Bull. Environ. Contam. Toxicol. (1986) 36:1–8

Environmental Contamination and Toxicology

Use of Adenylate Energy Charge as a Physiological Indicator in Toxicity Experiments

Zubir B. Din* and James M. Brooks

Department of Oceanography, Texas A&M University, College Station, TX 77843

For many years, the major criterion used to determine an organisms response to a toxicant stress has been the measurement of an LC_{50} value which is operationally defined as the concentration of a pollutant that will cause 50 percent mortality in the test organisms in a designated exposure period. These bioassay results are used by regulatory agencies such as the United States Environmental Protection Agency (EPA) to establish maximum allowable concentrations of pollutants in discharge effluents. Grice (1974) commented that the results of these and similar laboratory tests have limited ecological value since the results do not reflect the physiological state of the "unaffected" organisms. Consequently, these values cannot be used to predict the long-term effects of toxicants on organisms or ecosystems.

More recently, investigators have generally decreased the test levels of exposure, to concentrate instead on the physiological changes that are induced in organisms exposed to chronic or low levels of pollutants. Investigations with marine phytoplankton indicate that some chemical wastes which may seem dangerous, may cause no significant long-term effects at concentrations that can be detected in the ocean; in many instances these wastes may even stimulate algal growth (Dunstan et al. 1975; Murphy et al. 1981; and Din 1983).

One of the procedures that can be used in toxicity experiments to study organismal responses at the biochemical-cellular level is the measurement of Adenylate Energy Charge or AEC (Capuzzo 1981). The concept of Adenylate Energy Charge was popularized by Atkinson and Walton (1967) and is defined as:

$$AEC = \frac{(ATP) + (\frac{1}{2}\ ADP)}{(ATP) + (ADP) + (AMP)}$$

where ATP, ADP and AMP refer to the nucleotides adenosine triphosphate, adenosine diphosphate and adenosine monophosphate, respectively. Use of energy charge values as a physiological

*Present address: School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia

index has been extensive in recent years (Chapman et al. 1971; and Romano and Daumas 1981). Chapman and his co-workers found that for the bacterium, Escherichia coli, growth could occur only at energy charge values above 0.8. Bacterial viability was maintained at values between 0.5 and 0.8, but cells died at values below 0.5. The study also surveyed literature which indicated that these general limits are fairly characteristic of a wide variety of organisms.

This study was conducted to determine if values of Adenylate Energy Charge can be used as an index of sublethal stress for phytoplankton exposed to "toxic" materials. The results are contrasted with changes in biomass (chlorophyll a and ATP) as well as variations in primary production.

MATERIALS AND METHODS

The two chemical industrial wastes used as "toxic" materials in this study were the American Cyanamid waste (American Cyanamid Industrial Plant; Linden, New Jersey) and the DuPont Grasselli waste (E.I. duPont de Nemours and Company; New Jersey).

A centric diatom species Skeletonema costatum (Grev.) Cleve, was used as the test organism. This chain forming diatom of the family Thalassiosiraceae has been extensively used in various toxicity experiments (Atkinson et al. 1977) as well as in ocean dumping related research (Dunstan and Menzel 1971; Goldman and Stanley 1974; and Dunstan 1975). Droop (1962) proposed that this species should be ideal for toxicity studies since it has no absolute growth factor requirement other than for vitamin B_{12}.

The test diatom was first grown in "F/2" culture medium and maintained at log-phase of growth. At the beginning of each experiment, 100 µl aliquots of the diatom culture were inoculated into five 4-liter flasks each containing 3-liters of growth medium. The system was allowed to equilibrate, with stirring, for approximately one hour. Triplicate samples were then taken from each flask for measurement of primary production (^{14}C uptake), biomass (chlorophyll a and ATP) and adenylate energy charge (AEC). Aliquots of the waste were then added to the flasks, to give final concentrations of 0, 100, 500, 1000 and 4000 ppm by volume. Triplicate samples were taken at daily intervals for 4 days.

Radioactive carbon uptake was measured by the classic procedures of Steemann-Nielsen (1952), with modifications by Strickland and Parsons (1972). Samples were incubated for 5 hours and radioactivity was measured using a Model 6890 Tracor Analytic Delta 300 Liquid Scintillation system.

