Interim Report R&D Project 396

European Collaboration to Develop Standard Sediment Toxicity Test Methods

WRc plc
January 1993
R&D 396/4/T

EUROPEAN COLLABORATION TO DEVELOP STANDARD SEDIMENT TOXICITY TEST METHODS

R Fleming

Research Contractor:
WRc plc
Henley Rd Medmenham
PO Box 16 Marlow
SL7 2HD

National Rivers Authority
Rivers House Waterside Drive
Almondsbury Bristol BS12 4UD

NRA Interim Report 396/4/T

National Rivers Authority
Rivers House Waterside Drive
Almondsbury Bristol BS12 4UD
Tel: 0454 624400
Fax: 0454 624409

Dissemination status
Internal: Restricted
External: Restricted

Research contractor
This document was produced under R&D Contract 396 by:
WRc plc
Henley Rd Medmenham
PO Box 16 Marlow
SL7 2HD
Tel: 0491 571531
Fax: 0491 579094

WRc Report Nº NR 3259/1/4281

NRA Project Leader
The NRA's Project Leader for R&D Contract 396:
Dr D Tinsley - Thames Region

Additional copies
Further copies of this document may be obtained from Regional R&D Co-ordinators or the R&D Section of NRA Head Office.

396/4/T

CONTENTS

LIST OF TABLES

EXECUTIVE SUMMARY

This report describes progress in a programme of work to develop standardised test protocols for determining the toxicity of poorly soluble substances in sediments. The programme involves an interlaboratory comparison of the performance of three sediment toxicity tests using the test organisms *Corophium volutator*, *Chironomus riparius* and the freshwater mussel *Sphaerium corneum*.

The first stage of collaborative testing is scheduled to begin in November 1992 and to date preliminary work has been carried out in order to finalise protocols. Definitive protocols are included in the Appendices of this document.

Two poorly soluble substances have been chosen for the programme, lindane and PCB 118, which are both of high environmental concern. A test sediment from Poole Harbour is being used by all collaborators and the animals are being supplied by coordinating laboratories.

This report describes the work of WRc to date and briefly covers the work of other collaborators. Progress with NRA objectives is discussed.

KEY WORDS

Sediments, toxicity, freshwater, marine, standardization

1. INTRODUCTION

This report describes progress in a programme of work conducted by WRc and European collaborators on behalf of the NRA and EC. The aim of this programme is to develop standardised test protocols for determining the toxicity of poorly soluble substances in sediments, and provide guidelines for the use of such tests to help the NRA meet its business needs.

The project involves a European interlaboratory comparison of the performance of three sediment toxicity tests using the test organisms *Corophium volutator, Chironomus riparius* and *Sphaerium corneum*. In the first stage, comparison will be carried out using acute lethal tests with *Corophium* and *Chironomus* plus a sublethal test to determine the induction of the enzyme glutathione-s-transferase in *Sphaerium*. The second stage will involve a further comparison of these tests, possibly modified in the light of the results, and will also include chronic tests with *Corophium* and *Chironomus*. Testing will begin when protocols have been finalised for the toxicity tests and the amendment of sediment with poorly soluble substances has been completed. The protocols to be used in the first stage of testing can be seen in Appendices A - D.

WRc is coordinating all interlaboratory testing. Each species is being tested by at least three laboratories so that results can be compared. The participating laboratories and the tests they will carry out are as follows:

WRc	*Corophium* *Chironomus* Sphaerium
DGW (Rijkswaterstaat - Tidal Division - Netherlands)	*Corophium*
RIZA (Rijkswaterstaat - Inland Division - Netherlands)	*Chironomus* *Sphaerium*
UNU (University of Utrecht (Netherlands)	*Sphaerium* *Chironomus*
UNH (University of Hamburg (Germany)	*Sphaerium* *Chironomus*
INIP (Institute of Fisheries - Portugal)	*Corophium*

At the beginning of the programme, a meeting was held in the Netherlands to bring together all collaborators and to decide responsibilities, timetables and practical details. The outcome of this meeting is summarised below.

1. Main objective: The development of rapid and simple methods for assessing sediment toxicity and the production of suitable protocols for sediment spiking and assaying.

2. Chemicals: One chemical should be inexpensive, easy to handle and analyse and of known acute toxicity; one chemical should be truly 'poorly soluble'. Therefore all participants will use lindane and PCB congener 118.

3. Source of sediment: Poole harbour sediment from the UK will be used in all assays. Freshwater assays will be performed with desalinated sediment.

4. Preparation of stock solutions and spiked sediments: WRc will coordinate the preparation of all three stock solutions and spiking of sediments. The following organisations will be responsible for dosing sediments with particular chemicals:

WRc	Lindane
INIP	PCB 118

5. Bioassays: The following organisations will be responsible for coordinating work with the relevant test systems:

WRc	*Sphaerium corneum* mortality and GST induction
DGW	*Corophium volutator* mortality
RIZA	*Chironomus riparius* mortality, growth and development

The responsibility for coordinating work was allocated depending on the experience of the laboratories. WRc has carried out GST assays and developed a protocol for the NRA (Johnson *et al* 1989). WRc have also developed acute lethal test protocols for *Corophium* and *Chironomus* for the NRA (Project A12(89)024) which were reported in Report No. 024/1/T (Davies *et al* 1991). However, it was felt that the NRA would benefit from the experience of the Dutch participants and that their protocols should be used with any modifications suggested by WRc. The *Corophium* protocol produced by WRc in Report 024/1/T was initially developed in conjunction with DGW and therefore the Dutch protocol for use in this programme is the same as that reported to the NRA. The *Chironomus* protocol reported to the NRA has been modified by RIZA for testing in this programme but all changes are considered by WRc to be beneficial.

The following timetable for work was agreed at the initial meeting:

Time is expressed as months from start of contract.

1. Sediment collection and characterisation (1 month).
2. Develop spiking methods and examine effects of storage (3 months).
3. Develop bioassay techniques (6 months).
4. Complete first interlaboratory exercise (12 months).
5. Improve assay protocols.
6. Complete second intercalibration exercise (20 months).
7. Complete final reports and submit to NRA and EC.

The first six month period has been a developmental phase in which coordinating labs have been carrying out practical work to refine their protocols. These have been disseminated for comment amongst participants. In this document, the progress of WRc to date will be reported and then a brief section will be included on progress of the collaborators. Definitive protocols for the first stage are included in the Appendices. Finally, details of the next phase and any modifications to the original timetable will be covered.

2. EXPERIMENTAL WORK

2.1 Work completed at WRc

In this section, the following studies will be outlined with any results obtained so far:

2.1.1 Survival tests and characterisation of test sediment.

2.1.2 Preliminary toxicity test using marine sediment spiked with lindane.

2.1.3 Preliminary toxicity test using freshwater sediment spiked with lindane.

2.1.4 Induction of glutathione-s-transferase (GST) in freshwater mussels exposed to sediment spiked with lindane.

2.1.5 Preliminary toxicity test using freshwater sediment spiked with polychlorinated biphenyl (PCB 118).

2.1.6 Amendment of sediment spiking procedure.

2.1.1 Survival tests and characterisation of test sediment.

A natural marine sediment from Poole Harbour was collected and a full characterisation performed. Results of analysis for metals are shown in Table 2.1. An organic carbon content of 4.4% has been confirmed.

In previous tests with this sediment at WRc, control survival of both marine and freshwater species, primarily *Corophium volutator* and *Chironomus riparius*, has always been above 80%. Survival tests for this project carried out at Rijkswaterstaat (Inland Division) have shown *Chironomus* egg and larval development to be comparable to that in Dutch reference sediment. Studies with *Corophium* at Rijkswaterstaat (Tidal division) have resulted in over 90% control survival in the test sediment, even higher than that usually seen in the Dutch reference sediment.

