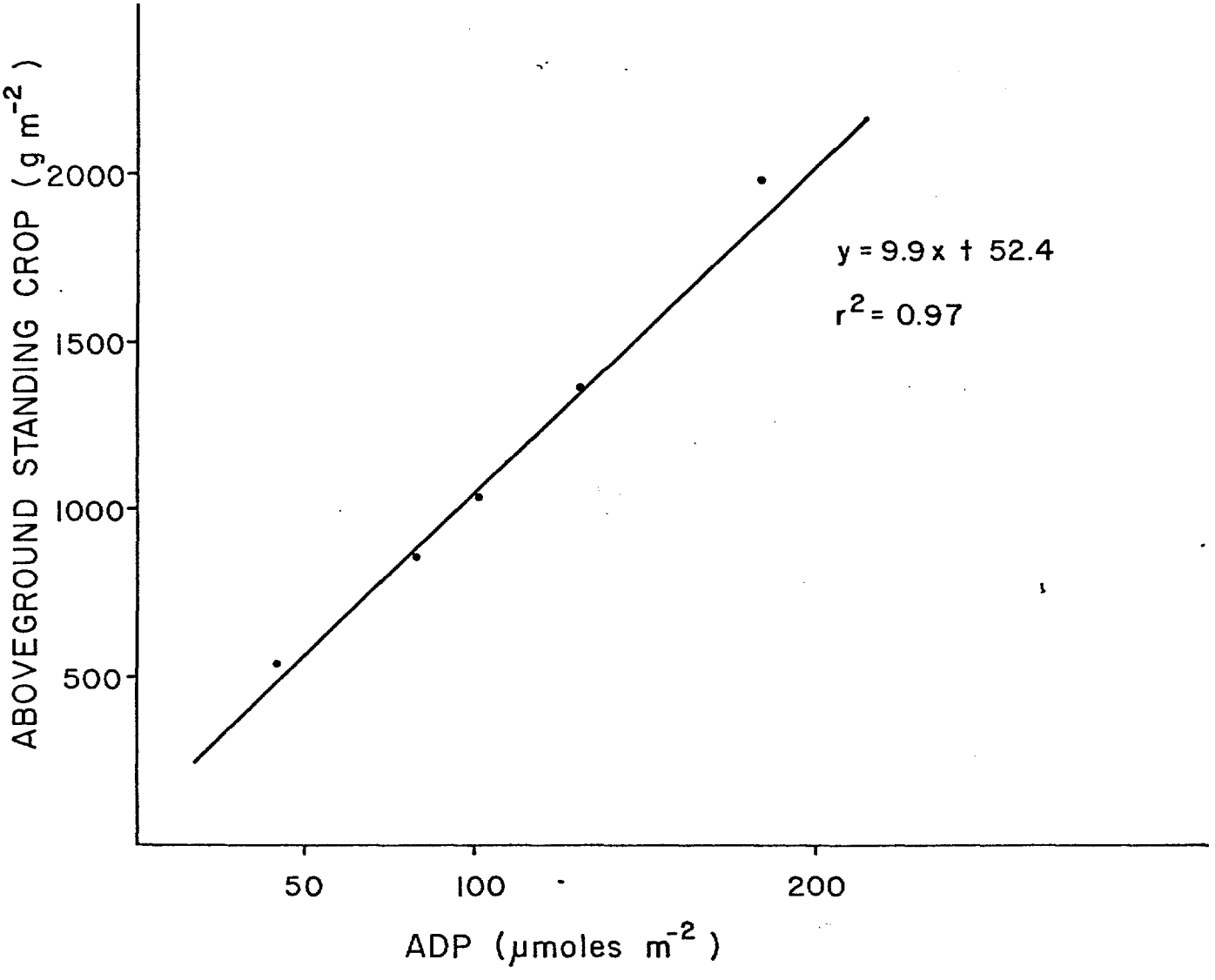

ABOVEGROUND STANDING CROP (g m^{-2})
2000
1500
1000
500
50
100
200
ADP (μmoles m^{-2})
y = 9.9 x + 52.4
r^2 = 0.97

Fig. 6. Relationship between aboveground standing crop (g m^{-2}) and Energy Charge Ratio for Spartina alterniflora sampled along a north-south transect west of Barataria Bay, Louisiana.

