Bull. Environ. Contam. Toxicol. (1989) 43:261–270

Bulletin of Environmental Contamination and Toxicology

Application of Adenylate Energy Charge and Adenine Nucleotide Measurements as Indicators of Stress in *Nephtys incisa* Treated with Dredged Material

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Adenylate energy charge is an indication of the amount of energy available to an organism from the adenylate pool. It is calculated from measured concentrations of three adenine nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), which are integral to the energy metabolism of all organisms (Atkinson 1971). The AEC, defined as (ATP + 1/2 ADP)/(ATP + ADP + AMP), has a maximum value of 1.0 when all adenylate is in the form of ATP and a minimum value of 0 when all adenylate is in the form of AMP (Atkinson and Walton 1967). The energy charge has been considered important in the control of key catabolic and anabolic pathways (Atkinson 1971). Values of energy charge correlate with physiological condition: energy charges between 0.8 and 0.9 are typical of organisms which are actively growing and reproducing, usually under optimal environmental conditions (Ivanovici 1980; Vetter and Hodson 1982; Zaroogian et al. 1982; Geisy et al. 1983; Hoya et al. 1983). Values in the range of 0.5 to 0.7 have been observed in stressed organisms, (Wijsman 1976; Ivanovici 1980; Vetter and Hodson 1982; Zaroogian et al. 1982) and whose growth and reproduction rates are reduced (Chapman et al. 1971). Values below 0.5 have been associated with irreversible loss of viability under detrimental conditions (Wijsman 1976; Vetter and Hodson 1982). If these relationships apply generally, a knowledge of the energy charge of key species with known responses to particular environmental conditions would provide a convenient measure to assess the extent to which these species are stressed. Sediment from a relatively clean site in Long Island Sound and a highly contaminated sediment from Black Rock Harbor which contained high concentrations of PCBs, PAHs and some metals were used to determine if any observable stressful effect as indicated by AEC was due to the physical action of the suspended material rather than to a toxic compound. Of the many studies that reported the use of AEC as a measure of stress, several have considered the importance of extraction and handling technique (Wadley et al. 1980; Mendelssohn and McKee 1981; Sklar and McKee 1984).

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Initial attempts at measuring AEC in Nephtys incisa yielded low values. A low energy charge most likely indicates a poor extraction of adenine nucleotides in tissue samples from individuals collected from a nonlimiting environment. Thus, there was a need to develop an extraction and handling procedure for N. incisa that consistently produced values for AEC ratios that were representative of in vivo values reported for actively metabolizing cells, (Atkinson 1977).

The objective of this study was to evaluate the applicability of AEC as a measure of stress in N. incisa treated with dredged material under laboratory conditions and to determine the degree of variability inherent in the test.

MATERIALS AND METHODS

Reference sediment (REF) for this study was collected from a reference site in Central Long Island Sound, U.S.A. (40° 7.95N and 72° 52.7W) and was reported to contain PCBs (39ng g^{-1}), PAHs (4500 ng g^{-1}) and the metals Cu and Cr at 60 and 50 μg g^{-1} dry weight respectively (Lake et al., 1988). The sediment was collected with a Smith-McIntyre grab sampler (0.1-m^2) and was returned to the laboratory where it was press sieved (wet) through a 2-mm mesh stainless steel screen, homogenized in a tub and stored in polypropylene or glass containers at 4°C until used in experiments.

Black Rock Harbor sediment (BRH), which was collected from 25 locations within the Black Rock Harbor (Bridgeport, Conn., U.S.A.) study area with a 0.1m^2 gravity box corer to a depth of 1.21m, contained PCBs (6400 ng g^{-1}), PAHs (142,000 ng g^{-1}) and the metals Cu and Cr at 2900 and 1480 μg g^{-1} dry weight respectively (Lake et al., 1988). It was homogenized, distributed to barrels, and stored at 4°C. The contents of each barrel were later homogenized in a tub, wet-sieved through a 2-mm sieve, distributed to glass jars, and stored at 4°C until used in experiments.

Nephtys incisa (3-4 cm in length) were collected for sediment exposure with a Smith-McIntyre grab sampler (0.1-m^2) from the reference site and acclimated in the laboratory for 5 days at 20°C in REF sediment.