Table 2.1 Concentration of metals in reference sediment

Metal	$\mu g\ g^{-1}$	Metal	$\mu g\ g^{-1}$
Ti	3200	Ag	<=2
V	85	Cd	<=1
Cr	62	Sn	9
Mn	115	Te	<=2
Fe	26000	I	66
Co	10	Ba	195
Ni	19	La	25
Cu	52	Ce	56
Zn	82	Pb	64
Rb	82		
Sr	62		
Y	23		
Zr	230		
Nb	13		
Mo	<=2		
Pd	<=2		

2.1.2 Preliminary toxicity test using marine sediment spiked with lindane.

The objectives of this study were:

- to determine a range of concentrations causing acute toxic effects in *Corophium volutator*;
- to assess the toxicity of stored sediment relative to freshly spiked sediment.

Ten-day tests were performed with sediment which had been aged for four weeks following contaminant addition, and also with sediment which had been freshly spiked. Nominal concentrations are shown in Table 2.2. These were based on 96 hr LC_{50} values for selected marine and estuarine Crustacea in the water phase (Verscheuren 1983). These values were converted into sediment concentrations ($\mu g\ g^{-1}$ dry weight) using the partitioning equation from Lyman *et al* 1982:

$$\text{IW concentration } (\mu g/ml) = \frac{\mu g \text{ adsorbed/g org C}}{K_{oc}}$$

IW = interstitial water

Calculation of the K_{oc} (organic carbon normalised partition coefficient) was carried out using the regression equation for chlorinated hydrocarbons (Chiou *et al* 1979):

$$\log K_{oc} = -0.557 \log S + 4.277$$

S (solubility) = freshwater 8 mg l^{-1}
seawater 2 mg l^{-1}

K_{oc} = 2990 (freshwater), 6454 (seawater)
org. C = 4.4%

Table 2.2 Nominal concentrations of water and sediment in seawater test systems

Treatment No.	1	2	3	4
IW conc. µg l^{-1}	0	1.00	5.00	10.00
Sed. conc. µg g^{-1}	0	0.26	1.29	2.58

IW - Interstitial water

Carrier solvent controls were also tested. All treatments were duplicated.

Measured sediment concentrations, shown in Table 2.3, were approximately twice nominal concentrations. This was later found to be caused by an under-estimation of sediment water content. Total sediment concentration was not significantly different in aged and fresh sediment showing that sediment-bound lindane does not degrade when stored at 4 °C over a four week period.

Table 2.3 Measured sediment concentrations in seawater systems

Nominal conc. µg g^{-1}	0.26	1.29	2.58
Aged sed. conc. µg g^{-1}	0.50	2.70	5.50
Fresh sed. conc. µg g^{-1}	0.58	2.50	5.90

2.1.4 Induction of glutathione-s-transferase (GST) in freshwater mussels exposed to sediment spiked with lindane

The objectives of this study were:

- to determine a range of concentrations causing GST induction in *Sphaerium corneum* over a 10 day test period;
- to determine inter-replicate differences in total sediment concentration.

Ten-day tests were carried out with freshly spiked sediment. Nominal concentrations were based on concentrations causing no mortality in the preliminary freshwater test (Section 3). Ten animals were used per vessel and treatments were duplicated. Chemical analysis was carried out on both replicates.

Measured concentrations are shown in Table 2.6

Table 2.6 Measured sediment concentrations for *Sphaerium* test

Nominal conc. $\mu g\ g^{-1}$	0.20	0.60	2.00
Measured conc. Rep 1 $\mu g\ g^{-1}$	0.13	0.54	1.03
Measured conc. Rep 2 $\mu g\ g^{-1}$	0.15	0.45	1.12

Measured concentrations were closer to nominal concentrations than in all previous tests. Any discrepancies in this case were probably caused by a lower than nominal stock concentration (933 $\mu g\ l^{-1}$ minstead of 1000 $\mu g\ l^{-1}$). This may have been due to adsorption to glassware which in future may be minimised by using a siliconising fluid.

Differences between replicates were small considering possible experimental error during the spiking procedure. This could be minimised by taking sediment for both replicates from the same spiking vessel.

Sphaerium survival was 100% in all treatments. GST analysis was carried out on five samples of two animals per treatment. Enzyme levels have not yet been spectrophotometrically determined.

2.1.5 Preliminary toxicity test using freshwater sediment spiked with a polychlorinated biphenyl (PCB 118)

The objectives of this study were:

- to determine a range of PCB 118 concentrations causing effects in *Chironomus* and *Sphaerium*;
- to evaluate the lindane spiking method for amending sediment with PCB 118.

Ten-day tests were carried out with freshly spiked sediment. Once again, *Sphaerium* and *Chironomus* were tested in the same vessels as a problem had not been identified at this point. No toxicity or solubility data were available for PCB 118 and so concentrations were based on the maximum achievable using this method. These were then determined analytically. Each treatment was duplicated.

Measured concentrations are shown in Table 2.7.

Table 2.7 Measured concentrations of PCB 118 in freshwater test systems

Treatment	1	2	3	4
Measured conc. ($\mu g\ g^{-1}$)	0	0.021	0.175	1.620

A nominal concentration of 500 $\mu g\ l^{-1}$ was expected for the spiking solution but only 50 $\mu g\ l^{-1}$ was obtained. This was due to problems in dissolving the PCB 118 in acetone. Only a small quantity of the substance was available and it is probable that most of this was not dissolved. In future, a greater volume of PCB must be used and further work must be carried out to determine its solubility in both acetone and water.

The results show that there was an increase in sediment concentration with increasing spiking solution concentration, but it would seem that only 5% of the contaminant was adsorbed onto the sediment at each treatment. This may have been due either to binding to glassware or insufficient rolling time. There is clearly a need for further work on PCB spiking techniques.

High mortality of *Chironomus* was seen in control vessels, therefore invalidating the acute lethal biological results. Again this is probably due to the presence of *Sphaerium corneum*.

One-hundred percent survival of *Sphaerium* was seen at all concentrations. Results of GST analysis show that there is a significant difference between levels of GST in the control animals and those exposed to the highest concentrations (See Table 2.8).

Table 2.8 GST activity (µktals) in mussels exposed to PCB 118

Treatment No.	1	2	3	4
Nominal conc. (ng g^{-1})	0	21	175	1620
GST Activity (µktals)	-0.00022	0.0017	0.0021	0.0037

Conc.1 and Conc.2 - not significant
Conc.1 and Conc.3 - $p<0.05$ ($q=4.196$)
Conc.1 and Conc.4 - $p<0.001$ ($q=7.089$)

This result could be due to the fact that control samples were prepared at the beginning of the sampling time and high exposure samples at the end. All animals were removed from test vessels at the same time and were left in dry conditions until sample preparation. It is possible that the additional stress from air exposure could have elevated GST levels.

In future, GST samples will be blocked, i.e. at each preparation time a sample from the control and each concentration will be prepared.

2.1.6 Amendment of sediment spiking procedure

The objectives of this study were:

- to optimise the procedure for sediment spiking by studying the effects of differences in:
  - rolling time
  - standing time
  - spiking volume
- to assess the toxicity of sediment bound lindane to first and second instar *Chironomus* in the absence of *Sphaerium*.

Ten day tests were performed with first instar larvae which are thought to be more sensitive than the second instars used so far in the programme. Nominal sediment concentrations of 0.15, 0.30 and 0.60 µg g^{-1} lindane were chosen on the basis of results from preliminary tests (see Section 2.1.3).

Three tests were carried out to look at the effects on total sediment concentration of rolling time, spiking volume and standing time. For rolling time, sediment was rolled for 1, 6 and 24 hours. For spiking volume two ratios of sediment:water volumes were rolled: 150:800 and 300:650 ml. In this case, the contents of rolling vessels containing 300 ml sediment were distributed evenly between two test vessels and a sample of each was taken for analysis. This was carried out to assess whether concentrations would be more repeatable if all replicates for a certain concentration were rolled together. For standing

time, sediments were stored either with clean overlying water or overlying spiking solution for 24 hours.

