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Biological effects of contaminants: *Paracentrotus lividus* sea urchin embryo test with marine sediment elutriates

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This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Abstract

This *ICES Techniques in Marine Environmental Sciences* describes a sediment elutriate bioassay using embryos of the sea urchin *Paracentrotus lividus*, a species widely distributed in both Atlantic and European Mediterranean waters. The proposed method is directly applicable to other echinoid species used in ecotoxicology worldwide, such as the *Strongylocentrotus* and *Arbacia* genus. The bioassay endpoint is a quantitative, observer-independent, automatically readable response. Statistical methods and assessment criteria to classify sediment samples according to their biological quality status are also included, consistent with the demands of the European Water Framework Directive.

Keywords. Sediment elutriate bioassay, *Paracentrotus lividus*, sea urchin, toxicity testing, environmental quality assessment.

1 Introduction

Early developmental stages of organisms are more sensitive to chemical stress than adults are; therefore, they are the weakest link in an organism's life cycle. The embryo-larval bioassays using marine invertebrates detect exposure to a broad spectrum of toxicants at comparatively low concentrations, on the order of 1 $\mu g\,l^{-1}$ for tributyltin (TBT) and other antifouling products; 10 $\mu g\,l^{-1}$ for Hg, Cu, and Zn; 100 $\mu g\,l^{-1}$ for Pb, Cd, and other metals; 0.1 $mg\,l^{-1}$ for organochlorine pesticides, detergents, and refined oil; and 10 $mg\,l^{-1}$ for crude oil (Kobayashi, 1995; His *et al.*, 1999).

The sea urchin embryo test (SET), described herein, has been used frequently as a rapid, sensitive, and cost-effective biological tool for the evaluation of marine sediment and seawater quality worldwide (Kobayashi, 1971, 1991; Bougis *et al.*, 1979; Vashchenko and Zhadan, 1993; Carr *et al.*, 1996; Beiras *et al.*, 2003a, 2003b; Losso *et al.*, 2007). Current standard methods (ASTM, 1995; Carr, 1998) choose as an endpoint a qualitative response, morphological normality of the larvae, which requires detailed microscopic inspection of each individual and knowledge of echinoderm embryology. This paper describes a procedure to conduct the SET using a simple quantitative response, size increase, as endpoint. This is an objective and automatically readable test result and facilitates the use of SET as a rapid routine tool for marine environmental quality assessment. The procedure described here also includes statistical methods for calculating toxic units (TU) and assessment criteria to classify sampling sites into discrete categories of biological quality according to their TU value. This approach provides a simpler interpretation for non-specialist decision-makers.

Sediments are commonly used as an environmental matrix in monitoring programmes, because they act as major reservoirs of persistent pollutants in coastal and estuarine systems. Concentrations of pollutants in sediments tend to be more stable over time and concentrations are generally orders of magnitude above those occurring in the water column. Currently, integrated monitoring, including not only sediment chemistry but also toxicity tests with early life stages of marine organisms, which respond to the bioavailable fraction of pollutants, is advocated.

2 Test method

2.1 Control seawater

Control seawater is used for *in vitro* fertilization, preparation of the sediment elutriates, serial dilutions of the testing samples, and control incubations. Control seawater is 0.22 μm filtered seawater (FSW) of oceanic characteristics, including full salinity and absence of chemical pollution. If natural seawater with these characteristics is not available, an alternative is the preparation of chemically defined artificial seawater (ASW) according to Lorenzo *et al.* (2002), by dissolving the salts by the order listed in Table 1 in deionized MilliQ water. All reagents must be of maximum quality (analytical grade or equivalent), and special care must be taken in the trace metal impurity content of the salts. Commercial mixtures or "purified grade" salts are not suitable for larval rearing.

Table 1. Composition of artificial seawater.