Chlorophyll a concentration was determined by the fluorometric method of Yentsch and Menzel (1963) as detailed by Strickland and Parsons (1972). Fluorescence measurements were carried out using a Perkin-Elmer Model 650-40 UV-Fluorescence Spectrophotometer.

For the determination of ATP concentration and AEC, subsamples (10 ml) were filtered through 0.45 µm Nuclepore polycarbonate filters, which were then placed in boiling Tris buffer solution for 3 minutes to extract the nucleotides and inactivate all enzymes. The samples were then frozen at -20°C prior to analysis (generally within one week).

ATP was analyzed using the methods of Holm-Hansen and Booth (1966), Shoaf and Lium (1976) and Hodson et al. (1976). Computation of the adenylate energy charge followed the methods of Wiebe and Bancroft (1975) and Holm-Hansen and Karl (1978). In these procedures, the nucleotides ATP, ADP and AMP were determined by the bioluminescence method using firefly luciferase. Firefly extract (Sigma Chemical Co., FLE-50) was used as a primary source of luciferase. Light intensities produced by the reactions were measured by a Model 3000 SAI Technology Integrating Photometer. Duncan's multiple range test ($\alpha = 0.05$) of replicate samples was used to determine statistical difference.

RESULTS AND DISCUSSION

The growth and production of Skeletonema costatum was inhibited at the highest concentration (4000 ppm) of the American Cyanamid waste, while enhancement was indicated at a level of 100 ppm and to a lesser extent at 500 ppm (Figures 1 and 2). This stimulation of growth was observed in both the time course changes in biomass as well as in radioactive carbon uptake.

The time course changes in AEC showed that the responses of the algae to this particular waste at a concentration of 4000 ppm could be irreversible since the AEC ratio dropped to about 0.70 at Day 1 and continued decreasing to about 0.60 on Day 4. This is contradictory to the changes in biomass, which showed chlorophyll and ATP synthesis at least on the first day of exposure to 4000 ppm waste concentration. Based on the AEC values, the few diatoms remaining in the 4000 ppm flask may have remained barely alive at the end of the experimental period, since the energy charge ratio was about 0.6.

When exposed to the DuPont waste, the responses of S. costatum were somewhat different. Figure 3 indicates that cell growth was similar to the control or retarded at all waste levels studied, although more so at the 4000 ppm level. However, even at 4000 ppm waste concentrations, the cell density on the last day of the experiment was similar to the value on the first day [first day the chlorophyll (µg/L), ATP (µg/L) and ^{14}C uptake (ppm) were 0.31±0.04 S.D., 0.06±0.01 and 540±80, respectively; fourth day they were 0.15±0.06, 0.05±0.01 and 370±45, respectively]. Changes in the AEC ratio tracked the time course changes in biomass and production closely (Figures 3 and 4). At the 4000 ppm level, the cellular biomass remaining in the flask at the end of the 4-day experiment maintained a AEC value in excess of 0.7 which indicated that they were viable even if there was little evidence of growth. It appears that the growth of this diatom

