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Development of tools for assessing genotoxicity

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Steve Killeen

Head of Science

Executive Summary

Background
In addition to the measurement of sets of single-substance standards, known as Environmental Quality Standards (EQSs), or Environmental Assessment Levels (EALs), the aqueous environment is protected by measurements of biological effect. Ecotoxicological tests and bioassays are also employed to determine the toxic hazard posed by chemicals and environmental samples. The Environment Agency currently uses such tests within its Direct Toxicity Assessment (DTA) programme to set toxicity targets for selected effluent discharges to surface waters.

Aims and objectives
A limitation of DTA tests and bioassays is that they usually measure acute toxicity and lethality and, as such, provide little information regarding sub-lethal biological effects, particularly at the genetic level. This report presents the results of a series of research and development studies, which aimed to coordinate laboratory and field exposure studies between the Environment Agency and the University of Plymouth.

Conclusions and recommendations
Investigations were carried out to assess the potential for biomarkers of genotoxicity to be applied in association with currently employed DTA methods in the same target species. The conclusions and recommendations from this report are as follows:

- The chromosome aberration (CAb) assay was successfully developed and validated against reference mutagens in the embryo-larval stages of the Pacific oyster, *Crassostrea gigas.*
- The CAb assay was successfully applied to test the genotoxic potential of whole effluents and sediment elutriates using the embryo-larval stages of *C. gigas.*
- As a method for routine monitoring of environmental samples, the CAb assay is somewhat laborious compared with some DTA methods. In addition, fully trained and experienced staff are required to observe and analyse the chromosomes. This restricts the testing of numerous water samples for genotoxic potential. But compared with other methods (Ames and GreenScreen® assays), it appeared to be the most sensitive in terms of detecting genotoxicity.
- The induction of metallothionein, inhibition of acetylcholine esterase activity, and ferric reducing antioxidant power may be useful measures of sub-lethal biological effects. Further optimisation and validation is required for these methods in *Arenicola marina*. Cause–effect relationships can only be extrapolated with additional inter-discipline data.
- Initial results indicate that other methods for detecting genotoxic effects such at the Comet assay can be applied in species such as *A. marina*. Application of this method requires further optimisation and validation.

Contents

1 Introduction

In England and Wales, effluents are traditionally regulated by means of sets of single-substance standards, known as Environmental Quality Standards (EQSs), or Environmental Assessment Levels (EALs). These are designed to protect the aquatic environment and specifically the aqueous compartment. Standards for sediment can also be set, but their application is generally less widespread than aqueous standards. The levels of contaminants permitted within discharges are based on compliance with the relevant chemical standard after initial dilution in the receiving environment. This method is known as the substance-specific approach.

Bioassay-based regimes have the advantage, especially when assessing the hazard of complex samples containing thousands of substances, of integrating the overall adverse effect attributable to all the substances, including those without an EQS or EAL. Within the Environment Agency, 'whole-sample' toxicity tests have been deployed for regulatory purposes under the auspices of its Direct Toxicity Assessment (DTA) programme. Such tests are now a requirement under the Integrated Pollution Prevention and Control (IPPC) regime for all chemical companies above a certain size that discharge directly to controlled waterways. Under the DTA risk assessment process, companies whose discharges are predicted by modelling to be toxic after dilution in the receiving environment (beyond a predefined point of protection) must undertake a programme of toxicity reduction as a condition of their IPPC permit to discharge.

The bioassays initially applied with DTA were selected from existing, internationally standardised methods adapted for use with effluents. But in addition to general toxicity, Annex VIII of the Water Framework Directive 2000/60/EC includes 'Substances and preparations, or the breakdown products of such, which have been proved to possess carcinogenic or mutagenic properties' in its indicative list of the main pollutants.

The implications of genotoxicity on ecosystems are highly important since genetic lesions may led to adverse effects such as pathophysiological changes or reduced fitness (including effects upon reproduction) in individuals and potential alterations in gene frequencies and genetic diversity in populations and communities (Depledge 1994, Bierkins *et al.* 2005). Carcinogenic or mutagenic properties of substances are mediated, in the majority of cases, by their ability to interact with cellular DNA either causing damage (strand breakage) or by altering its base-pair sequence. Genotoxic substances may also act indirectly, interfering with the proteins and enzymes that orchestrate normal DNA production, manipulation and repair. These interactions potentially interfere with normal DNA replication. In higher organisms, damage to DNA material has been linked directly to neoplasia and, in some cases, malignancy (Weinstein 1988).

This report documents a series of laboratory studies carried out at the University of Plymouth with the aim of developing and validating biomarker techniques. Particular emphasis was placed on applying methods in species currently used in DTA risk assessments. Particular attention was given to the measurement of sub-lethal biological effects at the genetic level in order to provide information regarding genotoxic effects, which is often not provided by standard DTA methods.

1.1 Structure of this report

The following three chapters of this report describe genotoxicity studies carried out in the following areas:

- determination of chromosome aberrations in the Pacific oyster, *Crassostrea gigas* – cell cycle kinetics, validation studies, application to effluents and sediment elutriates;
- assays based on the lugworm, *Arenicola marina*, using sediment samples collected by the Environment Agency from Seal Sands on Teesside as part of its work under the Habitats Directive – metallothionein induction, acetylcholine esterase activity and ferric reducing antioxidant power (FRAP);
- application of the Comet assay to *Arenicola marina* in sediment samples collected by the Environment Agency from the River Thames as part of the National Marine Monitoring Programme.

The aim, method, results and observations for each study are presented within the chapters, with conclusions and recommendations on the topic covered given for the whole chapter. The final chapter lists the publications prepared as a result of this Environment Agency Science Group project.

Appendix 1 presents the standard operating protocols (SOPs) used at the University of Plymouth and Appendix 2 the results of a series of experiments carried out by the Environment Agency's National Laboratory Service to develop and validate the OysteR Embryo Chromosome Aberrations (ORCA) assay.

2. Determination of chromosome aberrations

The metaphase chromosome aberration (CAb) assay is one of several cytogenetic tests that uses light microscopy to identify genetic lesions such as clastogenesis in mitotically dividing cells (Scott *et al.* 1990). Analysis of CAbs has been considered important because of their known role in the induction of malignancies, congenital abnormalities and foetal wastage (Natarajan *et al.* 1994, Tucker and Preston 1996). It is thus one of the standard tests employed within a battery of methods for assessing genotoxicity of pharmaceuticals in mammalian systems. Genotoxic agents produce structural chromosomal aberrations by a variety of mechanisms with the end-point being a discontinuity in the chromosomal DNA, which is usually observed during metaphase. The assay has more recently been applied in aquatic organisms to study the impact of genotoxic agents released into the aquatic environment (Jha 2004).

Larvae of the Pacific oyster, *Crassostrea gigas*, are used in standardised developmental bioassays in the UK to assess the hazard of complex industrial effluents from the chemical industry as part of Direct Toxicity Assessment within the IPPC regime. They are also used routinely within the UK National Marine Monitoring Programme carried out to fulfil OSPAR[1] obligations.

The aims of the studies described in this chapter were to:

- optimise, develop and validate the CAb assay in the early life stages of *C. gigas*;
- apply the CAb assay in *C. gigas* in order to quality the genotoxic potential of industrial effluents.

2.1 Cell cycle kinetics

Aim: To establish the cell cycle kinetics of *Crassostrea gigas* early life stages.

The average generation time (AGT) of the embryo-larval cells of *C. gigas* was not known. It was therefore necessary to calculate it before undertaking cytogenetic studies. Temperature is known to have an influence upon the rate of cell division and thus rates of embryonic development. The aim of this initial study was therefore to determine the proliferation rate index (PRI) and the AGT in the embryo-larval stages of *C. gigas* at two different temperatures, 15 and 20°C.

2.1.1 Methods

C. gigas embryo-larvae were collected by the strip spawning of adult oysters. A brood stock of conditioned *C. gigas* was supplied from a culture maintained at Guernsey Sea Farms. At least two males and two females were strip spawned as described by Thain

[1] 1992 Oslo and Paris Convention on the protection of the marine environment of the North-East Atlantic

(1991) and their gametes resuspended in separate vessels containing ~50 ml artificial seawater (20 ± 2°C). Before fertilisation, the gametes were checked both qualitatively and quantitatively. Viable eggs from one female were fertilised as described by in the ASTM standard guide (ASTM 1992). The embryos were resuspended in artificial seawater and incubated at a maximum density of 100 embryos per ml for 12 hours at the respective temperatures (15 or 20 ± 2°C) in the dark before the first treatments were exposed to 5-bromodeoxyuridine (BrdU).

A stock solution of BrdU was made up with filtered seawater at a concentration of 1.0 x 10^{-4} M and stored in a 500 ml amber glass bottle, which was further protected from photo-activation by covering with aluminium foil. The embryo-larvae were exposed to a working solution of 1.0 x 10^{-5}M BrdU for periods of 4, 6, 8 and 12 hours at each temperature (15 or 20ºC) before fixing. To synchronise the time at which the samples were fixed (when the embryo-larvae were 24-hours old), the times of addition of BrdU were staggered.

The methods of slide preparation, chromosome staining and chromosome analysis are presented in Appendix I. These were adopted from earlier studies by Harrison and Jones (1982) and subsequently modified by others (e.g. Jha *et al.* 1996, 2000). When the embryo-larvae were 24-hours old and had received 1.0 $\times$ 10^{-5} M BrdU exposure for 4, 6, 8 or 12 hours, they were poured through a 30 µm nylon mesh sieve and treated with 0.025 per cent colchicine solution (w/v) dissolved in seawater, hypotonic solutions and fixed as per the protocol described in Appendix I. Metaphase spreads were then prepared.

Sister chromatid differential (SCD) staining was conducted to allow the number of cell cycles that the metaphases had passed through to be identified. SCD staining was carried out following a procedure developed by Goto *et al.* (1975), with some modifications introduced by Jha *et al.* (1996). A 0.025 per cent (w/v) of Hoechst 33258 solution was prepared in phosphate-buffered saline (PBS) in an amber glass 250 ml bottle and stored in the dark at room temperature until ready for use. The slides with the chromosome spreads were arranged in glass coplin jars and rinsed twice with distilled water to remove any dust or artefacts that might have attached to the surface of the slides. The distilled water was drained away, replaced with 0.025 per cent Hoechst solution and the slides incubated in the dark for 20 minutes at room temperature. The slides were then rinsed twice with distilled water and allowed to air-dry. A few drops of PBS were added to each slide and coverslips applied. The slides were then placed on a hot plate maintained at 55°C and simultaneously exposed to black light (F18W-BLB lights; Sylvania, UK) for 25 minutes. The slides were rinsed a further twice, allowing the coverslips to slide off in the process and air-dried. The slides were then stained with 10 per cent Giemsa solution, and coverslips mounted with DPX as per the staining protocol for CAbs described in Appendix I.

Metaphase spreads were examined using a bright field microscope (Olympus Polyvar), at a magnification of $\times$1000 (with oil immersion). To determine the cell cycle kinetics, at least 100 cells per replicate were examined and classified as first (M1), second (M2) or third or subsequent (M3+) division cells. The cells were classed by taking note of the pattern of staining for the metaphase cell being examined.

- Cells in M1 consisted of chromosomes that were stained dark throughout the spread, with the staining of the sister chromatids of each chromosome being of equal depth of staining.

- Cells in M2 consisted of chromosomes that were stained differentially and of a 'harlequin' appearance.

- Cells in M3 consisted of a mixture of chromosomes that were differentially stained and chromosomes that consisted of sister chromatids which were both lightly stained.

The PRI was calculated using the following equation, which is based on the method proposed by Lamerti *et al.* (1983):

$$PRI = \frac{[(1 \times M1) + (2 \times M2) + (3 \times M3)]}{\text{Number of cells scored}}$$

Once the PRI had been calculated, the generation time (GT) could be calculated using the following equation as proposed by Ivett and Tice (1982).

$$GT = \frac{\text{hours in BrdU}}{PRI}$$

During attempts to score the chromosomes for PRI, it was noted that there were very few metaphase spreads present (approximately 1–20 metaphases per slide) compared with past observations of a minimum of approximately 200 metaphases per slide when similar experiments were conducted with *Mytilus edulis* embryo-larval cells. Of the metaphases present, many of the chromosomes were not condensed adequately and thus the staining was of poor quality. It was noted that many of the larvae had developed into the prodissoconch stage ('D-shell' larvae). From previous experience it had been concluded that, ideally, the samples should be treated with colchicine at the trochophore stage when cells are still dividing rapidly and a shell has not yet formed. Figure 2.1 illustrates the morphology of the eggs and embryo-larval stages of *C. gigas*. The experiments were therefore repeated with a modification to the periods of exposure to BrdU (2, 4, 6 and 8 hours) and the timing of the harvesting of the embryo-larvae reduced to 20-hours old post-fertilisation. A further repeat experiment was conducted at 20°C for further validation of the cell kinetics at this temperature.

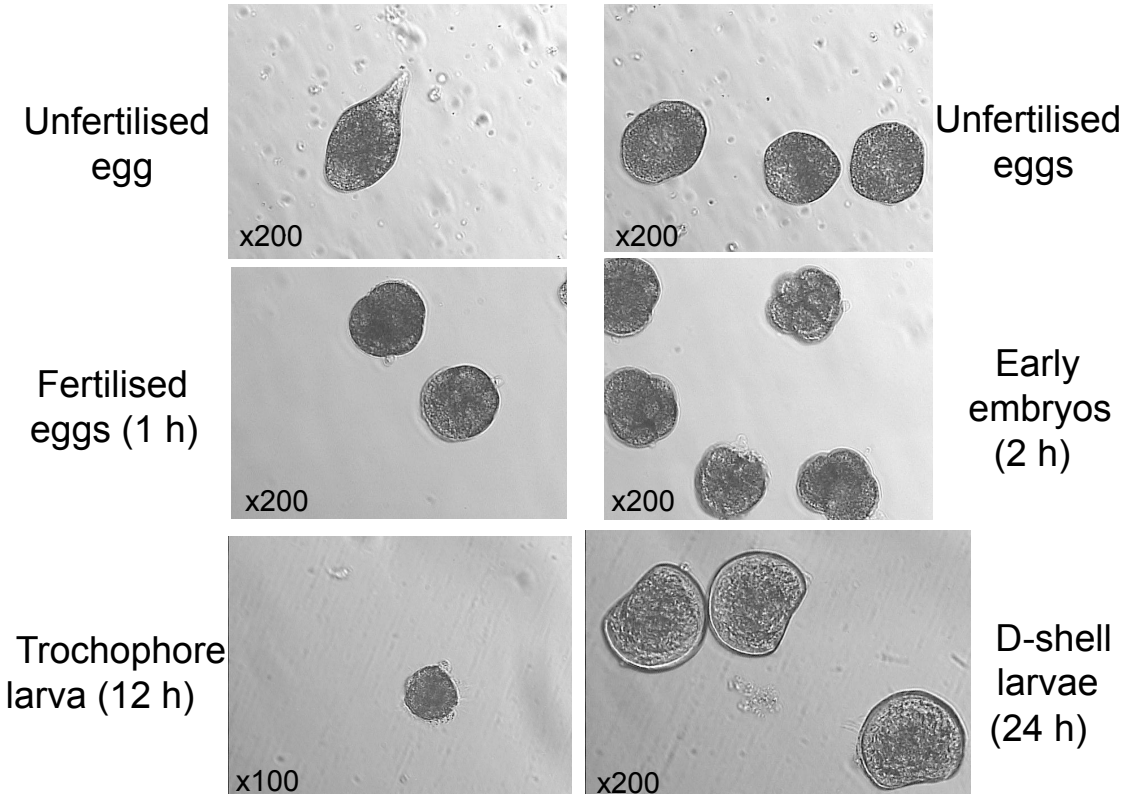


Figure 2.1 Morphology of eggs, embryos and larvae from *C. gigas*

2.1.2 Results and observations

Following a repeat of the cell cycle kinetics where the cells were harvested at 20 hours post-fertilisation, it was observed that chromosomes in metaphase spreads were plentiful for scoring and well condensed. Figure 2.2 illustrates the SCD staining, allowing the identification of cells that have passed through 1,2 and 3/3+ cell cycles.

From the PRI, it was calculated that the AGT of the early life stages from *C. gigas* incubated at 15ºC was 2.64 hours (Table 2.1). Embryo-larvae incubated at the higher temperature of 20°C resulted in a faster AGT of 2.47 hours, and 2.57 hours when the experiment was repeated (Table 2.1). From this result, one cell cycle for all subsequent experiments was considered to be approximately 2.5 hours.