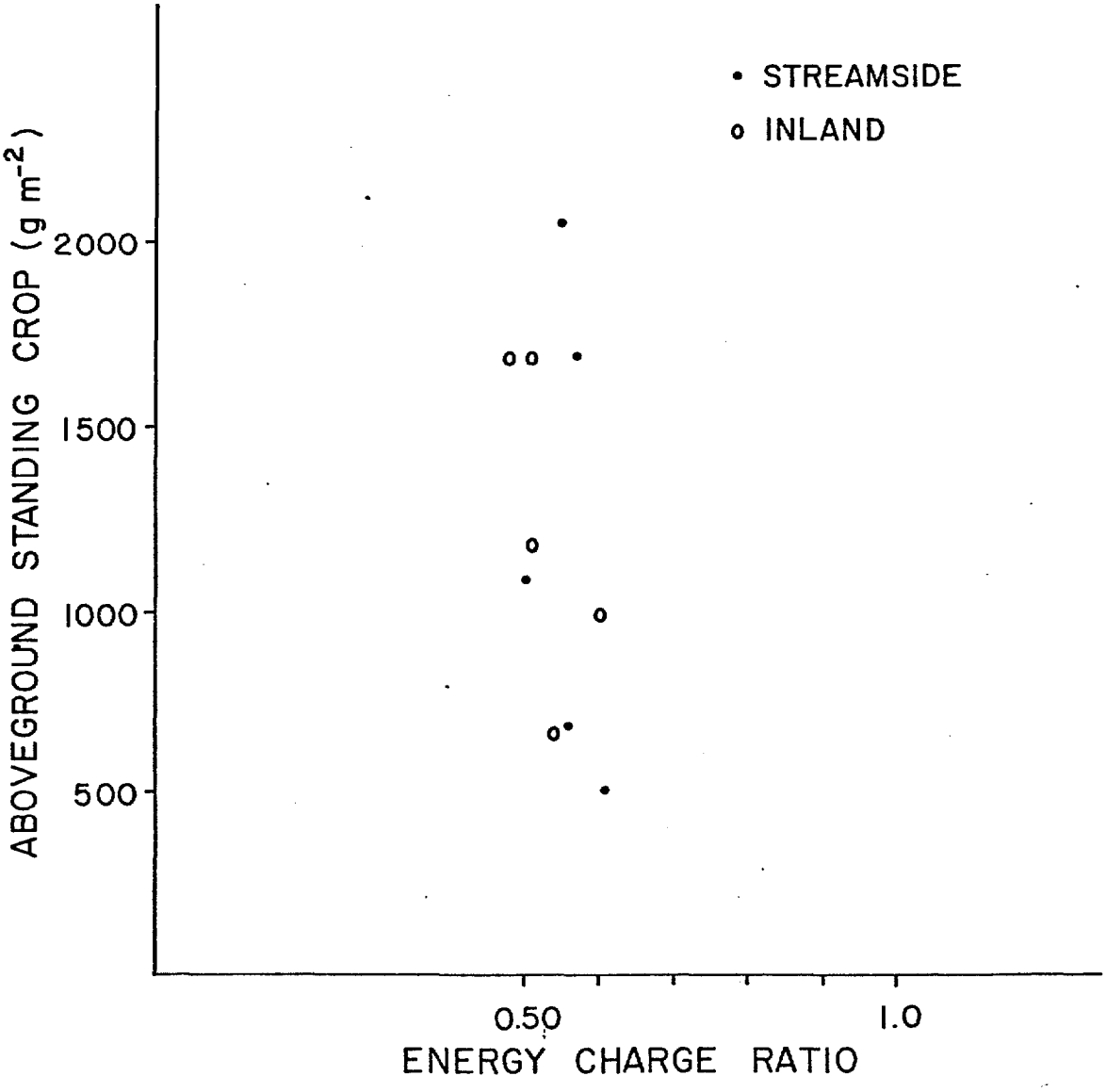

• STREAMSIDE
o INLAND
ABOVEGROUND STANDING CROP (g m-2)
2000
1500
1000
500
0.50
1.0
ENERGY CHARGE RATIO

Table 8

ATP, ADP, AMP, Total Adenylate Concentration (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio in Spartina alterniflora Leaf Tissue Collected from Streamside and Subsided-Inland Areas Near Leeville, Louisiana

Sampling site	ATP	ADP	AMP	Total adenylate	E.C. ratio
Streamside	131[a] (44)	234* (27)	23* (17)	379 (55)	0.64* (0.06)
Subsided-inland	126 (35)	140 (28)	186 (19)	452 (46)	0.41 (0.07)

[a]Each value is the mean of four observations. Value in parentheses is the $s_{\bar{x}}$.

*Asterisk indicates significant difference ($P < 0.05$) between streamside and subsided-inland values.

inland Spartina (Table 8) indicated further the greater degree of stress to which these plants are exposed. The actual environmental factors creating this stress can only be speculated upon, but probably include low nutrient conditions, constant waterlogging, and/or potentially toxic compounds (e.g. H_2S, ethylene, organic acids, etc.) formed in an anaerobic environment.

