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Biological effects of contaminants: Oyster (Crassostrea gigas) embryo bioassay

J.E. THAIN

Ministry of Agriculture, Fisheries and Food
Directorate of Fisheries Research
Fisheries Laboratory
Remembrance Avenue
Burnham-on-Crouch
Essex CM0 8HA
United Kingdom

INTERNATIONAL COUNCIL
FOR THE EXPLORATION OF THE SEA
Palægade 2-4 - DK-1261 Copenhagen K

INTERNATIONAL COUNCIL FOR THE EXPLORATION OF THE SEA
CONSEIL INTERNATIONAL POUR L'EXPLORATION DE LA MER
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BIOLOGICAL EFFECTS OF CONTAMINANTS: OYSTER (CRASSOSTREA GIGAS) EMBRYO BIOASSAY

1 INTRODUCTION

The oyster embryo bioassay was initially developed by Woelke (1972). The Ministry of Agriculture, Fisheries and Food (MAFF) in the UK have modified the method to improve the accuracy of the test and allow its use on board research vessels. This paper describes the modified method which has been used to obtain a measure of the deterioration in biological water quality in UK coastal areas receiving anthropogenic discharges. MAFF have successfully used this protocol since 1976 (see Lloyd and Thain, 1981; HMSO, 1982; Thain and Watts, 1984; Byrne et al., 1985; Utting and Helm, 1985; Byrne et al., 1986; Law et al., 1986; Byrne et al., 1988).

The phrase "deterioration in biological water quality" implies that a change in chemical, physical, and/or biological composition has occurred which is potentially harmful to aquatic organisms. A bioassay to measure such a deterioration should be based on a response by an organism which clearly represents a harmful effect at both the individual and the population level of organization. The lowest level at which such responses can be measured with certainty are the three 'scopes' for activity, growth, and reproduction.

The organism response used in this bioassay is the ability of the oyster embryo to develop normally and reach the 'D'-shaped larval stage (at which the paired hinged shells can be seen) within 24 hours. Although the exposure time is short, it encompasses a period of intense cellular activity during which the impairment of a number of critical physiological and biochemical processes may result in poor growth and development. The response measured is, therefore, similar to that used in other early life stage tests which record growth and development, and it has the advantage that exogenous feeding is not required, thus eliminating this source of variation in the test results.

2 TEST METHOD

2.1 Reference sea water

A large volume of sea water is taken during the winter months, filtered through Whatman GFC filter paper, and stored frozen (-18^{0}C) in acid-washed bottles for subsequent use in oyster gamete collection and control exposures. It is preferable to use offshore water for this purpose; the author uses water from the Western Approaches (N 48^{0} 29' W 08^{0} 02') to the English Channel. Clean natural sea water is recommended rather than artificial sea water. Experience has shown that artificial sea waters vary in quality and often produce inconsistent results in control embryo development.

2.2 Water samples for bioassay

Sample volumes should be 200 ml; aliquots of this sample are placed in the test vials immediately after sampling and bioassayed as soon as possible to avoid sample deterioration. If it is necessary to delay the assay, the samples in the vials should be stored at the ambient sea water temperature.

In some samples, it may be necessary to remove particulates and suspended solids which can affect embryo development (Davis and Hidu, 1969). At present, the exact particle size and particle loading which may affect embryo development are not known, but they are likely to be under 10 µm in size and in excess of 300 ppm loading. In estuaries where turbidity is often high, it is a serious factor for consideration. Ideally, filtration is not recommended. The author uses Whatman GFC filtration to facilitate a large through-put of samples, but this method may remove some of the dissolved contaminants. For small numbers of samples, centrifugation is recommended.

2.3 Organism

The test described here uses the Pacific oyster (Crassostrea gigas), but the method can be readily adapted for those other species of bivalve mollusc whose eggs are fertilized in the water column. Adult oysters (greater than 45 g live weight) are conditioned for spawning in the laboratory by maintaining them at 22^0C in flowing sea water to which a mixed algal diet supplement of Isochrysis, Tetraselmis, and Skeletonema sp. is added (Walne and Spencer, 1971; Helm et al., 1973). Maturation occurs within 1 week in summer and 8 weeks in winter.

2.4 Collection of oyster gametes

For each assay, mature oysters are opened and the gametes stripped from the first two males and three females, using clean Pasteur pipettes for each oyster. The gametes from each oyster are then transferred to separate 1 litre volumes of reference sea water at 24^0C. Sperm are identified by their milky appearance and eggs by their granular appearance, once they are in the water. After filtration through a 90 µm filter to remove tissue debris, the three batches of eggs are pooled and the egg density adjusted by eye with reference sea water to give a density of between 1000 and 4000 (and ideally between 3000 and 4000) eggs per 1 ml. Clearly, this comes through experience and some practice is essential before carrying out the assay. This density is then measured accurately using a Coulter Electronics particle counter or a counting chamber and microscope (preferably an inverted type). The two batches of sperm are also pooled, but not filtered. All the glassware used is sterilized in an autoclave.

