Qatar Univ. Sci J. (1999), 19:

ADENYLATE ENERGY CHARGE AS A TOOL FOR EVALUATING SUB-LETHAL TOXICITY

By

Picado A. and Le Gal Y.

Yves Le Gal : Laboratoire de Biologie Marine Muséum National d'Histoire Naturelle et Collège de France, 29900 Concarneau, France; Tel. 33 (0)2 98 97 06 59, Fax 33 (0)2 98 97 81 24, E-mail : ylegal@sb.roscoff.fr)

Running Title : *Adenylate energy charge and sub-lethal toxicity*

SUMMARY

Adenylate energy charge (AEC) is directly related to the cellular concentrations of the three adenylates nucleotides ATP, ADP and AMP. This biochemical index reaches high values (0.9) under optimal conditions but drops rapidly in the presence of stressing agents. In vertebrates, AEC is strongly regulated and maintained within narrow limits. In contrast, in invertebrates, AEC displays a wide range of values according to the importance of the internal stress or to the variations in the external environment of the organisms (natural or anthropogenic). This work concerns the use of adenine nucleotides concentrations, ATP/ADP ratios and AEC as physiological markers in different species. Our results show that AEC is modified in animals submitted to experimental pollution or sampled in polluted areas. Results of zinc contamination in adults of clam *Ruditapes decussatus* show that after one month exposure, AEC values change significantly.

The use of AEC in the bivalve *Ruditapes decussatus* for in *situ* evaluation allowed a classification among different sampling sites in what concerns environmental quality. In this way, AEC proved to be an accurate biochemical and physiological marker in monitoring the health of organisms exposed to pollution and can be used as a complementary tool in impact and environmental management studies.

INTRODUCTION :

Chemical surveys that monitor the quality of coastal and estuarine waters allow the inventory of overall pollutant levels but are inadequate *per se* for assessing the actual impact of pollutants on living systems [1].

Various approaches and procedures have been proposed for environmental hazard assessment [2,3]. Within this framework, the various techniques of biological monitoring proposed so far are based on different biological principles but have also different meanings. Mixed function oxydases enzymes [4], metallothioneins [5] mainly account, in a first approach, for the detoxification processes in response to the presence of environmental contaminants. Biochemical methods based on both systems are able to detect zones contaminated by hydrocarbons or by metals with a precision similar to or higher than that of chemical methods.

Another aspect of these indexes is that they are reflecting only the presence of molecules or substances of a particular type : PAH, metals, etc.

In addition to the classical tests and ecological surveys, it is thus necessary to establish techniques which measure the «well-being» of apparently normal organisms and which allow the assessment of possible future damages (or recovery) of biological system.

Because man-induced modifications of the environment are likely to stress organisms, ecophysiological or ecobiochemical-based diagnostics are needed [2].

In these conditions, methods such as scope for growth [6] appear very interesting as they have a global character and respond not only to specific contaminants but to a large series of natural or man-induced stressing agents generally present in the environment at low concentrations. Such total or global indexes do have their natural place in any analytical approach of long-term effects of low level contaminants present in marine environment [7].

An index based on the measurement of the metabolic energy pool offers several advantages, in particular, the university of the biological systems and therefore the adenylate energy charge was proposed as an index of environmental stress [8].

ACE is the ratio of the concentrations in ATP, ADP, and AMP and varies theoretically form 0 to 1. the value of AEC reflects the energy balance at a given time for an organism or a part of it [9].

Due to more loose regulations and particularly to the low efficiency of the enzyme AMP deaminase invertebrates are able to survive to lower energy charge (0.3-0.4) than vertebrates (0.5-0.6) [10].

AEC Values	Physiological conditions
$0.75 < AEC < 0.90$	Optimum : growth, reproduction
$0.50 < AEC < 0.75$	Perturbation : slow growth, no reproduction reversible effects.
$AEC < 0.5$	No growth, no reproduction, irreversible effects. Death.