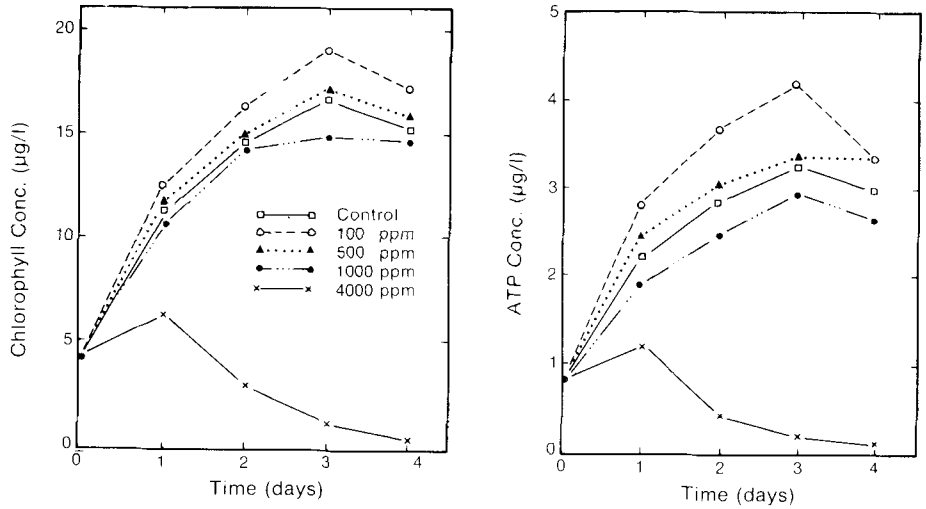

Figure 1. Changes in biomass, as measured by chlorophyll a and ATP concentrations of Skeletonema costatum exposed to the American Cyanamid Waste.

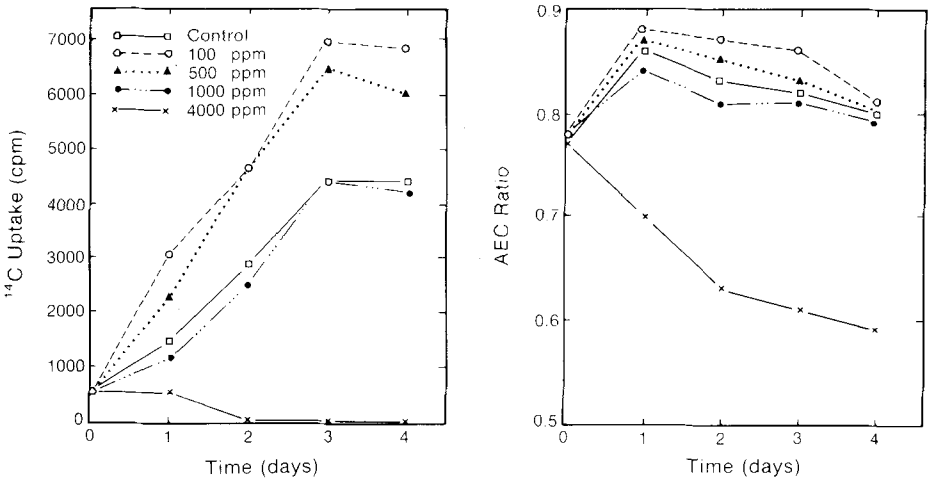

Figure 2. Changes in primary production (radioactive carbon uptake) and Adenylate Energy Charge ratios of Skeletonema costatum exposed to the American Cyanamid Waste.

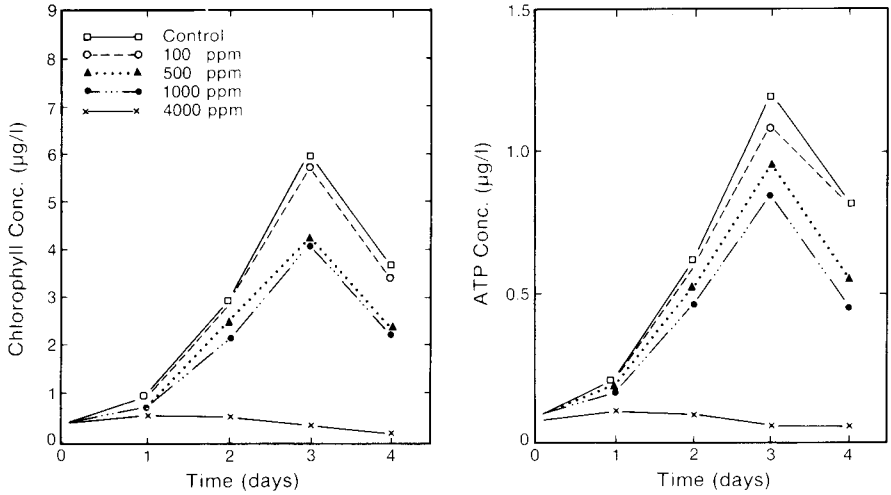

Figure 3. Changes in biomass, as measured by chlorophyll a and ATP concentrations of Skeletonema costatum exposed to the DuPont waste.