Chemical analysis of sediment was carried out for all treatments at each concentration. Toxicity tests were performed on rolling time and spiking volume sediments only.

Measured concentrations are shown in Table 2.9.

Table 2.9 Measured lindane concentrations in freshwater systems

Nominal conc. ($\mu g\ g^{-1}$)			0.15	0.30	0.60
Measured conc. ($\mu g\ g^{-1}$)					
Treatment:					
Rolling time	1 hr		0.10	0.18	0.41
	6 hr		0.11	0.21	0.47
	24 hr		0.14	0.14	0.45
Spiking volume					
	150:800 ml		0.08	0.17	0.38
	300:650 ml	Rep 1	0.11	0.21	0.44
		Rep 2	0.12	0.22	0.45
Standing time					
	Clean	Rep 1			0.53
		Rep 2			0.54
	Spiking	Rep 1			0.51
		Rep 2			0.57

All measured concentrations were below nominal values which once again highlights the need for siliconisation of all glassware.

- Rolling time

  There was no significant difference between concentrations after 6 and 24 hour rolling periods although 1 hour was not sufficient to achieve the highest concentration possible. The low concentration at a nominal level of 30 $\mu g\ g^{-1}$ after 24 hours was possibly due to experimental or analytical error.

- Spiking volume

  Measured concentrations were closer to nominal values when a large volume of sediment was rolled. Also, replicate concentrations were more similar when taken from the same rolling vessel than those rolled separately. Therefore, 300 ml sediment will be rolled in future and used for both replicates at each test concentration.

- Standing time

  There were no significant differences in sediment concentration whether the sediments were stored in clean overlying water or contaminant spiking solution.
  After a ten day test period, no larvae were recovered, even in control vessels. It is possible that the test sediment was not suitable for this life stage and in future, second instars will be used.

2.2 Work completed by other collaborators

2.2.1 DGW - Rijkswaterstaat (Tidal Division) - *Corophium*

Ten day tests have been performed to compare survival of the DGW population of *Corophium volutator* in Poole Harbour and Dutch reference sediment. This is the population that will be used for the first stage of collaborative testing. Percentage survival in Poole sediment was 97% compared with 88% in the Dutch sediment.

A preliminary range finding test has also been carried out with lindane spiked sediment prepared by WRc. A ten day LC_{50} value of 0.48 µg g^{-1} (based on nominal concentrations) was obtained with 95% confidence limits of 0.37 - 0.61 µg g^{-1}. This value is currently being recalculated following analytical confirmation.

The protocol for the ten-day test has been finalised and can be seen in Appendix B. A range of test concentrations for the first testing stage has been set between 0.25 and 2.00 µg g^{-1}.

2.2.2 RIZA - Rijkswaterstaat (Inland Division) - *Chironomus*

Tests have been performed to compare the development of *Chironomus riparius* larvae in Poole Harbour and Dutch reference sediment. Two methods were used, one using egg packages and the other using second instar larvae. In both cases, development in Poole sediment was good and comparable to that in Dutch reference sediment.

A comparison of interpopulation sensitivity has been performed using WRc and RIZA cultures. 3.4 dichloroaniline and potassium dichromate were used as the reference toxicants. Control mortality for the WRc larvae was high and 96 hr LC_{50} values of 6.0 (5.3 - 6.7) mg l^{-1} DCA and 44.9 (37.6 - 53.7) mg l^{-1} $K_2Cr_2O_7$ were obtained compared to

10.4 (8.8 - 12.4) mg l^{-1} DCA and 63.4 (54.8 - 73.3) mg l^{-1} $K_2Cr_2O_7$ respectively for the RIZA culture. Because of the increased mortality and sensitivity of the WRc animals, RIZA animals have been chosen for the first stage of interlaboratory comparison.

A preliminary range-finding test has been performed with lindane-spiked sediment prepared at WRc. Control survival was 100% although there was high mortality in all test concentrations. A further range-finding test is currently under way using lower lindane concentrations.

The protocol for the ten-day test has been finalised and can be seen in Appendix C.

2.2.3 Other Groups

Work at other organisations has involved setting up culture facilities for the test organisms and preliminary work leading to modifications of circulated protocols.

2.3 Technical recommendations

1. Start definitive tests with freshly spiked sediment (maximum storage period four weeks).

2. Carry out *Chironomus* and *Sphaerium* testing in separate test vessels.

3. Use second rather than first instar *Chironomus* larvae.

4. Use a blocking technique when preparing samples for GST analysis.

5. Minimise loss in stock solutions by siliconising all glassware.

6. Minimise differences in concentration between replicates by using sediment from the same spiking vessel. This could be achieved by:

   - spiking a larger volume which actually results in measured sediment concentrations closer to nominal values;

   - decreasing size of test vessels.

7. Reduce rolling time of lindane from 24 to 6 hours as there are no significant differences in resulting concentrations.

8. Carry out further work on PCB spiking techniques. It is essential to determine the solubility of this compound both in water and acetone. Greater volumes of contaminant should be handled in future.

3. NRA OBJECTIVES

In general, good progress has been made to date on the objectives set out by the NRA in the Project Investment Appraisal. Points (a), (c), (d) and (e) have been completed.

(a) Two sparingly soluble substances of high environmental concern have been selected.

(c) The test sediment has been selected and characterised.

(d) Test methods have been selected and protocols finalised.

(e) Range finding tests have been performed with lindane and test concentrations identified for definitive tests.

The work described in Point (b) i.e. the selection of methods for spiking sediment with poorly soluble substances, has been completed for lindane but has been slow in progressing for PCB 118. This has been due to difficulties with the organisation of INIP, the Portuguese collaborators, who were to have carried out work with PCB. The Contract Manager of this project is to visit Portugal in November to resolve difficulties. Collaborative testing with this substance has been rescheduled due to the delay and is now programmed to start at the beginning of 1993. Testing with lindane is to begin in November. These delays should have no effect on the original timetable outlined at the first coordinating meeting which specified that the first stage of testing would be completed by March 1993.

REFERENCES

Chiou C.T., Peters, L.J. and Freed, U.H. (1979) A physical concept of soil-water equilibria for non-ionic organic compounds. McGraw-Hill Book Company, New York.

Davies, R., Roddie, B. and Jerman, E. (1991) The development of chemical and biological methods for assessing the toxicity of sediment-bound contaminants. WRc report NR 2729/1.

Johnson, I., Crane, M., Garood, A.C., Beverly, M. and Roddie, B.D. (1989) New biological methods for assessing the effects of pollution. WRc report PRS 2399-M.

Lyman, W.J., Reehl, W.F. and Rosenblatt, D.H. (1982) Handbook of chemical property estimation methods, Environmental behaviour of organic compounds. McGraw-Hill Book Company, New York.

Macek, K.J., Lindberg, M.A., Sauter, S., Boxton, K.S. and Costa, P.A. (1976) Toxicity of four pesticides to water fleas and fathead minnows. EPA-600/3-76-099.

Verscheuren, K. (1983) Handbook of environmental data and organic chemicals. 2nd Edition. Van Nostran Reinhold Co.

APPENDIX A - PROTOCOL FOR THE AMENDMENT OF SEDIMENTS WITH POORLY SOLUBLE SUBSTANCES - Rachel Fleming, WRc

Introduction

This document describes a simple method for spiking uncontaminated sediment with a known concentration of a "poorly" soluble contaminant. It involves rolling wet sediment with a concentration of spiking solution, to give a sediment concentration expressed in $\mu g\ g^{-1}$ dry weight. The spiking solution contains saltwater or freshwater and a known volume of contaminant in an acetone carrier. Rolling is carried out in 1-litre glass bottles, preferably amber in order to minimise photodegradation. In each bottle 300 ml of wet sediment is rolled with 600 ml of spiking solution at a known concentration. One hundred and fifty millilitres of spiked sediment is used in each test vessel and so each rolling vessel contains enough sediment for two replicates.