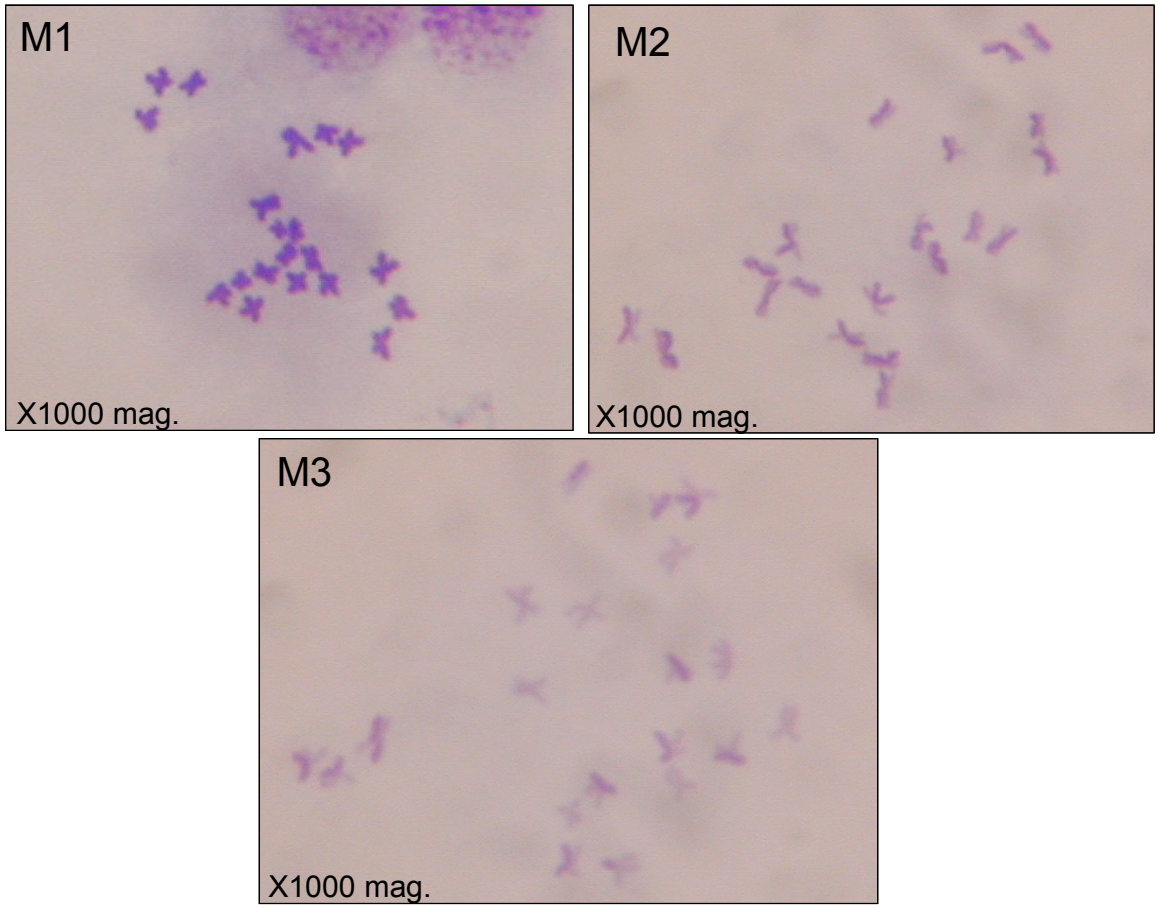


Figure 2.2 Metaphase chromosome spreads from *C. gigas* early life stages that have passed through 1 (M1), 2 (M2) or 3/3+ (M3) cell cycles showing sister chromatid differential staining

Table 2.1 PRI and AGT calculated for *C. gigas* embryo-larvae

Temperature(°C)	Hours in BrdU	Replicate	M1	M2	M3	Total cells	PRI	GT	AGT per Temp.	
15	2	A	59	22	19	100	1.60	1.25	**2.64**	15°C
15	2	B	70	19	11	100	1.41	1.42		
15	4	A	79	4	20	103	1.43	2.80		
15	4	B	59	23	18	100	1.59	2.52		
15	6	A	22	63	16	101	1.94	3.09		
15	6	B	22	51	27	100	2.05	2.93		
15	8	A	15	42	43	100	2.28	3.51		
15	8	B	22	35	44	101	2.22	3.61		
20	2	A	71	15	14	100	1.43	1.40	**2.47**	20°C
20	2	B	61	8	31	100	1.70	1.18		
20	4	A	23	27	50	100	2.27	1.76		
20	4	B	61	12	29	102	1.69	2.37		
20	6	A	14	48	38	100	2.24	2.68		
20	6	B	15	45	40	100	2.25	2.67		
20	8	A	19	53	29	101	2.10	3.81		
20	8	B	24	45	31	100	2.07	3.86		
20	2	C	52	18	30	100	1.78	1.12	**2.57**	20°C
20	2	D	60	19	21	100	1.61	1.24		
20	4	C	57	6	37	100	1.8	2.22		
20	4	D	54	10	36	100	1.82	2.20		
20	6	C	29	39	32	100	2.03	2.96		
20	6	D	28	31	41	100	2.13	2.82		
20	8	C	29	43	28	100	1.99	4.02		
20	8	D	27	44	29	100	2.02	3.96		

2.2 Validation studies

Aim: To validate the CAb in *C. gigas* early life stages using reference mutagens

In order to validate the CAb assay in the target species, experiments were performed to evaluate the genotoxic effects of two reference mutagens on the embryo-larval stages of *C. gigas*. The genotoxins used were:

- methylmethanesulfonate (MMS) – a direct acting alkylating agent;
- benzo(*a)*pyrene (B(*a*)P– a pro-mutagen considered to require metabolic activation.

2.2.1 Methods

Embryo-larval stages of *C. gigas* were collected as described in Section 2.1.1. The embryo-larvae were allowed to grow overnight in clean seawater and exposed to MMS at the following nominal concentrations, based upon similar concentrations used by Jha *et al.* (1996, 2000):

- 1.0×10^{-3} M = 1 mM
- 1.0×10^{-4} M = 0.1 mM
- 1.0×10^{-5} M = 0.01 mM
- 1.0×10^{-6} M = 1 µM
- 1.0×10^{-7} M = 0.1 µM

The nominal concentrations for B(*a*)P dissolved in dimethylformamide (DMF) were:

- 1.0×10^{-5} M = 0.01 mM
- 1.0×10^{-6} M = 1 µM
- 1.0×10^{-7} M = 0.1 µM
- 1.0×10^{-8} M = 0.01 µM

In addition, seawater controls were run and, for the B(*a*)P exposures, a 0.5 per cent DMF control was run in parallel. The embryo-larval samples were exposed for 1.5 cell cycles for the determination of CAb induction at the end of the initial incubation period (in clean seawater), as suggested by Scott *et al.* (1990). The exposures were terminated (i.e. the samples removed from the test agent and immersed in colchicine) at 20 hours post-fertilisation.

At 20 hours post-fertilisation, the samples were treated with colchicine, hypotonics and fixed in Carnoy's fixative as described in Appendix I (SOP for CAbs). The following day, chromosome spreads were prepared. They were subsequently stained for analysis and coverslips mounted in DPX as described in Appendix I for chromosome preparation and staining.

During attempts to score the chromosomes for CAbs, sister chromatid exchanges (SCEs) and PRI, it was noted that there were relatively few metaphase spreads present (approximately 40–50 metaphases per slide) compared with past observations of a minimum of approximately 200 metaphases per slide. Of the metaphases present, many

of the chromosomes were not condensed adequately and thus the staining was of poor quality. This was the case in both negative control samples, as well as those exposed to MMS.

It is likely that, during periods of manipulation of the embryo-larvae (e.g. at the start of the experiment during strip spawning and determination of fertilisation rate and embryo-larval density, and at the later stages during colchicine and hypotonic treatments), room temperature was high (approximately 28–30°C) due to the ambient weather conditions and enough to cause accelerated cellular proliferation. This resulted in many of the larvae reaching, or developing close to, the 'D-shell' stage, where cellular proliferation is slowed and metaphase spreads are difficult to prepare (Figure 2.1 shows D-shell larvae). This experiment was therefore repeated to reduce the total time of incubation.

It was also proposed that the protocol for exposure be amended to ensure earlier harvesting of the chromosomes. In addition to the problems with poor quality metaphase spreads, this protocol cannot be linked directly with standard Oyster Embryo-Larval(OEL) toxicity tests carried out by the Environment Agency where the samples are exposed from the 32-cell stage of the embryos. With this in mind, two simultaneous exposures were attempted. In the second attempt, the following timescales were applied to allow comparison between:

- long-term (early) exposure for the full period;
- pulse exposure for 1.5 and 2 cell cycles (for Cabs and SCEs respectively) as per the previous attempt.

Figures 2.3 and 2.4 illustrate the exposure scenarios for the long-term and pulse exposures respectively.

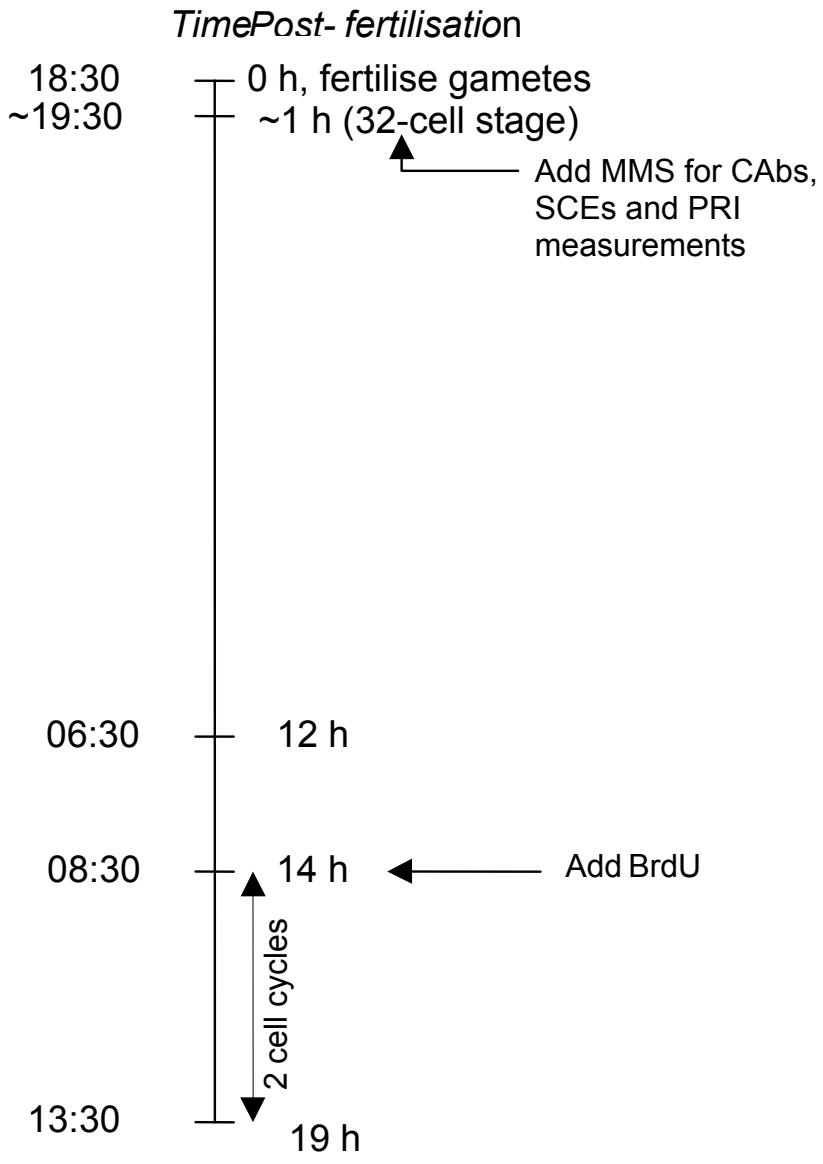


Figure 2.3 Validation studies: long-term (early) exposure of *C. gigas* to MMS

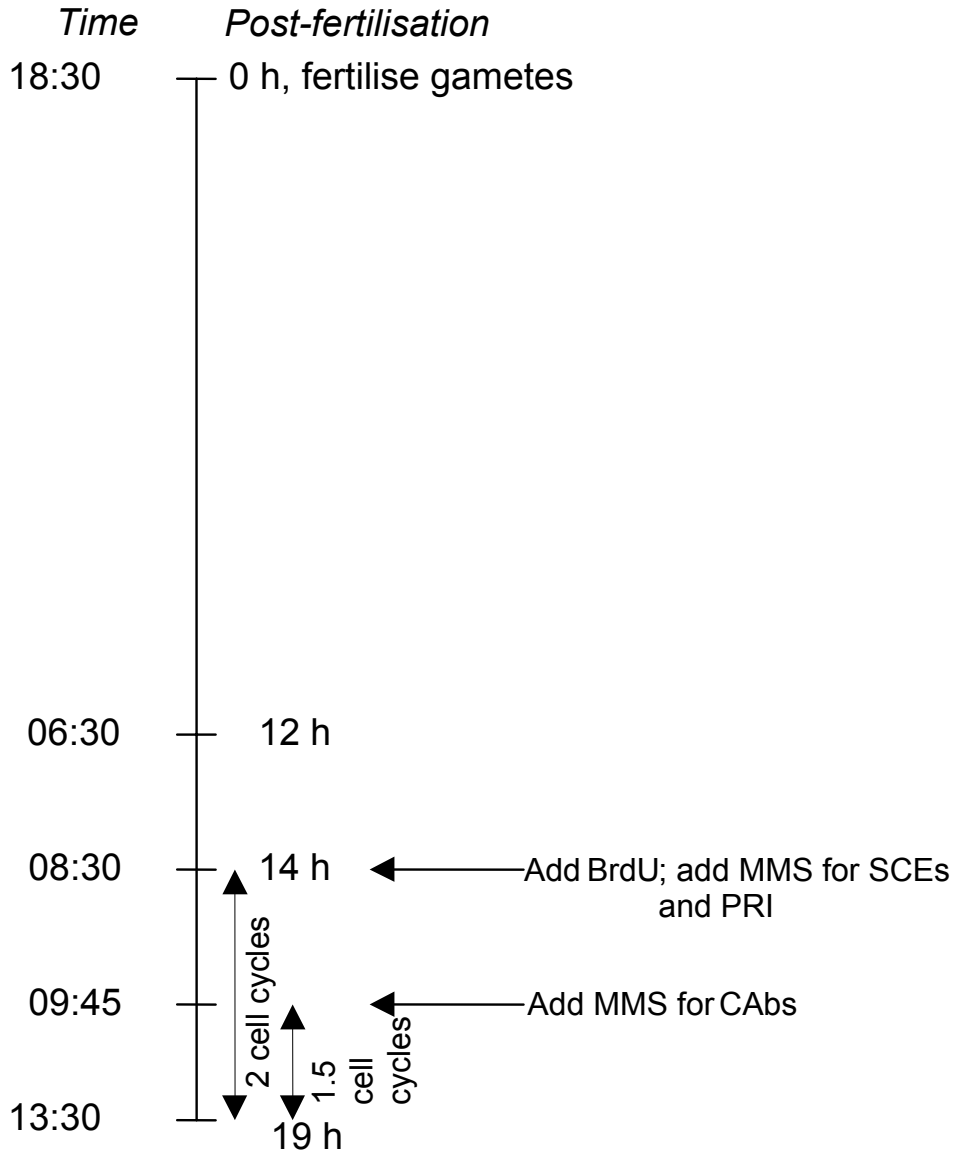


Figure 2.4 Validation studies: pulse exposure of *C. gigas* to MMS

2.2.2 Results and observations

Harvesting the cells at 19 hours post-fertilisation resulted in good quality and quantities of metaphase chromosome spreads. Figures 2.5 and 2.6 show the results of the two methods (long-term and pulse exposures). These suggest that a 'pulse' exposure as is carried out in standard mammalian CAb assays is more appropriate.

In the 'long-term' exposure, there was a poor dose–response relationship and insufficient dividing cells could be used for analysis at the highest dose (1.0×10^{-3} M). This protocol also allows time for damaged cells to induce DNA repair, which may result in either error-prone or error-free repair, both of which may mask the gross levels of DNA damage induced. Furthermore, cellular toxicity may result in a delay in the cell division. The advantage of undertaking a pulse exposure is that this allows quantification of DNA lesions that have occurred during a single cell division without the opportunity for cells to repair. The results of the pulse exposure provide a significant dose-dependent increase in the frequency of chromosome aberrations.

Statistical analysis of the data using chi-squared (λ^2) test indicates that there is no significant difference (P >0.05) between the MMS treated samples compared with the control in the 'long-term' exposure. In contrast, the data from the 'pulse' exposure showed there was a statistically significant increase in the two highest concentrations of MMS (1.0×10^{-4} M and 1.0×10^{-3} M) compared with the seawater (SW) control (Table 2.2).