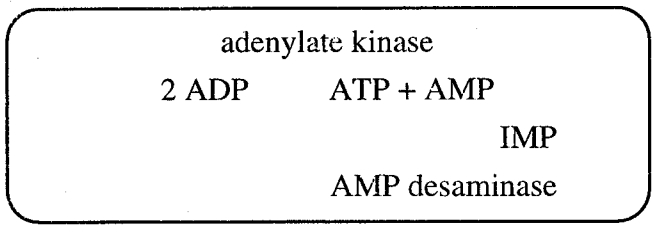
AEC measurements have been performed either on organisms submitted to experimental contaminations in the laboratory or directly sampled in the natural environment [11-14]. Generally these measurements lead to the conclusion that AEC used either in the laboratory or *in situ* constitutes a sensitive mean of appreciating sub lethal effects undergone by organisms living in polluted areas.

A large number of factors can affect ACE of organisms. Handling of organisms can, in some instances, modify drastically AEC. Bad conditions of conservation of the samples can also be at the origin of erroneous values of AEC. A rapid freezing or freezeclamping of the living samples in liquid nitrogen is an absolute condition of success for the subsequent treatments. The choice of the method for estimating adenylate nucleotides species is relatively unimportant and depends mainly of the availability of equipments. Bioluminescence is based on the enzymatic production of light in presence of ATP. It requires a prior enzymatic conversion of AMP and ADP into ATP. However it allows a simultaneous treatment of a large series of samples.

The second type of method is based on the separation of the adenylates nucleotides by HPLC and gives directly the measure of ATP, ADP and AMP.

A third method is based on the measure of ^{31}P by NMR techniques. This methods offers the advantage of giving also access to the phosphagens and to inorganic phosphate and can be used in some cases on small living organisms.

The interpretation of the results has also to take in account the overall physiology of the regulation of AEC, in which two key enzymes are involved : Adenylate kinase and AMP deaminase.



The result of the activity of these two enzyme systems is a protection of the cell against a drop of AEC at the expense of the global stock of adenylates. Invertebrates, AMP deaminase is particularly efficient and thus limitates any variation of AEC in stress conditions. However, in invertebrate species the low catalytic activity of AMP deaminases authorises large variations of AEC that can be used for testing the biological response of organisms to natural of man-induced stress modifications of their environment.

METHODS

Laboratory experiments

Adults of *Ruditapes decussatus* with an average length of 3.5 - 4.0 cm were sampled at Ria Formosa (Fig. 1- site B). After laboratory acclimation (35.6% pH 8.0, 15°C, aeration, medium renewal every other day and feed with *Phaeodactylum tricornutum*) organisms were exposed to sub lethal concentration of 1 mg/1 Zn Cl_2 for 15 and 30 days. After these period the

edible part was frozen under liquid nitrogen for adenylates analysis.

Field experiments : Individuals of *Ruditapes decussatus*. were sampled at sites A and B (Fig. 1) and frozen under liquid nitrogen till analysis.

Analysis of the nucleotides. For all tested organisms tissues of each individual were pulverised under liquid nitrogen and extracted with perchloric acid [8].
Analysis of adenylates was performed by high-performance liquid chromatography according to Leray [15] with LKB-Bromma equipment. The work column (5 µm) and pre-column (7 µm) were Lichrosorb RP 18 and the elution was carried out with 0.35 M phosphate buffer, pH 5.5. All samples and buffer were filtered (0.45 µm) prior to analysis. The adenylate concentrations were referred to the protein content of the extracts [16] with bovine serum albumin as standard. Values of the adenylate energy charge obtained at each concentration (laboratory experiments) or at each sampling station (field experiments) and defined [9] as the ratio of the molar concentrations of the adenylate nucleotides according to
AEC =
(ATP + 1/2ADP) (ATP + ADP + AMP)
are presented in the form of cumulated frequency plots.

RESULTS

Laboratory experiments. AEC values and total adenylates for *Ruditapes decussatus* exposed to a sub lethal zinc concentration for different times are presented in table 1. After a 30 days exposure a drop on AEC mean values is observed. Mean value of AEC for *in situ* individuals is 0.8 (s.e.m. 0.01 n=10).