Two 10-day tests with suspended particulates were performed. Essentially identical dosing systems, one for REF and one for BRH, provided a delivery of concentrated sediment slurry in seawater. Suspended particulates were constantly recirculated past a three-way valve. Argon gas was added to the reservoir of the dosing system to minimize oxidation of the slurry (Figure 1). Opening/closing of the three-way valve was controlled with a microprocessor programmed to deliver a pulse of slurry at periodic time intervals. In the mixing chamber, concentrated slurry was mixed with seawater to the proper concentration of suspended solids and distributed to the individual test chambers.

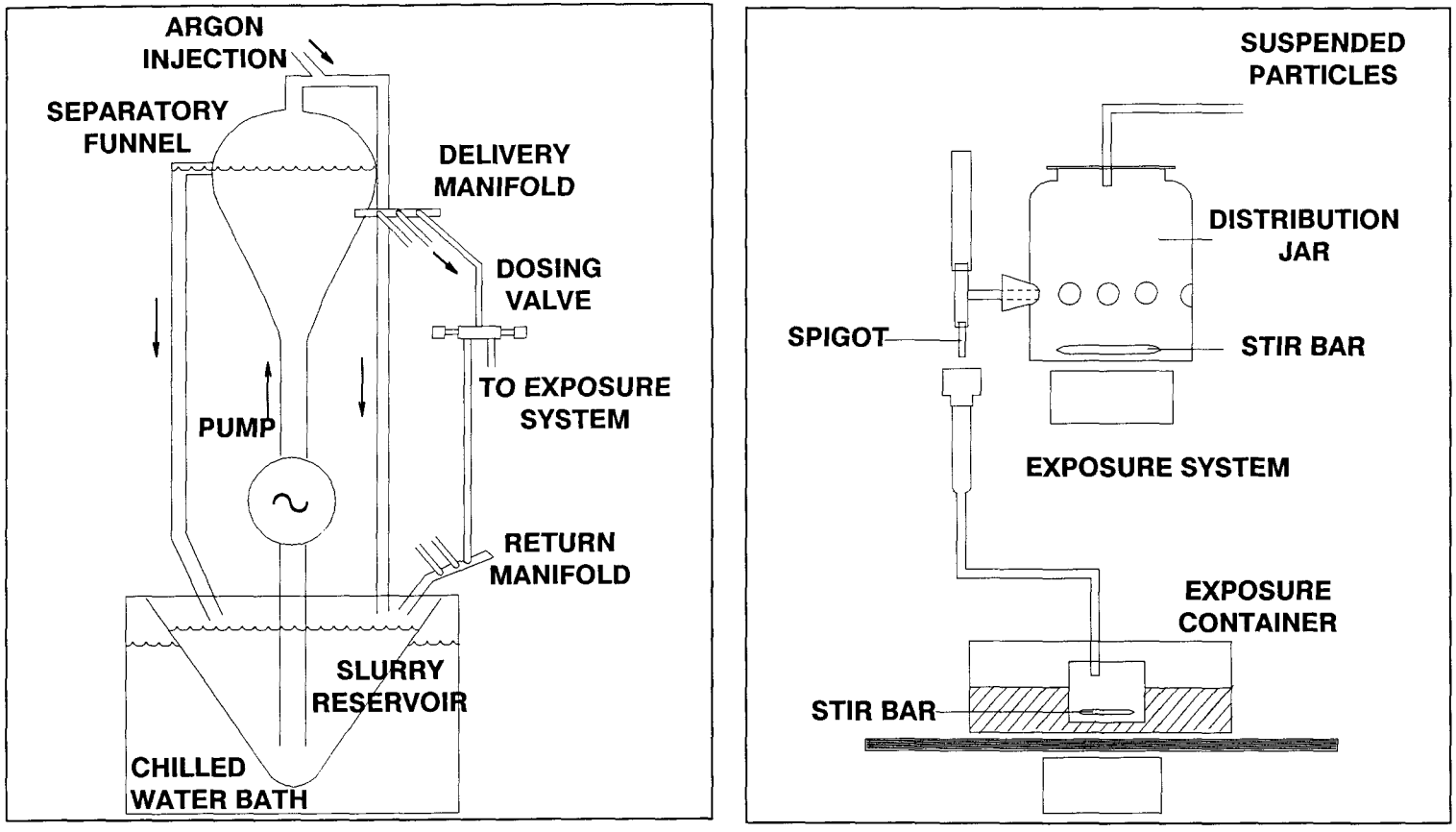

Figure 1. Left: Sediment dosing system with chilled water bath and argon gas supply. Right: Suspended sediment dilution system, distribution chamber and exposure chamber used for acute toxicity tests with N. incisa.