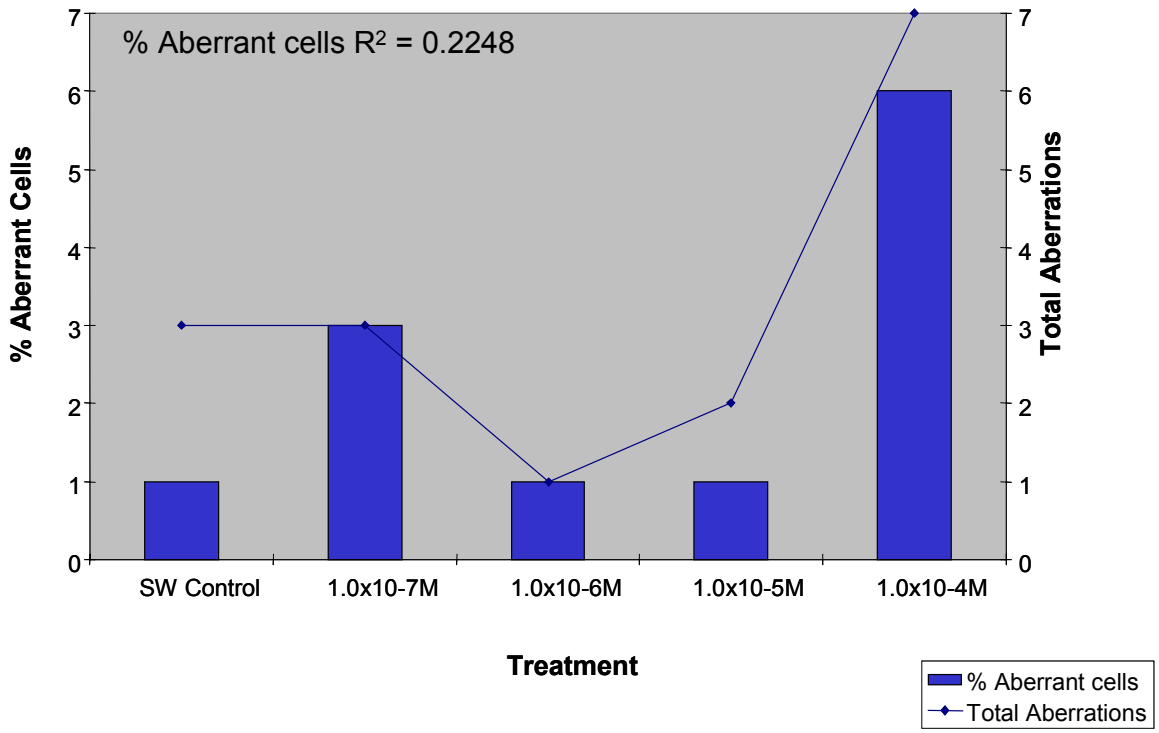


Figure 2.5 Chromosome aberrations induced in *C. gigas* embryo-larvae following 'long-term' exposure to MMS

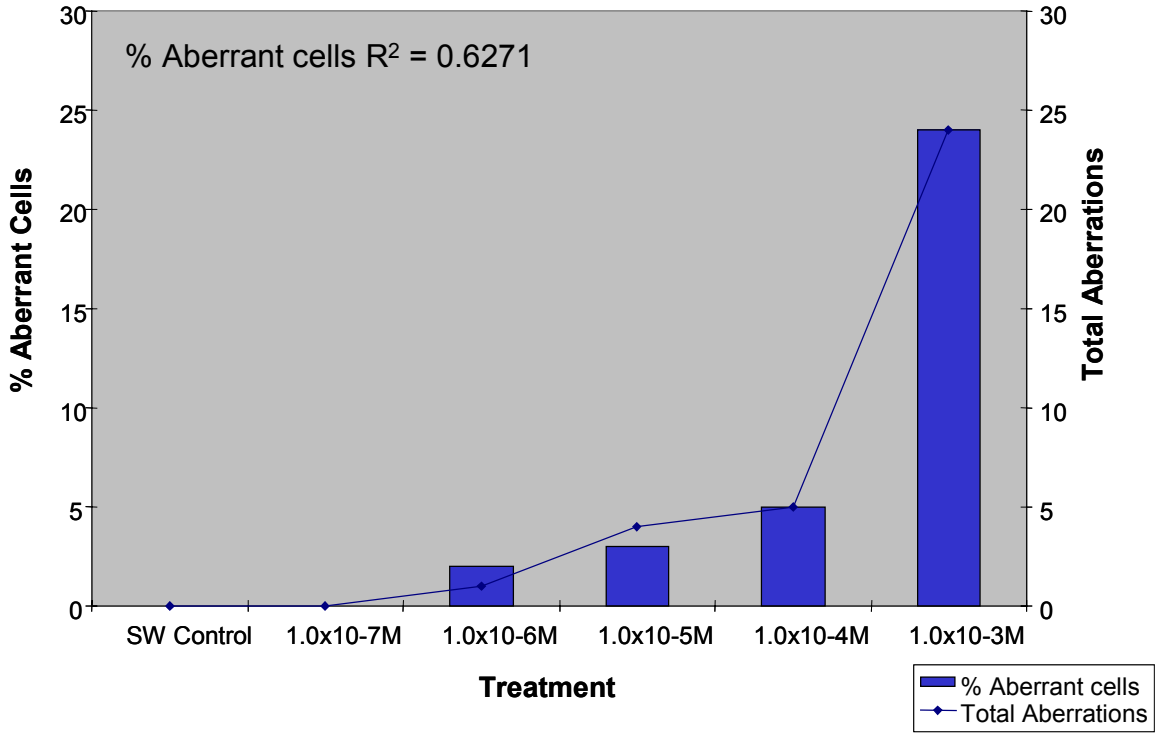


Figure 2.6 Chromosome aberrations induced in *C. gigas* embryo-larvae following 'pulse' exposure to MMS

Table 2.2 Application of chi-squared test to 'pulse' MMS exposure

Test	*Z*-value	Level of significance (*P*-value)
SW control vs. 1.0×10^{-7} M MMS	0	>0.05
SW control vs. 1.0×10^{-6} M MMS	–0.421	>0.05
SW control vs. 1.0×10^{-5} M MMS	–1.745	>0.05
SW control vs. 1.0×10^{-4} M MMS	–2.264	**<0.05**
SW control vs. 1.0×10^{-3} M MMS	–5.223	**<0.001**

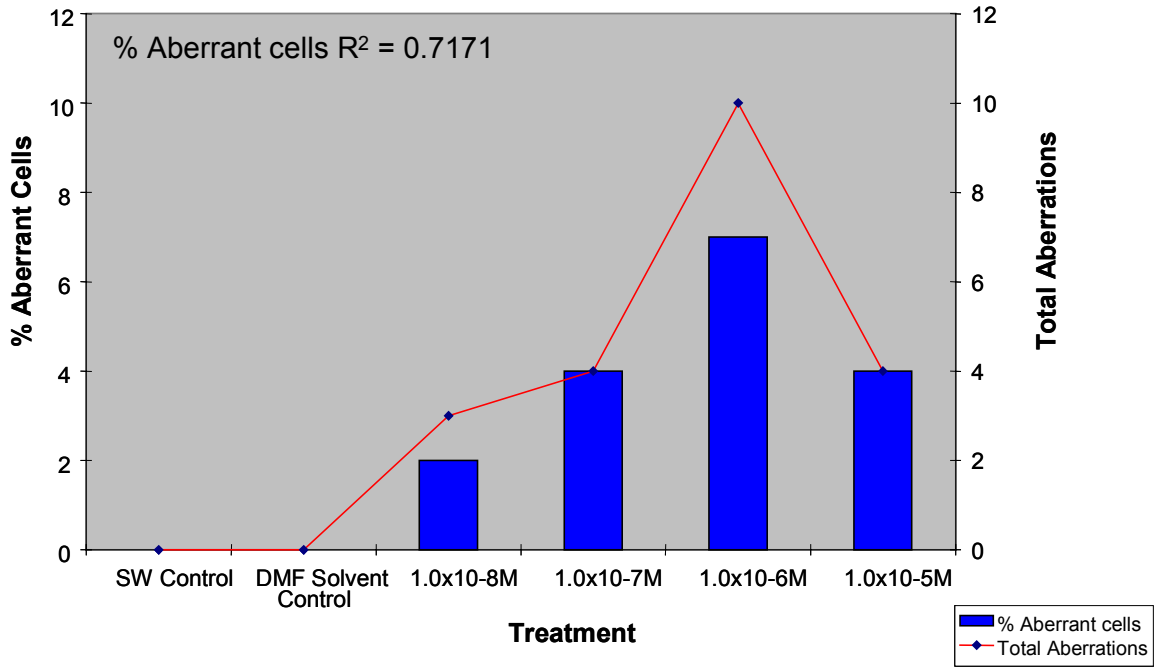


Figure 2.7 Chromosome aberrations induced in *C. gigas* embryo-larvae following 'pulse' exposure to B(*a*)P

Table 2.3 Application of the chi-squared test to B(*a*)P exposure

Test	*Z*-value	Level of significance (*P*-value)
SW control vs. DMF solvent	0	>0.05
SW control vs. 1.0×10^{-8} M B(*a*)P	–0.421	>0.05
SW control vs. 1.0×10^{-7} M B(*a*)P	–2.020	**<0.05**
SW control vs. 1.0×10^{-6} M B(*a*)P	–2.693	**<0.01**
SW control vs. 1.0×10^{-5} M B(*a*)P	–2.020	**<0.05**

Figure 2.7 shows the results of the exposures to the pro-mutagen B(*a*)P. Statistical analysis of the data using the chi-squared test indicates that the solvent has no significant effect on the induction of CAbs. There were significant differences (P <0.05) between the MMS treated samples compared with the control and in the three highest concentrations of B(*a*)P (≥1.0×10^{-7} M) compared with the seawater control (Table 2.3). However, there were fewer aberrations at the highest concentration than expected. It was also noted during the scoring of the chromosomes that there were fewer dividing cells at the highest dose. This suggests that the lower frequency at 1.0×10^{-5} M was due to cytotoxicity and that a reduction in the frequency of aberrations was therefore observed due to the lack of dividing cells as opposed to a reduction in the induction of aberrations. Furthermore, it can be postulated that the highest dose may have exceeded a toxicity threshold beyond which the cells were not able to metabolise the parent B(*a*)P compound.

Figure 2.8 illustrates the morphology of normal and aberrant metaphase cells. It was noted that chromatid type aberrations were more common than chromosome type aberrations in both MMS and B(*a*)P treatments.

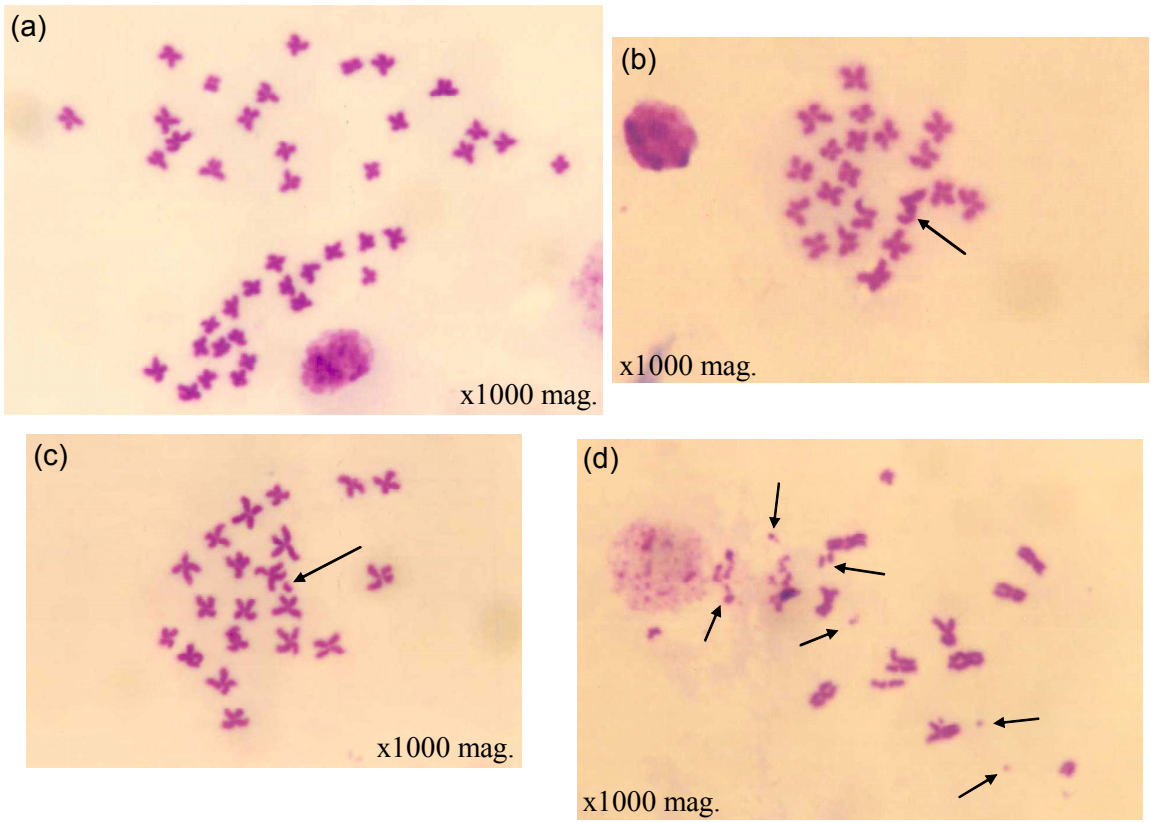


Figure 2.8 Metaphase chromosome spreads from *C. gigas* embryo-larvae: (a) two normal cells; (b) a chromatid type break; (c) a chromatid type break; (d) a highly damaged aberrant cell

2.3 Application – effluents and sediment elutriates

Aim: To employ the CAb assay in *C. gigas* early life stages in order to test the genotoxic potential of whole effluents and sediment elutriates

After the validation studies, a series of exposures were carried out on whole effluent discharges and elutriates prepared from sediments collected close to these discharges.

Final effluents were collected from two locations by the site operators and sediments were sampled from mudflats below each effluent discharge by Environment Agency staff in September 2004. Seawater elutriates were prepared by Environment Agency staff and frozen on-site.

Both effluents and elutriates were sent to the laboratories at the University of Plymouth. The thawed samples were tested with the CAb assay in *C. gigas* embryo-larvae to evaluate their genotoxic potential. Chemical analyses of the samples were conducted by the Environment Agency's National Laboratory Service.

In addition to the CAb assay, parallel studies were conducted to test the same samples for genotoxicity and general toxicity using methods such as the Ames test, the GreenScreen® assay and the Oyster Larval Development assay. These have been described elsewhere by Cahill *et al*. (2004) and Thain (1991).

2.3.1 Methods

Embryo-larval stages of *C. gigas* were collected as described in Section 2.1.1. The embryo-larvae were allowed to grow overnight in clean seawater and exposed to effluent or elutriate samples at 0, 6.25, 12.5, 25.0, 50.0 and 100 per cent based on similar concentrations used in standard toxicity tests in DTA.

The embryo-larval samples were exposed for 1.5 cell cycles for the determination of CAb induction at the end of the initial incubation period (in clean seawater) as described in Section 2.2.1. The exposures were terminated (i.e. the samples removed from the test agent and immersed in colchicine) at 19 hours post-fertilisation.

2.3.2 Results and observations

Harvesting the cells at 19 hours post-fertilisation resulted in good quality and quantities of metaphase chromosome spreads. Statistical analysis of the data using the chi-squared test indicates that the effluents from site 1 (X1385) had no significant effect on the induction of CAbs, with the exception of the samples exposed to 100 per cent effluent (P <0.05) (Table 2.4). A dose-dependent increase in the frequency of aberrant cells was observed in this treatment (R^2 = 0.6379), as shown in Figure 2.9.

No significant increases in the induction of CAbs was detected in any of the samples exposed to effluents from site 2 (Figure 2.11), elutriates from site 1 (Figure 2.10) and site 2 (Figure 2.12). No genotoxic effects for any of the whole effluent or elutriate samples were detected using the Ames test or the GreenScreen assay.

In contrast, the OEL development test detected a significant (*P* <0.05) toxic effect in the proportion of abnormal larvae when testing the elutriate samples from site 1 at concentrations ≥50 per cent (24-h EC50 = 61.2 per cent; NOEC = 25 per cent; LOEC = 50 per cent elutriate).[2] Furthermore, the effluent from site 2 also had a significant (*P* <0.05), albeit a small adverse effect upon normal development (24-h EC50 >100 per cent; NOEC = 50 per cent; LOEC = 100 per cent effluent).