Sediment to be used for spiking must be characterised in terms of heavy metal and organic compound content, particle size distribution and organic carbon content. It must also have been sieved to a particle size of less than 500 µm to remove organic debris and predatory macroinvertebrates.

Glassware to be used for spiking must have been first treated with a solution of siliconising fluid. This includes all transfer vessels used to make up stock solutions.

Apparatus and materials

Rolling machine or orbital shaker capable of 25 revs/min
Analytical balance
Top pan balance
Cold store or facilities maintained at 4 °C
1 litre amber glass bottles
1 litre pyrex tall form beakers
Range of pipettes and syringes measuring down to 10 µl

Acetone - no analytical grade necessary
Compound in solid form
Seawater/freshwater - artificial or natural (the same as that to be used in the test).
1% Aquasil™ siliconising fluid

Methods

1. Choosing test concentrations

Very often toxicity data are expressed in $\mu g\ l^{-1}$ because tests have been carried out in the dissolved phase. When choosing relevant concentrations for sediment tests it is therefore easier to select values that would occur in the interstitial water and then calculate the

sediment concentration that would give rise to these. For this, the following calculation is used from Lyman *et al.* (1982):

$$\text{IW conc. } (\mu g\ ml^{-1}) = \frac{\mu g \text{ adsorbed/g organic C}}{K_{oc}}$$

$$\log K_{oc} = -0.557\ (\log S) + 4.277$$

S = solubility
C = carbon

If a range of sediment concentrations can be selected directly for a test then there is no need for this step.

2. Achieving test concentrations

(i) Dry weight measurement

As sediment concentration is expressed in µg g^{-1} dry weight, a dry weight measurement of the test sediment must be taken prior to spiking. This must be carried out for every new batch of sediment to be spiked.

- Remove sediment from cold store and allow to warm up to room temperature before mixing to give a homogeneous texture.
- Take three aliquots of sediment (5 ml is usually sufficient) and place in three preweighed glass vessels.
- Measure the weight of sediment and glassware. From these figures the wet weight of the sediment can be calculated.
- Place vessels in the oven at 100 °C for 8 hours (longer time is required for lower temperatures).
- On removal, place in a desiccator and when cool reweigh.
- Replace containers in oven for a further 30 minutes and reweigh. Continue this procedure until there is no further weight loss.
- Calculate the mean percentage water content from the wet and dry weight values for the three replicates.

(ii) Weight of sediment

Before the concentration of spiking solution can be calculated, the weight of dry sediment in 300 ml of wet sediment must be measured.

- Measure out 300 ml of homogeneous, wet sediment into a measuring cylinder or graduated beaker.

- Place an empty amber glass bottle on the desk top balance, zero and add the 300 ml wet sediment.

- Calculate the weight of dry sediment in this volume using the weight of wet sediment and the percentage water content derived above.

- Use this weight of wet and dry sediment for every treatment.

(iii) Calculation of stock solutions

The weight in grammes of dry sediment in every 300 ml of wet sediment is known from the above calculation. The spiking solution must contain the contaminant concentration in µg that will give rise to the chosen test concentrations in µg g^{-1}. For example, if there are 120 g of dry sediment present, to achieve a test concentration of 2 µg g^{-1} would require 240 µg in the spiking solution. The spiking solution has a constant volume of 600 ml.

- From the weight of dry sediment in 300 ml, calculate the concentration of contaminant required in each spiking vessel to achieve the required test concentration.

- Calculate the concentration of contaminant required in an intermediate stock solution. For example, lindane stock solutions have had a concentration of 1 µg ml^{-1}.

- Calculate the concentration of contaminant required in the first stock solution which is made up in acetone to increase solubility.

NB. When carrying out these calculations, bear in mind that the OECD limit for acetone in toxicity tests is 100 µl l^{-1}. This must not be exceeded.

(iv) Calculation of spiking solution volumes

The spiking solution contains different volumes of seawater/freshwater, intermediate contaminant stock solution and acetone but must be kept at a constant volume of 600 ml. The volumes of each will be dependent on the volume of stock solution required to get the nominal spiking concentration. The total volume of acetone must be kept constant in all treatments and must also be spiked into a set of acetone controls.

(v) Preparation of stocks

All glassware must be pretreated with a 1% solution of siliconising fluid (see Appendix).

- Acetone stock

  — Weigh the required amount of contaminant in solid form into a glass boat using an analytical balance.

  — Wash into a volumetric flask with acetone - (in the preparation of the acetone stock, smaller volumetric flasks can be used (<250 ml) so that amounts of test substance can be reduced).

  — Fill up to the mark with acetone and shake well.

  — Store in a spark proof refrigerator until required.

- Intermediate stock

  — Fill a volumetric flask half full with seawater or freshwater.

  — Inject in the required amount of acetone stock solution.

  — Fill to the mark and shake well.

  — Store in a spark proof refrigerator until required.

3. Preparation of rolling vessels

- Add 300 ml by weight to pretreated, labelled amber bottles. (Use the same weight as the wet weight determined above).
- Add the required amount of water to each bottle.
- Inject in required volumes of intermediate stock solution and acetone.
- Cap the bottles and shake well.
- Place on roller or orbital shaker at 25 revs/min for 6 hours.

4. Preparation of test vessels

- Remove vessels from roller.
- Shake well and immediately pour 300 ml of slurry into pretreated, labelled, 1-litre tall form beakers. Each amber bottle contains enough sediment for two replicates.
- Place all beakers in a cold store until sediment has settled (usually 4 to 5 days).

- Siphon off overlying water and take samples of sediment for chemical analysis.

- Use vessels in the test as soon as possible. When required, carefully add overlying water with care not to disturb the sediment. Leave for 24 hours to allow the sediment to settle and equilibrate before adding animals. Up to this point no aeration is required.

APPENDIX

Preparation of siliconising fluid

1. Fill a 1-litre volumetric flask half full with deionised / distilled water.

2. Pipette in 10 ml of full strength aqueous siliconising fluid and fill to the mark with deionised water.

3. Shake well and store in a refrigerator. A new solution should be prepared every week.

Preparation of glassware

1. Add an aliquot of siliconising fluid solution to each vessel.

2. Shake vessels until walls are evenly coated.

3. Dispose of excess fluid by pouring down the sink with plenty of water.

4. Leave glassware to dry at room temperature for 24 hours or in an oven at 60 °C for 1 hour before using.

APPENDIX B - PROTOCOL FOR SEDIMENT BIOASSAYS WITH THE AMPHIPOD *COROPHIUM VOLUTATOR* - Silvana Ciarelli and Peter van den Hurk, DGW

B1 INTRODUCTION

In water pollution evaluations, physical and chemical analyses alone are not sufficient to assess potential effects of toxic substances on aquatic biota. Biological responses are needed to provide further information about the effects of contaminants on aquatic organisms.

Therefore, in the last twenty years useful bioassay techniques have been developed in many countries in order to measure the biological responses of organisms to known and unknown concentrations of materials after exposure either in fresh or in saline waters.

Aquatic sediment bioassays are assays in which the responses of aquatic organisms are used to detect or measure the presence or/and effects of chemicals and wastes accumulated in the sediment. Where toxicity tests are concerned, by definition, with adverse effects of a pollutant, in bioassays the effect may be either beneficial or adverse (Brown 1973).

This protocol describes the procedure to carry out a solid-phase sediment bioassay using the benthic marine amphipod *Corophium volutator* (Pallas).

B1.1 Aims of sediment bioassays

Bioassay techniques are commonly applied in laboratories of universities, industries and research institutes for:

(a) routine monitoring of sediment quality;
(b) the evaluation of impacts from pollution incidents;
(c) assessing the bioavailability of contaminants in organisms;
(d) predicting ecological effects of new chemicals or other stressors and effluent discharges.

