Phuket mar. biol. Cent. Res. Bull. **60** : 87-93 (1995)

WATER QUALITY BIOASSAYS AND THEIR APPLICATION TO MARINE POLLUTION STUDIES IN THE EAST ASIAN REGION

By I. R. B. McFadzen[1] and N. Bussarawit[2]
[1] *Natural Environmental Research Council, Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth, Devon, PL1 3DH, United Kingdom*
[2] *Phuket Marine Biological Center, P.O. Box 60, Phuket 83000, Thailand*

ABSTRACT

The rapidly increasing anthropogenic activities in the East-Asian region exert considerable stress on the aquatic environment. The principal stresses are related to the pressures on the exploitation of marine resources (coral reefs, mangroves, fisheries and endangered species) from problems pertaining to the pollutants released to the marine environment. Since the ultimate concern is the capacity of the sea to support life, it is desirable to measure water quality in terms of biological response. The response in the laboratory of suitable test organisms to water samples taken from polluted areas can be used as an index of water quality. Echinoderm embryos and larvae have been routinely used in water quality assessment due mainly to their ease of culture, distinct developmental stages and relative sensitivity to environmental variables. Historically, water quality assessments have been conducted in temperate waters and the effectiveness of such methodologies in tropical conditions, are tested in this study. Bioassays utilising the blue-spot sea urchin *Diadema setosum* (a representative species of coral reef communities), can be exploited as valuable training tools to assess and demonstrate environmental impacts in the East Asian region using survival and developmental success as indicators.

INTRODUCTION

Water quality is often assessed chemically in terms of concentrations of known (or detectable) toxic contaminants. This method may be satisfactory when there are a limited number of contaminants whose biological effects are known and predictable, but effluents are often extremely complex and may contain numerous synthetic organic compounds. Effluents from industry entering estuarine or coastal waters may include thousands of individual elements and compounds, making it impractical to define and costly to monitor water quality by chemical analysis alone. Many factors influence the toxicity of contaminants on entering sea water. Some contaminants degrade quickly into harmless products, some may be bound by organic material, with changes in their biological availability and some may interact chemically to become more, or less, toxic in combination than separately.

Since the ultimate concern is the capacity of the sea to support life, it is desirable to measure water quality in terms of biological response. The response in the laboratory of suitable test organisms to water samples taken from polluted areas can be used as an index of water quality. In this way, all the biologically relevant variables that affect water quality are integrated in terms of the responses of individual organisms. Among the advantages of this approach is the integration it provides, not only of the effects of toxicants and modifying variables that are known and understood, but also those that are not yet known (Stebbing, 1985).

The rapidly increasing population and developing agro-industrial activities in the East-Asian region exert considerable stress on the aquatic environment. Often untreated municipal wastes are discharged directly or indirectly to the marine environment (Phromlert, 1992). The principal stresses are related to the pressures on the exploitation of marine resources (coral reefs, mangroves, fisheries and endangered species) stemming from the rapid population growth of the region (Gomez, 1988). Further problems pertain to the pollutants released to the marine environment, namely heavy metals (Menasveta *et al.*, 1985; Hungspreugs, 1988), pesticides (Siriwong *et al.*, 1991) and oil derivatives (Chansang, 1988; Siripong, 1988). A range of biological effects of pollutants have been addressed in the region, including studies to assess metal tolerance of corals (Harland and Brown, 1989), accumulation of heavy metals in adult bivalve molluscs (Menasveta *et al.*, 1985) and microbiological qualities of bivalve-culture areas (Lovetelli, 1988; Hungspreugs *et al.*, 1991).

The latest predictions concerning the rapid pace of development and industrialization in the countries of the East Asian Seas region are that these environmental insults will continue for the foreseeable future, with the inevitable deterioration of the marine environment. This fact resulted in the implementation of a project by the Co-ordinating Body on the Seas of East Asia: COBSEA (Kuala Lumpur, November 1991), aimed at the development of management plans for endangered coastal and marine resources in East Asia. Additionally, it was deemed necessary to implement scientifically based assessment schemes to complement management-oriented projects. As a result of the COBSEA report a "training" workshop on the Biological Effects of Pollutants was approved; to be held at the Phuket Marine Biological Center (PMBC) of the Royal Thai Government. The objectives of the workshop were based on the following considerations:

- the most up-to-date studies of the effects of pollutants on marine flora and fauna were usually conducted in temperate and not tropical countries. The effectiveness and efficiency of these methodologies needed to be tested and ascertained for the tropical conditions.