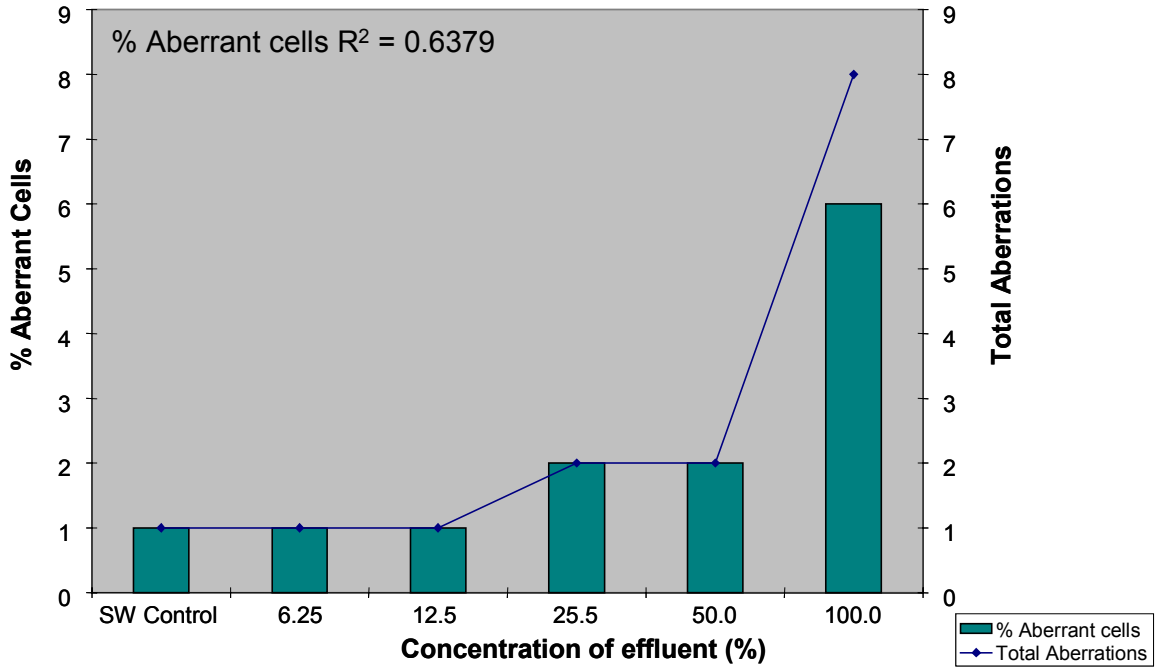


Figure 2.9 Chromosome aberrations induced in *C. gigas* embryo-larvae following exposure to effluents from site 1 (X1385)

Table 2.4 Application of chi-squared test to effluents from site 1 (X1385)

Test	*Z*-value	Level of significance (*P*-value)
SW control vs. 6.25% effluent	1.003	>0.05
SW control vs. 12.5% effluent	1.003	>0.05
SW control vs. 25.0% effluent	–0.582	<0.05
SW control vs. 50.0% effluent	–0.582	>0.05
SW control vs. 100.0% effluent	–1.923	**<0.05**

[2] LOEC = lowest observable effect concentration; NOEC = no observable effect concentration

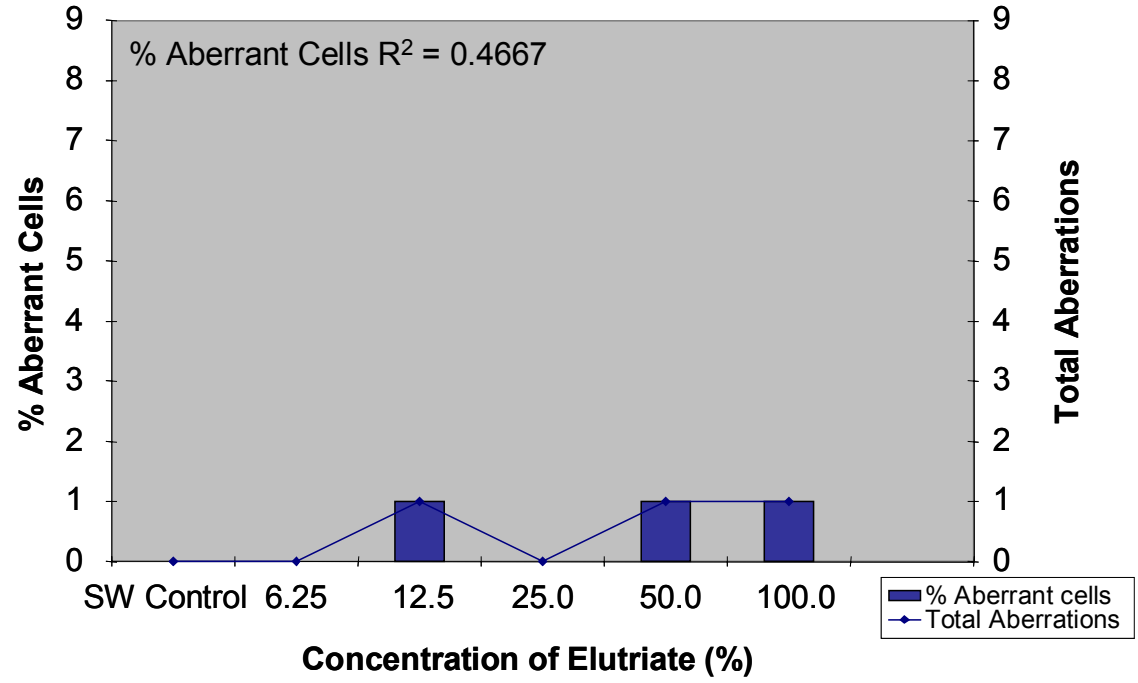


Figure 2.10 Chromosome aberrations induced in *C. gigas* embryo-larvae following exposure to elutriates from site 1 (X1472)

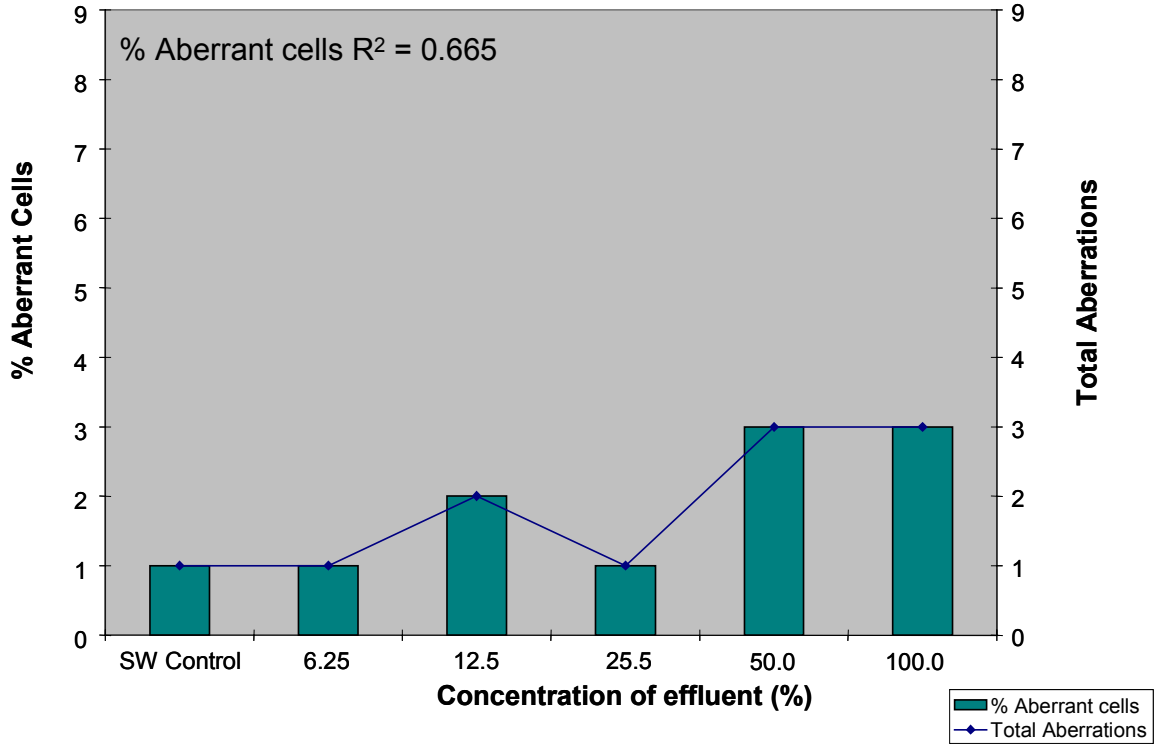


Figure 2.11 Chromosome aberrations induced in *C. gigas* embryo-larvae following exposure to effluents from site 2 (X1386)

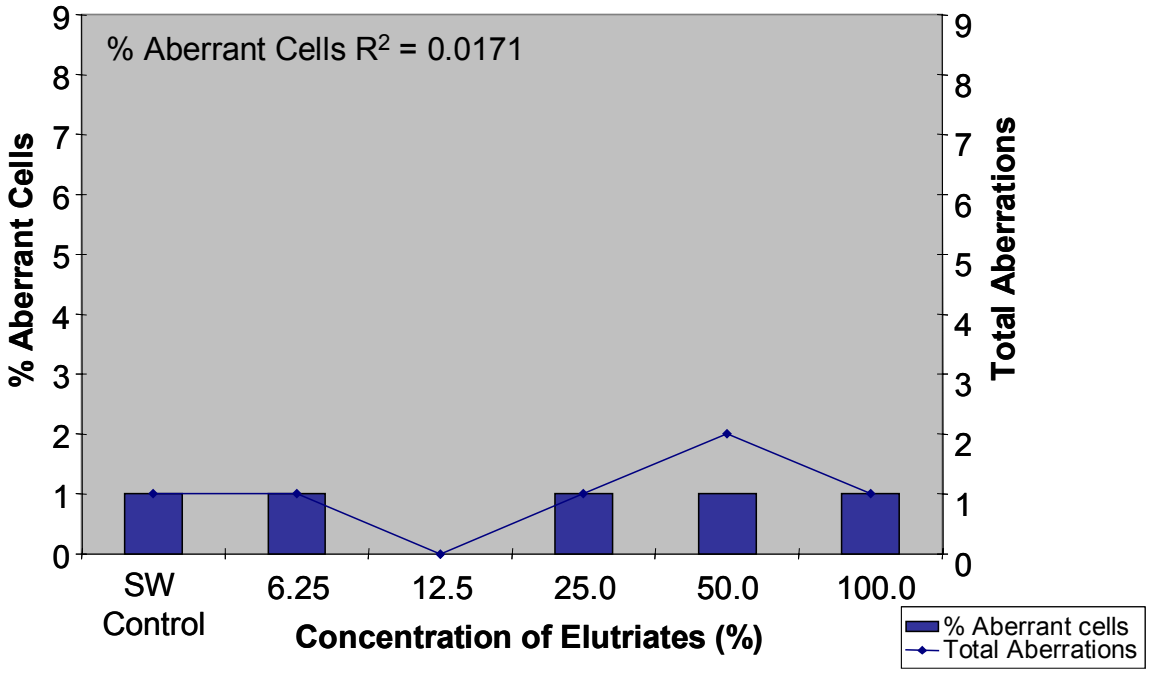


Figure 2.12 Chromosome aberrations induced in *C. gigas* embryo-larvae following exposure to elutriates from site 2 (X1471)

2.4 Conclusions and recommendations

This series of studies has demonstrated that it is possible to use the metaphase cells from the early life stages of *C. gigas* to detect genotoxic effects by means of the CAb assay. As observed in other marine invertebrate species such as *Mytilus edulis* and *Platynereis dumerilii*, the karyotype and morphology of the chromosomes from *C. gigas* have shown *C. gigas* to be a suitable test species.

Since *C. gigas* is already used in routine DTA, there is potential for the CAb assay to be used for detecting sub-lethal biological effects at the cytogenetic level. However, due to the cell cycle kinetics and timing of the protocol, a reduced temperature of ~20°C is the maximum temperature at which the embryo-larvae should be incubated. The standard temperature for the OEL development test is ~24°C; at this temperature, the embryo-larvae develop too quickly and the metaphase chromosome spreads are of poor quality for examination.

The dose-dependent responses to two mutagens (MMS and B[*a*]P) have validated the responses in the species and the method has been successfully applied to test both whole effluents and sediment elutriates. When compared with two other methods, the Ames test and GreenScreen assay, the CAb assay was able to detect genotoxic effects not quantified by the other *in vitro* methods. However, the maximum concentration of whole samples that the GreenScreen assay is able to test is 50 per cent, thus limiting its test range. An advantage of employing the CAb assay in *C. gigas* is that it is an *in vivo* method and thereby takes into account the bioavailability of contaminants in whole samples and the metabolic activation that occur in whole organisms, thus increasing the environmental relevance. But the CAb is relatively laborious and, as indicated by the

records presented in Appendix II, requires some practice and fully trained personnel in order to interpret the samples.

3. Habitats Directive study

Aim: To apply a series of biochemical biomarkers to *Arenicola marina* used in assessing the direct toxicity of sediments

Within Europe, natural habitats are continuing to deteriorate and an increasing number of wild species face a serious threat mainly as a result of development and agricultural intensification. The EC Habitats Directive introduces robust protection for those habitats and species considered of European importance. Its aim is to promote biodiversity by requiring Member States to take measures to maintain/restore natural habitats and wild species at/to a favourable conservation status. In applying these measures, Member States are required to take account of economic, social and cultural requirements and regional and local characteristics.

As part of work to fulfil the requirements of the Habitats Directive in the UK, the Environment Agency conducted a field survey in an area of the Tees estuary called Seal Sands. The primary aim of the study was to collect indigenous *Nereis* (ragworm) from 20 sampling points and to measure body burden contaminants (metals and organics). During the sampling programme, insufficient indigenous animals were found for the application of biomarker studies.

In addition, the Environment Agency performed whole sediment assays in the laboratory from the same 20 sites using the lugworm, *Arenicola marina*. The end-points measured were lethality, cast formation and bioaccumulation; the results are presented by Johnson *et al.* (2004).

Due to the inadequate number of indigenous animals, some of the *A. marina* used in the sediment bioassays were frozen and used for a variety of biomarker end-points, i.e. measurements of metallothionein induction, acetylcholinesterase activity and ferric reducing antioxidant power. These experiments are described in this chapter.

The animals were exposed to sediment samples at the Environment Agency's laboratories for 10 days. Subsequently, five individuals from each treatment (with the exception of site number 12 where only three individuals were provided) were transferred to clean artificial seawater for 24 hours to allow the animals to eject their gut contents. The animals were then snap-frozen in liquid nitrogen and stored at –20°C until needed for biomarker measurements.

3.1 Metallothionein induction

Metallothioneins (MTs) are widely distributed low molecular weight (6–7 kDa for vertebrates), cysteine-rich, metal-binding proteins thought to be present in most if not all animals (Livingstone 1993). MTs are thought to have several functions due to their high metal affinity and ability to chelate essential (Zn, Cu) and non-essential metals (Cd, Hg, Ag). This includes intracellular regulation of endogenous metals and detoxification of excess levels of pollutant metals. Other roles include general stress responses, e.g. temperature stress and free radical scavenging.

Detoxification of metals occurs by metal-mediated transcriptional activation of MT genes, increased MT synthesis resulting in sequestration of free metals by binding to MT. MT induction in response to cellular concentrations of metal ions forms the premise for the use as a biomarker for metal exposure. Hormones such as progesterone and glucocorticoids can also induce MT synthesis; therefore, variations may occur between species, reproductive condition, diet and season. MT in fish is considered as a general stress response due to metals. In molluscs (particularly mussels), MTs are specifically involved in responses to heavy metals and considered as a biomarker of exposure to heavy metal pollution (Viarengo *et al.* 1999).

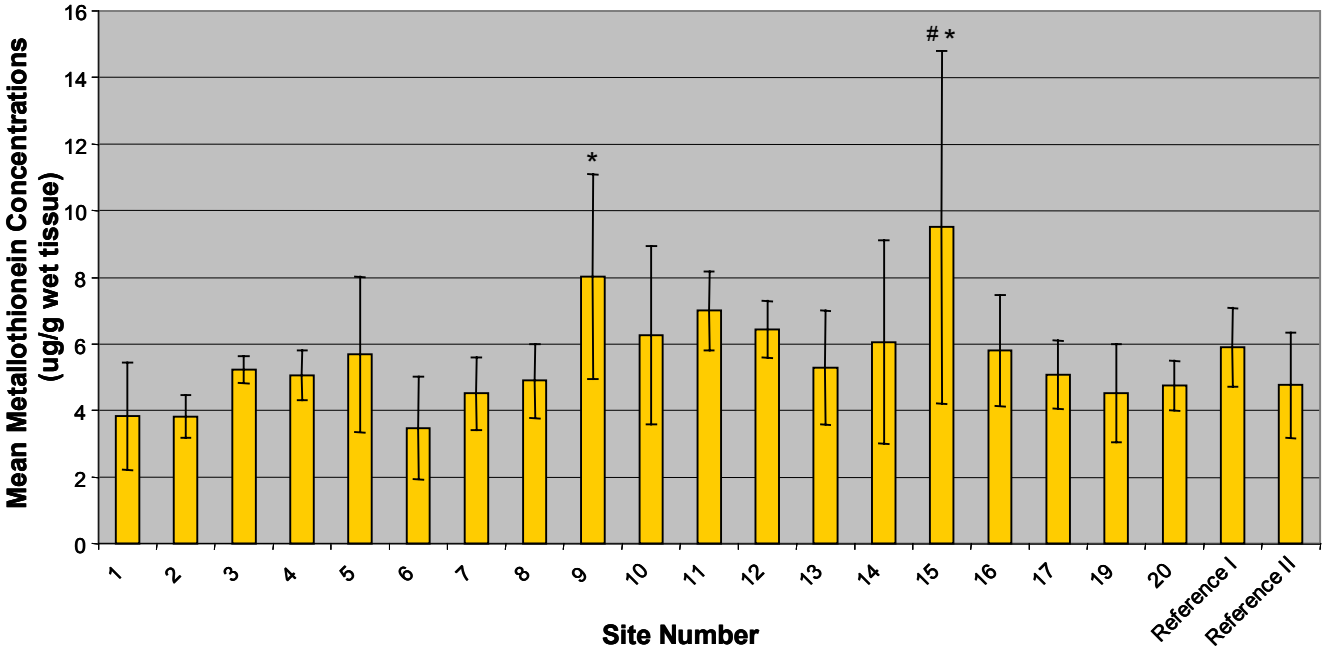
3.1.1 Methods

The MT concentration in total tissue was determined by a spectrophotometric method using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) based on a method described by Viarengo *et al.* (1997). The following modifications were made to this method during preparation of the sample.

The samples were prepared by grinding approximately 1 g wet weight of whole tissue to a fine powder in liquid nitrogen before adding 3 ml ice-cold 1 mM dithiothreitol (DTT) and 30 µl phenylmethylsulphonylfluoride (PMSF) solution. The mixture was sonicated (3 $\times$ 15 seconds) at 40 per cent output power and a further 30 µl PMSF added. The resulting suspension was subjected to ultracentrifugation at 100,000*g* and 4°C for 70 minutes, and the cytosolic fraction in the resulting supernatant stored at –80°C until analysis. The absorbance of the samples was read at 412 nm and the MT levels quantified by comparison with a reduced glutathione standard. A full description of the protocol can be found in Appendix I (Determination of Metallothionein).

3.1.2 Results and observations

Figure 3.1 shows the mean levels of MT determined in the whole tissue of *A. marina*. One-way analysis of variance (ANOVA) demonstrated a significant higher level of MT in samples exposed to sediments from site number 15 compared with the levels measured in the samples exposed to the two reference sediments. Samples exposed to sediments from site number 9 had a significantly higher level of MT compared with those exposed to reference II sediments. These results suggest that metal exposure may have been higher at these two sites than the other locations.