Salt	Amount (g l^{-1})
NaF	0.003
$SrCl_2.6H_2O$	0.024
$Na_2B_4O_7.10H_2O$	0.0475
KBr	0.100
KCl	0.700
$CaCl_2.2H_2O$	1.47
Na_2SO_4	4.00
$MgCl_2.6H_2O$	10.78
NaCl	24.50
$NaHCO_3$	0.200

2.2 Sediment elutriation

Sediment toxicity can be tested with water column organisms by either obtaining an elutriate from the sediment (mixed with control seawater) or by directly obtaining the interstitial porewater from the sediment. Elutriates are obtained by rotatory mixing of 100 g of sediment and 500 ml of control FSW at 60 rpm for 30 min in airtight polypropylene flasks with no head space. After overnight settling at 20 °C in the dark, the liquid phase (elutriate) is siphoned into a separate beaker, then aerated for 10 min to remove any H_2S. There are several advantages to the elutriate method, including smaller amounts of sediment, simpler equipment needs, and the fact that the environmental characteristics of the elutriate (dissolved oxygen, pH, salinity, ammonia, and sulphides) are closer to those of the natural water column than for porewater, in particular when dealing with anoxic or hypoxic sediments. These chemical characteristics are the most common source of false positives (see Section 3.2). In addition, when using porewater, they must be adjusted prior to testing to the optimum range for the test species. Conversely, porewater has the advantage that no control seawater is needed and the dilution of the potential toxicants present is lower, enhancing sensitivity. The choice of the method can depend on sampling constraints and sample availability because, when the confounding factors are taken into account, both methods yield comparable results (Beiras, 2002).

2.3 Test species

Because of its abundance and broad geographical distribution in European waters, the sea urchin, *Paracentrotus lividus,* is recommended as test species. Alternatively, echinoid species that are locally or regionally abundant can be used, e.g. *Strongylocentrotus droebachiensis* and *Echinus esculentus* in northern Europe, *S. intermedius*, *S. nudus*, and *Hemicentrotus pulcherrimus* in the Asian Pacific coast, *Arbacia punctulata* and *S. purpuratus* in the Atlantic and Pacific coasts of North America, and *Echinometra lucunter* and *Loxechinus albus* in the Atlantic and Pacific coasts of South America.

2.4 *In vitro* fertilization and delivery

Gametes are obtained from mature adults either by direct stripping of the gonad or osmotic-shock-induced spawning. The latter is done by injecting 1 ml of 0.5M KCl through the peristomal membrane into the coelom; spawning typically starts a few minutes after the injection. Females are inverted over a beaker containing FSW and

left to release eggs. Sperm is aspirated "dry" from the gonopores of the males, and it must not be diluted until fertilization.

Prior to fertilization, gamete viability is assessed under the microscope by placing the gametes in a drop of FSW and checking for egg roundness and sperm motility. Eggs from a single female are collected in a 100 ml measuring cylinder with FSW and a few microlitres of undiluted sperm collected with a glass Pasteur pipette from a single male are added with gentle stirring provided by a plastic plunger. Four aliquots of 20 µl are taken, and the total number of eggs and number of fertilized eggs, identified by the fertilization membrane, is counted in a 1 ml counting cell. Calculations are made to deliver with an automatic pipette between 20 and 40 fertilized eggs per ml^{-1} in the test chambers.

2.5 Preparation of experimental treatments

The following treatments are made up in quadruplicate, except when otherwise stated.

- Fertilized eggs fixed after delivery (time 0) to know the initial size
- Controls of FSW ($n = 8$)
- Undiluted elutriates
- ½, ¼, and 1/10 dilutions in FSW

At least one of the elutriates should be obtained from a reference site with no pollution, but otherwise with similar characteristics to the experimental sediments, in particular grain size and organic content.

2.6 Incubations

Typical standard test chambers that allow miniaturization of the test are 4 ml polypropylene vials placed in a rack or 3 ml microwell plates. After 48 h incubation at 20°C in the dark, samples are fixed with two drops of 40% formalin.

2.7 Reading

Observations of the maximum dimension of the first 35 individuals per vial are made directly on the test chambers using an inverted microscope. The individuals may be fertilized egg (Figure 2a), embryos (Figure 2b and c), prism larvae (Figure 2d), or pluteus larvae (Figure 2e). This method avoids time-consuming, observer-dependent judgements about the degree of morphological normality of the larvae. Image analysis can be used to collect these data.