Figure 2 present in the form of cumulated frequency plots individual AEC values for different exposure times. Figure 3 presents AEC variation versus ATP/ADP ratios. Contaminated organisms present very dispersed values either for AEC or for ATP/ADP ratio.

Field experiments. Individuals of *Ruditapes decussatus* sampled in different sites of the portuguese coast were utilised for site monitoring study. In table II results on mean AEC values and total adenylates are presented.

Results presented in the form of cumulated frequency plots for individual AEC values in different sites are presented in figure 4. Results obtained shows different site conditions.

In decreasing order : Faro, Aveiro and Culatra. Organismes form Culatra present some high values concerning total adenylates and great dispersion. Figure 5 present AEC variation with ATP/ADP ratio. The lowest ATP/ADP values were obtained for organisms sampled at Culatra.

Discussion

One of the major issues arising from ecotoxicological studies concerns the relationship between the effects noted during short-term laboratory experiments

and the future of the organisms in their environment. Adenylate energy charge analyses is seen to resolve this issue.

At the laboratory level and for zinc exposure AEC is able to detect contamination effect on the bivalve.

Similar results have been described for different organisms placed under stressing conditions [8,17] experiments with a paper mill effluent not causing mortality within 96 hr, lead to significant variations in AEC values after 24 hr in the case of *Cerastoderma edule* [4].

The use of AEC for field studies allowed a classification of different sites according environmental conditions. Culatra, considered a priori as a reference showed the lowest quality. Also, tranfert experiments from non polluted areas to contaminated sites allow the use of this biomarker to detect effects on C. *edule* due to effluent discharges [14].

Cellular AEC seems to be strictly controlled. It can be stabilized by a reduction in the total adenylate nucleotide pool through a degradation of AMP catallyzed by AMP deaminase [1] considered a crisis enzyme by Raffin [18]. Results demonstrate that under stress conditions and after 24 hr a series of regulations processes operates to ensure a stabilized AEC level. Thus, we can assume that area the evolution of the nucleotide pool is more important than the AEC itself [12, 19].
AEC is not a signal of discomfort but an index of reaction or of counteraction to adverse situation. Appearance of the reaction signal is only possible within a given interval of intensity of the stressor. The study of this signal can thus indicate the limits of active response of an organism (counteractive capacity).

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Table I - Mean AEC values and total adenylates for *Ruditapes decussatus* exposed to a concentration of 1mg/1during 0, 15 and 30 days

Day	AEC s.e.m	(ATP)+(ADP)+(AMP) (nmole/mg prot.) s.e.m
0	0.79 (n=5) 0.01	33.30 2.05
15	0.70 (n=9) 0.02	89.10 9.77
30	0.67 (n=13) 0.02	67.56 4.4

Table II - Mean AEC values and total adenylates for *Ruditapes decussatus* sampled in different sites.

Site	AEC s.e.m	(ATP)+(ADP)+(AMP) (nmole/mg prot.) s.e.m
Aveiro (A)	0.75 (n=7) 0.02	40.43 6.20
Culatra (C)	0.67 (n=8) 0.04	53.63 9.86
Faro (F)	0.81 (n=7) 0.02	27.86 2.10

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0	0,79 (n=5)	33,30
	0,01	2,05
15	0,70 (n=9)	89,10
	0,02	9,77
30	0,67 (n=13)	67,56
	0,02	4,4

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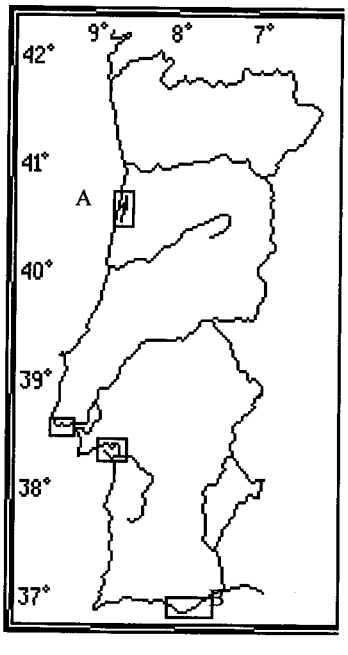

A - Ria Aveiro

B - Ria Formosa – 2 sites, Culatra, Faro

Fig. 1 : Sampling sites for Ruditapes decussatus.