Error bars represent 2 SEM (standard error of the mean)
= statistically significant difference compared with reference I sediments
* = statistically significant difference compared with reference II sediments

Figure 3.1 Mean metallothionein concentrations determined in whole tissue of *A. marina* following 10 days exposure to sediments from Seal Sands

3.2 Acetylcholinesterase activity

The acute toxicity of organophosphates (OPs) or carbamates is due to the inhibition of acetylcholinesterase (AChE), a serine hydroxylase found in the central and peripheral nervous systems. Other cholinesterases exist in plasma, the erythrocyte membrane and other tissues, although their function is unknown. AChE acts in the neuromuscular junction to cleave and inactivate the neurotransmitter acetylcholine. Reactions of OP/carbamate compounds with the enzyme results in the formation of either a phosphoryl or carbaryl intermediate, both of which are resistant to subsequent hydrolysis, leading to inactivation of the enzyme. As a consequence, the build-up of AChE at the synapse results in overstimulation and depolarisation of the post-synaptic membrane.

In vertebrates, inhibition of AChE in the parasympathetic and central nervous systems and at neuromuscular junctions leads to an acute cholinergic syndrome characterised by muscle weakness, bronchoconstriction, convulsions and, ultimately, respiratory paralysis and death by asphyxiation (Marrs 1996). In invertebrates, the localisation and classification of cholinesterases is broader, with various forms present in different tissue types, involving both excitatory and inhibitory sensory function and possibly detoxification reactions (Habig and Di Guilio 1991). Symptoms may be apparent at anything from 10–100 per cent inhibition and can include a general depression of behaviour and sensory function, decreased body weight, hypothermia and disruption of other atonomic processes (Grue *et al.* 1990). The onset and duration of symptoms and susceptibility to

the toxic effects of OP/carbamates will vary between species as there are numerous different molecular forms of cholinesterase, each with its own characteristics of inhibition and reactivation.

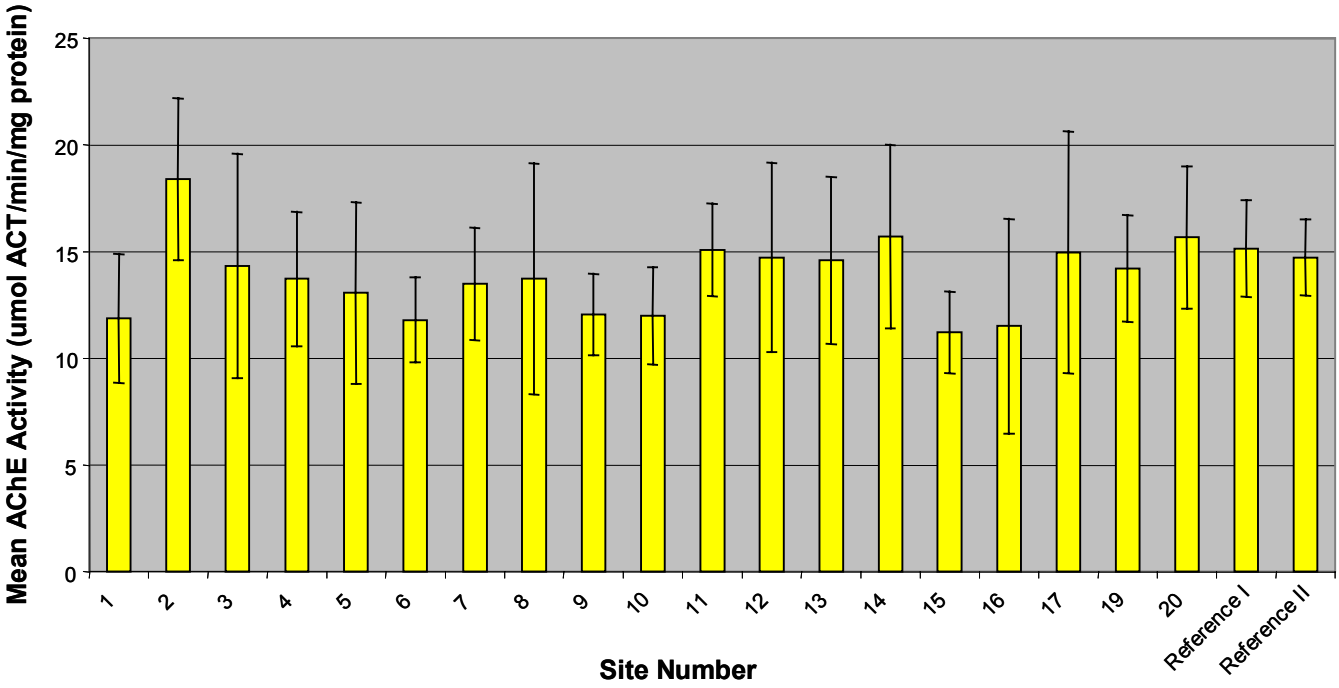
AChE activity is measured using the substrate analogue acetylthiocholine iodide, which is converted to thiocholine. The reaction of thiocholine with the chromogenic substrate dithionitrobenzoic acid (DTNB) leads to the formation of a yellow anion, nitrobenzoic acid, which absorbs strongly at 405 nm.

3.2.1 Methods

AChE activity was determined in whole tissue samples using a method described by Galloway *et al.* (2002). A full description of the method is given in Appendix I. In brief, whole tissue samples were ground to a fine powder in liquid nitrogen and resuspended in homogenising buffer. Following centrifugation, samples or buffer blanks (50 µl) were incubated in 96-well microtitre plates with 150 µl DTNB and the endogenous reaction measured at 405 nm for 5 minutes at 30-second intervals. Following the addition of 50 µl 3 mM acetylthiocholine iodide (ACTI), the absorbance was measured for 5 minutes at 30-second intervals. The results were expressed as specific activity (µ moles substrate hydrolysed per minute per mg relative to the total protein in the sample). Total protein was determined using a commercial kit (BioRad) with bovine serum albumin (BSA) as the standard.

3.2.2 Results and observations

Negligible amounts of endogenous activity occurred in these samples. Figure 3.2 shows the mean levels of AChE activity determined in the whole tissue of *A. marina*. One-way ANOVA indicated that there was no significant statistical difference between the samples exposed to sediments from the 20 sites and the reference sediments, suggesting that no biological effects were caused by OP or carbamate exposure.



Error bars represent 2 SEM

Figure 3.2 Mean acetylcholinesterase activity determined in whole tissue of *A. marina* following 10 days exposure to sediments from Seal Sands

3.3 Ferric reducing antioxidant power (FRAP) assay

It is widely documented that oxidative stress has the potential to cause harmful effects in the marine environment. Oxidative stress may occur if the balance between the generation of reactive oxygen intermediates (ROIs) associated with aerobic processes exceeds the defensive processes that have evolved in all organisms to limit the extent of oxidative damage.

Oxidative damage can affect lipids, proteins and genetic material leading to reduced metabolic activity and, ultimately, to cell death. Increased exposure to ROIs may:

- be chemically induced and associated with anthropogenic contaminants;
- result from photo-oxidative stress from ultraviolet radiation;
- result from hyperbaria from photosynthetically generated oxygen by algal symbionts (zooxanthellae).

Many marine invertebrates may be exposed to more than one of the above situations, in some cases simultaneously. Special adaptive mechanisms to ameliorate oxidant stress have evolved to cope with the extreme conditions that may be encountered in aquatic habitats.

Antioxidant defences take many forms and the study of oxidative stress has typically measured individual specific responses such as the antioxidant enzymes superoxide dismutase and catalase, or measured the concentration of low molecular weight

antioxidants such as ascorbate, glutathione, tocopherols and uric acid in different biological tissues. However, medical and environmental researchers sought a simple means of quantifying the net antioxidant status of a sample, i.e. an index of its ability to resist oxidative damage.

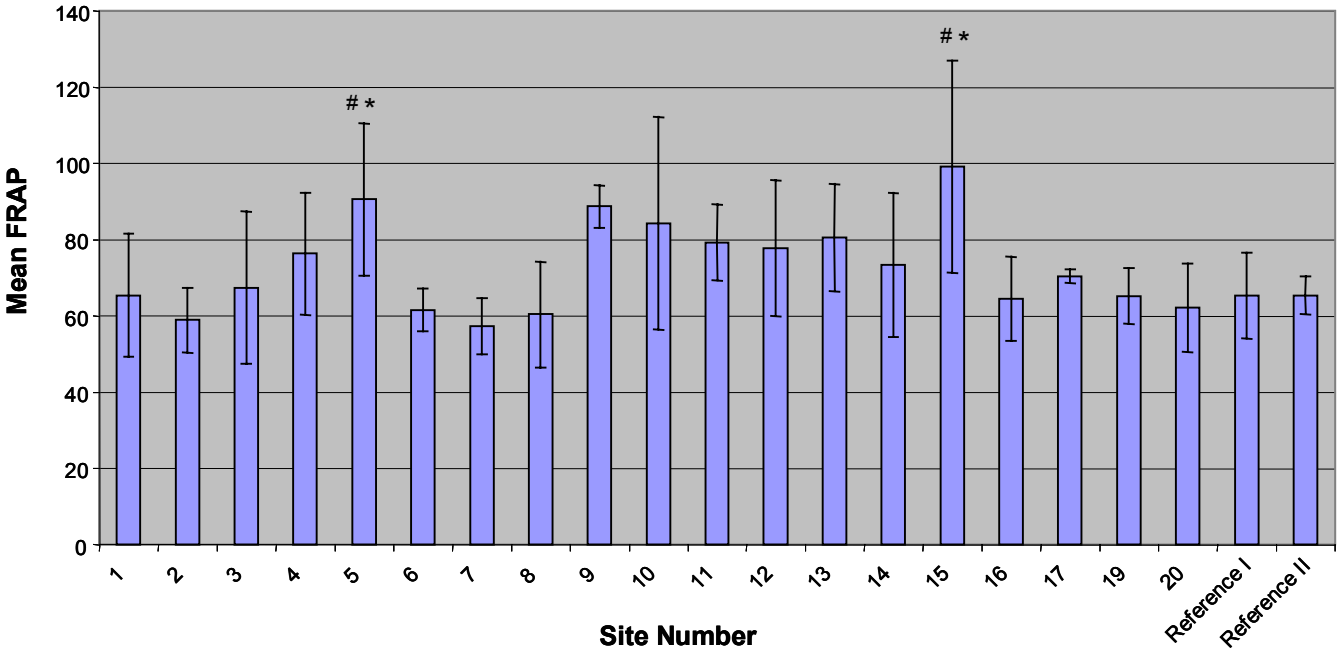
The ferric reducing antioxidant potential (FRAP) assay described by Benzie and Strain (1996, 1999) is quick, reproducible and does not require expensive or technically demanding equipment. Antioxidants in the sample are used as reductants in a redox-linked colorimetric assay employing a stoichiometric excess of the easily reduced oxidant ferric tripyridyltriazine [Fe(III)-TPTZ]. At low pH, reduction to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorbance at 593 nm. The reaction is non-specific in that any half reaction that has a lower redox potential under reaction conditions than that of the ferric/ferrous half reaction, will drive the ferric [Fe(III)] to ferrous [Fe(II)] reaction. The change in absorbance is therefore related directly to the combined reducing power of the electron-donating antioxidants present in the reaction mixture. The assay is suitable for measurements using many types of biological sample including tissue homogenates or haemolymph.

3.3.1 Methods

The FRAP of whole tissue homogenate was determined using the method described by Benzie and Strain (1996). A full description of the method is given in Appendix I (FRAP Assay). In brief, whole tissue samples were ground to a fine powder in liquid nitrogen and resuspended in homogenising buffer at a 1:5 ratio (w/v). The homogenate was centrifuged and the supernatant used for determining FRAP. Working FRAP reagent was prepared from 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-*s*-triazine (TBTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ mixed at a 10:1:1 ratio. Supernatant (50 µl) from each sample was transferred to wells (in duplicate) in a 96-well plate, to which 200 µl of freshly prepared FRAP reagent was added. The absorbance of the samples was read immediately at 593 nm and again after 10 minutes incubation at 25°C. The change in absorbance (ΔA_{593nm}) was calculated and the FRAP quantified by comparison with a range of $FeSO_4.7H_2O$ standard solutions.

3.3.2 Results and observations

Figure 3.3 shows the mean FRAP determined in the whole tissue of *A. marina*. Bartlett's test for variance indicated that there was a statistically significant difference amongst the standard deviations at the 95.0 per cent confidence level. Therefore, the non-parametric Kruskal–Wallis test was applied to compare the medians between samples. This test demonstrated a significant higher FRAP in samples exposed to sediments from site numbers 5 and 15 when compared with the levels measured in the samples exposed to the two reference sediments. These results suggest that oxidative stress, possibly due to contaminants in the sediments was higher at these two sites than the other locations.



Error bars represent 2 SEM
= statistically significant difference compared with reference I sediments
* = statistically significant difference compared with reference II sediments

Figure 15 Mean FRAP determined in whole tissue of *Arenicola marina* following 10 days exposure to sediments from Seal Sands)

3.4 Conclusions and recommendations

These initial results indicate that the measurements of MT, AChE activity and FRAP can be used in whole tissue samples of *A. marina.*

The Habitats Directive study originally considered the possibility for applying biomarkers in indigenous samples of *Nereis,* but the distribution and density of this species was not consistent between sampling sites and, in many of the sites, it was absent due to the distribution of *Enteromorpha* in the area. It was therefore necessary to measure biological effects in *A. marina* that had been exposed to the sediments in laboratory conditions. For a more robust study, more individuals were required from each of the treatments; in this study, $n = 5$ ($n = 3$ at site number 12; $n = 0$ from site 18).

There were significant effects on the level of MT measured in the whole tissues of *A. marina* exposed to sediments from sites 9 and 15. But without a full set of data detailing the chemical analysis of the sediments and/or body burden of the organisms, it is difficult to deduce any cause–effect relationships. The FRAP results also indicated that there was a biological effect in samples exposed to sediments from site 15, in addition to site 5.

Further investigations to qualify and quantify the contaminants from these sites are recommended in order to identify the agents likely to be causing the biological effects observed.

None of the sites detected detrimental effects on survival, using *A. marina* in DTA tests, with the exception of site 12. Sediments from sites 13 and 18 measured reduced feeding rates, while animals exposed to sediments from sites 3, 12, 13 and 15 resulted in reduced biomass. However, many other factors should have been considered in this study such as particle size distribution and organic carbon concentrations in the sediments. Further studies are required to fully validate the biomarkers measured in *A. marina* as a test species.

4. Work under the National Marine Monitoring Programme

Aim: To assess the genotoxic potential of sediments using the 'Comet assay' in *Arenicola marina*

The National Monitoring Plan (NMP) was initiated in the late 1980s to coordinate marine monitoring in the UK between a number of organisations including the Environment Agency's precursors. It is now called the National Marine Monitoring Programme (NMMP).

The biological effects monitoring included within this programme indicated that biological effects are greatest in estuaries known to be contaminated. The initial survey provided a useful overview of the spatial distribution of contaminants in UK waters and highlighted locations where biological effects methods should be focused.

As part of the ongoing monitoring for the NMMP, sediment samples were collected by Environment Agency staff from the River Thames. The primary aim was to test the whole sediments using *A. marina* and measuring the standard end-points of lethality and cast formation (Johnson, 2004). In addition, the Comet assay was carried out to measure DNA strand breaks caused by exposure of the animals to contaminants that may have been present in the sediment samples. This rest of this chapter discusses the work involving the Comet assay.

4.1 Comet assay

The Comet assay or single-cell gel electrophoresis assay is a rapid, versatile and easy-to-use tool for collecting data on DNA strand breakage and has been widely performed on vertebrates and invertebrates.

The assay measures the electrophoretic migration of relaxed or fragmented DNA away from the nuclei of cells immobilised in agarose gel. It requires only small samples of any eukaryotic cell population and allows the quantitation of single cells, allowing heterogeneity and subcellular population responses to be examined.

The assay has been reported to be a good indicator of general DNA damage in organisms for which further studies could then be focused to elucidate the nature of the DNA lesions and the mechanisms from which they have been induced (Birmelin *et al.* 1998, Mitchelmore and Chipman 1998a, Mitchelmore and Chipman, 1998b, Mitchelmore *et al.* 1998, Steinert 1999).

4.1.1 Methods

To extract haemocytes and coelomocytes from *A. marina*, the body wall of the animals was perforated using the tip of a 200 µl pipette. The animals were then placed in individual chilled 25 ml beakers placed on ice while the cells extruded from the body cavity for 10 minutes. Samples of cell suspension were then collected from the beakers

and transferred to microcentrifuge tubes, avoiding collection of gametes wherever possible; this was observed in some samples such as those illustrated in Figure 4.1.

Samples were centrifuged for 3 minutes at 10,000 rcf (relative centrifugal force) at 4°C. The supernatant was transferred to another microcentrifuge tube and centrifuged a second time at 10,000 rcf for 3 minutes at 4°C to further remove contamination with gametes (as illustrated in Figure 4.2). The supernatant was discarded and the small pellet of cells was then resuspended in 200 µl 1.0 per cent low melting point agarose (LMPA).

The Comet assay protocol was then followed as described by Mitchelmore *et al.* (1998). This protocol was modified by:

- using dry coated normal melting point agarose (NMPA);
- omitting the final layer of LMPA;
- conducting electrophoresis for 25 minutes.

A full description of the protocol is given in Appendix I. One slide, each with duplicate microgels, was prepared per treatment.