2.8 Expression of SET results

For every incubation unit, experimental treatments, and controls, size increase is calculated as mean ($n = 35$) maximum dimension minus mean egg size at $t = 0$. Size increase values in the experimental treatments are expressed as percentages of the control.

For every sediment sample, including reference samples, two toxicity parameters are calculated, namely percentage net response (PNR) and toxic units (TU; Figure 1). The PNR value is the response (length increase) in the undiluted elutriates divided by the control, calculated according to Thain (1991). The TU value is calculated using the responses in all of the experimental dilutions; in this case, four dilutions (see Section 2.5). The theoretical dilution of the elutriate causing an inhibition of 50% in the

response, ED_{50}, is obtained by linear regression of the control-corrected response vs. dilution in logarithmic scale. Then TUs are calculated as $TU = 1/ED_{50}$.

The TU values have two advantages compared with PNR. First, they summarize more toxicological information (four or more dilutions) from each sampling site than PNR (a single dilution). Second, their comprehension is more intuitive because, unlike PNR or ED_{50}, TU values increase as toxicity increases. In contrast, PNR values are directly comparable with control responses and they are, therefore, more useful to detect significant differences with control.

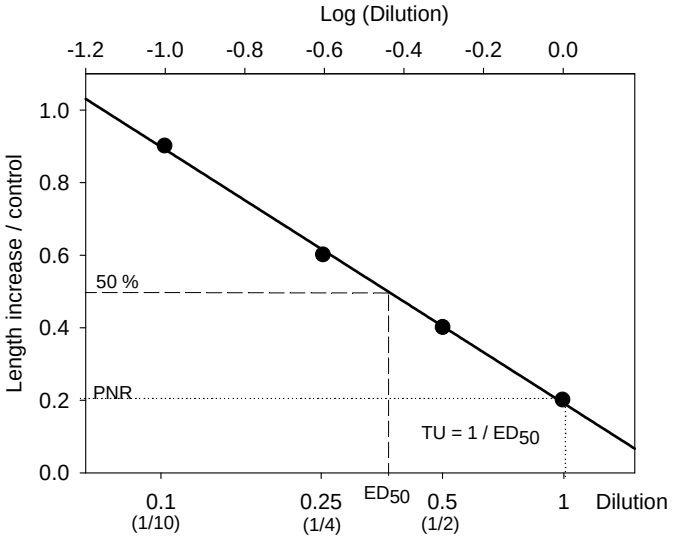

Figure 1. Example of calculation of TU and PNR from bioassay results.