B1.2 Use of Amphipods in sediment bioassays

The use of Amphipods as standard sediment test organisms is quite widespread in North America. Amphipods are considered to be appropriate species for sediment bioassays for the following reasons:

(a) Wide geographical distribution and high abundance of organisms in the field allow a universal application of these species in different laboratories.

(b) *Corophium* is an important element in the food chain as a major prey of many fishes, birds and larger invertebrate species.

(c) The animal ingests sediment particles and is therefore, in direct contact with sediment-associated contaminants.

(d) The animal is easy to handle in the laboratory and is sensitive to contaminated sediments.

B2 MATERIALS AND METHODS

B2.1 Experimental design

The basic scheme of a test with *Corophium volutator* can be as follows:

Test duration - 10 days
Test temperature - 15 °C ± 1
Test organism - mature adults of *Corophium* (of 5-10 mm)
Test endpoint - mortality
Exposure method - 1.5 or 2 cm of clean or contaminated sediment in 1-litre tall-form borosilicate glass beakers, with 750 ml of overlying filtered seawater
Replication - each treatment has 3 replicate test vessels (prelim.)
Number of animals per replicate - 20 (prelim.)

B2.2 Experimental controls

The first negative control consists of clean sediment from the amphipod collection site, This has previously been frozen for 12 h in order to kill all the macroinvertebrates, potential predators of *Corophium*, and to ensure the sediment integrity. The second negative control consists of a clean reference sediment from Poole Harbour (England).

Both types of sediment are assumed to be uncontaminated, and characterised by a grain size that is environmentally realistic. They have been shown to produce less than 20% mortality in 5-10 mm *Corophium* adults during a 10-day period.

A solvent control for the chemical used in the procedure of spiking onto the sediment, should be carried out prior to testing in order to ensure the inocuity of the solvent for the test animals. The solvent concentration in the sediment should be the maximal value used in the spiking procedure. In bioassay experiments with lindane spiked sediment a solvent control with acetone has been performed. The solvent showed no effect in the concentration used.

A toxic reference control (positive): a dissolved-phase test consisting of five beakers containing different concentrations of a reference toxicant (Cd) has to be performed. The concentration of Cd that can be used in each beaker is: 0, 2.5, 5.0, 10.1 and 10.1 mg l^{-1}. The sensitivity of each batch of test organisms for Cd is thus established, and has the function to inform about the health condition of the animals. Reference toxicants also provide information about increased sensitivity that may occur as a result of disease or, on the contrary, insensitivity or stress tolerance developed during acclimatization or in the handling process.

In the first ring test the following concentrations of lindane-spiked sediment will be used:

Nominal concentrations ($\mu g\ g^{-1}$)

Control	
Control + acetate	
Concentration 1	0.25
Concentration 2	0.50
Concentration 3	1.00
Concentration 4	2.00

B2.3 Collection of animals

The following procedure is used for collecting organisms from the field:

(a) identify a clean intertidal site showing a large population of *Corophium volutator*;
(b) before low tide collect seawater in a 25-l plastic bin;
(c) scrape off the top 2-4 cm of the sediment with a scoop and collect it in a 10-l bucket;
(d) wet sieve the sediment containing the animals through a stainless steel sieve (1 mm mesh) in seawater;
(e) the animals which have been sieved are transferred to the 25-l bin;
(f) after collection, transport the bin with seawater and animals and the bin with sediment back to the laboratory (part of the collected sediment is used for the negative control);
(g) transfer the animals to a tank with a flow-through system which allows the shells and algae to separate from the animals. These can then be collected in a 500-µm sieve;
(h) after that, transfer the collected sediment in a bin; fill it with seawater, add the animals and place the bin in a waterbath where the water temperature is the same as the test, in order to allow the acclimatization process of the animals;
(i) the animals should be acclimatized for 3 days to the same salinity (32 prom.) and temperature (15 °C) conditions as to be used in the test;
(j) the air supply in the bin can be realised with a couple of Pasteur pipettes.

B2.4 Procedure for the set-up of a test

(a) Fill 1-l borosiliocate glass beakers with 1.5 or 2 cm sediment which has to be tested (in the case of hydrophobic toxicants a water soluble siliconizing fluid for glassware should be applied 24 h before filling in order to minimise adsorption to glass surfaces).
(b) Fill the beakers carefully with 750 ml overlying seawater without resuspending the sediment, transfer the beakers to the waterbath having a constant temperature of 15 °C . The air supply in each glass beaker is realised with a Pasteur pipetter.
(c) Next day, sieve the animals in a plastic try and transfer them to a vessel with seawater in which they can swim.

(d) Catch the animals one at a time with a rigid piece of mesh and transfer them to small plastic beakers. The number of plastic beakers is the same as the number of borosilicate beakers. The animals of each plastic beaker are then transferred to the borosilicate beakers. In this way, a random (homogeneous) population in each beaker will be obtained. Only healthy and swimming organisms of similar size should be used.
(e) Before filling the beakers with animals record T, pH, DO and salinity.
(f) Put the borosilicate glass beakers into the waterbath and leave them for 10 days.

B2.5 Maintenance of the experiment and response criteria

(a) The air supply of the test beakers is checked every day. If any of the pipetter tips are blocked they are replaced by new ones.
(b) The activity of *Corophium* in each vessel is recorded at the start of the test (swimming or crawling). At the beginning of the test, the amphipods swim first around and then quickly swim to the bottom and burrow completely into the sediment. In the absence of stress, they remain buried during the 10-day exposure period.

Three response criteria are possible:

1. Mortality after 10 days and, thus, calculation of the LC50 and determination of dose-response relations at the end of the test.
2. Ability of survivors to bury in clean sediment.
3. Recording of emergence of amphipods during exposure.

B2.6 Abiotic parameters

Oxygen content, salinity, pH and temperature at the start and at the end of the experiment are recorded.

Oxygen: should be maintained at the saturation point through the supply of a Pasteur pipette. Turbulence on the sediment surface should be avoided.

pH: a range between 7.8 and 8.5 can be considered as the optimum one.

Salinity: 31 ± 2 prom. is the standard range although *Corophium* seems to be very tolerant to salinity variations.

Sediment type: from pervious experiments a range of sediment from fine silt to very coarse beach sand (5.5 µm-2 mm) seems to be acceptable for the test.

(Light does not seem to be very important for amphipods since they live mostly buried in the sediment. Nevertheless, a normal day/night regime is advised.

B2.7 Laboratory requirements

Inert materials such as stainless steel, teflon and glass should be used for the test in order to minimize sediment contamination. The borosilicate glass beakers are cleaned with a 5% solution of HCL in order to remove metals, sulfides and carbonates. They they are rinsed first with fresh water and then with deionised water. Just before setting up the test the beakers are rinsed with filtered seawater. A stainless steel spoon or spatula can be used for scooping the sediment.

B3 CALCULATION METHODS

The LC50 or EC50 and their 95% confidence interval should be calculated on the basis of the (nominal) concentration which has been measured and on the basis of the mortality data. Several methods can be used for the LC50 or EC50 calculations but in most cases the trimmed Spearman-Karber and the Litchfield-Wilcoxon methods are applied.

In the case of replicates of field specimens the means of survivals of the different locations and of the controls have to be statistically compared through an analysis of variance t-test (ANOVA) followed by a multiple comparison test (Dunnett's procedure). The former test will be used in order to decide whether the observed differences between the concentrations of all the specimens are statistically significant.

When the ANOVA is not significant ($P>0.05$) the conclusion is that the observed effects of the toxicants are not large enough to consider them statistically significant. In case of a significant ANOVA all the effect concentrations can be compared with the controls by using one of the following methods: the Dunnett's procedure, the Williams' method, or the Fisher method.