The design provided four treatments whereby REF or BRH suspended sediment in seawater flowed over a bed (solid phase) of 100% reference or Black Rock Harbor sediment to create the following combination of treatments: REF/REF, REF/BRH, BRH/REF and BRH/BRH. This would simulate possible field conditions but not field concentrations. The exposure conditions for these experiments were chosen on the basis of previous experiments and were expected to be sublethal for the 10-day exposure period.

Test chambers were glass crystallizing dishes (150 by 75 mm), which contained 400 ml of sediment (2.5 to 3.5 cm deep). A smaller glass crystallizing dish (60 by 35 mm) was placed in the center of the larger dish. A Teflon[1]-coated stir bar was used in the small dish to keep the particulate material in suspension. The inflow water flowed out of the central dish over the sediment surface, and overflowed the edge of the large crystallizing dish (Figure 1).

The worms were fed prawn flakes (ADT-Prime, Aquatic Diet Technology, Brooklyn, N.Y.) suspended in seawater and pumped by peristaltic pump into the distribution chamber of the dosing system. Amount fed was 127 mg dry weight per test chamber per day. This amount of food was determined optimal in prior feeding studies with N. incisa. (P. Schauer and C. Pesch, unpublished data).

All tests were conducted with sand-filtered Narragansett Bay seawater at 20°C at approximately 30 ppt salinity. Flow rates were 35 ml min^{-1}. The photoperiod was a 14:10 hr light-dark cycle.

After 10 days treatment, worms were collected by replicate on a fine mesh sieve (0.9 mm mesh) and immediately anesthetized by immersion of sieve and worms into a 7% solution of $MgCl_2$ in seawater for 2-1/2 min (Dean and Mazurkiewicz, 1975). Worms were washed by immersion of the sieve in clean seawater and then removed from the sieve and placed into a Carolina[2] dish (75 mm diam.) containing approximately 50 ml clean seawater. One or two anesthetized worms (> 0.1 g wet wt.) were placed on a Millipore filter pad (25 mm, 1.2 μ) and as much seawater as possible was removed by vacuum. Anesthetized worms were gently removed from the filter pad onto a labelled polythene strip and freeze clamped at -196°C. The extraction procedure was identical to that used for nucleotide extraction from M. edulis (Zaroogian et al. 1982) except that the homogenized tissues were doubly extracted with 6% PCA containing 0.33% ethylenediaminetetraacetic acid (EDTA) to give a final weight/volume ratio of 1:10. EDTA and the tissue

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[1] Registered trademark. Mention of trade names of commercial products does not constitute endorsement or recommendation for use.

[2] Registered trademark.

extracts were assayed within two hrs of extraction. The concentrations of ATP, ADP, and AMP were determined spectrophotometrically (340 nm) with hexokinase (Lamprecht and Trautschold 1974), pyruvate kinase and myokinase (Adam 1963), respectively. All enzymes, chemicals and reagents (analytical grade) were obtained from Boehringer Mannheim, Indianapolis, Indiana.

Data were analyzed by analysis of variance (ANOVA) to detect differences among treatments and to determine the reproducibility of AEC among treatments within a test. Fisher's Least Significant Difference was used to make pairwise comparison of means between treatments (Snedecor and Cochran 1980).

RESULTS AND DISCUSSION

The double extraction of whole Nephtys incisa with PCA-EDTA gave extraction efficiencies consistently greater or equal to 96±0.3%.