- to assess the applicability of and refinement, of methods for assessing the harmful biological effects of pollutants in a tropical environment.

- to develop the scientific skills of workshop participants engaged in studies and assessment of biological effects of pollutants in the East Asian Sea region.

- the necessity for comparable studies and results in the relevant institutions of the participating countries of the region.

The Phuket Biological Effects of Pollutants workshop was therefore designed to emphasize the above considerations whilst the specific objectives of the Water Quality Bioassay component were to concentrate on:

1. - identifying an indigenous invertebrate species in a gravid condition.
2. - demonstrating practical methods for obtaining and assessing gamete quality.
3. - assessing developmental success in the early life stages of marine inverte-brates.
4. - identifying lethal and sub-lethal end-points to determine biological effects of pollutants.

The early life stages of marine invertebrates, especially pelagic larvae, are extremely sensitive to variations in water quality (Wilson, 1951), and are therefore suited for short-term exposure experiments. Many embryonic/larval stages have the advantage that they are lecithotrophic (nourishment is gained from endogenous feeding on yolk) for up to several days, thus avoiding the problems and inconvenience of culturing food for the duration of the test. Echinoderm embryos and larvae have previously been used in water quality assessment (Kobayashi, 1971; Pagano *et al.*, 1986, 1992; Bressan *et al.*, 1991), due to their ease of culture, distinct developmental stages and relative sensitivity to environmental variables (Itoh, 1992). Native blue-spot sea urchins *Diadema setosum*, were recommended by staff at the PMBC as a suitable test species, and an evaluation of their potential for biological effects monitoring was undertaken.

METHODS

1. Test species: Indigenous stocks of gravid adult blue-spot sea urchins *Diadema setosum*, were identified and collected from a "clean" local bay, close to the PMBC, for demonstration purposes during the workshop, and to fulfil the objectives of the bioassay component. Twenty adults were collected at low water and transferred to the laboratory sea water system prior to spawning.

2. Artificial spawning of *Diadema setosum:* Adult sea urchins were held at ambient sea water temperatures (26°C) in the laboratory in glass aquaria in clean aerated sea water for no more than 24h prior to spawning. To ease the handling procedures all large spines were trimmed from the adult's test prior to induction of spawning. Each adult was then rinsed with flowing sea water to remove detritus and any possible spermatozoa that may have been released from other specimens in the holding tanks. Individual adults were induced to spawn, as required by injecting 1 ml of 0.75M KCl solution into the coelom through the peristomial membrane, as described by Pagano *et al.,* (1986). Immediately after injection, the sea urchins were inverted and held in contact with a beaker of filtered sea water to allow released gametes to fall clear of the gonopores and settle on the base of the beaker. Five minutes after injection, the quality of released gametes was assessed by microscopic examination, any adults failing to release gametes after 5 minutes were discarded. Spermatozoa quality among spawning males was based on relative motility of released gametes. Males with poor quality spermatozoa (low motility) were discarded, while selected males were retained. Determination of egg quality was dependant on regularity of shape (high quality eggs were spherical) and uniformity of the size. Only females releasing large regular oocytes were selected for subsequent matings. Eggs from each female were held on a 53µm nytex mesh to enable subsequent rinsing of the embryos.

3. Assessing embryonic and larval development: *Fertilization.* Egg densities from three individual females were estimated by eye, then each suspension adjusted to give approximately equal densities. Single male/ female crosses were achieved by adding 1 ml of motile spermatozoa to 100 ml of oocyte suspension. This was repeated with separate pairings, to yield 3 distinct parental crosses (crosses A, B & C). All fertilizations were conducted at 26°C ± 1°C, under static conditions using 0.45µm filtered sea water (aerated prior to use) from a "clean" site off the jetty at the PMBC. Each fertilization was conducted within 20 minutes of the first signs of gamete release.

Twenty minutes after the addition of spermatozoa to the eggs, the sample was carefully rinsed 3 times in clean reference sea water to remove coelomic fluids liberated during gamete release and excess redundant spermatozoa, reducing the risk of polyspermy and possible bacterial growth. Thirty minutes after the addition of spermatozoa to the egg suspension, fertilization success was determined as the percentage of eggs with a visible fertilization membrane (Table 1). Simultaneously, total numbers of embryos were assessed for each parental cross using a Sedgewick Rafter Cell.