B3.1 Confidential limits and acceptability

A 10-day exposure sediment toxicity test is not acceptable when more than 20% mortality in the controls has been obtained. In the following cases a toxicity test cannot be considered statistically valid:

(a) the vessels have not the same experimental conditions;
(b) the population of the test organisms is not homogeneous and randomly established;
(c) the negative controls, the positive controls and the reference toxicant controls are missing;
(d) the test organisms are not in a healthy condition and/or have not been acclimatised for at least 48 hours in advance;
(e) the oxygen saturation value is below the 60% and the other abiotic parameters (salinity, temperature, pH and the sediment type are not in the acceptable range mentioned before).

REFERENCES

Brown, V.M. (1973) In: *Bioassay Techniques and Environmental Chemistry*, edited by G E Glass, Ann Arbor Science Publishers, Ann Arbor. Chap. 2, 73-95.

Cairns, J. Jr. and Pratt, J.R. (1989) The scientific basis of bioassays. *Hydrobiologia*, 188/189: 5-20.

Chapman, P.M. (1988b) Marine sediment toxicity tests. In: *Chemical and Biological Characterization of Sludges, Sediments, Dredge Spoils and Drilling Muds*, edited by J.J. Lichtenberg, F.A. Winter, C.I. Weber and L. Fadkin. American Society for Testing and Materials, ASTM STP 976, 391-402.

Ditsworth, G.R., Schults, D.W. and Jones, J.K.P. (1990) Preparation of benthic substrates for sediment toxicity testing. *Environ. Toxicol. Chem.*, 9, 1523-1529.

Hansen, D.J. (1984) Utility of toxicity tests to measure effects of substances on marine organisms. In: *Concepts in Mar. Poll. Measurements*, edited by H.H. White, Maryland Sea Grant, Publisher.

Hurk, van den P. and Smit, T. (1991) Bepaling van de toxiciteit van sedimenten met behulp van mariene en estuarience gravende amphipoden. Report for Bureau Waardenburg b.v. advisers for the environment and ecology. In charge of Tidal Waters division. Project 87.03.

Samoiloff, M.R. (1989) Toxicity testing of sediments: Problems, trends and solutions. In: *Aquatic Toxicology and Water Quality Management*, edited by J.O. Nriagu and J.S.S. Lakshminarayana. Wiley, New York (Advances in Environmental Science and Technology, Vol. 22).

Stephan, C.E. (1977) Methods for calculating LC50. *Aquatic Tox. and Hazard Evaluation*, ASTM STP 634, edited by F.L. Mayer and J.L. Hamelink. American Society for Testing and Materials, 65-84.

Swartz, R.C. (1989) Marine sediment toxicity tests. Contaminated Marine Sediments - Assessment and Remediation. Marine Board/Nat. Res. Council. Nat. Acad. Press, Washington DC, 115-129.

Standard Test method: marine and estuarine sediment toxicity assessment using *Corophium volutator*. Protocol of WRc, Medmenham.

ASTM)American Society for Testing and Materials) (1990) Standard guide for conducting 10-day static sediment toxicity tests with marine and estuarine amphipods. In: *Jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate; subcommittee E47.03 on Sediment Toxicology.*

APHA (American Public Health Association((1981) Bioassay methods for aquatic organisms. Part 800 of *Standard Methods for the Examination of Water and Wastewater*. - - APHA Publishers. Library of Congress Catalogue Number 55-1979.

APPENDIX C - PROTOCOL FOR SEDIMENT BIOASSAYS WITH THE FRESHWATER DIPTERAN *CHIRONOMUS RIPARIUS*

EXPOSURE OF *CHIRONOMUS RIPARIUS* TO LINDANE-SPIKED SEDIMENT OVER A TEN DAY TEST PERIOD - Liesbeth Grootelar, RIZA

OBJECTIVE

To investigate the effects of lindane-spiked sediment on *Chironomus riparius* by observing survival and the larval development (by difference in headcapsule width).

TEST ANIMALS

Species: *Chironomus riparius*
Source: laboratory culture, maintained as described by Grootelaar *et al.* (1991)

The four different larval instars are separated on the basis of headcapsule width (Appendix 1) using a lightmicroscope. Late second instar larvae (length 2.6-3.4 mm; 10 days old) are used as test organisms. The sensitivity of the cultured midge larvae must be controlled by an acute test in water with potassium dichromate ($K_2Cr_2O_7$) or 3,4-dichloraniline as reference compound (Appendix 2).

TEST SEDIMENT

Character:	Clean, desalinated
Source:	Newton Bay, Poole Harbour, UK
Collection date:	October 1992
Storage conditions:	4 ± 1 °C, dark

TEST CONCENTRATIONS

For the first ring test, four concentrations and both clean and acetone controls will be tested. All treatments will be triplicated with 25 larvae in each test vessel.

Nominal concentrations (lindane $\mu g\ g^{-1}$)

Control	
Control + acetone	
Concentration 1	0.012 $\mu g\ l^{-1}$
Concentration 2	0.060 $\mu g\ l^{-1}$
Concentration 3	0.120 $\mu g\ l^{-1}$
Concentration 4	0.600 $\mu g\ l^{-1}$

EQUIPMENT

250 ml tall-form pyrex beakers
Small glass containers containing 70% alcohol
Aeration equipment
Aeration bottles containing sediment

Siliconising fluid - Aquasil^{tm}
Sieve 250 µm
Temperature controlled room or water bath 20 ± 1 °C
Glass tube ø 2 mm
Light microscope

METHOD

The test method must be carried out at a temperature of 20 ± 1 °C with a photoperiod of 16 hours light and 8 hours dark and a light intensity of 2000-2500 lux. The survival in the control must be at least 8-% after 10 days. During the test period aeration of the overlying water is continuous and the animals are not fed.

1. Siliconise all glassware to minimise adsorption of a lindane onto test vessels. This is carried out by preparing a 1% solution of siliconising fluid (e.g. 10 ml in one litre deionised water) and coating the walls of test vessels. Any excess fluid is discarded and glassware is left to dry for 24 hours at room temperature or in an oven at 60 °C for 20 minutes.

2. Remove sediment bottles from cold store and decant off any overlying water. Mix the sediment within the bottle and place 40 ml sediment in each of the triplicate test vessels.

3. Place test vessels on the bench in random positions. Add 160 ml Dutch Standard Water (DSW, Appendix 3) taking care to minimise sediment disruption and leave for 3 days to allow any suspended sediment to resettle and allow some degree of partitioning to take place between sediment and water phases.

4. Commence aeration for four hours before recording dissolved oxygen, pH, temperature and $NO_3/NO_2/NH_4$ levels in each test vessel. A proposal is given for the boundary conditions that overlying water must satisfy in order to be considered suitable as a test medium for *C. riparius* (Appendix 4).

5. Place 25 late second instar larvae in each test vessel by a glass tube (ø 2 mm).

6. Make observations every day to whether the animals have left the sediment or died.

7. After four days and on Day 10 record DO, pH, temperature and NO_3/NO_2 - and NH_4 - levels.

8. Sieve the sediment from each test vessel using a 250 µm sieve and put the surviving larvae in small glass containers continuing 70% alcohol.

9. Observe the larval instar development (difference in headcapsule width) using a light microscope.

Take eventually 5 ml sediment to analyse lindane and store the samples at -70 °C.

STATISTICS

The LC50, EC50 and NOEL will be calculated if appropriate

APPENDIX 1

The four different instars of *C. riparius* separated on basis of headcapsule width using a lightmicroscope,

Instar	Headcapsule width µm	Colour
1	73 - 107	white
2	133 - 201	pink
3	257 - 347	red
4	429 - 583	red

APPENDIX 2

Acute toxicity test to control the sensitivity of the cultured midge Chironomus riparius

METHOD

The test must be carried out at a temperature of 20 ± 1 °C with a photoperiod of 16 hours light and 8 hours dark and a light intensity of 2000-2500 lux. The survival in the control must be at least 80% after 96 hours.