Brackish marsh habitat

Spartina patens and S. alterniflora collected from a newly created dredge-spoil area had significantly higher ATP, ADP, and total adenylate concentrations than those plants from an adjacent natural marsh (Table 9). However, only the E.C. ratio of S. alterniflora was significantly higher in the dredge-spoil area than the natural marsh, while that for S. patens was not significantly different.

The data indicated that those plants growing on the dredge-spoil site were of a higher energy status (i.e. less stressed) than those on the natural marsh. Newly deposited dredge material may indeed provide a more suitable site for plant growth than a mature natural marsh since 1) dredge material may be a source of unexploited nutrients, 2) at the higher elevation of the dredge site, nitrogen (often a limiting nutrient to marsh plant growth) mineralization will be greater due to the more aerobic environment, and 3) a new dredge site is initially unvegetated, biotic competition is at a minimum. The higher ATP, ADP, and total adenylate concentrations of the dredge-spoil vegetation would tend to support this theory.

Fresh water marsh habitat

The E.C. ratio of Sagittaria falcata collected from an impounded fresh water marsh was significantly higher than that from an unimpounded adjacent marsh (Table 10). The ATP, ADP, AMP, and total adenylate

Table 9

ATP, ADP, AMP, Total Adenylate Concentration (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio of *Spartina patens* and *Spartina alterniflora* Leaf Tissue Collected from a Dredge Spoil Site and a Natural Salt Marsh Near Leeville, Louisiana

Sampling site by species	n	ATP	ADP	AMP	Total adenylates	E.C. ratio
S. patens						
Dredge	8	171* (34)[a]	180* (13)	39 (12)	370* (44)	0.66 (0.02)
Marsh	8	73 (15)	75 (10)	19 (7)	202 (39)	0.65 (0.04)
S. alterniflora						
Dredge	4	195* (42)	250* (27)	44 (36)	390* (119)	0.69* (0.05)
Marsh	4	51 (4)	86 (17)	36 (5)	235 (65)	0.54 (0.01)

[a]Value in parentheses is the $s_{\bar{x}}$.

*Asterisk indicates significant difference ($P < 0.05$) between dredge and marsh values.

Table 10

ATP, ADP, AMP, Total Adenylate Concentration (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio in Sagittaria falcata Leaf Tissue from an Impounded and Non-Impounded Fresh Water Marsh Near Galliano, Louisiana

Sampling site	n	ATP	ADP	AMP	Total adenylates	E.C. ratio
Impounded	10	332* (32)[a]	369 (23)	101 (16)	802 (54)	0.65* (0.02)
Non-impounded	10	243 (29)	400 (31)	114 (20)	708 (53)	0.58 (0.02)

[a]Value in parentheses is the $s_{\bar{x}}$.

*Asterisk indicates significant difference ($P < 0.05$) between impounded and non-impounded values.

concentrations of Sagittaria leaf tissue from the two areas were not significantly different, although ATP and total adenylate concentrations were somewhat greater in the impounded marsh (Table 10).

The results from the fresh water marsh habitat depict a case in which our preconceived ideas of what constituted a stressed environment disagreed with what the E.C. ratio specified as being stressed. Sagittaria falcata is a fresh water marsh species which is well adapted to a waterlogged environment. The constant waterlogging conditions of an impounded marsh may not constitute a stress for this species. Also, the possibility exists that the "natural" marsh might be in some way, undetected by the investigators, stressed to a greater extent than the impounded site. We must also consider that not all stresses will necessarily reduce the energy charge ratio. If a stress increases respiration but does not significantly reduce photosynthesis, the ATP concentration and E.C. ratio may increase. Pell and Brennan (1973) found that ozone stress stimulated ATP production in Pinto bean leaves while photosynthesis decreased and respiration increased.

The results found in the fresh water marsh show the necessity of testing under controlled conditions the response of this index to a variety of stresses. In this way, the effect of specific stresses on adenylate patterns may be discerned.

Cypress swamp habitat

No significant differences were found in adenylate concentrations or E.C. ratio of bald cypress, Taxodium distichum, trees from an impounded swamp and an adjacent swamp managed as a crayfish farm (Table 11). Primary productivity data for bald cypress also showed little difference (William H. Conner, personal communication, Center for Wetland Resources, Louisiana State University, Baton Rouge) between these two areas.