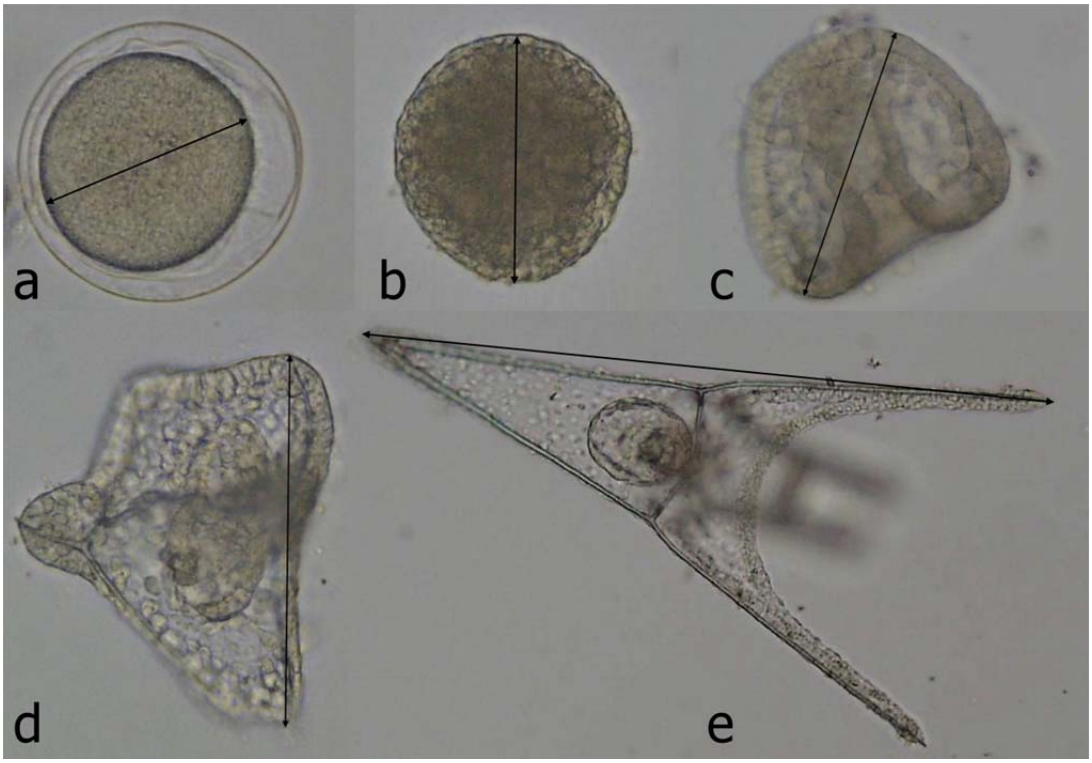

Figure 2. Measurement of maximum dimension in (a) *P. lividus* sea urchin fertilized eggs, (b) morulae, (c) gastrulae, (d) prism larvae, and (e) pluteus larvae.

3 Sources of error

3.1 Quality assurance of the biological material

Quality assurance (QA) is important for the development of robust ecotoxicological tools. The need for updating QA for biological measurements to ensure comparability of data has been identified (ICES, 2007). An important aspect of QA for an embryo-larval bioassay is the control treatment, which gives essential information regarding biological quality of the test organisms. Currently, the main limitation of these bioassays is the availability of reliable, good-quality biological material all year-round, particularly outside the natural spawning season of the species.

Acceptability criteria for the SET, i.e. minimum control response for a test to be considered reliable, have been inferred from the 5th percentile of the control data accumulated over several years in our laboratory (Table 2). Controls incubated at 20°C in the dark, where *n* was >130, were selected and results were grouped according to the type of water, either FSW or ASW. The use of ASW consistently produced larger larvae. According to that, a test is acceptable when mean response in the control exceeds a size increase of 218 µm for FSW or 253 µm for ASW.

Table 2. Acceptability criteria, calculated as the 5th percentile of the normal distribution of frequencies in controls (*n* >130). Data from controls of natural 0.22 µm-filtered seawater (FSW) and artificial seawater (ASW) are illustrated both independently and pooled together.

	SIZE INCREASE (µM)		
water	FSW	ASW	FSW and ASW
Mean (95% CI)	287.9 (272.8; 291.0)	345.1 (335.5; 354.6)	312.3 (306.0; 318.7)
n	167	139	226
5th percentile	218	253	245

A second identified source of variability in the control response was season. When data from several years are pooled together and plotted on a monthly basis (Figure 3), a peak in larval size during summer (from June to October) can be seen. This is probably the result of riper gonads and better reproductive condition in the adults of the local population at that time of year.

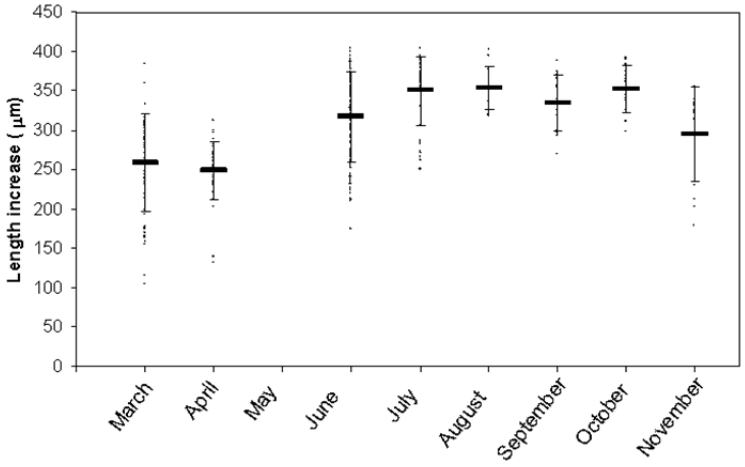

Figure 3. Annual distribution of control mean length increase in *P. lividus* sea urchin early developmental stages incubated at 20°C for 48 h. Note the trend towards higher growth in summer.