Figure 4.1 Body fluids collected from adult *A. marina* following perforation of the body wall: (A) spermatocytes; (B&C) oocytes overlain by coelomocyte and haemocytes suspension

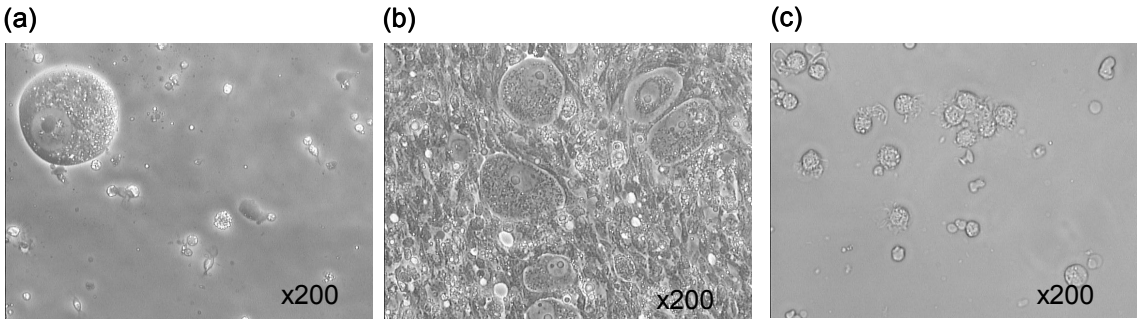


Figure 4.2 (a) Large coelomocyte and spermatocytes; (b) oocytes; (c) haemocytes, collected from *A. marina*

4.1.2 Results and observations

Figure 4.3 shows the level of DNA damage measured as tail moment (the product of the tail length × tail per cent DNA) in the circulatory cells of *A. marina*. Bartlett's test for

variance indicated that there was a statistically significant difference among the standard deviations at the 95.0 per cent confidence level. Therefore, the non-parametric Kruskall-Wallis test was applied to compare the medians between samples. This test indicated that there was no significant increase in the level of DNA damage in the *A. marina* exposed to sediments from the two sites compared with the reference sediment. In fact, there was a significantly lower level of DNA damage in animals exposed to the sediments from site X1349 compared with the reference sediments.

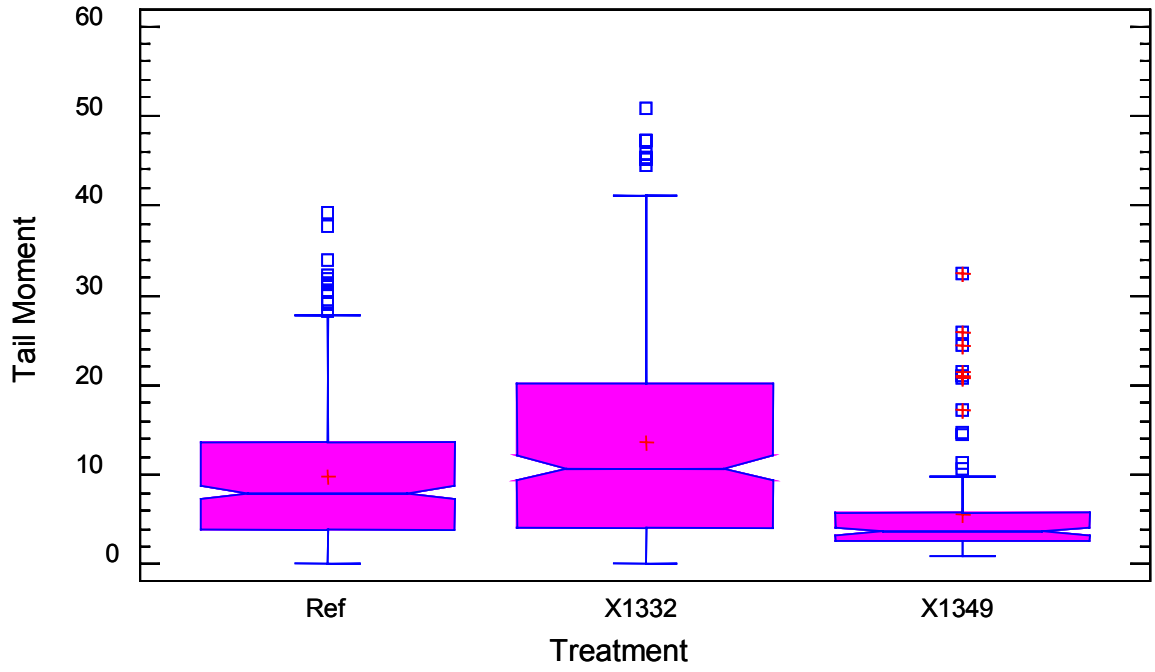


Figure 4.3 Tail moment measured with the Comet assay in circulatory cells from *A. marina* exposed to sediments from the River Thames

4.1.3 Conclusions and recommendations

The difference in sexual maturity of the *A. marina* may have influenced the level of contamination of the cell suspensions collected from the samples from the River Thames. Therefore, further optimisation is required for obtaining a single cell suspension. However, samples that appeared to have no gamete contamination produced cells which could be stained with ethidium bromide and measured using an image analysis system (Komet v. 5.0; Kinetic Imaging). In addition, studies of *in vitro* and *in vivo* exposures of the organisms to known genotoxins are required to validate the methodology in this species.

These initial results indicate that it is possible to apply the Comet assay in *A. marina* but that further research and development of the method is required.

5 Publications and presentations

The work described in this report formed the basis for the following articles and conference papers.

Peer reviewed publications

Cheung V V, Jha A N, Owen R, Depledge M H and Galloway T S, 2006 *Development of the* in vivo *chromosome aberration assay in oyster (*Crassostrea gigas*) embryo-larvae for genotoxicity assessment*. Marine Environmental Research, **62**, Suppl. 1, S278-282.

Cheung V V, Depledge M H and Jha A N, 2006 *An evaluation of the relative sensitivity of two marine bivalve mollusc species using the Comet assay.* Marine Environmental Research, **62**, Suppl. 1, S301-305.

Cheung V V, Simpson P, Knight A W, Cahill P A, Charles A K, Depledge M H, Owen R and Galloway T S *The application of prokaryotic, eukaryotic, cytogenetic and direct toxicity assays to evaluate the toxicity of industrial effluents in England and Wales.* [in preparation]

Book chapters

Cheung V V, Galloway T S and Depledge M H (2004) *Chemical disruption of biological phenomena* [online]. In Encyclopedia of Life and Sustainable Development. Oxford: EOLSS Publishers and UNESO Publishing. Available from: http://www.eolss.net [Accessed 2 August 2006]

Conference presentations

Cheung V V, Sanger R C, Browne M A, Dissanayake A, Brown R J, Galloway T S, Jha A N and Depledge M H, 2003 *An evaluation of the relative sensitivity of two bivalve mollusc species as bioindicator organisms for environmental monitoring.* International Union of Biological Sciences, Hong Kong, December 2003.

Cheung V V, Depledge M H and Jha A N, 2004 *The relative sensitivity of cyto- and genotoxic biomarkers in two marine bivalve mollusc species.* Society for Experimental Biology, Edinburgh, Scotland, March 2004.

Cheung V V, Owen R, Depledge M H and Galloway T S, 2005 *Optimisation and validation of an* in vivo *genotoxicity test using the embryo-larval stages of the Pacific oyster,* Crassostrea gigas*.* SETAC-Europe. Lille, France, May 2005.

Cheung V V, Simpson P, Knight A W, Cahill P A, Charles A K, Depledge M H, Owen R and Galloway T S, 2005 *A comparison of prokaryotic, eukaryotic, cytogenetic and direct toxicity assays to evaluate the toxicity of industrial effluents*. Pollution Responses in Marine Organisms, Alessandria, Italy, June 2005.

List of abbreviations

AChE	acetylcholinesterase
ACTI	acetylthiocholine iodide
AGT	average generation time
ANOVA	analysis of variance
ASW	artificial seawater
BSA	bovine serum albumin
B(*a*)P	benzo(alpha)pyrene
BrdU	5-bromodeoxyuridine
CAb	chromosome aberration
CI	confidence interval
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DTA	Direct Toxicity Assessment
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
FRAP	ferric reducing antioxidant power
GT	generation time
IPPC	Integrated Pollution Prevention and Control
LMPA	low melting point agarose
NMPA	normal melting point agarose
MMS	methylmethanesulfonate
MT	metallothionein
OP	organophosphate
PMSF	phenylmethylsulphonylfluoride
PRI	proliferation rate index
rcf	relative centrifugal force
SCD	sister chromatid differential (staining)
SD	standard deviation
SEM	standard error of the mean
SW	seawater
v/v	volume/volume
w/v	weight/volume

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Appendix I – University of Plymouth protocols

The following protocols used for measuring biomarkers at the University of Plymouth are presented:

- Preparation of metaphase chromosomes from embryo-larva of *Myrilus edilis* or *Crassostrea gigas* for the determination of chromosome aberrations (CAbs)
- Determination of metallothionein (MT) in marine invertebrate tissue
- Acetylcholinesterase activity
- Determination of total protein: BioRad Bradford assay
- Ferric reducing antioxidant power (FRAP) assay
- Comet assay

Preparation of metaphase chromosomes from embryo-larvae of *Mytilus edulis* or *Crassostrea gigas* for the determination of chromosome aberrations (CAbs)

Preparation of embryo-larvae

1. Spawn at least two pairs of adult bivalves as per protocol for embryo-larval development test. Check viability of gametes (seawater held at 20 ± 2°C).
2. Allow eggs to be fertilised as per protocol for embryo-larval development test. Determine fertilisation rate 1 hour after addition of sperm to egg suspension. Age zero is taken to be the time at which >90 per cent eggs are fertilised. If this fertilisation rate is not achieved within 3 hours of placing sperm and eggs together, the gametes should be discarded and alternative adults should be used for spawning.
3. Allow embryos to grow in clean seawater at a maximum density of 20 embryos per ml seawater for *M. edulis* and 100 embryos per ml seawater for *C. gigas*, for 14 hours post-fertilisation (15°C for *M. edulis*; 20°C for *C. gigas*).
4. Exposure of the embryos to the test solution should be for 1.5 cell cycles: one cell cycle is approximately 4 hours for *M. edulis*; and approximately 2.5 hours for *C. gigas.* Therefore if testing *M. edulis*, the exposure should commence at 14 hours post-fertilisation and be terminated at 20 hours post-fertilisation. If testing *C. gigas*, the exposure should commence at 15 hours 15 minutes post-fertilisation and be terminated at 19 hours post-fertilisation.
5. Sieve the embryo suspension from each through separate 30 µm nylon sieves (the embryos should remain on the sieve).
6. Immerse the embryos (within the sieve) into 0.025 per cent (w/v) solution of colchicine for 30 minutes (NB Colchicine is dissolved in seawater) held at room temperature, e.g. 25 mg in 100 ml seawater.
7. Immediately transfer the embryos (within the sieve) into a series of hypotonic solutions. These are made of 0.56 per cent KCl solution, e.g. 5.6 g in 1 litre distilled water. Hypotonic solutions are made up to the following ratios:

Seawater	:	KCl solution
(a) 2	:	1
(b) 1	:	1
(c) 1	:	2
(d) 1	:	3

   The embryos are transferred into the hypotonic solutions following the sequence above (from a to d) for 10 minutes in each solution at room temperature. Carefully transfer the embryo-larvae into 1.7 ml siliconised microcentrifuge tubes from the surface of the sieves using plastic Pasteur pipettes.
8. Centrifuge for 5 minutes at 2000 rpm (revolutions per minute).
9. Using a pipette, remove and discard the supernatant to leave a loose pellet of embryo-larvae.
10. Add cold (4°C) Carnoy's fixative (acetic acid: methanol in a ratio of 1:3 v/v), drop by drop to the embryos.
11. Allow the embryos to settle out down in the microcentrifuge tube (approximately 20 minutes), remove the Carnoy's fixative using a pipette and replace with fresh Carnoy's fixative. Repeat once more. Leave overnight before preparing chromosome spreads.

Preparation of metaphase chromosome spreads

1. Once again remove the Carnoy's fixative using a pipette and replace with fresh cold (4°C) Carnoy's fixative. Resuspend the embryo-larvae by gently pipetting with plastic Pasteur pipettes. Add approximately 4–5 drops of the fixed embryo-larvae suspension to a microscope slide, ensuring even distribution over the slide surface.
2. Add approximately 4–5 drops of 60 per cent (v/v) glacial acetic acid to the slide.
3. Place onto a hot plate set at 40°C and allow the slide to dry while rotating the slide by hand to ensure an even distribution of the chromosomes.
4. Check the spreading of the metaphases with a phase-contrast microscope at ×400 total magnification. Store the slides at room temperature in a dust-free environment overnight to allow the chromosomes to adhere to the slides.

Staining of the chromosomes

1. Rinse each slide with distilled water.
2. Arrange the slides in a coplin jar/staining trough.
3. Immerse the slides in 10 per cent Giemsa solution (e.g. 10 ml in 90 ml Giemsa buffer) at room temperature and allow to stain for 15 minutes.
4. Remove the slides from the stain and rinse twice in distilled water.
5. Allow to air-dry at room temperature.
6. Once dry, mount coverslips onto the slides with DPX. Place 3–4 drops of DPX mountant on the slide and apply a coverslip avoiding trapped air bubbles.
7. The DPX mountant should be allowed to dry thoroughly before slide analysis under the microscope.

Scoring of chromosomes

1. Examine complete metaphase spreads (± 2 chromosomes from 2n) for CAbs at a total magnification of ×1000 (with oil immersion). NB. 2n in *M. edulis* = 28 chromosomes; 2n in *C. gigas* = 20 chromosomes. Score 100 metaphase cells for aberrations as defined by Scott *et al.* (1990).
2. Present results as percentage of aberrant cells and total frequencies of aberrations.

Determination of metallothionein (MT) in marine invertebrate tissue

STAGE 1 – Preparation of tissue

1. Grind up frozen tissue in a cold mortar with liquid nitrogen.
2. Place approximately 1 g of ground tissue in a 10 ml beaker. Record the exact wet weight of the sample.
3. Add 3 ml of cold 1 mM DTT and 30 µl PMSF solution. Stir with spatula. Keep sample on ice at all times.
4. Sonicate for 3 × 15 seconds (40 per cent duty cycle, output control 4), placing 10 ml beaker inside a 50 ml beaker containing ice in case of the smaller beaker breaks.
5. Add another 30 µl PMSF solution.
6. Ultracentrifuge at 55,000 rpm at 4°C for 70 minutes.
7. Transfer 550 µl of the supernatant (avoiding lipid surface layer) into three eppendorf tubes. Freeze at –80°C for a maximum of 7 days before stage 2 (purification of MT).

Stage 1 reagents:
1 mM DTT = 7.71 mg made up fresh in 50 ml distilled water
0.1 mM PMSF solution = 17.4 mg in 10 ml ethanol

STAGE 2 – Purification of MT

NB Keep samples on ice at all times.
1. Set the centrifuge up before starting to get the temperature down to 4˚C:
   Set key: univ
   Speed: 2000 rpm
   Time: 12 minutes
   Temp: 4˚C Excess temp: 25˚C
   Ensure that the correct Number (rotor radius) has been entered.
   Close lid to allow temperature to be maintained.
2. Take the samples out of the freezer and allow them to thaw in an eppendorf rack on ice.
3. Prepare relevant number of nalgene centrifuge tubes (label and arrange in rack).
4. To each centrifuge tube add: 500 µl of thawed sample (cytosolic fraction/supernatant), 500 µl of –20°C absolute ethanol (keep this in the freezer until needed) and 40 µl chloroform. Mix using vortex.
5. Centrifuge at 6,000*g* (7,000 rpm) for 12 minutes.
6. Pour the supernatant into a new centrifuge tube (pellet can be discarded). To this supernatant, add three times its volume of –20˚C absolute ethanol = i.e. 3 ml vortex.
7. Place the samples in a test tube rack, cover with parafilm (or put lids on if available) and place in –20°C freezer for 1 hour.
8. Meanwhile, take Na_2-P buffer out of fridge and place on a magnetic mixer (NB It may have started to freeze at fridge temperatures).
9. Take samples out of freezer, centrifuge at 6,000*g* for 12 minutes.
10. Make washing buffer, store at –20°C.
11. Pour away supernatant, then wash the pellet (and sides of tubes) using 2 ml of washing buffer (wash two times with 1 ml each time). Vortex the mixture.
12. Centrifuge at 6,000*g* for 12 minutes.
13. Meanwhile, make standard curve, with GSH solution.

14. Make up DTNB and keep in the dark. Make enough for samples and standards (7.1 mg DTNB in 42 ml Na_2-P buffer).
15. Pour away supernatant from each sample. Dissolve each pellet in 300 µl Tris-EDTA buffer. Vortex thoroughly so that the pellet has completely dissolved.
16. Add 4.2 ml (0.43 mM) DTNB solution and vortex (samples and standards).
17. Incubate at room temperature for 15 minutes.
18. Set up spectrometer/plate reader to read the absorbance at 412 nm.

Calculating MT concentration:

$$MT = \frac{\text{Concentration in µg}}{\text{Volume of sample used (i.e. 0.5 ml)}} \times \frac{\text{Volume of DDT used (i.e. 3 ml)}}{\text{Weight of sample weighed out in first stage (g)}}$$

This gives the amount of MT expressed as µg/g wet weight tissue.