Table 11

ATP, ADP, AMP, Total Adenylates (nmoles g^{-1} dry wt) and Energy Charge (E.C.) Ratio in Bald Cypress, Taxodium distichum and, Red Maple, Acer rubrum var. drummondii Leaf Tissue from an Impounded Swamp and a Swamp Managed as a Crayfish Farm Near Lower Vacherie, Louisiana

Plant species	n	ATP		ADP		AMP		Total adenylates		E.C. Ratio	
		I[a]	C[b]	I	C	I	C	I	C	I	C
Cypress	10	165 (8)[c]	173 (15)	140 (10)	122 (16)	42 (8)	34 (12)	350 (9)	319 (30)	0.67 (0.02)	0.71 (0.02)
Maple	10	306* (17)	161 (22)	163 (16)	146 (17)	48 (11)	55 (13)	514* (18)	355 (31)	0.76* (0.03)	0.64 (0.03)

[a] Impounded swamp

[b] Crayfish farm

[c] Value in parentheses is the $s_{\bar{x}}$.

* Asterisk indicates significant difference ($P < 0.05$) between impounded swamp and crayfish farm values with a plant species.

Red maple, Acer rubrum var. drummondii, a dominant understory tree, possessed significantly higher ATP and total adenylate concentrations and E.C. ratio in the impounded site than in the crayfish farm (Table 11). Unlike the impounded fresh water marsh habitat, the reason for the higher E.C. ratio in the impounded cypress swamp was apparent. The cypress canopy in the impounded site was much more open than in the crayfish farm; thus more light penetrates to the understory vegetation. The maple leaves from the impounded site were visibly a lighter color green than those from the crayfish farm. Leaves exposed to high insolation are generally a light green color due to photooxidation of chlorophyll. However, at high light intensities the amount of chlorophyll has a negligible effect upon the rate of photosynthesis (Bannister, 1978). Thus, the high E.C. ratio for red maple in the impounded area reflects the more suitable light conditions to which this plant was subjected.

These results demonstrate the point that the adenylate composition within a plant is a net result of all environmental variables impinging upon that plant. Thus, a potential stress may be counteracted by beneficial levels of another factor. In this case, beneficial light conditions tended to be most important in determining the difference in red maple E.C. ratios between the two sites.

CONCLUSIONS

We have developed a method for measuring sublethal stress via changes in adenylate composition and energy charge ratio for the dominant macrophytes in each of the major coastal plant communities in Louisiana. This method, which utilizes bioluminescent light generation resulting from the reaction of ATP with the luciferin-luciferase substrate-enzyme complex, is extremely sensitive, reproducible, and is suitable for field sampling. Extraction of the adenylates from frozen-lypholized tissue in a boiling

EDTA-PVPP solution produced the highest ATP concentrations and recovery rates.

The results of this study indicate that adenylate patterns and/or E.C. ratio can delineate sublethal stress resulting from point sources of pollution such as petroleum hydrocarbon and from the impact of cumulative stresses such as found in subsided-inland Spartina marshes.

Adenylate composition proved to be very sensitive to petroleum hydrocarbon exposure. We feel that this index has great potential for defining the degree of sublethal stress associated with oil spills in wetland communities. Future research will be directed toward fine-tuning this index for monitoring stress due to petroleum hydrocarbon exposure.

Habitats that are naturally stressed such as subsided-inland Spartina marshes are easily delineated by the E.C. ratio. Although from visual observation alone it is obvious that the subsided-inland area is stressed, this example provides further evidence for the validity of this method.

Sampling of the dredge spoil site and the impounded cypress swamp demonstrated that more subtle differences in energy status of plants can be detected using adenylate composition. The apparently more favorable environment of the dredge spoil site and the higher solar penetration occurring in the impounded swamp was readily detected by E.C. ratio methodology, even though these differences were not evident from field observation.

The investigation of the energy status of Sagittaria falcata in a fresh water marsh demonstrated another important point. The E.C. ratio of this plant from the impounded site was significantly higher than that of the natural marsh. This observation was in opposition to our preconceived concept of impounded marshes as stressed habitats. As discussed in the RESULTS and DISCUSSION section of this report, there may be a number

of reasons for this discrepancy. This type of results makes evident the need for controlled studies of the effect of a number of natural environmental stresses on adenylate composition.

The strong relationship found between aboveground standing crop and E.C. ratio of Spartina alterniflora along the transect east of Barataria Bay suggests that the E.C. ratio may be a suitable means of measuring very subtle changes in the growth potential of this species. If so, this index may provide a means by which coastal habitats could be mapped on the basis of their potential for primary production.

In general, this study has provided preliminary information indicating the suitability of adenylate composition and/or E.C. ratio as a monitor of environmental stress in coastal plant communities. However, further testing of this method under both field and laboratory conditions is required before a final conclusion concerning its ultimate use can be stated.

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