1. Make a concentration range of potassiumdichromate (K_2 Cr_2O_7) or 3,4-dichloro-aniline in 50 ml DSW in 100 ml glass test vessels.
   Conc. range (K_2 Cr_2O_7)
   Control - 10 - 18 - 32 - 56 - 100 mg l^{-1}

2. Use duplicate test vessels. Add 25 second instar larvae in each test vessel.

3. Feed the larvae at t=0 and t=48 h with 200 µl of a 2% (2 g/100 ml) foodsuspension (Trouvit).

4. Observe every day the behaviour and survival of the midge larvae.

5. Record at t=0 and t=96 h Dissolved Oxygen, pH and NO_3 /NO_2 - and NH_4 - levels (testkit) in each test vessel. Measure these conditions also if high starvation is observed in a concentration.

6. Do not aerate during the test.

   Calculate the LC50, EC50 and NOEL if appropriate.

Compound	Test duration (h)	LC/EC50 (mg l^{-1})
$K_2Cr_2O_7$	96	$20 < x < 75$
3,4 DCA	96	$4 < x < 12$

APPENDIX 3 - PREPARATION AND COMPOSITION OF DUTCH STANDARD WATER (DSW)

Stocksolutions:

Stocksolution	I:	$CaCl_2H_2O$	100 g per litre
Stocksolution	II:	$MgSO_4.7H_2O$	90 g per litre
Stocksolution	III:	$NaHCO_3$	50 g per litre
		$KHCO_3$	10 g per litre

Sterilize Stocksolutions I and II for 20 minutes in an autoclave at 121 °C.
Sterilize Stocksolution III by filtration over a membrane filter of 0.2 µm.

Preparation:
Add 2 ml of each solution to one litre demineralized water for preparation of the DSW. This demineralized water is prepared by filtration of Cu-free tapwater by a column (Elgastat; C224, Laméris, Breulelen).

Aerate DSW-water at least one hour properly before use.
The equilibrium pG after aeration is about 8.2. The hardness is 210 mg l^{-1} as $CaCO_3$.
The water can be prepared in a large amount. You can keep it for one month provided that the water is aerated properly.

APPENDIX 4

Boundary conditions for toxicity testing with *Chironomus riparius*.

A proposal is given for the boundary conditions, with concentrations of no acute effect, that overlying water must satisfy in order to be considered suitable as a test medium for *C. riparius*.

pH	5-9
O_2	>3 mg l^{-1}
NO_2	1 mg l^{-1}
NH_4	32 mg l^{-1}
Cl	5.5 g l^{-1}
Conductivity	1200 mS/m

APPENDIX D - PROTOCOL FOR SEDIMENT BIOASSAYS WITH THE FRESHWATER MUSSEL *SPHAERIUM CORNEUM*

(i) EXPOSURE OF *SPHAERIUM CORNEUM* TO LINDANE-SPIKED SEDIMENT OVER A TEN DAY TEST PERIOD - Rachel Fleming, WRc

OBJECTIVE

To investigate the sublethal effects of lindane-spiked sediment on *Sphaerium corneum* by measuring the induction of Glutathione-s-transferase (GST).

TEST ANIMALS

Species	: *Sphaerium corneum*
Source	: River Derwent, Stamford Bridge, UK.
Collection date	: 21.11.92
Storage conditions	: Aerated groundwater

Approximately 130 animals will be supplied to each collaborator.

TEST SEDIMENT

Character	: Clean, desalinated
Source	: Newton Bay, Poole Harbour, UK.
Collection date	: October 92.
Storage conditions	: 4 °C, dark

Sediment will be spiked with lindane according to the Sediment Spiking Protocol

TEST CONCENTRATIONS

Test concentrations of lindane spiked sediment will be used that have been shown to cause no mortality in *Sphaerium* over a ten day test period but have caused 100% mortality in *Chironomus riparius* over this time. Three concentrations and both clean and acetone controls will be tested. All treatments will be duplicated with ten animals in each vessel. For the first ring test the following concentrations will be tested.

Nominal concentrations (lindane $\mu g\ g^{-1}$):
Control
Control + acetone
Concentration 1 0.12
Concentration 2 0.60
Concentration 3 1.20

Samples will be taken for analysis before distribution to collaborators and analysed by capillary gas chromatography seven days before the beginning of the test. Bottles will be supplied for each treatment containing enough sediment for both replicates.

EQUIPMENT

One-litre tall-form pyrex beakers
Crystallising dishes or other small glass containers
Aeration equipment - e.g., plenum chambers
Amber bottles containing sediment
Siliconising fluid - Aquasil™
Sieve > 500 µm
Temperature controlled room or water bath - 15 ± 1 °C.

METHOD

1. Siliconise all glassware to minimise adsorption of lindane onto test vessels. This is carried out by preparing a 1% solution of siliconising fluid (e.g. 10 ml in 1 litre deionised water) and coating the walls of test vessels. Any excess fluid is discarded and glassware is left to dry for 24 hours at room temperature or in an oven at 60 °C for 20 minutes.

2. Remove sediment bottles from cold store and decant off any overlying water. (At this point dry weight measurements can be carried out if required). Mix the sediment within the bottle and place equal amounts in the duplicate test vessels.

3. Place test vessels on the bench in random positions. Add overlying water, taking care to minimise sediment disruption, and leave for 24 hours to allow any suspended sediment to resettle and allow some degree of partitioning to take place between sediment and water phases.

4. Record dissolved oxygen, pH and temperature in each vessel.

5. Place ten *Sphaerium* in each vessel by hand and leave for two hours before commencing aeration.

6. Make observations every day as to whether the animals have left the sediment or are gaping. Remove any "gapers" and push "escapees" back down into the vessel.

7. On Day 10 record DO, pH and temperature. Remove overlying water and place a small amount into labelled crystallising dishes for holding *Sphaerium* before the GST assay.

8. Sieve the sediment from each test vessel and place surviving *Sphaerium* in the crystallising dishes.

9. Make an estimate of survival and carry out GST analysis as in Protocol 2. Prepare *Sphaerium* individually removing two animals from each treatment for every run.

STATISTICS

Mortality will be analysed using the appropriate Generalised Linear Model. The EC_{50} and NOEL will be calculated if appropriate. Statistics for GST are outlined in Protocol 2.

(ii) DETERMINATION OF THE GLUTATHIONE-S-TRANSFERASE ACTIVITY (GST) OF *SPHAERIUM CORNEUM* - Ian Johnson and Rachel Fleming, WRc

1. INTRODUCTION

This Standard Operating Procedure describes the method to be used for preparing and analysing enzyme extracts from the freshwater mussel, *Sphaerium corneum*, for glutathione-s-transferase (GST) activity. Glutathione-s-transferase are a ubiquitous family of enzymes involved in the detoxification of accumulated organic compounds. Elevations in GST activity can indicate exposure to organic chemicals. The technique can also be linked with sub-lethal measurements of toxic effect.

2. HAZARDS AND PRECAUTIONS

The test substances contained in stock solutions or present in samples of test media may be hazardous. Always check the relevant safety information (COSHH hazard assessment) beforehand.

A COSHH assessment should be prepared for all test substances and guidance given regarding the protective clothing required.

3. PRINCIPLE

1. Whole tissue mass is removed from individual *Sphaerium* and sonicated or homogenised at 4 °C in phosphate buffer. The homogenate is centrifuged in a refrigerated centrifuge to obtain an enzyme extract.

2. The GST activity in the enzyme extract is measured spectrophotometrically at 340 nm based on the catalysis of the conjugation of glutathione and 1,chloro-2,4-dinitrobenzene (CDNB) substrates in the assay media.

4. MATERIALS

4.1 Preparation of enzyme extracts

(a) A top loading balance accurate to 0.01 mg, such as the Sartorius 1712 MP 8.

(b) Ice granules in a polystyrene container.