One hour prior to inoculation, water samples were vigorously aerated for 15 minutes to obtain high oxygen saturation levels, then 200 ml aliquots of reference water (PMBC jetty) were dispensed into clean 250 ml glass beakers. All beakers were arranged at random in a thermostatically controlled incubator and allowed to equilibrate to the incubation temperature (18°C ± 1°C and 26°C) prior to the addition of the embryos. Embryos were adjusted to a final density of 50 embryos ml^{-1} then carefully transferred to the test water via Oxford pipette.

Parental cross A for both rearing temperatures was used to assess development stages reached at the times specified in Table 2. The remaining 2 crosses were then used to assess survival, and developmental stages reached after 48 hours (Table 3). Beakers were maintained under static conditions with a lose fitting top for up to 48 hours at each rearing temperature ± 1°C. Each beaker was then spiked with 1 ml of 4% buffered formalin to preserve the larvae for subsequent analysis. Developmental stages reached after the 48 h period were recorded, based on the first 200 larvae encountered from a random subsample.

RESULTS & DISCUSSION

Artificially induced spawning of field collected *D. setosum* was successfully achieved in the laboratory. Fertilization success was >98% for all three parental crosses (Table 1) with rapid progression through all developmental stages (Table 2). During early embryogenesis of *D. setosum*, the symmetrical cleavages, large blastomere size and distinct morphological stages of development, provided unequavacol sublethal end-points, ideal for teaching purposes. The embryogenesis of two indigenous bivalves, *Perna viridis* and *Chlamys senatoria*, (both with asymmetrical blastomere formation) was also studied, but rapid progression through the early development stages proved difficult for the novice to follow when compared with *D. setosum*.

Differences in *D. setosum* embryo development rate were observed between the two rearing temperatures (Table 2), with slower rates at the cooler 18°C. Overall developmental success was lower for embryos reared at 18°C, compared to those at 26°C (Table 3), with 65% of embryos at the late gastrula stage as opposed to the echinopluteus. Abnormal embryonic development was observed at both rearing temperatures; consisting predominantly of asymmetrical blastomere formation, gut evagination and in older larvae skeletal deformities were common (Table 4).

The aim of this comparison was to demonstrate to the participants the dramatic effects of temperature on developmental success without the addition of environmantal contaminants and to identify a range of sublethal end-points for determining biological effects.

Table 1. Fertilization success of three separate parental crosses of the blue-spot sea urchin (*Diadema setosum*) at 26°C, 30 min after the addition of spermatozoa to the egg suspension. the presence of a fertilization membrane was considered to be indicative of a successful fertilization.

Spawning cross	Fertilization success
A	98% n = 200
B	98% n = 200
C	89.5% n = 200

Table 2. Developmental stages of the blue-spot sea urchin (*Diadema setosum*) at two temperatures. t=0 is the time at which the spermatozoa were added to the egg suspension. Time of embryo addition to the cooler water (*32 min).

Time (approximate)		Dominant developmental stage	Unequivocal morphological characters
18°C	26°C		
	0	Unfertilized egg	Spherical egg, distinct nucleus
*	2min	Fertilized egg	Fertilization membrane separating from the egg
2h	1h	1st Cleavage	2 equal blastomeres (cells)
12h	6h	Blastula	End of cleavage, hollow ball of cells
20h	8-10h	Early gastrula	Early mouth, 1st mesenchyme cells
26h	12h	Late gastrula	2nd mesenchyme cells, presence of archenteron and triangular spicule
36h	18h	Prism larva	Presence of skeletal spicules
48h	24h	Pluteus	Distinct mouth, anus and oral arms

The response in the laboratory of suitable test organisms, such as *D. setosum*, to environmental variables can be used as an index of water quality. In this way, all the biologically relevant variables that affect water quality are integrated in terms of the responses of individual organisms. The blue-spot sea urchin *D. setosum,* is common through out east Asia, and is easy to spawn and rear in the laboratory; all factors promoting the species as a potential sentinal test organism for the region. Among the advantages of determining biological effects on early life stages, is the integration it

Table 3. Developmental stage after 24 hrs. post-fertilization of the blue-spot sea urchin (*Diadema* setosum) reared at two temperature.

Temperature	% gastrula	% prism	% pluteus
18°C	65.0	29.3	5.7
26°C	0.0	3.4	96.6

provides, not only of the effects of toxicants and modifying variables that are known and understood, but also those that are not yet known (Stebbing, 1985). The results obtained with the sea urchin embryos in this pilot study show great potential for the application of similar studies in the field.

