**Offshore Industry Series**

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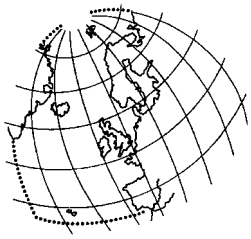
# OSPAR

# Protocols on Methods for the Testing of Chemicals Used in the Offshore Oil Industry[1]



OSPAR Commission

2006

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[1] *Also an OSPAR agreement (reference number: 2005-11, a revised version of agreement 1995-07). Part B of the agreement was amended by OSPAR 2006 (see OSPAR summary record 06/23/1, §9.7)*

The Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention") was opened for signature at the Ministerial Meeting of the former Oslo and Paris Commissions in Paris on 22 September 1992. The Convention entered into force on 25 March 1998. It has been ratified by Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Sweden, Switzerland and the United Kingdom and approved by the European Community and Spain.

*La Convention pour la protection du milieu marin de l'Atlantique du Nord-Est, dite Convention OSPAR, a été ouverte à la signature à la réunion ministérielle des anciennes Commissions d'Oslo et de Paris, à Paris le 22 septembre 1992. La Convention est entrée en vigueur le 25 mars 1998. La Convention a été ratifiée par l'Allemagne, la Belgique, le Danemark, la Finlande, la France, l'Irlande, l'Islande, le Luxembourg, la Norvège, les Pays-Bas, le Portugal, le Royaume-Uni de Grande Bretagne et d'Irlande du Nord, la Suède et la Suisse et approuvée par la Communauté européenne et l'Espagne.*



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# contents

# Preface

In 1995 OSPAR[2] adopted a Harmonised Offshore Chemical Notification Format (HOCNF) together with guidelines for completing the format. This was a first step towards a harmonised mandatory control system for the use and the reduction of the discharge of offshore substances/preparations

To implement a full system, OSPAR adopted in 1996 PARCOM Decision 96/3 on a Harmonised Mandatory Control System for the Use and Reduction of the Discharge of Offshore Chemicals which contained the HOCNF in appendix.

This Decision 96/3 was revised and superseded by OSPAR Decision 2000/2, OSPAR Recommendation 2000/4 and OSPAR Recommendation 2000/5[3]OSPAR Decision 2000/2 and OSPAR Recommendation 2000/5 were amended in 2005 by OSPAR Decision 2005/1 and OSPAR Recommendation 2005/3 respectively.

In order to provide harmonised data sets from the testing of offshore substances/preparations, OSPAR organised four ring–tests, which resulted in the agreement of four protocols, namely:

Growth Inhibition Test Using the Marine Alga *Skeletonema costatum*

Acute Toxicity Test Using the Marine Copepod *Acartia tonsa*

A Sediment Bioassay Using an Amphipod *Corophium sp*

Protocol for a Fish Acute Toxicity Test

The first two of these protocols are ISO standards. The documentation for them should therefore be obtained from ISO. This agreement sets out the other two protocols.

With regard to the sediment reworking tests, OSPAR agreed that, all tests already performed on sediment reworker species and offshore chemicals and oil-based muds should be accepted as long as they were carried out in accordance with the PARCOM Guidelines of 1991 regarding harmonisation of procedures of approval, evaluation and testing of offshore chemicals and drilling muds.

OSPAR also agreed that the turbot juvenile (96 hour) is the recommended species in the fish acute-toxicity test for assessing the environmental hazards of offshore chemicals, but that the juvenile sheepshead minnows can be used when the turbot juvenile is unavailable for practical reasons.

In 2005 OSPAR agreed to amend the heading and paragraphs 1, 2, 11, 12, 13, 14, 15, 18, 19 of Annex 1 of Part B of these Protocols, in order to reduce the number of fish used for toxicity testing of offshore chemicals, while maintaining a mandatory fish test.

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2 The Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention) was opened for signature at the Ministerial Meeting of the Oslo and Paris Commissions in Paris on 22 September 1992. On its entry into force on 25 March 1998, the OSPAR Convention replaced the Oslo Convention and the Paris Convention. However, all Decisions, Recommendations and other agreements adopted under those Conventions continue to be applicable, unless they are terminated by new measures.

3 The full titles of these instruments are the OSPAR Decision 2000/2 on a Harmonised Mandatory Control System for the Use and Reduction of the Discharge of Offshore Chemicals, the OSPAR Recommendation 2000/4 on a Harmonised Pre-Screening Scheme for Offshore Chemicals and the OSPAR Recommendation 2000/5 on a Harmonised Offshore Chemical Notification Format.

# Part A: A Sediment Bioassay using an Amphipod Corophium sp

## 1. Introduction

The results of the sediment bioassay ring-test organised by PARCOM in 1991 were inconclusive. The follow-up workshop in Wageningen concluded that a further ring-test would be needed to evaluate sediment reworker tests. One of the recommendations, based on results produced by van den Hurk was that an infaunal amphipod, preferably *Corophium volutator*, should be included in any future ring test. Burrowing amphipods have been used for testing sediments for some time in North America and standard guidelines for such tests have been produced by ASTM (1990). Several European laboratories have evaluated the guidelines using European species such as *Corophium* sp. It has been specifically designed and compiled for the testing of chemical products that are liable to incorporation into seabed sediments. The method was ring tested in 1993 alongside three other species, *Abra alba*, *Arenicola marina* and *Echinocardium cordatum*, and was chosen as the most suitable species.

In the test adult *Corophium* are exposed to spiked sediments for 10 days. During this period burrowing behaviour may be assessed by counting the number of amphipods on the sediment surface or actively swimming. At the end of the experiment the amphipods are sieved from the sediment and the number of surviving animals recorded. Survival is then analysed statistically, comparing the controls with the treatments.

The objective is to determine the initial concentration which, in 10 days, kills 50% of the exposed animals ($10_d$ $LC_{50}$).

At present *Corophium* is the most worked with genus, but it should be borne in mind that the procedure described below can be used with any infaunal amphipod and indeed in the future other species may prove to be of equal value.

An important conclusion of the ring test was the need for guidance to provide a basis for the choice of chemicals for which a sediment test will be required and the spiking method to be employed: these are addressed in appendices 2 and 3.

## 2. Materials

The following materials and equipment are required to set up and run the toxicity test:

a. test protocol;

b. reference sediment collected from an area known to be free from significant contamination;

c. reference seawater: natural seawater is preferred, but artificial seawater is acceptable. It must be free from significant contamination;

d. adult *Corophium*, greater than 5 mm in length, collected from an area known to be relatively free from contamination. Either *Corophium volutator* or *Corophium arenarium* is acceptable, but an attempt should be made to identify which species is being used, and at least to ensure that a mixture of species is not used;

e. 500 μm (approximately) sieve;

f. an orbital shaker or roller;

g. shaker bottles of the appropriate type and capacity, (non-leaching plastic are generally the most practical);

h. test vessels; 1l tall-form glass beakers are most commonly used, but any vessel of a suitable material is acceptable which permits a minimum sediment depth of 15 mm;

i. aeration system. Individual air stones or plastic pipette bubblers can be used for each test container. However, these easily become blocked and require daily checking and maintenance. For routine testing an example of a more practical option, has been the use of the aeration system described in Annex 1;

j. automatic 5 ml dispensing pipette or 10 ml pipettes;

k. scoops/serving spoons for handling sediments;

l. pH meter (to 0,1 pH units);

m. dissolved oxygen meter (mg/l or as %);

n. thermometer (to 0,1°C);

o. salinity meter (directly/conductivity or otherwise);

p. aquaria for holding *Corophium* (approximate size 10 to 30 l);

q. a balance that will weigh to two decimal places (i.e. 0,01 g), or to a precision greater than or equal to 1% of the quantity being weighed;

r. clean 100 ml beakers (glass or of a suitable inert material). One beaker is required for each test vessel (cf. § 2.8).

# 3. Method

## 3.1 Field collection and storage of animals

Typical densities that occur on the shore are 10 000-50 000 $m^2$, but population densities can fall dramatically in winter to below 1 000 $m^2$. A single test may require between 150-600 animals depending on the design and degree of replication.

**Procedure A**

Collection of animals: *Corophium sp* should be collected from a clean intertidal shore (mud or muddy sand). The animals should be collected by removing the top 5 cm of sediment with a spade and laying this carefully in plastic trays. Sea water may be poured over the sediment and they should then be returned to the laboratory as soon as possible.

Holding conditions: on returning to the laboratory the overlying water should be aerated and maintained at about 15°C, under flow through or static conditions. Temperature, pH, salinity and dissolved oxygen should be monitored according to the appropriate laboratory practices. Mortalities during holding should be acceptably low, and if possible less than 10%.

**Procedure B**

Collection of animals: an alternative to the above procedure can be used. *Corophium sp* are sieved at the site of collection. This may be an advantage, particularly where natural densities are not so great that a check can be made on the numbers collected. Animals collected in this way are transferred to the laboratory in natural sea water.

Holding conditions: On returning to the laboratory the animals are held in aquaria (about 10 to 30 l) in the presence of a small amount of detrital material under static or flow through conditions, and should be used within 5-10 days. Temperature, pH, salinity and dissolved oxygen should all be recorded according to the appropriate laboratory practices. Mortalities during holding should be acceptably low, and if possible less than 10%.

Whichever method of collecting the animals is used it is essential to measure the salinity at the point of collection. If the salinity is low the animals should be acclimated to full salinity seawater (at least 25‰) at a maximum rate of approximately 3 per day.

## 3.2 Preparation of *Corophium* for use in the toxicity tests

*Corophium* collected and held in the laboratory as described in procedure 'A' above should be prepared by sieving them out of their native sediment prior to initiating the test. Care should be taken to ensure that any animals damaged during sieving are not included in the test, either through the use of a quarantine period (e.g. two days) or by visual inspection, or both. The holding trays should be drained down and sediment gently dug out with a scoop and transferred to a 500 μm sieve (e.g. stainless steel or nylon). The sediment should then be sieved and gently washed away with seawater. It is very important to take care at this point to avoid damaging the animals. Damage can be minimised by keeping the sieve mesh in water as much as possible. The animals that remain on the sieve should be gently washed into a clean aquarium containing flowing or static seawater and aeration must be provided.

Animals collected and held in the laboratory as described in procedure 'B' may be sieved or pipetted directly from the holding tank (cf. § 3.6).

Salinity, dissolved oxygen, pH and temperature should all be recorded at regular intervals, and must stay within the range specified for the test (cf. § 3.7 below). Mortality should be acceptably low and preferably not exceed 10% during the holding period.

## 3.3 Reference sediment collection

The reference/base sediment should have the following approximate characteristics:

1. an organic content of between 0,5 and 4%;
2. a silt/clay fraction (<63 μm) of between 5 and 20%;
3. a median grain size of 90 to 125 μm.

A muddy fine sand should be used, not a mud nor a coarse sand.

Sediment (approximately 40 kg wet weight or sufficient for the needs of the study) must be collected from an area known to be clean, and preferably from the same location from which the animals were collected. The aerobic layer of sediment (usually the top 5-10 cm) should be removed with a spade, and transferred to polythene bags or suitably cleaned vessels. The bags or containers should then be sealed, after excluding as much air as possible.

On return to the laboratory the sediment should be sieved to 500 μm using reference seawater. Sieving in this way serves to adjust the interstitial salinity of the sediment and excludes any benthic organisms which might interfere with the test or eat the test animals. It is very important that the sediment is washed in a closed system e.g., a container with a limited volume of water, and that the slurry is left to settle for 24 hours before decanting the overlying water. The sediment should be carefully mixed before storage. The sieved sediment should then be placed into clean polythene bags, and after as much air as possible has been excluded, the bags should be sealed and stored in the dark at 4°C until they are required for use.

## 3.4 Preparation of test sediments

This should be carried out as described in Annex 3.

## 3.5 Test preparation

a. **Replication**: The minimum acceptable for the test is 20 animals per concentration and it is recommended that at least two replicates are used. The raw mortality data must be provided in the formal report.

   ***Note:***
   ***If it is desired to compare control with treatment mortality then the degree of replication should reflect this requirement.***

b. **Addition of sediments**: After a mixing bottle (see paragraph 3.4) has been removed from the shaker/roller it should be shaken by hand and the slurry poured into the test vessels. Care should be taken to ensure that the sediment suspension is evenly distributed (including the fines) between any replicates. The beakers should then be left overnight to allow the sediment to settle. Some surface water will appear during this procedure.

   The depth of sediment may vary in the test beakers. This is not critical except that the minimum depth must NOT be less than 15 mm, and the total sediment depth should not give rise to a sediment to water ratio of greater than about 0,2.

c. **Addition of water**: The addition of water directly to the sediment surface causes considerable disturbance and resuspension of the sediment. This must be avoided, for example, by using discs of polythene or other inert material as a buffer (other methods may be suitable). Discs (approximately 6 are needed per test substance), are cut to the internal diameter of the test beakers and threaded with either polypropylene string or cable ties. Within 24 hours of introducing the sediment to the test vessels, one disc should be carefully placed over the sediment surface in each vessel and reference sea water carefully added to bring the total volume (i.e., sediment plus water) up to the 850 ml graduation mark. The polythene disc is then carefully removed by gently pulling it through the water column by the attached string or cable tie. The beaker is then left to allow any resuspended sediment to settle.

When the sediment has settled, aeration should be applied. The level of aeration should be adjusted so that no sediment resuspension occurs. In some cases some resuspension may be observed. This is particularly

true when fine sediments are used and in these cases aeration should be adjusted to minimise this. The beakers should then be left to aerate for at least twelve hours prior to initiating the test.

## 3.6 Addition of experimental animals to test tanks

Animals are prepared for each test as previously described (see § 3.2.).

*Corophium* should be transferred to a tank or shallow tray in the reference sea water so that they can be selected more easily. This is accomplished by pouring the water from the holding tank through a 500 μm sieve and gently washing the contents of the sieve into the tray. If animals have been held during acclimation in the absence of sediment they may be sorted directly from the holding tanks.

Individual animals are selected using an automatic or standard pipette. In each case the opening of the pipette should be 8 mm in diameter, the ends of which should be heat smoothed to avoid damaging the animals. Animals of a size of greater than approximately 5 mm are selected by gently sucking them up into the pipette and randomly assigning them to reference seawater in the 100 ml beakers (one 100 ml beaker is required for each test vessel). Randomly assigning animals ensures that there is no biasing for size or activity. When each beaker contains 10 (or 20 as appropriate) *Corophium* the volume of the beakers is adjusted to an appropriate mark, e.g., 40 ml, using reference seawater. The animals are then ready to be added to the test vessels. At this point the aeration in the test vessels should be temporarily stopped.

The test is initiated by randomly placing the groups of animals into the test container. This is best achieved by gently moving the beaker to a horizontal position where the rim is under the water surface and then gently pouring out the contents of the beaker.

***Note: Always check that no animals have been left in the 100 ml beakers***

When all the animals have been transferred to the test vessels the test has been initiated and aeration should be restored.

## 3.7 Routine monitoring and data collection

The following parameters should be measured at the start (before introduction of the test animals), after 24 hours, and at least twice further during the test, including day 10, and noted on appropriate record sheets:

| | |
|---|---|
| pH: | (normal range 7,5-8,5) |
| dissolved oxygen: | (normally >85%) |
| temperature: | (15 ± 2°C) |
| salinity: | must be measured at the start and finish of the test (normally range of ± 4 during the experiment). |

The salinity of the overlying test water should be maintained close to day 0 value throughout the test by the addition of distilled water. Salinity can readily be monitored by marking the final level on the test beaker after the animals have been added on day 0; addition of distilled water can be made to this mark when required. A 5mm drop in level is equivalent to an approximately 2% increase in salinity and a change of this magnitude may be used as a practical threshold for restorative action.

Other observations: Records may be kept on the burrowing behaviour of the animals, although this may not be possible if the water in the test containers is turbid. Animals that are dead on the surface of the sediment are opaque in appearance. These mortalities should be recorded together with the number of animals that are not buried, but may be seen swimming in the water column or browsing on the sediment surface. Where possible, dead animals should be removed.

### 3.8 Termination of the test

The test is continued for 10 days, and on the 10th day all readings should be taken before the test is terminated. Termination is achieved by gently stirring up the sediment in the test beaker to form a slurry, and pouring the slurry into the 500 μm sieve. This is best achieved by keeping the sieve immersed in seawater. Any sediment left in the sieve should be gently washed away with reference seawater and the number of animals alive and dead recorded. Death is defined as the absence of movement after gentle stimulation with forceps.

### 3.9 Treatment of results

The endpoint of the test is mortality as defined as the initial addition minus the number of surviving animals for each treatment. Dead animals may decompose or be consumed during the test and for this reason "missing" animals are presumed and counted as dead.

The 10d $LC_{50}$ should be calculated using an appropriate statistical method. The raw mortality data must be provided in the final report.

## 4. Bibliography

1. ASTM (1993). Standard guide for conducting 10-day static sediment toxicity tests with marine and estuarine amphipods. In 1993 Annual Book of Americal Society for Testing and Materials (ASTM) Standards: E 1667-92, pp. 1138-1163.
2. SEBC (1991). Standard European Behaviour Classification (SEBC) System, Bonn Agreement, Counter Pollution Manual, Vol. 2, Chapter 25, pp. 1-8.
3. Stortelder et al., (1989). Kansen voor waterorganismen, 1989, Normstelling, Ministerie van Verkeer en Waterstaat, the Netherlands.

# Annex 1: Aeration System for Amphipod Bioassay

The system was developed by Dr. B. Roddie at the Institute of Offshore Engineering. The main advantage of this system is that it provides uniform aeration to each of the test chambers.

**Components** (to provide aeration for 10 test chambers).

1 of 63 mm external diameter ABS/PVC tubing 130 cm in length;

1 of threaded tube connector;

1 of piece of clear acrylic sheet 15 x 130 cm, 5 mm thick;

2 of No. 51 rubber/neoprene bungs;

10 of 3 cm lengths of 8 mm external diameter vinyl/plastic tubing;

10 of 1 ml pipette tips.

**Assembly Instructions**

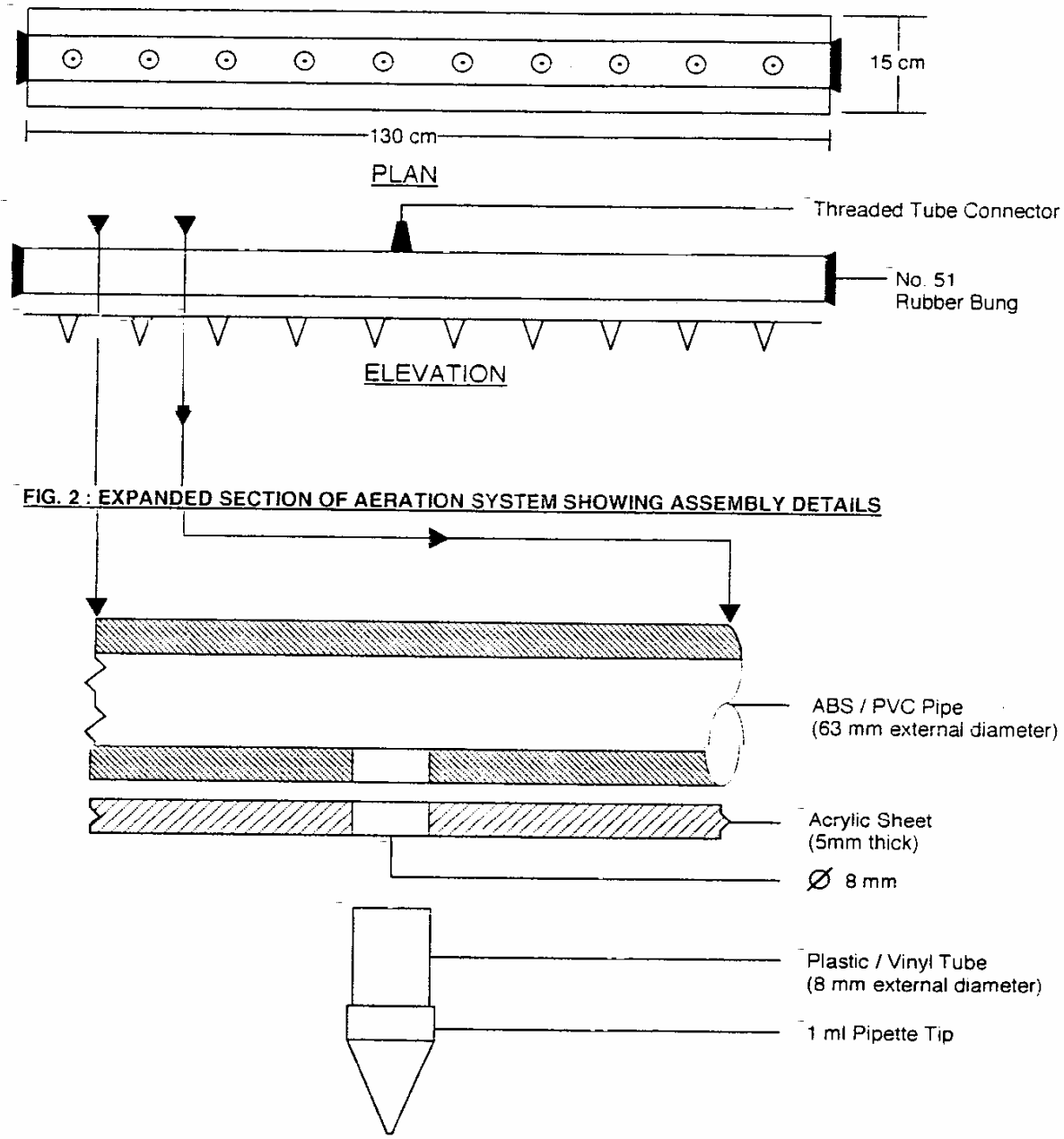
1. A centre line should be drawn longitudinally on the acrylic sheet and the centre marked.
2. Marks should then be made from the centre of the sheet. The initial centres should be 6,5 cm from the centre mark, thereafter the marks should be made 13 cm from the preceding one.
3. These central marks then provide the centres for the holes that have to be drilled in the acrylic sheet. They should be drilled out with an 8 mm drill.
4. It is essential that the first sheet should be drilled and marked accurately, as it can then be used as a template for further systems.
5. The ABS pipe should then be firmly clamped to the template sheet and drilled with the same 8 mm drill that has been used previously.
6. Another 8 mm hole should be drilled in the centre of the pipe, on the opposite side, and tapped to receive a threaded tube connector, which will provide the inlet for the air supply.

**Assembly**

1. 2-3 cm lengths of the 8 mm OD tubing should be cut.
2. One end of the cut length of tubing should be pushed into the end of each of the 1 ml pipette tips. This process should be continued until 10 of these units are assembled (see Annex 1, Figure 1).
3. The Acrylic sheet and the pre-drilled pipe should be arranged so that the holes match, and the pipette tip/tubing assemblies should then be pushed into the holes. The fit should be sufficiently tight to ensure that the unit stays together and should remain air-tight. However, this can be achieved by using a silicon sealant, suitable for aquarium use.
4. The final stage is to insert the rubber bungs into the end of the ABS pipe. They should be pushed in as far as possible and should give an airtight fit.
5. The end product should look something like Figure 1.

Figure 1

## Schematic of Toxicity Test Aeration System



# Annex 2: Guideline to define physical and chemical properties of substances to be tested with a sediment test

### Introduction

This guideline was prepared by Dr D M M Adema (previously of TNO, the Netherlands) to provide a basis for the choice of chemicals for which a sediment test will be required. One condition essential in a scheme for selecting chemicals (for sediment reworked testing) is that it should be done on the basis of physico-chemical properties which are already required in existing regulatory schemes.

As a matter of principle, a test with a sediment reworked species should be carried out with chemicals which will at least partly end up at the sea bottom.

### Chemicals for which a sediment test is required:

1. Chemicals which directly sink, i.e. chemicals with a density greater than that of sea water and with a low water solubility. The impact of these parameters on the actual behaviour of the chemicals will depend on environmental factors such as weather conditions. For a first discussion, it is recommended that the "Standard European Behaviour Classification (SEBC) (see Appendix A) be followed and that all chemicals with the indication "S" (sinker) (SEBC, 1991) should be included.

2. Chemicals which will end up at the sea bottom after transportation via biotic or abiotic particles. These are chemicals which adsorb to particles i.e. chemicals with a high partition coefficient between water and organic matter or other particles. Such partition coefficients are not widely known, but they are related to the well known log $P_{o/w}$, which has to be reported anyhow as an indication for bioaccumulation potential.

   In the Netherlands, a log $P_{o/w}$ of 5 is taken as a threshold for setting criteria for sediment rather than for the aqueous phase (Stortelder et al., 1989). According to the EC or OECD-guidelines a low log $P_{o/w}$ of 3 is the borderline for tests on bioconcentration. It is generally accepted that from a $logP_{o/w}$ of 3, the partitioning between water and biota might lead to appreciable amounts of chemicals in biota. Such plant- or animal-material might end up on the sea bottom. It is suggested that a test with a sediment reworker species be required for chemicals with a $logP_{o/w}$ of 4 and higher. Appendix B lists $logP_{o/w}$, values with regard to chemicals tested.

3. Surface active and particulophile (e.g. Vantocil) substances unless full and reliable information indicates the opposite of assumed adsorbability.

### Preparations

It is generally preferable to test substances. Should, however, preparations be tested, then a sediment test would be required if any of the intentionally added substances (at a concentration of >1%) in the preparation falls within one of the categories above.

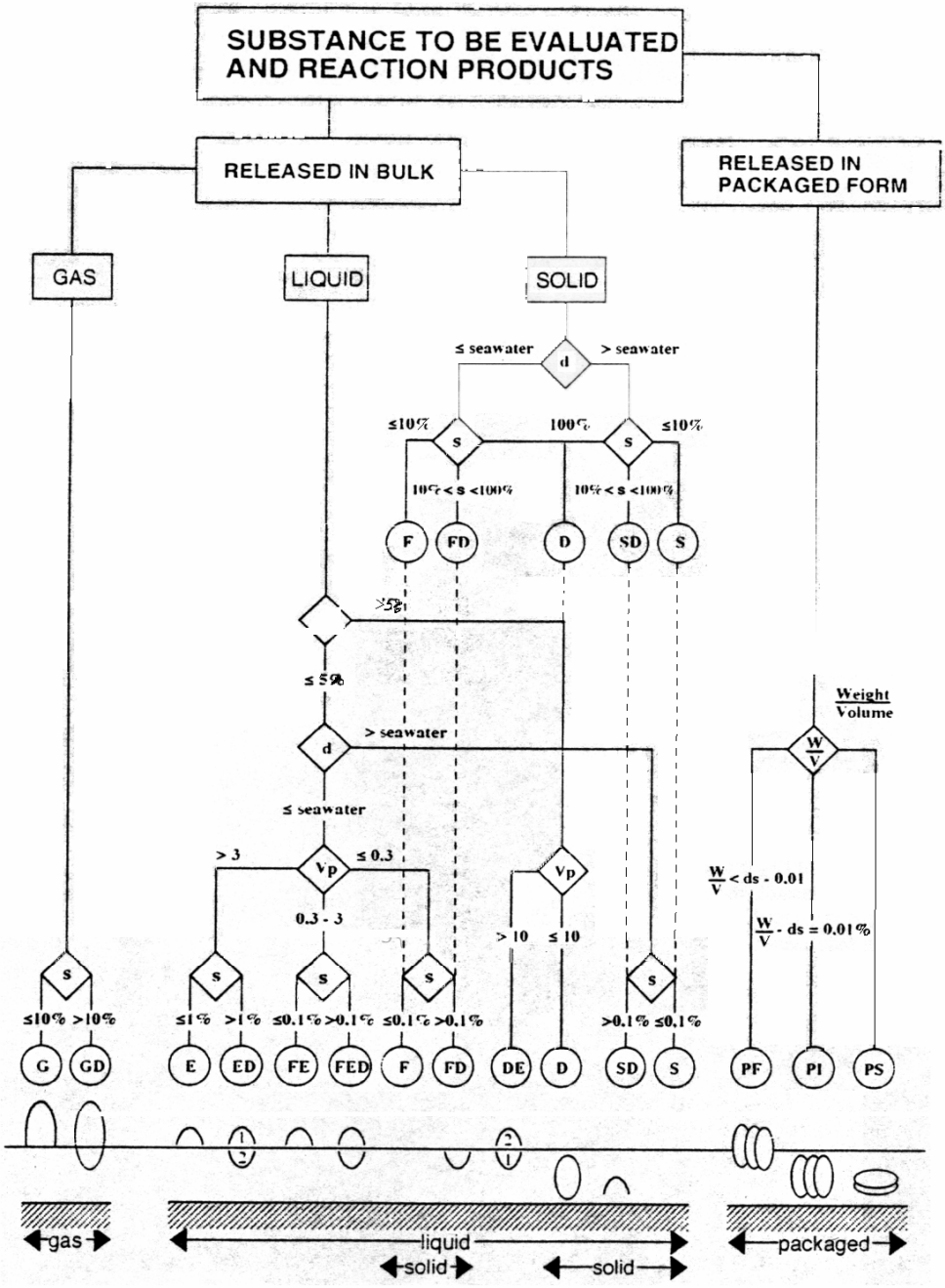
### Summary

Chemicals for which a sediment reworker test is required are those which:

- are partly or completely classified as sinkers according to the SEBC (Appendix A)
- have a $logP_{o/w}$ of 4 or higher
- are surface-active chemicals (unless full and reliable information indicates the opposite of assumed adsorbability)
- are known to adsorb to particles (are particulophile, e.g., Vantocil) (unless full and reliable information indicates the opposite of assumed adsorbability).

Appendix A

## European Classification System - Flow Diagram (SEBC)




**Air and water temperature: 10 or 20°C**
**Atmospheric pressure: 100 kPa**
**s : solubility (%)**
**d : density**
**ds : density of seawater**
**Vp : Vapour pressure (kPa)**

**D : dissolver**
**E : evaporator**
**F : floater**
**G : gas**
**P : package**
**S : sinker**

Appendix B

**Some $logP_{o/w}$ values:**

| **chemical** | **$logP_{o/w}$** |
|---|---|
| benzene | 2,13 |
| toluene | 2,59 |
| m-xylene | 3,09 |
| ethylbenzene | 3,13 |
| isopropylbenzene | 3,66 |
| n-butylbenzene | 4,28 |
| di-isopropylbenzene | 5,01 |
| n-dodecylbenzene | 8,40 |
| n-hexane | 3,51 |
| cyclohexane | 3,18 |
| n-nonane | 5,09 |
| n-decane | 5,62 |
| 1-octanol | 3,03 |
| 1-nonanol | 3,53 |
| 1-decanol | 4,03 |
| 1-undecanol | 4,53 |
| 1-dodecanol | 5,00 |
| 1-tridecanol | 5,51 |
| nonanone | 2,88 |
| decanone | 3,40 |
| undecanone | 3,93 |
| dodecanone | 4,46 |
| tridecanone | 4,98 |
| hexachlorobutadiene | 4,63 |
| monochlorobenzene | 2,81 |
| 1,2-dichlorobenzene | 3,53 |
| 1,2,4-trichlorobenzene | 4,20 |
| 1,2,3,4-tetrachlorobenzene | 4,94 |
| pentachlorobenzene | 5,69 |
| hexachlorobenzene | 6,44 |
| lindane | 3,53 |
| trichlorobiphenyls | 5,9 |
| tetrachlorobiphenyls | 6,6 |
| naphthalene | 3,5 |
| phenanthrene | 4,5 |
| chrysene | 5,6 |
| benzo(a)pyrene | 6,0 |

# Annex 3: Preparation of Test Sediments

### