Proc. Nat. Acad. Sci. USA
Vol. 72, No. 6, pp. 2112–2115, June 1975

Use of the Adenylate Energy Charge Ratio to Measure Growth State of Natural Microbial Communities

(ATP–ADP–AMP ratios/microbial growth/estaurine sediments/assessment of environmental changes/ocean water)

WILLIAM J. WIEBE AND KEITH BANCROFT

Department of Microbiology, University of Georgia, Athens, Ga. 30602

Communicated by Eugene P. Odum, March 7, 1975

ABSTRACT **Measurement of the adenylate energy charge ratio is proposed as a means of determining the growth state of natural microbial communities and the effect of environmental changes on them. Observations on microbial cultures and on natural microbial populations from the Western North Atlantic Ocean water and from sediments of a coastal salt marsh show that energy charge measurements do show the metabolic state of communities as well as species populations.**

The extension of the adenosine triphosphate (ATP) assay to measure community microbial biomass (1) has stimulated a great deal of research on the natural distribution of microorganisms. The method is rapid, simple, and extremely sensitive, if somewhat lacking in accuracy. In this paper, we propose the use of the adenylate energy charge (E.C.) relationship (2) so that, in addition to biomass, a comparative "state of growth" of natural microbial communities can be ascertained.

The concept and biochemical application of the E.C. phenomenon have been described in recent years, primarily by Atkinson and colleagues (2–4). The ratio is calculated by the formula:

$$\text{E.C.} = \frac{\text{ATP} + {}^1/_2\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

The derivation of the formula has been explained by Atkinson (3). The basic premise is that an organism attempts to maintain a particular cellular ratio of ATP to adenosine diphosphate and monophosphate (ADP, AMP) and that this ratio depends upon the state or phase of growth (i.e., lag, logarithmic, stationary, death phase). Chapman *et al.* (2) demonstrated that a wide variety of organisms, both eukaryotes and prokaryotes, maintain essentially the same E.C. ratio when in the same growth phase. Actively growing and dividing cells have an E.C. ratio of 0.8–0.95; cells in stationary growth phase maintain a ratio of about 0.6; and scenescent or resting phase cells have a ratio below 0.5. Since this appears to be a general property of organisms, one should be able to estimate the relative or average growth state for an entire microbial community. In this paper we examine the E.C. ratios of natural and manipulated communities and discuss the possible applications and limitations of this technique.

METHODS AND MATERIALS

Adenylate Extraction and Analysis. Adenylate was extracted by the boiling $NaHCO_3$ method of Bancroft *et al.* (5). For extraction from marine sediments this technique was superior to all those tested and for extraction from water it was equivalent to the boiling Tris buffer method of Holm-Hansen and Booth (1). The efficiency of adenylate extraction from each natural material was determined by the method of Christian *et al.* (6). The methods of adenylate analysis were those described by Chapman *et al.* (2). Background concentrations of ATP, ADP, and AMP were measured for all samples.

Open Ocean Samples. Samples of open ocean water were taken in a transect from the Bahamas to Puerto Rico; the station locations are shown in Table 1. Material was collected in 30-liter Niskin samplers and filtered through 0.45-μm Gelman glass fiber filters. Filtration took up to 30 min. Just before the filter was pulled to dryness, it was removed and rapidly placed in 5 ml of boiling 0.1 M $NaHCO_3$ for 1 min. The filter was removed and the extract was immediately frozen at −20° for subsequent analysis.

Sediment Samples. Sediment cores were taken from two biologically and physically distinct zones in the intertidal *Spartina alterniflora* salt marsh at Sapelo Island, Georgia. The material was screened through a 1 mm nylon mesh to eliminate macro-organic matter, particularly the living and dead roots of *S. alterniflora* (6). The screened samples were immediately extracted, processed by the method of Christian *et al.* (6), and frozen at −20° for subsequent analysis.

Sediment Slurries. Aerobic sediment slurries consisted of two parts 0.22-μm membrane-filtered artificial seawater (ASW) with one part sediment. This material was placed in flasks and stirred. When the E.C. values had stabilized, amendments to the material were made. At day 0 all flasks received 1 mg of $(NH_4)_3PO_4$ per g dry weight of sediment. Experimental vessels received 1.25 mg of glucose in ASW per g dry weight of sediment; control vessels received an equal volume of filtered ASW. The $(NH_4)_3PO_4$ was added to prevent these inorganic nutrients from becoming limiting. Measurement at the end of 12 days confirmed the presence of free NH_3 and PO_4^{3-} in the medium. Slurries were incubated at 25°.

Pure Culture Studies. The E.C. ratios of suspensions of *Enterobacter aerogenes* were measured at intervals over growth cycles in an ASW/peptone/yeast extract medium (8). Suspensions of *Pseudomonas aeruginosa* were assayed during diauxic growth in the mineral salts/succinate/glucose medium described by Hylemon and Phibbs (7). In all cases cultures were incubated at 25°. Growth was assayed by optical density using a Klett-Summerson colorimeter (no. 66 filter).

Abbreviations: E.C., energy charge; ASW, artificial seawater.

TABLE 1. *Location of sampling stations*

Station no.	Location	Longitude	Latitude	Depth (m)
1	Tongue of the Ocean	24° 00'N	77° 20'W	1420
2	NE Providence Channel	25° 25'N	77° 13'W	3600
3	E of Eleuthera Island	25° 18'N	75° 50'W	4480
4	E of Mayaguana Passage	23° 23'N	73° 00'W	5125
5	E of Turks Island	21° 49'N	70° 45'W	6500

Listed are locations in the Bahamas epicontinental sea and in the Western North Atlantic and total water depth of stations of Research Vessel *Eastward* Cruise E-1C-74, Jan. 21–30, 1974, at which ATP content and energy charge of the microbial community were measured. See Fig. 3.

RESULTS

Over the growth cycle E.C. ratios for *E. aerogenes* and *P. aeruginosa* (Fig. 1) were identical to those noted by Chapman *et al.* (2). The effects of diauxic growth on the E.C. ratio of *P. aeruginosa* are shown in Fig. 2. During the transitional period, when succinate was depleted and enzymes enabling growth at the expense of glucose were synthesized, growth ceased and the E.C. ratio dropped to about 0.7. Then as growth resumed the E.C. ratio rose to 0.8.

At five open ocean stations ATP content and E.C. ratios were correlated with depth of sampling. ATP was highest in samples from the surface and then dropped to a low constant value (Fig. 3a and b). The E.C. ratios clustered near 0.6, which coincides with the ratio characteristic of a stationary phase of growth for pure cultures of organisms. The only exception was at 400 m, where the E.C. ratio increased to 0.7–0.8 at stations 1, 3, and 4.

The E.C. ratios for salt marsh sediment micro-organisms are shown in Fig. 4a and b. The values for samples taken in February were low, regardless of depth, but rose in samples obtained during April and July to approach 0.6. While there are major biological and physical differences between the high marsh and levee zones (6), too few samples have been analyzed thus far to determine whether there are significant differences in the E.C. ratios of samples from these two zones.

The effect of substrate addition on the E.C. ratio of sediment micro-organisms is shown in Table 2. When sediment samples were homogenized and stirred, there were some fluctuations in the E.C. ratios but values remained below 0.6. After the addition of substrate there was an increase in the E.C. ratio, which reached about 0.9 by the third day. By the twelfth day the E.C. ratios of the communities had returned to less than 0.6. There was also a real but lower increase in the E.C. ratios in control flasks. This was most likely due to the addition of $(NH_4)_3PO_4$. In preliminary studies this addition produced a transient stimulation of CO_2 evolution (R. R. Christian and W. J. Wiebe, unpublished).

DISCUSSION

The results of studies with pure culture were similar to those of other workers and were undertaken to provide a "control" on the method. However, one aspect of these results has not received attention. The break between log and stationary E.C. values precedes the observed change in growth rate. This same "anticipation" phenomenon was observed when

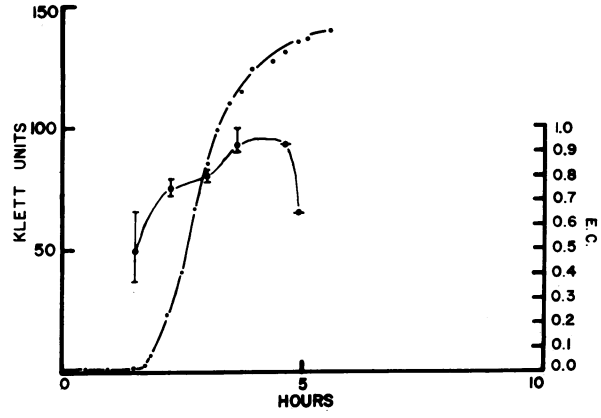

FIG. 1. Adenylate energy charge ratios for *E. aerogenes* during various phases of the growth cycle in ASW/peptone/yeast extract medium (●, Klett units; ⊺, energy charge).

ATP alone was measured (5) or when a different metabolic parameter, respiration, was examined (9). It appears that even before the apparently linear logarithmic growth ended there was a shift in metabolic function.

The E.C. ratios for cells undergoing diauxie (Fig. 2) provide an example of the sensitivity of the E.C. ratio assay for following cellular metabolism. While the shift-over from succinate to glucose metabolism takes only 10–15 min, there is rapid adjustment in the E.C. ratio. Chapman *et al.* (2) noted that a variety of physical changes (centrifugation, filtration, deoxygenation) affect the E.C. ratio. Observing cultures during diauxie revealed that the E.C. ratio can be used to track subtle and rapidly adjusted biological phenomena.

The distribution of ATP in open ocean water columns (Fig. 3a) was similar to that reported for recent examinations of oligotrophic ocean waters (10–12). The E.C. ratios (Fig. 3b) deviated from 0.6 only at the 400 m depth. Hamilton *et al.* (11) showed that an increase in particulate nitrogen and carbon often occurs at 400–600 m in Pacific Ocean waters. The increase in particulate matter was "not reflected in the distribution of either ATP or DNA with depth." We also found that ATP concentrations at 400 m were similar to those of the samples from deeper waters. Two points are illustrated

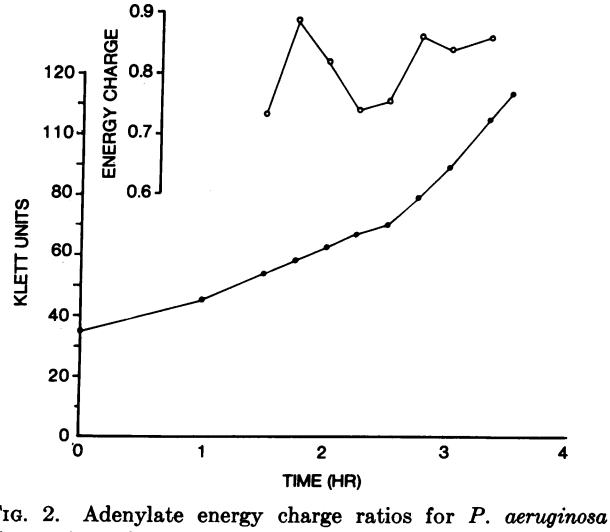

FIG. 2. Adenylate energy charge ratios for *P. aeruginosa* during various phases of a diauxic growth cycle in mineral salts/succinate/glucose medium (●, Klett units; ○, energy charge).

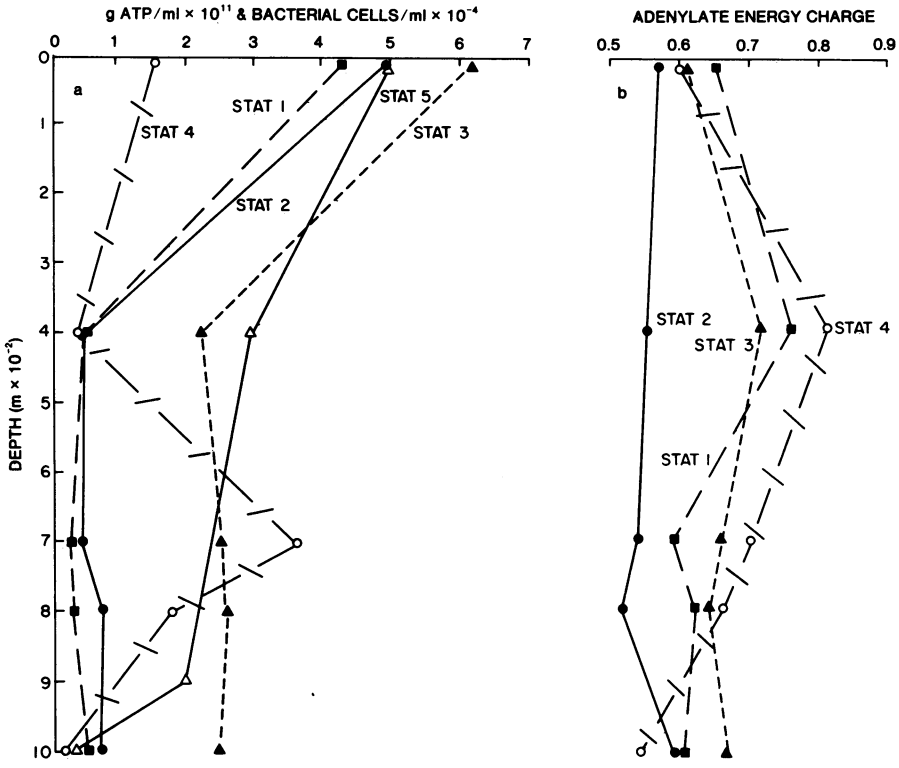

FIG. 3. ATP and estimated number of bacterial cells (a), and adenylate energy charge ratios (b) of micro-organisms in samples representing ocean profiles of upper 1000 m (for locations see Table 1).

by these observations. First, community density need not change for the E.C. ratio to change. Growth state is independent of population density. Second, by using the E.C. ratio, one can rapidly identify specific regions of microbial activity. This screening process permits investigators to focus their activity on potentially important regions and examine them in more quantitative metabolic terms, such as oxygen uptake or substrate metabolism.

According to our E.C. ratio data, deep oceanic microbial communities appeared on the average to be in the stationary growth phase. These observations represent the first direct evidence that such communities need not represent scenescent populations. There has been some controversy for years (compare ref. 13) as to whether deep-sea micro-organisms were simply sinking scenescent populations of surface organisms, resting in stationary phase, or actively growing. Because filtration lowers the E.C. of at least pure cultures of bacteria (2), we were unable to distinguish between logarithmic and stationary growth phases in these populations. Since filtration would probably reduce the E.C. ratio of the communities as well, the micro-organisms in the samples are probably at least in stationary phase.

Microbial communities in Sapelo Island sediment responded differently. Populations were dense (around 10^9 bacterial cell equivalents per g dry weight of sample). (See ref. 6 for an explanation of this calculation.) The E.C. ratios were lowest in winter and rose in spring and summer. The rise coincided with an increase in plant growth and (presumably) root exudation into the rhizosphere. While individual microbial populations, for example methane producers, probably became active, the community average always stayed below 0.6. This low estimate of growth activity agrees with results of other assays on salt marsh sediment. Calculations based on the work of Christian *et al.* (6) and the modelling efforts of Wiegert *et al.* (14) suggest that the realized generation time of micro-organisms in the sediments is of the order of 50–60 days. While this community maintained a high standing stock of organ-

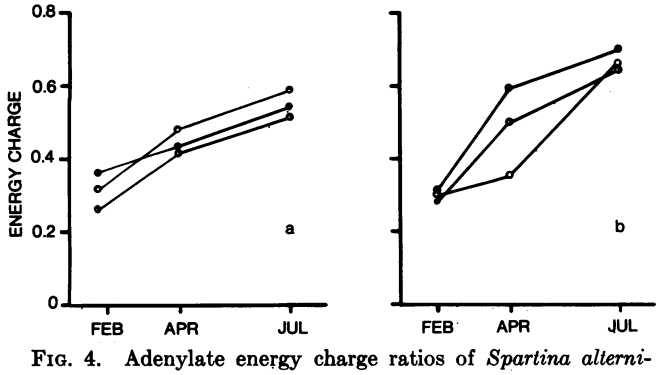

FIG. 4. Adenylate energy charge ratios of *Spartina alterniflora* salt marsh sediments. (a) High marsh; (b) levee zone (●, 0–1 cm; ◑, 5–10 cm; ○, 15–25 cm).

TABLE 2. *Glucose amendment increases energy charge of micro-organisms in stirred sediment*

Flask no.	Glucose added	$(NH_4)_3PO_4$ added	Days before and after				
			−1	0*	1	3	12
1,2	+	+	0.25	0.51	0.54	0.95	0.52
3,4	−	+	0.40	0.38	0.36	0.68	0.57

* Glucose and $(NH_4)_3PO_4$ added.

isms, little overall growth was noted. The effect of substrate addition on the sediment E.C. ratio (Table 2) was consistent with the hypothesis that the system comprised a high biomass at low growth rate. There was a transient increase in the E.C. ratios followed by a return to the initial state.

These preliminary studies illustrate the types of information that monitoring E.C. ratios can provide. As a qualitative index, it provides insight into the growth activity of systems. E.C. ratio changes due to perturbations of a natural community may rapidly reveal, for example, the effects of pollutants such as mercury, herbicides, and others on microbial communities. The effect on populations of encountering new environments could also be examined (e.g., freshwater organisms entering an estuary or micro-organisms enduring changes in soil). Because the E.C. ratio precisely mirrors an organism's metabolic status, such studies should prove valuable in predicting the outcome of altering environmental features.

The use of the E.C. ratio directly to assess the growth state of populations is difficult and needs more work before unquestionably consistent interpretations are possible. Drastic alteration of the E.C. ratio by centrifugation complicates interpretation of data on micro-organisms in the water column. In these studies, samples were raised from depth and filtered. Even so some E.C. values (400 m) approached those of an active growth state. However, we do not know whether natural populations change E.C. ratios as rapidly, or even in the same direction, as pure cultures.

Another problem is the presence of animals in natural samples. Multicellular animals appear to maintain a high E.C. ratio until they are moribund (3). Any samples with a significant number of animals might yield falsely high community E.C. ratios. To our knowledge E.C. ratios for protozoa have not been obtained.

We propose that the analysis of E.C. ratios for natural microbial communities offers promise as a diagnostic tool for the estimation by microbial ecologists of community growth state. In addition, a rapid and sensitive method for examining total community growth response is now available for measurement of the effects of environmental changes. Even so, determination of E.C. ratios should not be considered an end in itself. Quantitative assays of oxygen consumption, substrate uptake, and other phenomena should still be carried out. Finally, as a practical matter, it is no more difficult logistically to extract total adenylates than ATP. Thus, for only a little extra analytical effort one can determine E.C. ratios for the same sample and with the same equipment used to measure ATP.

We gratefully acknowledge Dr. L. R. Pomeroy's aid in arranging the oceanographic portions of this work, and the assistance of W. B. Campbell, R. R. Christian, J. E. Yetka, and B. Mannes. This work was done in part on cruise E-1C-74 of the Research Vessel *Eastward*, operated by Duke University with support from the National Science Foundation. Other aspects of this work were supported by Grant DES72-01605-A02 from the National Science Foundation and by grants from the Sapelo Island Research Foundation. This is Contribution no. 290 from the University of Georgia Marine Institute.

1. Holm-Hansen, O. & Booth, C. R. (1966) *Limnol. Oceanogr.* **11**, 510–519.
2. Chapman, A. G., Fall, L. & Atkinson, D. E. (1971) *J. Bacteriol.* **108**, 1072–1086.
3. Atkinson, D. E. (1971) *Annu. Rev. Microbiol.* **23**, 47–68.
4. Atkinson, D. E. (1971) in *Metabolic Pathways*, ed. Greenberg, D. M. (Academic Press, New York), 3rd Ed., Vol. 5, pp. 1–20.
5. Bancroft, K., Paul, E. A. & Wiebe, W. J. (1974) *Bacteriol. Proc.* **74**.
6. Christian, R. R., Bancroft, K. & Wiebe, W. J. (1975) *Soil Science*, in press.
7. Hylemon, P. B. & Phibbs, P. V., Jr. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1041–1048.
8. Lyman, J. & Fleming, R. H. (1940) *J. Mar. Res.* **3**, 134–146.
9. Yetka, J. E. (1974) *Appl. Microbiol.* **28**, 1033–1039.
10. Holm-Hansen, O. (1969) *Limnol. Oceanogr.* **14**, 740–747.
11. Hamilton, R. D., Holm-Hansen, O. & Strickland, J. D. H. (1968) *Deep-Sea Res.* **15**, 651–656.
12. Holm-Hansen, O., Strickland, J. D. H. & Williams, P. M. (1966) *Limnol. Oceanogr.* **11**, 548–561.
13. Vaccaro, R. F. (1969) *Limnol. Oceanogr.* **14**, 726–735.
14. Wiegert, R. G. Christian, R. R., Gallagher, J. L., Hall, J. R., Jones, R. D. H. & Wetzel, R. L. (1975) in *Recent Advances in Estuarine Research*, ed. Costlow, J. (Academic Press, New York), in press.