It may also be useful to run a reference toxicant test to assess the biological quality of the test organisms. Intralaboratory control charts with reference toxicants (copper and zinc) indicated a coefficient of variation (*CV*) for percentage of normal larvae from 12 to 20% (Phillips *et al.*, 1998; Volpi Ghirardini *et al.*, 2005). Intercalibration exercises among laboratories are urgently needed in the field of marine ecotoxicological bioassays.

3.2 Confounding factors: salinity, pH, dissolved O_2, NH_3, and H_2S

In addition to the use of good biological material, several environmental parameters of the elutriates, frequently regarded as confounding factors, must be checked prior to dilution to avoid interference with the results. The aim of the ecotoxicological bioassays is to identify chemical pollution using sensitive organisms. The test is positive when a significant inhibition in the biological endpoint is detected and it is assumed that the inhibition is the result of the presence of anthropogenic toxicants. However, inhibition could also be the result of natural conditions of the samples, which may be not suitable for the sensitive test organism. This is of particular concern when testing elutriates from highly reduced sediments, whose characteristics may differ from the optimum environmental values for water column organisms. Therefore, before the incubations, salinity, pH, and dissolved oxygen of the sample must be checked and corrected where necessary, to make sure that they fall within optimum values for the test organisms. Unionized ammonia and other reduced compounds have been identified as the main sources of false positives in sediment elutriate toxicity testing (Cardwell *et al.*, 1976; Matthiesen *et al.*, 1998). Furthermore, the levels of these compounds generally increase with storage time. We recommend a maximum storage period for sediments of one week at 4 °C in the dark.

The *P. lividus* embryos are very sensitive to changes in salinity (stenohaline) and pH. Salinities below 31‰ and above 35‰ significantly reduce embryo development and early larval growth ($p < 0.05$; Saco-Álvarez *et al.*, 2010). The same was found for pH for values below 7.0 or above 8.5 ($p < 0.01$). In contrast, the embryos are tolerant of low dissolved oxygen concentrations down to 2 mg l^{-1}, but below that level, a sharp decrease in development takes place.

Unionized ammonia is highly toxic to sea urchin embryos, with a toxicity threshold delimited by a no observed effects concentration (NOEC) of 40 µg l^{-1} and a lowest observed effects concentration (LOEC) of 80 µg l^{-1} (Saco-Álvarez *et al.*, 2010). The EC_{10} calculated according to a logistic model is 68.4 µg NH_3 l^{-1} (95% confidence intervals 52.8–88.3) and the EC_{50} 178.9 µg NH_3 l^{-1} (95% CI 161.0–198.9). These values are similar to those found for *Arbacia punctulata*, with a NOEC and LOEC of 30 µg NH_3 l^{-1} and 90 µg NH_3 l^{-1} (Carr *et al.*, 1996) and for *Strongylocentrotus purpuratus*, with a range of NOEC of 66–38 µg NH_3 l^{-1} for a pH range of 7.7–8.4 (Greenstein *et al.*, 1996).

H_2S may also cause false positives, but unlike NH_3, it is easily eliminated with gentle aeration (see Section 2.2). Sulphide levels must be checked to make sure they are below the toxicity threshold for sea urchin embryos (NOEC = 0.1 mg l^{-1}; Losso *et al.*, 2004), and additional aeration must be provided if necessary.

4 Interpretation of results

To classify the sampling sites in different categories of sediment quality status, in agreement with the European Water Framework Directive, assessment criteria (AC) were obtained from the ecotoxicological database ($n = 183$), based on our earlier studies in the Galician Rias (Table 3). First, to obtain the AC values, reference stations

were identified, based on both chemical and ecotoxicological data. A single reference station per cruise was selected. Experiments not meeting the acceptability criteria for the SET (see Section 3.1.) were excluded from the database. Second, the stations that were not significantly different from the reference station from each cruise (*t*-test) were added to the reference database, and the 95th percentile was calculated. This value of 0.694 PNR (or 0.27 TU), was considered as the AC1 (the limit between good and moderate sites). The AC1 is also useful in screening surveys where only undiluted elutriates are tested. Samples with a PNR value below AC1 require further testing by serial dilutions to calculate TU values.