Adenylate energy charge for the treatment BRH/REF was significantly different (α = 0.05) from all other treatments within a test for each sample (Table 1). This extremely high charge would indicate that a highly oxidative and metabolically active state existed in these individuals. Apparently, more so than in Nephtys from other treatments, although AEC values obtained for all treatments are indicative of healthy individuals. No other significant difference occurred among the other treatments in either test I or II (Table 1).

The ATP/ADP ratios and AEC values were highest for the BRH/REF treatment. Analysis of variance indicated that worms from the BRH/REF treatment had significantly higher (α = 0.05) ATP/ADP ratios that were consistent with changes in AEC.

Lowest values for the adenylate pool among the four treatments were obtained with worms from the BRH/REF treatment. Due to variability, no significant differences (α > 0.05) were obtained between adenylate pool size and treatment. Changes in pool size were not consistent with changes in AEC.

Nephtys incisa is a possible species with which AEC can be used to assess their metabolic state and health condition when exposed to sublethal environmental insults or perturbations.

The reference AEC values for N. incisa from both Tests I and II were indicative of actively metabolizing cells in a nonlimiting environment. Small differences in AEC (0.03 in this study) between treatments that are significantly different can be detected with this method, because of the low variability associated with the AEC values. However, although these small differences are statistically significant, biological significance is not always implied. Thus, the statistically significant difference between tests for the BRH/REF treatments does not necessarily infer a difference in health condition.

Table 1. The response of adenine nucleotides in N. incisa after treatment with BRH sediment for 10 days under laboratory conditions

Treatment		μmol/g Wet Weight Tissue				
Suspended Solid	n	ATP	ADP	AMP	AEC	
			Test I			
REF/REF	10	1.36(0.04)*	0.35(0.02)*	0.06(0.005)*	0.87(0.01)*	A**
BRH/REF	10	1.33(0.09)	0.19(0.02)	0.02(0.001)	0.92(0.01)	B
REF/BRH	10	1.37(0.04)	0.35(0.01)	0.07(0.006)	0.86(0.01)	A
BRH/BRH	10	1.36(0.10)	0.42(0.02)	0.02(0.009)	0.87(0.01)	A
			Test II			
REF/REF	10	1.42(0.06)*	0.31(0.01)*	0.04(0.01)*	0.88(0.01)*	A**
BRH/REF	9	1.30(0.16)	0.20(0.04)	0.03(0.005)	0.92(0.01)	B
REF/BRH	11	1.17(0.07)	0.24(0.01)	0.03(0.003)	0.89(0.01)	A
BRH/BRH	12	1.37(0.05)	0.34(0.01)	0.05(0.005)	0.87(0.01)	A

* Mean value of each sample with standard error of the mean in parentheses.
** Means with different letters are significantly different within a test at $\alpha = 0.05$.

When BRH sediment was suspended over the REF sediment the worms were actively burrowing and feeding, thus they were metabolically active and the highest AEC values were obtained with this treatment. This would suggest that BRH is somewhat higher in nutritive value than the REF sediment.

The ATP/ADP ratio, which is considered a reflection of the metabolic status of the cell (Beis and Newsholme 1975; Harpold and Craig 1975), was significantly higher in worms from the BRH/REF treatment. This is indicative of higher metabolic activity in worms from this treatment. However, the quantitative relationship between statistical significance and biological significance has to be refined. Vetter and Hodson (1982) reported that changes in AEC were always accompanied by changes in the total adenylate concentration. Our results support these observations. The changes, however, did not parallel each other. This was apparent in worms from treatment BRH/REF where low pool concentrations were associated with the highest AEC values in both tests.
Excellent agreement occurred between the AEC and ATP/ADP ratios as indicators of the metabolic status in worms from the various treatments, whereas poor agreement occurred between pool size and these indicators. Atkinson (1977) reported that stress conditions responsible for a decrease in AEC usually relate to a decrease in adenylate pool size. Ordinary metabolic uses of ATP generate equimolar amounts of ADP or AMP so that no change in adenylate pool size would be expected. A decrease in the total adenylate concentration could result from deamination of AMP by adenylate deaminase which, in turn, would explain the increase in AEC. If adenylate deaminase were responsible for the pool-size decrease in the BRH/REF treatment, a fall in AEC would also occur, as reported by Atkinson (1977), and not the increase in AEC as observed in this study. Increased action of adenylate kinase would result in an increased concentration of ATP and AMP accompanied by a loss of ADP which would increase the ATP/ADP ratio. We observed this in both tests with the BRH/REF treatment. However, this does not explain the diminished pool size.