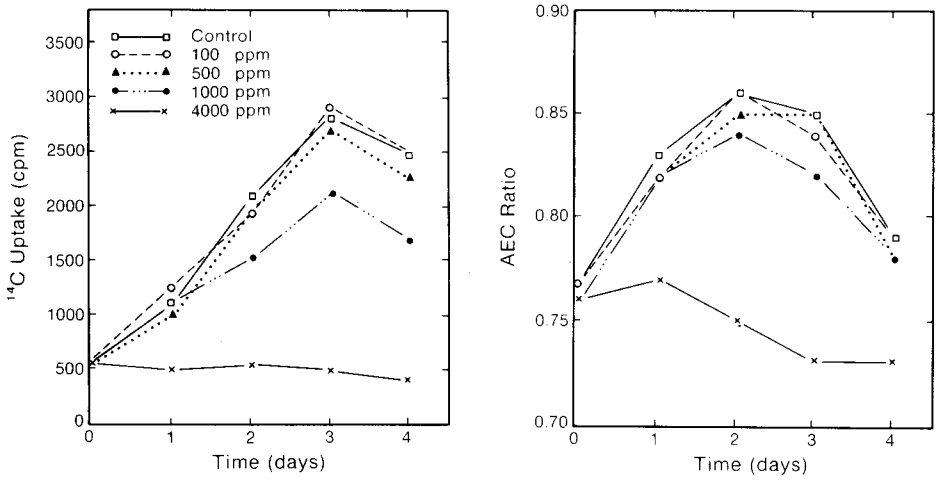

Figure 4. Changes in primary production (radioactive carbon uptake) and Adenylate Energy Charge ratios of Skeletonema costatum exposed to the DuPont waste.

may only be temporarily inhibited by the presence of the DuPont waste up to a level of 0.4% and that no irreversible physiological damage was incurred.

Both wastes at a concentration of 4000 ppm caused reductions in cellular biomass and primary production of the test diatom relative to the control. From the values of the AEC ratio it could be inferred that these reductions were the consequences of both cell death and reduced cell growth. When exposed to 4000 ppm of the American Cyanamid waste the AEC ratio of the algae decreased to about 0.6 at the end of the experimental period. According to Chapman et al. (1971) organisms with an AEC ratio of 0.60 are already at their lower limits of viability. Moribund cells have values of 0.50 or less. By contrast, when exposed to 4000 ppm of the DuPont waste the AEC ratio of the diatoms was reduced to only about 0.72, well within the range of viable cells. If these diatoms were to be transferred to a "clean" medium, immediate recovery would be anticipated.

At a level of 100 ppm, the presence of the American Cyanamid waste caused an excess of cellular biomass and primary production of the diatom relative to the control. Stimulation of growth at low levels of exposure of industrial wastes have been reported by Dunstan et al. (1975) and Murphy et al. (1981). The AEC values for the diatoms exposed to this concentration of the waste were generally found to be slightly higher than the control. However, these values were not statistically different ($\alpha > 0.05$).

The results from this study suggest the potential of using the AEC ratio as an index of physiological stress in toxicity experiments involving organisms such as phytoplankton. Its importance as an ecophysiological indicator has also been emphasized by Wiebe and Bancroft (1975) and Romano and Daumas (1981). These studies also illustrate the usefulness of AEC ratios as a qualitative index of growth activity. AEC data can be exceedingly important in supplementing routine measurements of biomass and primary production as well as LC_{50}. In addition, Wiebe and Bancroft (1975) have indicated that AEC ratios are independent of community density.

As with any new procedure, determination of AEC ratios has its share of problems. Although its potential in laboratory bioassays is clearly demonstrated, its use in the natural environment still needs further investigation. AEC values precisely reflect an organism's metabolic status; yet it is difficult to use them directly to assess the growth state of a population for several reasons. First, the presence of zooplankton and other animals in a natural population complicates interpretations. Atkinson (1971) reported that multicellular animals appear to maintain a high AEC ratio until they are moribund and any sample with a significant number of animals might yield a false community AEC ratio. Second, proper sample handling is essential to ensure that the AEC values are not altered during sample collection and analysis. Drastic alteration of the AEC ratio by

physical stresses such as centrifugation or filtration has been noted by Wiebe and Bancroft (1975).