Stripping the gametes from the oysters allows the best broodstock to be chosen for a test and, furthermore, permits one to carry out the assay when desired. Synchrony in gamete collection and fertilization is imperative. Gamete stripping does not appear to affect the quality or viability of the embryos (Allen et al., 1988).

2.5 Fertilization

The mixing of gametes is done at a ratio of approximately 2 ml of sperm to 1 litre of egg suspension. After mixing, they are left for 1-2 hours at 24°C without aeration, during which time a sample is examined under a microscope to confirm that the early stages of cleavage are occurring. If cleavage is not observed within 2 hours, the eggs should be discarded and other oysters stripped for gametes.

2.6 Test sample

At least four 30 ml aliquots of each sample of sea water from the survey area are placed in 50 ml polystyrene vials (stoppered or capped), and the temperature is raised to 24°C.

2.7 Control sample

At least eight 30 ml aliquots of reference sea water at 24°C are placed in 50 ml polystyrene vials (stoppered or capped).

2.8 Start of exposure

Using a sterilized Gilson (or similar model) automatic dispensing pipette, 1500 eggs at the early stage of cleavage are added to each 30 ml sample; the added volume of water containing the eggs is between about 0.37 and 1.50 ml (preferably between 0.37 and 0.50 ml), depending on the density of the egg suspension. The samples are incubated at 24°C for 24 hours; no aeration is necessary unless the samples have a high oxidizable organic content. Measurements of dissolved oxygen and pH are necessary only if they are likely to deviate from natural values, for example, when sampling in areas of known industrial waste contamination.

2.9 End of exposure

At the end of the 24-hour exposure period, each vial is gently shaken, and a 2 ml aliquot of sample is removed and 2 drops of 8% formalin added. This sample, which should have originally contained 100 eggs, is transferred to a gridded shallow dish or slide, and the number of normal 'D'-shaped larvae counted. Where large numbers of samples have been taken, the contents of the vials can be preserved for future counting by the addition of 0.5 ml of 20% buffered formalin.

2.10 Calculation of results

The number of 'abnormal' embryos is calculated to be 100 minus the number of normal 'D'-shaped larvae. 'Abnormal' includes those eggs which were not fertilized, and those which died at an early stage of development or became malformed. The additional abnormalities in the

test samples, compared with those in the control, is expressed as the Percent Net Response (PNR).

$$PNR = \frac{\%\ \text{test abnormality} - \%\ \text{control abnormality}}{100 - \%\ \text{control abnormality}} \times 100$$

Data derived in this way should be arcsine transformed prior to statistical treatment using analysis of variance.

3 SOURCES OF ERROR

3.1 Number of exposed embryos

The calculation of the results is based on the assumption that there were 100 embryos exposed in each 2 ml of test or control sample. This assumption may not be valid because of errors in:

(i) the measurement of the original egg density;

(ii) pipetting the aliquot containing the calculated 1500 eggs into each 30 ml in the sample vials;

(iii) the extra dilution which this aliquot gives to the 30 ml sample;

(iv) pipetting out the 2 ml aliquot for embryo examination.

Errors in (i) and (iii) should be constant between the test and control samples and, therefore, lost to some extent in the calculation of the PNR. For example, if in practice only 95 eggs were present in each 2 ml sample, this would be equivalent to an extra 5 per cent of non-fertilized eggs. In one series of tests, the mean number of eggs transferred to the sample vials was found to be 96 ($n = 58$, $SD = 8.0$, $SE = 1.05$); this slightly low value may reflect an error in the measurement of the original egg density, and the variation between vials can be reduced by ensuring that the eggs are evenly distributed in the stock suspension. Errors in (ii) and (iv) are random, and the overall error can be reduced by the use of replicate samples.

While additional procedures could be introduced into the method which would help to achieve the nominal egg density, they would be time consuming. Speed of operation is essential at the start of the exposure period, particularly when a large number of samples are being assayed, and the small errors which occur may be considered acceptable.

Annex 1 shows the results of a typical experiment; it is clear that, with experience in the techniques, the variation between replicates can be small. With a precision of this order, a statistically significant reduction in biological water quality can be shown when the PNR exceeds 5.

3.2 Dilution of sample

The procedure described above will lead to a dilution of the test sample by up to 5 per cent. Such a dilution effect may not be important in field surveys, where a variation between PNRs is looked for in relation to a known or suspected source of pollution (see Figure 1). However, if the toxicity of a chemical is being measured, it will be necessary to take this additional dilution into account when calculating the nominal exposure concentrations. If the volume of egg inoculum is below 0.5 ml, the error from this source is minimal.