Biological effects of sediment and surface water toxicity could be addressed in the east Asian region. Bioassay studies in temperate regions have demonstrated that the sea surface microlayer represents a unique physical and chemical environment, with enriched levels of contaminants compared to the subsurface water mass. Embryonic and larval stages of marine organisms can reside or visit the microlayer and are therefore vulnerable to accumulation of enriched levels of contaminants. Considering the importance of surface waters for the accumulation and transfer of pollutants, water quality bioassays should be conducted to ascertain the biological effects of microlayer samples from the east Asian region.

Table 4. Common malformations of the early life stages of the blue-spot sea urchin (*Diadema setosum*).

Malformation	Affected developmental stage
Asymmetrical blastomeres	1st cleavage to morula
Gut evagination	Early gastula to prism
Pre-pluteus blockage	Prism
Skeleton deformities	Plutrus

In order to ascertain the significance of both sediment and microlayer toxicity on the early life stages of marine organisms, future studies should consider the possible pathways of contaminant uptake. The exposure of early life stages to contaminants could be by direct uptake from the water column, sediments and microlayer, or possibly indirectly through the accumulation of contaminants in maternal gonads during oogenesis. Additional information on the occurrence and abundance of embryos and larvae in the water column, and the frequency of contact with the microlayer would aid extrapolation of results from laboratory studies to the field.

In many organisms cellular responses to environmental stress may be detected before stress becomes apparent in the physiology of the whole organism. Biochemical/cellular stress responses may provide a rapid and sensitive indicator of environmental alterations (Moore, 1980, Lowe, in press) and potentially can provide direct evidence of biological deterioration and the probable aetiology of the biological damage (Moore, 1990). At the other end of the scale of biological complexity, analysis of community structure (Warwick and Clarke, in press) offers a retrospective picture of responses to general environmental changes (Stebbing *et al.*, 1992). The results from the Phuket workshop are encouraging, but they demonstrate the need for a high level of integration between disciplines, to interpret fully the results of environmental toxicology. The use of sublethal biomarkers used in conjunction with a range of conventional bioassays, such as the embryo development of *D. setosum*, proposed here, could provide a powerful tool for biological effects monitoring in the future.

Many of the participants felt that the workshop had opened their eyes to the range of methods that had previously been perceived as being either impossibly difficult or, outside their scientific experience and expertise. Direct discussion with the course participants identified the importance of simplicity for the methods being taught. A fact that has long been recognised by the staff of the Plymouth Marine Laboratory and, hence a prerequisite in the development of effective biological effects indicators. The techniques demonstrated in the bioassay component of the workshop are readily transferable to a variety of marine environments and between sentinel species or target ecosystems in the East Asian region.

ACKNOWLEDGEMENTS

We are grateful to the United Nations Environment Programme and the Phuket Marine Biological Center for sponsoring and hosting the Workshop on Biological Effects of Pollutants (16-26 November 1993). Thanks go to all the staff of the PMBC, especially Drs. Prawin Limpsaichol and Somkiat Kholiattiwong. Special thanks go to Dr. Jintana Nugranad (Coastal Aquaculture Development Center) for coming to our rescue at such short notice, to offer her expertise with the spawning of native bivalves.

REFERENCES

Bressan, M., M.G. Marin and R. Brunetti. 1991. Effects of linear alkylbenzene sulphonate (LAS) on skeletal development of sea urchin embryos (*Paracentrotus lividus*). - Water Research. **25**: 613-616.

Chansang, H. 1988. Effects of oil pollution in marine and coastal living resources of the East Asian Seas region. In: Oil pollution and its control in the East African Seas region. - UNEP Regional Seas Report. **96**: 41-55.

Gomez, E. D. 1988. Overview of environmental problems in the East Asian Seas region. - Ambio., **17**: 166-169.

Harland, A. D. and B.E. Brown. 1989. Metal tolerance in the scleractinian coral *Porites lutea*. - Mar. Pollut. Bull., **20**: 353-357.

Hungspreugs, M. 1988. Heavy metals and other non-oil pollutants in Southeast Asia. - Ambio., **17**: 178-182.

Hungspreugs, M., S. Dharmvanij and W. Utoomprurkporn. 1991. Water quality of Ban Don Bay. *In*: - Chou, Thia, Khoo, Lim, Paw, Silvestre, Valencia, White, Wong. (*eds.*). Towards an integrated management of tropical coastal resources. International Center for Living Aquatic Resources Management (ICLARM*)*. **22**: 153-165.

Itoh, Y. 1992. Environmental variables desirable for the growth of early pluteus larvae of three sea urchin species. - Océnanis. **18**: 19-27.