Stage 2 solutions:

Washing buffer (make up fresh):

	For 20 ml
Absolute ethanol (at –20°C)	17.4 ml
Chloroform	200 µl
Tris–Sucrose solution*	2.4 ml

**Tris–sucrose (20 mM, 0.5 M) solution:*

	For 100 ml (keep in fridge)
Trisma-base	0.242 g
Sucrose	17.104 g
Distilled water	q.s. to 100 ml
pH 8 (adjust with HCl)	

Tris-EDTA buffer (5 mM, 1mM):

	For 500 ml (keep in fridge)
Trisma-base	0.302 g
EDTA	0.146 g
Distilled water	q.s. to 500 ml
pH 7 (adjust with HCl)	

0.43 mM DTNB (make up fresh and keep in the dark):

7.1 mg DTNB in 42 ml Na_2-P buffer

Na_2-P buffer (0.2 M):

	For 500 ml (keep in fridge)
Na2HPO4	14.2 g
Distilled water	q.s. to 500 ml
pH 8	

For standard curve:

Stock GSH solution:

Glutathione (reduced) (Sigma G4251) – dissolve 5 mg in 10 ml distilled water

Standard	GSH (µl)	Tris-EDTA (µl)	Concentration (µg)
1	0	300	0
2	5	295	2.5
3	10	290	5
4	20	280	10
5	40	260	20
6	80	220	40
7	160	140	80

Acetylcholinesterase activity

Tissue preparation

1. Grind up frozen tissue in a cold mortar with liquid nitrogen.
2. Dilute with a 1:5 w/v ratio of homogenisation buffer (0.1 M Tris-HCl, pH 7.2, 0.25 M sucrose). Centrifuge at 10,000 rpm for 10 minutes. Retain supernatant and store on ice.

Reagents

1. Prepare the phosphate buffer solution by dissolving:
   - 7.584 g $NaHPO_4$ (Sigma S-5136)
   - 1.8 g KH_2PO_4 (Sigma P-8416)

   In 1 litre of distilled water, make a 50 mM solution and adjust pH to 7.4. Alternatively, dissolve a saline buffer tablet (Sigma P 4417) in 200 ml of distilled water. This makes a 10 mM solution. Adjust pH to 7.4

2. Dissolve 0.043 g of ACTI (Sigma A-5751) in 50 ml of distilled water in a smoked amber bottle (to protect solution from the light) and place on ice. This produces a 3.0 mM solution.

3. Dissolve 0.0107 g of DTNB (Sigma-D8130) in 100 ml phosphate buffer in a smoked amber vial to produce a 270 μM solution and place on ice.

Determination of acetylcholinesterase activity

1. Prepare the microtitre plate reader as follows:
   - Absorbance at ΔA_{405nm}
   - Run time 5 minutes at 30 second intervals.

Performing the assay

There may be some non-enzymic (endogenous) reaction between the sample and the DTNB, which may interfere with the analysis. To control for this, a pre-incubation of DTNB and sample is performed before adding ACTI (see below). The use of a multi-pipette or repeat dispenser for the addition of ACTI is recommended as the reaction proceeds quickly. Pipette the reagents into a microtitre plate in the following order:

Addition	Blank	Sample
Haemolymph	-	50 μl
Buffer	50 μl	-
DTNB	150 μl	150 μl
Record A_{405nm} of endogenous reaction for 5 minute at 25°C		
ACTI	50 μl	50 μl
Record A_{405nm} for 5 minute at 25°C		

1. Press ‘read’ on KC4.
2. Results are recorded as ΔA_{405nm}/minute.
3. Results are expressed as specific activity (μ moles substrate hydrolysed per minute per mg total protein).

Determination of total protein: BioRad Bradford assay

Prepare protein standards

1. Start with a BSA standard solution of 2 mg/ml (Sigma P0834). Prepare standards as follows, mixing well with a vortex:

Standard (mg/ml)	Stock (µl)	Buffer (µl)
0 (blank)	0	100
0.2	10	90
0.6	30	70
1.0	50	50
1.4	70	30

2. Dilute BioRad (catalogue no. 500-0006) Bradford reagent 1 in 5 with distilled water (e.g. 5 ml + 20 ml distilled water).
3. Prepare microtitre plate reader:
   - Wavelength setting is 595 nm, single wavelength
4. Pipette 4 µl standards, blanks and samples into microtitre plate. Add 200 µl BioRad protein reagent. Incubate at 18-25°C for 10—30 minutes.
5. Place plate in reader and press 'read' on SOFTMAX PRO.
6. Results are expressed as mg/ml, correcting for original dilution

Calculation of specific activity

Enzyme activity can be standardised by volume or tissue weight, but the preferred method is to express units of activity relative to total protein.
one unit = amount of enzyme that hydrolyses 1 µmole ACTC per minute per mg protein

$$\text{AChE activity (units)} = \frac{\Delta A_{405} \times Vol_T \times 1000}{(1.36 \times 10^4) \times \text{light path} \times Vol_S \times [\text{protein}]}$$

$$= \quad \mu\text{mol ACTC per minute per mg protein}$$

where:

ΔA_{405} = change in absorbance (OD) per minute, corrected for spontaneous hydrolysis
Vol_T = total assay volume (DTNB + sample in ml)
1.36×10^4 = extinction coefficient of TNB (per M per cm)
light path = microplate well depth (1 cm)
Vol_S = sample volume (in ml)
[protein] = concentration of (mg/ml)

NB The only variables are ΔA_{405} and [protein]; all other factors are constant for each experimental format

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay provides a direct measure of the total antioxidant status of a sample. Antioxidants in the sample reduce ferric [Fe(III)] TPTZ to ferrous [(FeII)] TPTZ, which has a strong absorbance at 593 nm.

Materials
sodium acetate, $C_2H_3O_2Na.3H_2O$
glacial acetic acid
2,6,6-tripyridyl-s-triazine (TBTZ) (Fluka 93285)
HCl
$FeCl_3.6H_2O$ (Sigma F2877)

Optional standards

$FeSO_4.7H_2O$	Sigma F7002
L-+-ascorbic acid ultra pure	Merck
Uric acid	BDH
BSA fraction V	Sigma
Trolox	Aldrich 23881-3

Equipment
spectrophotometer or microtitre plate reader
0.5 ml cuvettes or microtitre plates capable of holding 300 µl per well
needles
syringes
eppendorf tubes for collection of samples

Preparation of FRAP reagent
1. 300 mM acetate buffer:
$C_2H_3O_2Na.3H_2O$ (molecular weight = 136.1) 3.1 g
glacial acetic acid 16 ml
Make up to 1 litre with distilled water and adjust pH to 3.6.

2. 10 mM TBTZ in 40 mM HCl:
TBTZ (molecular weight = 312.3) 312 mg
HCl (1 molar) 4 ml
Make up to 100 ml with distilled water.

3. 20 mM $FeCl_3$.6H2O (molecular weight = 270.3) 0.54 g
Make up to 100 ml with distilled water.

Prepare FRAP reagent fresh as required by mixing the above in the ratio 10:1:1.

4. Standards:
FeSO4.7H2O (molecular weight = 278)
1000 µM = 27.8 mg/100 ml
Prepare standard curve from 0 to 1000 µM

Concentration (µM)	Volume of 1mM $FeSO_4.7H_2O$ (µl)	Volume of distilled water (µl)
0	0	1000
50	50	950
100	100	900
200	200	800
400	400	600
800	800	200
1000	1000	0

5. Sample preparation:
   - Grind whole tissue to a fine powder in liquid nitrogen.
   - Transfer approximately 1 g of tissue to chilled 10 ml beakers.
   - Add physiological saline to each sample at a ratio of 1:5 (w/v) tissue: physiological saline.
   - Centrifuge the homogenates at 1,000 rpm for 10 minutes. Discard any particulate matter prior to assay.
   - Use the supernatant for subsequent analysis.

6. To perform assay:
   - Add 200 µl FRAP reagent and 50 µl sample, standard or buffer to a plastic cuvette or microtitre plate and mix well.
   - Start recording the absorbance at 593 nm immediately (time 0).
   - Incubate for 10 minutes at 25°C.
   - Take a reading every 15 seconds or monitor continuously. There is no need to record for longer than 6 minutes.

7. Calculate FRAP using the following equation:

$$\frac{\text{10 – 0 minute } \Delta A_{593nm} \text{ test sample}}{\text{10 – 0 minute } \Delta A_{593nm} \text{ standard}} \times \text{FRAP value of standard* (µM)}$$

* Blank corrected signal given by 100 µM $FeSO_4.7\ H_2O$ (FeII) is equivalent to a FRAP value of 100 µM.

Comet assay

Preparation of Kenny's salt solution (for LMPA)
Ingredients per 1000 ml:

	Final concentration	*Weight*
NaCl	0.4 M	23.37 g
KCl	9 mM	0.6709 g
K_2HPO_4	0.7 mM	0.12194 g
$NaHCO_3$	2 mM	0.16802 g

q.s. to 1,000 ml distilled water

Low melting point agarose (LMPA) (1.0 per cent)
To make 1.0 per cent LMPA, dissolve 10 mg/ml Kenny's salt solution by heating in a microwave for 1–2 minutes at full power until dissolved. Aliquot 2 ml samples into microcentrifuge tubes and store in a refrigerator at 4°C. When required, melt immediately before use.

Preparation of TAE solution (for NMPA)
Ingredients per 1000 ml:

	Final concentration	*Weight*
Tris-Acetate	40 mM	7.248 g
EDTA	1 mM	2 ml 0.5 M solution

q.s. to 1,000ml distilled water

Normal melting point agarose (NMPA) (1.0 per cent)
To make 1.0 per cent NMPA, dissolve 1.0 g/100 ml TAE solution by heating in a microwave for 1–2 minutes at full power until dissolved. Store in a refrigerator at 4°C. When required, melt immediately before use.

Lysing solution
Ingredients per 1,000 ml:

	Final concentration	*Weight*
NaCl	2.5 M	146.4 g
Na_2EDTA	100 mM	37.2 g
Tris Base	10 mM	1.2 g
NaOH to pH 10 (approximately 8.0 g)		
N-Lauroyl-sarcosine 1 per cent		10 g

Add pure water to give a final volume of 890 ml.
Filter sterilise, store at 4°C and adjust to room temperature before using.

Added immediately prior to use:

TRITON X100	1 per cent (e.g. 0.5 ml per 50 ml)
DMSO	10 per cent (e.g. 5 ml per 45 ml)

Electrophoresis buffer

	Final concentration	*Weight*
NaOH	1 N	40.0 g

q.s. to 1,000 ml with distilled water, filter sterilise. Store at room temperature.

EDTA 200 mM

If using 0.5 M EDTA solution, dilute 0.5 M Na_2EDTA solution by 2.5 times, e.g. 1 ml EDTA plus 1.5 ml millipore water (i.e. water previously passed through a 0.45 µm millipore filter). Store at room temperature.

Make up electrophoresis buffer immediately prior to use. Use freshly made electrophoresis buffer for each set of gels run. For 1-litre electrophoresis buffer, mix 300 ml 1N NaOH, 5 ml 200 mM EDTA and 695 millipore water.

Neutralisation buffer

	Final concentration	*Weight*
TRIS	0.4 M	48.44 g

q.s. to 1,000ml with distilled water. Adjust to pH 7.5 using concentrated HCl acid. Store at room temperature.

Ethidium bromide staining solution

Preparation of stock ethidium bromide solution:
Stock solution is made up of 2.0 mg/ml ethidium bromide in distilled water (giving a 5x dilution), e.g. 0.5 ml ethidium solution + 2ml distilled water. Store at room temperature in the dark.

Working solution = 0.05 ml of 2.0 m/ml stock + 4.95 ml distilled water (giving a 100x dilution of the stock solution). Store at room temperature in the dark.

Procedure

1. Bring LMPA and NMPA to melting point by microwaving or holding in a water bath.
2. Label slides with diamond pen.
3. Dip slides into molten NMPA and wipe excess from underside of slide. Dry slides in an incubator at 40°C for a minimum of 2 hours (can be prepared the previous day).
4. Centrifuge cells for 3 minutes at 200*g*.
5. Remove supernatant from centrifuged cells. Resuspend cells in 180µl LMPA by gently pipetting. (NB There should be no more than ~10,000 cells per sample).
6. Apply two drops of 85 µl LMPA/cell suspension on top of the base layer of NMPA (thus providing two replicates per cell sample).
7. Apply 22 × 22 coverslips and return to ice/fridge for at least 10 minutes.
8. Prepare the working lysing solution (add Triton X-100 and DMSO together).
9. Gently slide off coverslips and place slides in coplin jars/staining trough.
10. Add lysing solution to fully immerse slides.
11. Incubate for 1 hour at 4°C in the dark to lyse the cells.
12. Meanwhile make up electrophoresis buffer and pour into the electrophoresis chamber. Check the chamber is completely horizontal beforehand.
13. Run tap water through the electrophoresis chamber to maintain temperature.
14. Remove slides from the lysing solution and rinse in distilled water three times for 2 minutes.
15. Transfer slides into the electrophoresis chamber (preferably with the labelled end towards the anode), ensuring the gels are completely covered with the electrophoresis buffer.
16. Allow the slides to incubate in the alkaline electrophoresis buffer for 20 minutes to allow the DNA to unwind.
17. Turn on power supply and set to 20 V and 300 mA (may need to adjust volume of buffer). Run for 30 minutes. NB The voltage will only reach 17 V at 300 mA.

18. Remove slides from electrophoresis chamber, add ~5 drops of neutralisation buffer and leave for 5 minutes. Repeat a further twice.
19. If the slides are not to be analysed within 24 hours, then fix (dehydrate) for 5 minutes in 100 per cent methanol. To rehydrate, apply 100 μl distilled water (on each area of agarose) and apply coverslip – allow to rehydrate for 10 minutes before adding stain.
20. For analysis within 24 hours, apply 40 μl ethidium bromide stain (working solution) to each replicate, apply coverslips, and store in airtight containers in the fridge.
21. Analyse at least 50 cells per replicate (100 cells per slide).

Evaluation of DNA damage

1. For visualisation of DNA damage, observations should be made of fluorochrome-stained DNA using a 20× or 40× objective (depending on the cell size) on a fluorescent microscope. NB Avoid analysing cells at the edges of the gel, where it has been observed that high levels of damage can be seen.
2. There are several image analysis systems commercially available for quantification of data, e.g. Komet analysis system developed by Kinetic Imaging Ltd and Comet Assay II developed by Perceptive Instruments.

It is also possible to analyse comets quantitatively without image analysis software. A scheme has been developed by Andrew Collins and colleagues (University of Oslo, Norway) for visual scoring based on five recognisable classes of comet, from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). Each comet is given a value according to the class it is put into, so that an overall score can be derived from each gel.

Appendix II – OysteR Embryo Chromosome Aberrations (ORCA) assay: method development and validation

This appendix presents the aims, method, results and learning points of four experiments conducted at the Environment Agency's Waterlooville Laboratory on:

- 1 July 2004
- 27 July 2004
- 3 August 2004
- 10 August 2004.

Learning points are given in italics.

Experiment 1: 1 July 2004

Aim
To attempt oyster embryo chromosome extraction and staining procedures at the Biological Effects Laboratory at Waterlooville.

Method
1. Conditioned adult oysters were supplied by GSF (Batch No. Arch 04/01 5/5). Oysters arrived on the day of the test.

2. Oysters exposed to reference seawater (filtered artificial seawater (ASW)) at a density of 100 embryos per ml; 2 litres of oyster suspension was prepared.

3. Oyster embryos were incubated at 24°C in glass beakers for ~4 hours during early development to promote development to 16–32 cells. Once they had reached 16–32 cells, oyster embryos were inoculated from their developmental density (6,000 embryos/ml) to their test density (100 embryos/ml). Oyster embryos were then incubated at this density for 20 hours at 20°C.

4. After 20 hours, oyster embryos were exposed to colchicine and subsequent isotonic solutions as per the Plymouth method, but using three treatments of test solution volume: 500, 250 and 100 ml (50,000, 25,000 and 10,000 nominal embryos respectively). This was done to investigate the minimum amount of test solution required to yield enough embryos for slide production/scoring.

5. Fixed embryos were left for between 3 days and 3 weeks before slide preparation and staining

6. A standard zinc reference test was performed alongside the mesh-based chambers, but the exposure duration was extended to 24 hours to allow for development of larvae to D-stage.

Results/*Learning points*
Harvesting of embryos from test solutions using meshes and microcentrifuge tubes was successful.

Colchicine and hypotonic solutions should be poured into the centre of the mesh tubes and allowed to fill the crystallising dishes, rather than adding meshes to crystallising dishes previously filled with colchicine or hypotonic solutions. This minimises the presence of bubbles in the underside of the mesh.

Staining was successful. Good chromosome staining was observed in a few cells under oil immersion (×1000).

Spent Giemsa stain must be disposed of in a non-chlorinated waste solvents bottle. It should be possible to obtain one from the NLS.

Mounting of slides using DPX was reasonably successful. If too much DPX is used, the slides take too long to dry (layer is too thick). If too little is used (layer is too thin), subsequent observation under oil immersion becomes difficult as a large amount of oil is

needed to obtain the correct focal conditions. There also seem to be fewer bubbles forming under the cover slips when less DPX is used.

Continued practising with DPX and oil immersion is necessary to strike the correct balance between DPX thickness, drying time and ease of observation under oil immersion. It may also be possible to experiment with different immersion oils with greater viscosity.