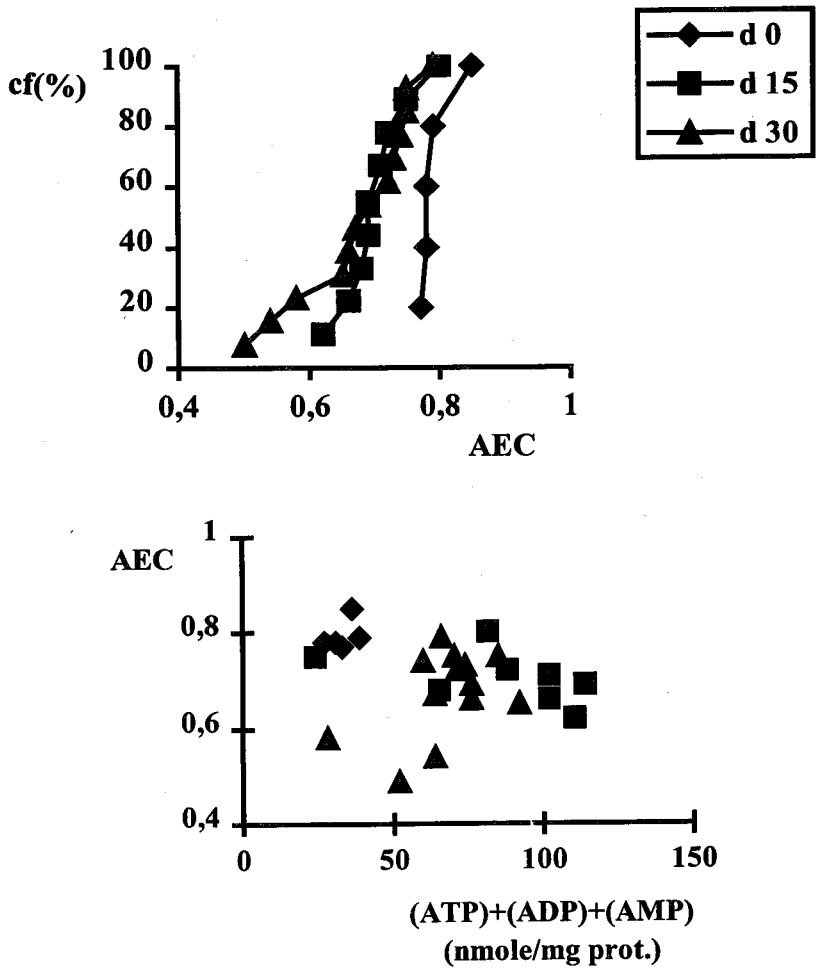

Fig. 2 : Cumulated frequencies versus individual AEC values and total adenylates evolution for Ruditapes decussatus exposed to a concentration of 1mg/1during 0,15 and 30 days.