We have had success in using PCA to extract adenine nucleotides from Mytilus edulis, as exemplified by high concentrations of ATP (> 2.80 μmol/g wet wt) with high AEC values (0.88) which were obtained consistently with untreated laboratory-held M. edulis (Zaroogian et al. 1982). This extraction procedure, however, did not appear to be as suitable for extraction of adenine nucleotides from N. incisa since low concentrations of ATP (0.52-0.57 μmol/g wet wt) with low AEC values (0.73-0.74) were consistently obtained with both field-collected and laboratory-held worms. Initially, the extraction of adenine nucleotides, particularly ATP, was thought to be incomplete. However, loss of ATP with corresponding decrease in AEC occurred in neutralized N. incisa homogenates which were stored for 1 week at -20°C. This indicated that ATPases were not being inactivated during the extraction process. Skjoldal and Bamstedt (1977) reported that 96% of the ATP in frozen zooplankton stored at -26°C degraded to

AMP in 8 days. Wijsman (1976) found that when M. edulis tissues were homogenized in PCA, only part of the ATP was recovered and that recovery was dependent upon the time between homogenization and assay. He also determined that the ATP was not hydrolyzed by PCA itself. We did not see any decrease in ATP or AEC upon storage of neutralized, PCA-extracted adductor muscle tissue of M. edulis for as long as 4 weeks at -20°C in a previous study (Zaroogian et al. 1982). Ivanovici (1980) reported that ATP was stable for 4 weeks in neutralized PCA extracts of an estuarine mollusc (Pyrazus ebininus) when stored at -30°C. Wijsman (1976) used the total soft parts of M. edulis in his study, whereas we used adductor muscle tissue and Ivanovici (1980) used columnar muscle tissue. The ATP degrading enzymes that Wijsman (1976) reported to be resistant to PCA inactivation may be found in tissues other than adductor muscle. Such remaining ATP degrading enzymes (ATPases) in PCA extracts have also been reported when PCA was used with microorganisms (Davison and Fynn 1974; Lundin and Thore 1975).

Lundin and Thore (1975) reported that the enzymes responsible for the loss of ATP could be irreversibly inactivated by EDTA in combination with PCA, TCA or heat and suggested that EDTA acts by destabilizing the enzymes by complexing metal co-factors. Methods incorporating EDTA for extraction of adenine nucleotides have been used with plants (Guinn and Eidenback 1972; Mendelssohn and McKee 1981), bacteria (Lundin and Thore 1975; Thore et al. 1975) and mussels (Skjoldal and Barkati 1982). In each case, highest concentrations of ATP were reported for the respective tissues analyzed. We also found it to be true in this study since, the highest concentrations of adenine nucleotides were extracted when EDTA was included. The data, however, do not indicate that the ATP degrading enzymes are inactivated by the EDTA as suggested by Lundin and Thore (1975) since no differences in ADP and AMP concentrations occurred between the same tissue extracted with PCA or PCA containing EDTA. If, in fact, ATP concentrations were due to degradation of ATP, then an equivalent increase in ADP and/or AMP should occur if no AMP degrading enzymes are present. Thus it would appear that EDTA is facilitating the extraction of adenine nucleotides. Since additional work is required to elucidate the effect of EDTA in the extraction and stabilization of adenine nucleotides from N. incisa, the possibility of EDTA inactivating ATPases cannot be disregarded. The fact that ATP was lost in our neutralized extracts of N. incisa when stored at -20°C lends credence to the ATPase tenet and the effect of EDTA during storage remains to be tested.

Acknowledgments. We thank Dr. Paul Schauer and Ms. Carol Pesch for their assistance in maintenance of the experimental system. Contribution No. 747 of the U.S. Environmental Protection Agency, Environmental Research Laboratory, Narragansett, R.I. 02882

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Received November 12, 1988; accepted February 10, 1989.