After 20 hours, the majority of oyster embryos had partially formed shells. This resulted in poor disaggregation of cells after hypotonic treatments and staining, and poor chromosome spreads. Not many cells could be scored individually as many were 'clumped'. The presence of shells was probably the result of two factors:

- total development time was ~24 hours (including early development time and exposure time);
- early development was conducted at 24°C promoting rapid cell division and development.

Future exposures should be timed from fertilisation rather than inoculation. They should be conducted at a single temperature only (20°C). This would make it more likely that good spreads would be prepared from the exposures.

Slides that were prepared within a few days of fixing in Carnoy's gave better spreads than those prepared after the same batch of embryos had been stored for an additional two weeks in Carnoy's.

This may be the result of cells becoming too 'brittle' after extended storage in Carnoy's fixative. Recommend that slides are prepared within a week of fixing in Carnoy's or within 48 hours if possible.

At this early stage, there seemed to be no difference in the quality of slides produced from 500, 250 or 100 ml of test solution.

It is too soon to recommend a preference for one volume of test solution over another. However, 250 ml seems a reasonable compromise in the short term. This density should be used in forthcoming exposures and until more information is available with which to make decisions.

Experiment 2: 27 July 2004

Aim
To repeat chromosome extraction and staining procedure detailed as experiment 1, specifically to improve the quality of chromosome spreads through use of a shortened exposure duration

Method
Nominal exposure timetable developed as detailed below:

Date	Time	Time post-fertilisation (hours: minutes)	Activity
27 July	16:45	0	Oyster eggs fertilised
28 July	09:00	16:15	Exposure to test substance
28 July	12:45	20:00	Colchicine
28 July	13:15	20:30	Hypotonic 1
28 July	13:25	20:40	Hypotonic 2
28 July	13:35	20:50	Hypotonic 3
28 July	13:45	21:00	Hypotonic 4
28 July	13:55	21:10	Centrifuge and Carnoy's fixative

1. Conditioned adult oysters were supplied by GSF (Batch no. Arch 04/03 7/7). Oysters arrived on the day of the test

2. Oysters were fertilised and grown on in reference seawater (filtered ASW) at 20°C at a density of 6,000 embryos per ml. Three batches of oyster egg suspension were prepared using different females.

3. Oyster eggs were fertilised at 17:00 (27 July 2004, 15 minutes behind nominal schedule). Sperm quality was poor on initial inspection. All subsequent timings are based on time from fertilisation.

4. Oyster embryos were gently resuspended at 30–45 minute intervals and examined for the presence of polar bodies. No polar bodies were present until18:30, 1.5 hours post-fertilisation, although one embryo had three cells at this point.

5. Oyster embryos were inoculated into ASW at test density (100 embryos/ml) at 19:30 (2.5 hours post-fertilisation); embryos were at 1–4 cells at this stage with ~90–95 per cent fertilisation rate. Three 250 ml replicates were prepared. Embryos were then incubated at 20°C in the dark for a further 17.5 hours.

6. At 20 hours post-fertilisation, embryos were exposed to colchicine and subsequent isotonic solutions as per the Plymouth method. Embryos were spun out of suspension and fixed in Carnoy's.

7. Slides were prepared and fixed the same week.

Results/*learning points*
Nominal exposure timetable worked reasonably well. Fertilisation was 15 minutes behind schedule, but later treatments were delayed to accommodate this.

No evidence of D-shells in embryos exposed for 20 hours at 20°C.

Quality of chromosome spreads was poor. Although no D-shells were present, cell disaggregation did not occur during slide preparation, resulting in 'clumping' and poor resolution of cells.

Neat glacial acetic acid was used for slide preparation, rather than the 80 per cent solution. This may have contributed to the poor chromosome spreads.

Experiment 3: 3 August 2004

Aim:
To repeat chromosome extraction and staining procedure (detailed in experiments 1 and 2) to:

- determine reason for poor quality of chromosome spreads;
- trial methodology and response of oyster embryos after exposure to genotoxic reference substance MMS for 1.5 cell cycles.

Method
Nominal exposure timetable developed as detailed below:

Date	Time	Time post-fertilisation (hours: minutes)	Activity
03 August	16:45	0	Oyster eggs fertilised
04 August	09:00	16:15	Exposure to test substance
04 August	12:45	20:00	Colchicine
04 August	13:15	20:30	Hypotonic 1
04 August	13:25	20:40	Hypotonic 2
04 August	13:35	20:50	Hypotonic 3
04 August	13:45	21:00	Hypotonic 4
04 August	13:55	21:10	Centrifuge and Carnoy's fixative

1. Conditioned adult oysters were supplied by GSF (Batch no: unknown). Oysters arrived on the day of the test.

2. Oysters were fertilised and grown-on in reference seawater (filtered ASW) at 20°C at a density of 6,000 embryos per ml. Three batches of oyster egg suspension were prepared using different females.

3. Oyster eggs were fertilised at 16:55 (03 August, 10 minutes behind nominal schedule). All subsequent timings are based on time from fertilisation.

4. Oyster embryos were gently resuspended at 30–45 minute intervals and examined for the presence of polar bodies.

5. Oyster embryos were inoculated into ASW at test density (100 embryo/ml) at 19:15 (2 hours 20 minutes post-fertilisation); embryos were at 1–4 cells at this stage with ~90–95 per cent fertilisation rate. Ten 250ml replicates were prepared (25,000 embryos per replicate).

6. Embryos were then incubated at 20°C in the dark for a further 17 hours, until exposed to the test substance.

7. Oyster embryos were exposed to four concentrations of MMS (in ASW) in duplicate:

    - 1.0×10^{-3} = 1 mM

- 1.0×10^{-4} = 0.1 mM
- 1.0×10^{-5} = 0.01 mM
- 1.0×10^{-6} = 0.001 mM

7. Exposure was scheduled to begin at 09:10 until 12:55 (3 hours 45 minutes corresponding to 1.5 cell cycles, as previously calculated by V. Cheung).

8. MMS concentrations were based on those used previously by V. Cheung and are known to induce CAbs and aberrant cells in oyster embryos. On the evening of 3 August, 500 ml of each test solution was prepared by serial dilution of the 1 mM test solution (prepared by dissolving 50.65mg of MMS in 500 ml of ASW). Test solutions were stored at 4°C in the dark until required on 4 August.

9. Exposure to test solutions began at 10:00 (50 minutes behind schedule in order to bring test solutions up to temperature). As a result, oyster embryos were only exposed to MMS for 2 hours 55 minutes (~0.9 cell cycles).

10. At 20 hours post-fertilisation, embryos were exposed to colchicine and subsequent isotonic solutions as per the Plymouth University method. Some difficulty was encountered due to the length of time it took to transfer the embryos from the test solutions to the colchicine (it took 30 minutes to put all replicates into Colchicine). As a result, some replicates were exposed to MMS and colchicine for less or more time than others.

11. Embryos were spun out of suspension and fixed in Carnoy's.

12. Slides were prepared and fixed the same week.

Results/*learning points*
Nominal exposure timetable worked reasonably well. MMS exposures were delayed as test solutions were not up to test temperature when required.

Chromosome spreads of better quality, but still some clumping. It was possible to distinguish some individual disaggregated cells along 'lines across slides'. There was no obvious evidence of CAbs, or aberrant cells across the MMS concentration range.

The appearance of 'lines across slides' of disaggregated cells may be evidence of improved disaggregation after more vigorous or repeated slide swirling during initial slide preparation. In previous experiments, large numbers of slides were prepared simultaneously and each slide may not have been swirled sufficiently to disaggregate the cells. In subsequent experiments, it will be important to swirl slides more frequently or for longer than at present to ensure that cells are disaggregated and distributed evenly across slides.

The lack of obvious CAbs or aberrant cells may be the result of a number of factors:
- *the interval between MMS test solution preparation and exposure;*
- *the curtailed MMS exposure duration (only ~0.9 cells cycles);*
- *the problems encountered when transferring replicates to colchicine to MMS.*

MMS degrades in solution and the overnight storage period, although short, could have resulted in MMS concentrations below nominal. In future experiments, solutions of MMS should be prepared on the day of the test.

As the MMS exposures were ~0.9 cell cycles, there may have been a reduction in assay sensitivity as fewer cells had entered metaphase.

The inefficiency of transfer from test solutions to MMS affected the exposure duration of certain replicates to MMS and colchicine. In future exposures, timing of the exposure to test solutions and colchicine should be undertaken using smaller discrete groups (maximum size 4–5 replicates), which can be managed more easily than the overall group.

Experiment 4: 10 August 2004

Aim
To repeat oyster embryo MMS exposure with chromosome extraction, staining and slide preparation procedure (detailed in experiments 1, 2 and 3) with modifications based on previous learning points.

Method
Nominal exposure timetable developed as detailed below:

Date	Time	Time post-fertilisation (hours: minutes)	Activity
10 August	16:45	0	Oyster eggs fertilised
11 August	09:00	16:15	Exposure to test substance
11 August	12:45	20:00	Colchicine
11 August	13:15	20:30	Hypotonic 1
11 August	13:25	20:40	Hypotonic 2
11 August	13:35	20:50	Hypotonic 3
11 August	13:45	21:00	Hypotonic 4
11 August	13:55	21:10	Centrifuge and Carnoy's fixative

1. Conditioned adult oysters were supplied by GSF (Batch no: unknown). Oysters arrived on the day of the test.

2. Oysters were fertilised and grown on in reference seawater (filtered ASW) at 20°C at a density of 6,000 embryos per ml. Three batches of oyster egg suspension were prepared using different females.

3. Oyster eggs were fertilised at 16:55 (10 minutes behind nominal schedule). All subsequent timings are based on time from fertilisation.

4. Oyster embryos were gently resuspended at 30-45 minute intervals and examined for the presence of polar bodies.

5. Oyster embryos were inoculated into ASW (from oyster 1 and 2) at test density (100 embryos/ml) at 19:15 (2 hours 20 minutes post-fertilisation); embryos were at 1–4 cells at this stage with ~90–95 per cent fertilisation rate. Ten 250 ml replicates were prepared (25,000 embryos per replicate).

6. Embryos were then incubated at 20°C in the dark for a further 17 hours until exposed to the test substance.

7. Oyster embryos were exposed to four concentrations of MMS (in ASW) in duplicate:

   - 1.0×10^{-3} = 1 mM
   - 1.0×10^{-4} = 0.1 mM
   - 1.0×10^{-5} = 0.01 mM

- 1.0×10^{-6} = 0.001 mM

8. Exposure was scheduled to begin at 09:10 until 12:55 (3 hours 45 minutes corresponding to 1.5 cell cycles, as previously calculated by V. Cheung).

9. On the morning of 10 August, 500 ml of each test solution was prepared by serial dilution of the 1 mM test solution (prepared by dissolving 55.065 mg of MMS in 500 ml of ASW).

10. Exposure to test solutions began at 9:40 (30 minutes behind schedule due to a delay preparing the test solutions). As a result, oyster embryos were only exposed to MMS for 3 hours 15 minutes (~1.3 cell cycles). Exposure took place in 300 ml crystallising dishes containing ~250 ml of test solution. Test organisms were contained within each dish by a polypropylene tube with a 30 µm mesh bottom. This tube allowed the oysters to be transferred to the colchicine and hypotonic solutions relatively easily. However, using these tubes resulted in the (physical) density that the oysters experienced of >100 per ml though the aqueous density remained at 100 per ml.

11. At 20 hours post-fertilisation, the embryos were exposed to colchicine and subsequent isotonic solutions as per the Plymouth University method. The replicates were spit into three groups of four replicates, with colchicine and hypotonic timings based on these groups. No problems with timings were encountered.

12. Two aliquots of control oysters were allowed to develop at 20°C for an additional 24 hours beyond the 20 hours experiment:
    - one exposed in mesh tubes at a density equivalent to the test (>100 per ml);
    - one exposed at 100 embryos per ml.

    This was to investigate whether:
    - the oysters would develop into normal D-shaped larvae if given sufficient time;
    - containment within mesh tubes (at a density >100 per ml) influenced normal development.

13. Embryos were spun out of suspension and fixed in Carnoy's. Two speeds were used (5 minutes) – the standard speed of 2,000 rpm used in previous experiments and an increased speed of 5,000 rpm. One replicate from each test concentration was spun at each speed; the remaining control replicate was spun at the standard speed. The increased speed was to counter the embryos swimming up the relatively short microcentrifuge tubes and being discarded with the supernatant. This initial experiment would investigate if subsequent slide preparation was affected.

14. Slides were prepared and fixed the same week.

Results/*learning points*
MMS exposures were delayed as test solutions were not ready when required, resulting in exposure duration <1.5 cell cycles

Ensure that fume cabinets, volumetric flasks and weighing boats are prepared on the day before exposures, in order that test solutions can be prepared without delay on the day of exposure.

Normal development of oysters after a further 24 hours exposure at 20°C is detailed in table 1 below.

Development time (hours post-fertilisation)	Percentage 'D-shaped'					Mesh (SD)
	Exposure type	1	2	3	4	
20	Beaker	0	0	0	0	0
	Beaker	37.0	28.6	28.4	29.6	30.9 (4.1)
24	Mesh tube	5.1	8.7	13.8	9.1	9.18 (3.6)
	Beaker	56.3	62.0	47.4	39.8	51.38 (9.78)
44	Mesh tube	74.0	64.9	61.5	69.9	67.58 (5.50)

SD = standard deviation

There are significant differences (P <0.05, see statistical analysis at end of this appendix) between the development of oysters in beakers and those in tubes at both time points. At 24 hours, development was better in the beakers but, at 48 hours, development was better in the mesh tubes.

Mesh tubes do not affect the normal development of oysters and, in fact, may improve development over longer time periods.

Increased centrifuge speed produced better pellets in the microcentrifuge tubes, with the larvae less likely to swim up into the supernatant.

Slides do not store well in the vertical (as used by current slide boxes). Vertical storage results in a gradual migration of DPX towards the bottom of the slide, producing a variable thickness of DPX across the slide and associated difficulties focussing on chromosomes.

Investigate alternative storage boxes that store slides on the horizontal rather than the vertical.

Chromosome spreads are of a much better quality than obtained in previous experiments. On initial inspection, there appeared sufficient numbers of metaphases with complete chromosome compliments (20 ± 2) on each slide to allow a proper CA assessment (see table below).

MMS (mM)	Replicate	Slide	Vernier position label to left (X, Y)	Chromosome compliment (n)	Comments
Control	A	1	750, 1040	15	Incomplete – normal
	A	1	750, 1043	20	Complete – normal
	A	1	750, 1078	9	Incomplete – normal
	A	1	751, 1150	20	Complete – normal
	A	1	751, 116	20	Complete – normal
	A	1	611, 116	20	Complete – normal
0.001	A	1	462, 1092	17	Incomplete – normal
	A	1	449, 1092	20	Complete – normal
	A	1	445, 1092	19	Complete – normal
	A	1	381, 1092	19	Complete – normal
	A	1	320, 1092	20	Complete – normal
	A	1	343, 1092	16	Incomplete – normal
0.01	A	1	431, 940	20	Complete – normal
	A	1	461, 940	20	Complete – normal
	A	1	461, 940	16	Incomplete – normal
	A	1	362, 940	17	Incomplete – normal
	A	1	325, 940	20	Complete – normal
	A	1	158, 939	20	Complete – normal
	A	1	158, 940	20	Complete – normal
	A	1	150, 9400	13	Incomplete - normal
0.1	A	1	432, 940	20	Complete – normal
	A	1	551, 950	20	Complete – normal

	A	1	546, 950	20	Complete – normal
	A	1	440, 950	-	Aberrant
	A	1	551, 946	20	Complete – normal
	A	1	551, 946	20	Complete – normal
	A	1	551, 946	20	Complete – normal
	A	1	551, 946	20	Complete – normal
	A	1	209, 951	-	Aberrant
	A	1	366, 1077	20	Complete - normal
1.0	A	1	482, 969	20	Complete – normal
	A	1	482, 962	20	Complete – normal
	A	1	475, 1065	-	Aberrant
	A	1	140, 1112	-	Aberrant

There was also some evidence of aberrant cells and CAbs at the highest MMS concentrations (0.1 and 1 M). However, it was difficult to score chromosomes definitively for aberrations because of their small size.

The only means to improve the magnification of the current high-power light microscope is to use a magnifying C-mount and view images via the computer. One should be obtained (either a X2 or zooming) in order to allow slides to be scored with accuracy.

No assessment of cytotoxicity has been undertaken to date.

Mitotic index is the simplest means of assessing this. This method should be attempted as soon as possible, as this can be achieved without the magnification required for scoring chromosomes.

Statistical analysis of oyster development in controls and mesh tubes

Two-sample *t*-test and confidence interval (CI): A 24-B, A 24-T

Two-sample *t*-test for A 24-B vs A 24-T

	N	*Mean*	*SD*	*SEM*
A 24-n	4	0.3144	0.0434	0.022
A 24-t	4	0.0919	0.0359	0.018

Difference = μA 24-B –μA 24-T

Estimate for difference: 0.2225

95 per cent CI for difference: (0.1500, 0.2949)

t-test of difference = 0 (vs not =): $t = 7.90$; $P = 0.001$ (DF = 5)

Two-sample *t*-test and CI: A 44-B, A 44-T

Two-sample *t*-test for A 44-B vs A 44-T

	N	*Mean*	*SD*	*SEM*
A 44-n	4	0.542	0.114	0.057
A 44-t	4	0.7439	0.0751	0.038

Difference = μA 44-B – μA 44-T

Estimate for difference: –0.2015

95 per cent CI for difference: (–0.3771, –0.0258)

t-test of difference = 0 (vs not =): $t = -2.95$; $P = 0.032$ (DF = 5)

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