(c) Polythene centrifuge tubes capable of holding 5 to 10 ml of homogenate and being spun at 40 000 g.

(d) Phosphate buffer: A 0.1 M solution of pH 6.5 made up from 0.1 M solutions of 'Analar' Potassium dihydrogen orthophosphate and 'Analar' Di-Potassium hydrogen orthophosphate in distilled water.

(e) Micropipetters capable of accurately dispensing 5 and 10 ml volumes.

(f) Non-toxic disposable tips for each micropipetter

(g) Homogeniser with 0.8 cm blade capable of operating at 10 000 rpm such as the Ultra-Turrex T-25 (Janke and Kunkel) or an appropriate sonicator.

(h) Centrifuge capable of centrifuging at a minimum of 12 000 g for 10 mins at 5 °C, such as the Kontron Centrikon T-1065 or Sorvall AC-2B.

(i) Glass pasteur pipette.

(j) Polythene flip cap tubes of 5 to 10 ml capacity to store the supernatants resulting from the centrifugation of the homogenates.

(k) Refrigerator set at 4 ± 2 °C.

(l) Non-self defrosting freezer at - 20 ± 2 °C.

4.2 Measurement of GST activity

(a) Spectrophotometer capable of measuring at 340 nm in the ultraviolet region of the spectrum. Single or dual beam machines can be used.

(b) One centimetre quartz or plastic micro-cuvettes.

(c) Glutathione solution: A 20 mM solution of 'Analar' reduced glutathione in distilled water. The solution should be prepared in a volumetric flask and stored at 4 ± 2 °C.

(d) 1-chloro-2,4-dinitrobenzene solution: A 50 mM solution of 'Analar' 1-chloro-2,4-dinitrobenzene in 95 % ethanol. The solution should be prepared in a volumetric flask and stored at 42 °C.

(e) Micropipetters capable of accurately dispensing 50, 100, 200 and 750 µl volumes.

(f) Non-toxic disposable tips for each micropipetter.

5. EXPERIMENTAL PROCEDURES

The procedures for preparing enzyme extracts and analysing the extracts for glutathione-s-transferase (GST) activity are shown in Figure 1.

5.1 Preparation of enzyme extracts

(i) Remove the whole body tissue from individual *Sphaerium* in a container of ice granules.

(ii) Remove excess water by blotting the tissue dry with absorbent paper.

(iii) Weigh the whole tissue or specific tissue on a top pan balance to the nearest 0.01 mg. For each tissue sample a centrifuge tube should be pre-weighed, the tissue is added to the tube which is re-weighed and the weight of the tissue is obtained from the difference. The data should be recorded on data sheet 1. Body tissue weighing less than 15 mg should not be used in the assay.

(iv) Add 5 ml of ice-cold 0.1 M Phosphate (kpi) buffer to each centrifuge tube containing tissue using an appropriate micropipetter.

(v) Prepare a homogenate of the tissue either by homogenising the tissue in the buffer at 10 000 rpm with an appropriate homogeniser or by sonicating the tissue using an appropriate sonicator. This is continued until all the tissue has disintegrated. During the homogenisation or sonication of tissue samples the tubes should be held in a bucket of ice granules to restrict the action of proteolytic enzymes released during tissue disruption.

(vi) Centrifuge the homogenates at a minimum of 12 000 g at 5 °C in an appropriate centrifuge for a period of 10 - 30 minutes. All tubes have to be of the same weight to ensure the rotor is balanced and the centrifuge operates efficiently and safely during this step.

(vii) After centrifugation the supernatant from each tube is transferred to a polythene flip-cap tube using a clean pasteur pipette. Care should be taken to avoid contaminating the supernatant with material from the pellet. The supernatants should then be analysed for GST activity immediately or stored in freezer at -20 °C for subsequent analysis.

5.2 Analysis of the enzyme extracts for GST activity

(i) An appropriate single or dual beam spectrophotometer capable of measuring absorbance at 340 nm in the ultraviolet region of the spectrum is switched on 30 minutes before the samples are to measured.

(ii) Samples which have been stored in a freezer should be given sufficient time to defrost before measurements are made.

(iii) For spectrophotometers with a single beam, prepare a blank in a 1-cm quartz micro-cuvette of cm^3 capacity. The blank contains 0.75 ml of phosphate buffer (pH 6.5), 0.1 ml of 20 mM glutathione solution and 0.05 ml of 50 mM CDNB solution. The reagents are thoroughly mixed by shaking the cuvette with a piece of parafilm over the opening. The cuvette is placed in the spectrophotometer and the absorbance is calibrated to zero. The cuvette is then removed and an aliquot of enzyme extract is added and thoroughly mixed. The cuvette is replaced in the spectrophotometer and the rate of change of absorbance is measured continuously over a 3-minute period. Two replicates are carried out for each enzyme extract. All measurements are made at a constant 25 °C.

(iv) For dual beam spectrophotometers, prepare blank and sample cuvettes containing phosphate buffer, glutathione and CDNB in the same way as described above. The absorbance of the sample cuvette is zeroed against the blank. An aliquot of enzyme extract is then added to the sample cuvette, mixed and the rate of absorbance change measured continuously over a 3-minute period at 25 °C.

(v) The volume of enzyme extract added depends on the concentration and should be adjusted so that there is a linear change in absorbance over the three minute recording period.

(vi) Because the absorbance of the blank is not constant, the rate of change must be measured in the same way as the enzyme extracts. Prepare a blank cuvette and measure the rate of change continuously over a 3-minute period. This must be carried out at the beginning of every run and again after every five samples.

6. DATA HANDLING

6.1 Recording raw data

It is vital that tissue weights and volumes of phosphate buffer used to prepare enzyme extracts are recorded on data sheet 1 to enable enzyme activities to be calculated (see Section 6.2). Chart recordings or computer printouts of absorbance changes for an enzyme extract can be generated, but in all cases values at 1, 2 and 3 minutes should be recorded on data sheet 2.

6.2 Treatment of results

6.2.1 Calculating the GST activity of enzyme extracts

For a tissue of given wet weight or protein concentration the mean absorbance change per minute in the enzyme extract is initially calculated from the two replicates. The enzyme activity (in mMoles substrate consumed/minute/litre of assay volume) is then calculated using the mM absorbance coefficient for CDNB (Habig *et al.* 1974):

Enzyme activity = Mean absorbance (340 nm)/minute
mM absorbance coefficient for CDNB

$$= \text{Mean } \frac{Abs_{340}}{9.6} \qquad \text{mMoles min } l^{-1}$$

$$= \text{Mean } \frac{Abs_{340}}{9.6} \times 1000 \qquad \text{mMoles min } l^{-1}$$

$$= \text{Mean } \frac{Abs_{340}}{9.6} \times \frac{1000}{1000} \qquad \text{mMoles min } l^{-1}$$

$$= \text{Mean } \frac{Abs_{340}}{9.6 \times 60} \qquad \text{mMoles min } l^{-1}$$

$$= \text{Mean } \frac{Abs_{340}}{9.6 \times 60} \qquad \text{uKatals ml}^{-1}$$

since 1 ukatal = 1 umole of substrate used per second

The enzyme activity has to be corrected for the volume of extract used (v_{ext}) and the total assay volume (v_{tot})

$$\text{Specific enzyme activity} = \text{Mean } \frac{Abs_{340}}{9.6 \times 60} \times \frac{v_{tot}}{v_{ext}}$$

Enzyme activities and tissue weights or protein concentrations are logarithmically transformed and regression equations of the form:

$$\log GST = a + b \log Wt$$

From this equation enzyme activities are corrected to a given weight using the equation:

$$\log GST_c = \log GST - (b \log Wt - b \log Wt_s)$$

where GST_c = weight corrected GST activity
GST = measured GST activity
Wt = measured tissue wet weight
Wt_s = standard wet weight

Protein concentrations can be used in place of wet weights in the equation.