Finally, stations with a response significantly different from the corresponding reference station were pooled, and the 50th percentile was calculated. This value, 0.508 PNR (or 0.86 TU), was considered as the AC2 (the limit between moderate and poor or polluted sites).

Table 3. Assessment criteria to classify sampling sites in three different categories of sediment-quality status according to the SET results in toxic units (TU), taking into account all of the dilutions of each elutriate, or percentage net response in the undiluted elutriate (PNR).

	SET results		
Sediment quality status	**TU**	**PNR**	**"Traffic-light" colour code**
high or good	<0.27	>0.694	green
moderate	0.27 to 0.86	0.508 to 0.694	yellow
poor or bad	>0.86	<0.508	red

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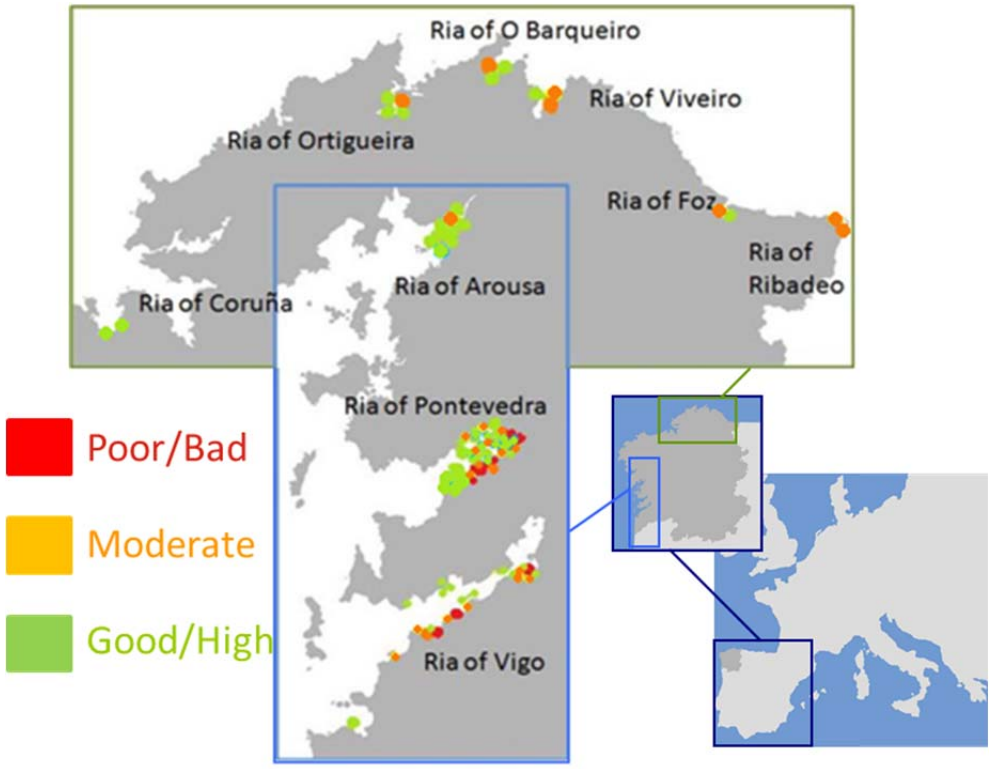
6 Annex 1

Assessment of coastal pollution in the Galician Rias (northwestern Iberian Peninsula), using the *Paracentrotus lividus* sea urchin embryo test (SET) with marine sediment elutriates. Case study.



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8 Abbreviations

AC	assessment criteria
ASW	artificial seawater
CI	confidence interval
CV	coefficient of variation
FSW	filtered seawater
LOEC	lowest observed effects concentration
Milli-Q water	simple water purified using a Millipore Milli-Q lab water system
NOEC	no observed effects concentration
PNR	percentage net response
QA	quality assurance
SET	sea urchin embryo test
TBT	tributyltin
TU	toxic units