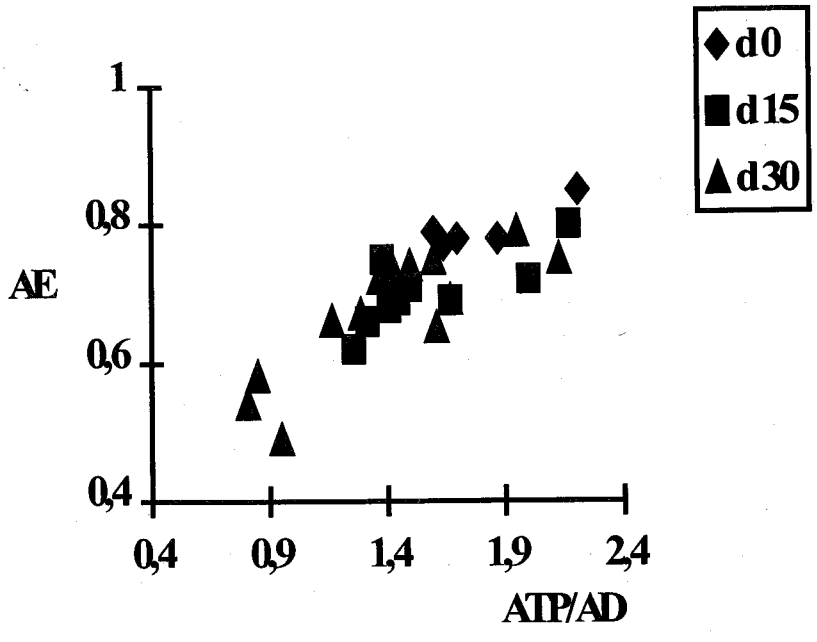

Fig. 3 : AEC evolution versus ATP/ADP ratio for Ruditapes decussatus exposed to a concentration of 1mg/1during 0,15 and 30 days.

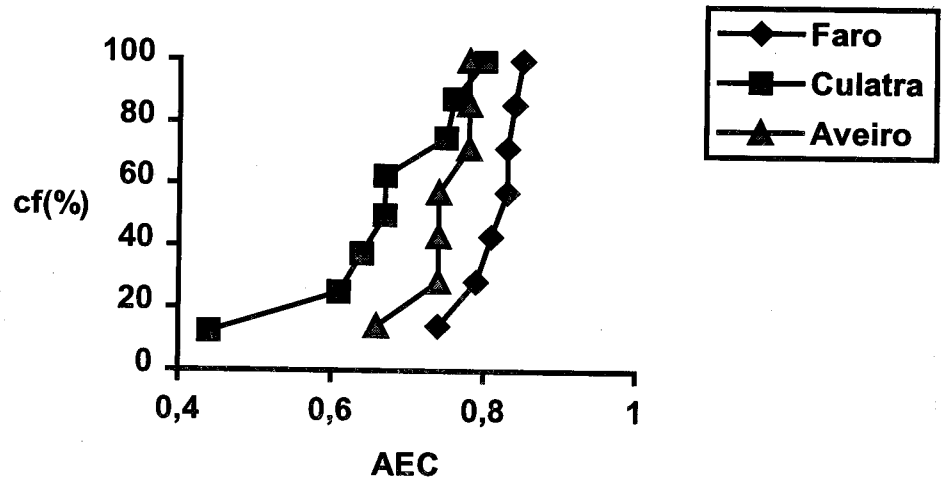

Fig. 4 : Cumulated frequencies versus individual AEC values and total adenylates evolution for Ruditapes decussatus in different sampling sites.

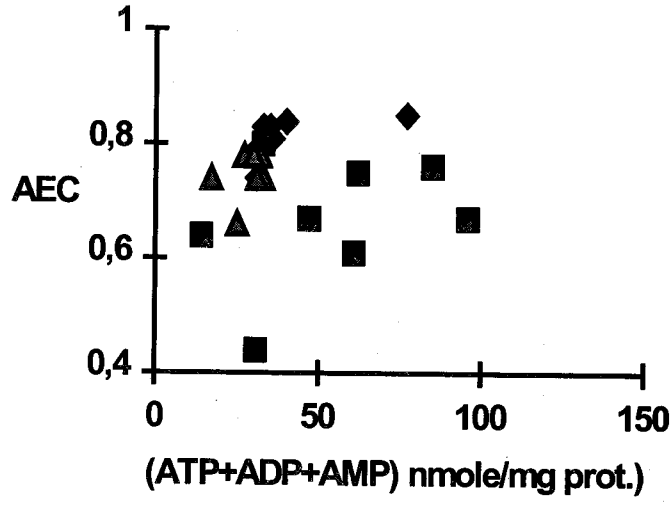

Fig. 5 : AEC evolution versus ATP/ADP ratio for Ruditapes decussatus sampled in different sites.