In conclusion, the AEC ratio is a reliable index for determining sublethal and chronic effects of "toxic" materials to phytoplankton. The analytical method is rapid, simple and extremely sensitive. The results from this particular study show that AEC values greatly complement data pertaining to cellular biomass (chlorophyll a and ATP concentrations) and primary production. Based on the AEC values, we were able to infer to some extent, the growth conditions of the organisms. The AEC values also provided information as to whether or not any irreversible physiological damage had been incurred. These are the kinds of results that would be most useful to regulatory agencies which evaluate problems such as waste disposal and aquatic pollution.

Acknowledgements. This research was primarily supported by NOAA Ocean Dumpsite Research and Monitoring Program under grant numbers DOC-NOAA-NA80AA-D-00037 and 04-M0155. Instrumentation support was provided by the Center for Energy and Mineral Resources at Texas A&M University.

REFERENCES

Atkinson DE (1971) Regulation of enzyme function. Annu Rev Microbiol 23:47-68

Atkinson DE, Walton GM (1967) Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J Biol Chem 242:3239-3241

Atkinson LP, Dunstan WM, Natoli JG (1977) The analysis and control of volatile hydrocarbons concentrations during oil bioassays. Water, Air and Soil Poll 8:235-242

Capuzzo JM (1981) Predicting pollution effects in the marine environment. Oceanus 24:25-33

Chapman AG, Fall L, Atkinson DE (1971) Adenylate energy charge in Escherichia coli during growth and starvation. J Bacteriol 108:1072-1086

Din ZB (1983) Sublethal effects of industrial wastes on the growth and productivity of marine phytoplankton in a flow-through laboratory system. PhD Dissertation, Texas A&M University, 168 pp

Droop MR (1962) Organic micronutrients. In: Lewin RA (ed) Physiology and biochemistry of algae. Academic Press, New York, pp 141-159

Dunstan WM (1975) Problems of measuring and predicting influence of effluents on marine phytoplankton. Environ Sci Technol 9: 635-638

Dunstan WM, Menzel DW (1971) Continuous cultures of natural populations of phytoplankton in dilute, treated sewage effluent. Limnol Oceanogr 16:623-632

Dunstan WM, Atkinson LP, Natoli J (1975) Stimulation and inhibition of phytoplankton growth by low molecular weight hydrocarbons. Mar Biol 31:305-310

Goldman JC, Stanley HI (1974) Relative growth of different species of marine algae in wastewater-seawater mixtures. Mar Biol 28:17-25

Grice GD (1974) Controlled ecosystem pollution experiment. Oceanus 18:29-31

Hodson RE, Holm-Hansen O, Azam F (1976) Improved methodology for ATP determination in marine environments. Mar Biol 34:143-149

Holm-Hansen O, Booth CR (1966) The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol Oceanogr 11:510-519

Holm-Hansen O, Karl DM (1978) Biomass and adenylate energy charge determination in microbial cell extracts and environmental samples. Methods in Enzymology 57:73-85

Murphy LS, Hoar PR, Belastock RA (1981) The effect of industrial wastes on marine phytoplankton. In: Ketchum BH, Kester DR, Park PK (eds) Ocean dumping of industrial wastes. Plenum Press, New York, pp. 299-340

Romano JC, Daumas R (1981) Adenosine nucleotide "energy charge" ratios as ecophysical index for microplankton communities. Mar Biol 62:281-296

Shoaf WT, Lium BW (1976) The measurement of adenosine triphosphate in pure algal cultures and natural aquatic samples. J Res US Geol Survey 4:241-245

Steemann-Nielsen E (1952) The use of radioactive carbon (C^{14}) for measuring organic production in the sea. J Cons Int Explor Mer 18:117-140

Strickland J.D.H., Parsons TR (1972) A practical handbook of seawater analysis, 2nd edition. Bull Fish Res Bd Canada 167: 310 pp

Wiebe WS, Bancroft K (1975) Use of adenylate energy charge ratio to measure growth state of natural microbial communities. Proc Natl Acad Sci USA 72:2112-2115

Yentsch CS, Menzel WD (1963) A method for the determination of phytoplankton chlorophylls and phaeophytin by fluorescence. Deep-Sea Res 10:221-231

Received November 26, 1984; accepted January 31, 1985.