3.3 Acceptable control abnormality

In the original method of Woelke (1972), it is recommended that control abnormalities should not exceed 5 per cent. However, these recorded abnormalities at the end of the exposure period do not include mortalities at the early stage of development, nor do they include non-fertilized eggs. It is common hatchery experience that, with good management practice, at least 80-85 per cent of oyster eggs develop successfully to the 'D'-shaped stage, although sometimes this falls to 50 per cent (Loosanoff and Davis, 1963). Experience with the oyster embryo bioassay indicates that control 'abnormalities' of up to 20 per cent are normal, and that up to 50 or even 60 per cent is acceptable. Higher percentages of abnormalities may be caused by contaminated reference sea water, or the use of immature gametes from oysters which have not reached sexual maturity, or gametes from oysters in poor condition.

4 REPRODUCIBILITY OF THE TEST

In recent years, tributyl tin has been used as a reference toxicant. Exposure of developing oyster embryos to a range of concentrations showed that 1.65 µg TBT/l would give a PNR of 50, and this concentration has been used in conjunction with subsequent field surveys. PNRs recorded for the reference toxicant in eight successive surveys have ranged from 37 to 71, with a mean value of 51; using data from the initial calibration test, this range of PNRs is equivalent to a concentration range of 1.25 to 1.95 µg TBT/l. Although the control abnormality in the separate tests ranged from 13 to 44 per cent, there was no apparent correlation between these levels and the calculated PNRs. This bioassay, therefore, has a reasonable reproducibility.

5 QUALITY ASSURANCE

This protocol has been used routinely at MAFF for many years and all aspects of quality control and sources of error have been covered above. In the author's experience, particular attention should be given to the source of reference sea water (Section 2.1, above), the storage of water samples, and the choice of method for the removal of suspended solids (Section 2.2, above). A minimum of eight control and four test samples is required, and it is important that a suitable reference chemical be included with each test. Persons using

the bioassay for the first time should obtain conditioned broodstock from an oyster hatchery; oysters poorly conditioned for spawning usually produce embryos with poor development to the 'D' larval stage.

6 EXAMPLE OF SURVEY DATA

Annex 1 gives details of the oyster embryo bioassay data obtained in 1984 from a survey of the sewage sludge disposal ground between Plymouth and the Eddystone lighthouse, Devon, UK. This shows the degree of reproducibility between replicate analyses of single samples.

Analyses of the toxicity of the sewage sludge to oyster embryos showed that the field PNRs correlated well with the concentration of sludge at the disposal ground, as calculated from suspended solids concentrations (Thain and Stebbing, in prep.).

As an example of the type of information obtained, Figure 1 shows the variation in water quality at another sewage sludge disposal ground when discharges were being made.

The design of such surveys should follow the same pattern, and be conducted with the same rigour, as that used for chemical sampling in similar circumstances.

7 INTERPRETATION OF DATA

The results of this bioassay cannot be used to predict the effects of a small measured deterioration in biological water quality on oyster populations, and even less on the general aquatic biota. This is especially the case where the natural biota are exposed to poor biological water quality for only a short period of time, whereas in the bioassay the embryos are exposed to the water sample for 24 hours. The results can be used to measure the gradients and distribution of poor biological water quality in the vicinity of pollutant inputs; where a gradient is found, it can be assumed that the potential for harm to aquatic biota will increase with the degree of the bioassay response, but the nature and extent of the effect which may occur in practice cannot be predicted. The bioassay is sensitive to a wide range of chemicals; it is also sensitive to a deterioration in biological water quality caused by algal blooms (Thain and Watts, 1987).

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ANNEX 1

Data from a survey of a transect between Plymouth and the Eddystone lighthouse. The sewage disposal ground is at stations 5 and 6. This information is given to show the variation obtained between replicates.

Sample		Number of D larvae in a 2 ml sample	$\bar{x}$	SD	PNR
Before dumping					
Station 1	Plymouth	73, 75, 70, 74 77, 69, 69, 67	72	3.5	-4.3
Station 4		69, 67, 66, 73 67, 74, 70, 70	69	2.9	0
Station 6		67, 69, 72, 68 70, 70, 74, 68	70	2.3	-1.4
Station 8	Eddystone	69, 70, 70, 72 76, 69, 65, 71	70	3.1	-1.4
Control		74, 68, 68, 70 65, 69, 68, 73 71, 66, 69, 66	69	2.7	-
Station 6 was then sampled at intervals during the disposal of sewage sludge					
6.1		22, 18, 24, 20 28, 29, 19, 21	23	4.1	67.0
6.2		35, 40, 40, 32 33, 34, 38, 35	36	3.1	48.0
6.3		0, 0, 0, 0, 0, 0, 0, 0	0	-	100.0
6.4		46, 49, 53, 42 42, 47, 42, 53	47	4.6	32.0
6.5		76, 70, 71, 73 65, 69, 72, 71	71	3.2	-2.9

Figure 1. Oyster embryo bioassay, Liverpool Bay, August 1981. Per cent net response fo surface water samples.

Underlined values are significantly different from the control value.