Lowe, D. M. (in press). Lysosomal membrane impairment in blodd cells of Perna viridis: an invitro marker of contaminant induced damage. - J. Phuket Marine Biological Center.

Loosanoff, V. l. and H.C. Davis. 1963. Rearing of bivalve molluscs. - Advances in Marine Biology. **1**: 1-136.

Lovatelli, A. 1988. Site selection for mollusc culture. - Proceedings of the Workshop on GIS applications in aquaculture, Bangkok, Thailand. 25 pp.

Kobayashi, N. 1971. Fertilized sea urchin eggs as an indicatory material for marine pollution bioassay, preliminary experiments. - Publs. Seto mar. Biol. Lab., **18**: 379-406.

Menasveta, P., V. Cheevaparanapiwat and C. Wongwit. 1985. Heavy metals in bivalves collected from river estuaries of Thailand. - Asian Mar. Biol., **2**: 31-36.

Middelfart, P. 1992. Early life stages of the muricid gastropods *Chicoreus ramosus, C. torrefactus* and *C. brunneus* from Phuket Island, Thailand. *In*: - J. Hylleberg, K. Ayyakkannu and S. Khokiattiwong (*eds.*). Proceedings of the second workshop of the tropical marine mollusc programme (TMMP), Annamalai University, IndiaPhuket Marine Biological Center. No **10**: 113-122.

Moore, M. N. 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. - Rapp. P.-v. Réun. Cons. int. Explor. Mer., **179**: 7-15.

Moore, M. N. 1990. Lysosomal cytochemistry in marine environmental monitoring. - Histochemical J., **22**: 187-191.

Pagano, G., M. Cipollaro, G. Corsale, A. Esposito, E. Ragucci, G. Giordano and N. Trieff. 1986. The sea urchin: Bioassay for the assessment of damage from environmental contaminants. *In*: - J. Cairns (*ed.*). Community toxicity testing Philadelphia, Association for Standard Testing and Materials, p. 67-92.

Pagano, G., A. Esposito, M. Guida, G. Melluso and N. Trieff. 1992. Sublethal toxicity testing in sea urchin fertilization and embryogenesis: A study of polluted water and sediment from two rivers in Campania, Italy. - MAP Technical Report. **69**: 209-215.

Phromlert, C. 1992. Local government initiatives in waste management. *In*: - T.E. Chua and L.R. Garces (*eds.)*. Waste management in the coastal areas of the Asian region. Roles of governments, banking institutions, donor agencies, private sector and communities. International Centre for Living Aquatic Resources Management (ICLARM), Manila-Philippines. **33**: 185-188.

Siripong, A. 1988. Oil and marine environment. *In*: - Oil pollution and its control in the east African Seas regions. UNEP-Regional Seas Report. Study. **96**: 255-272.

Siriwong, C., H. Hironaka, S. Onodera and M.S. Tabucanon. 1991. Organochlorine pesticide residues in green mussel (*Perna viridis*) from the Gulf of Thailand. - Mar. Pollut. Bull., **22**: 510-516.

Stebbing, A. R. D. 1985. Bioassay. *In*: - B.L. Bayne, D.A. Brown, K. Burns, D.R. Dixon, A. Ivanovici, D.R. Livingstone, D.M. Lowe, M.N. Moore, A.R.D. Stebbing and J. Widdows. (*eds*.). The effects of stress and pollution on marine animals. Praeger, New York. p. 133-137.

Stebbing, A. R. D., V. Dethlefsen, R.F. Addison, M. Carr, P.M. Chapman, W.P. Cofino, C. Heip, L. Karbe, M.N. Moore and A.D. Vethaak. 1992. Overall summary and some conclusions from the Bremerhaven workshop. - Mar. Ecol. Prog. Ser., **91**: 323-329.

Thain, J. E. 1991. Biological effects of contaminants: Oyster (*Crassostrea gigas*) embryo bioassay. - ICES report. Techniques in Marine Environmental Sciences. No. **11**.

Warwick, R. M. and K.R. Clarke. (in press). Multivariate measures of community stress and their application to marine pollution studies in the East Asian region. - J. Phuket Marine Biological Center.

Wilson, D. P. 1951. A biological difference between natural sea waters. - J. Mar. Biol. Ass. U.K., **30**: 1-22.

Woelke, C. E. 1972. Development of a receiving water quality bioassay criterion based on the 48 hour Pacific oyster (*Crassostrea gigas*) embryo. Washington Department of Fisheries, - Technical Report. **9**: 1-93.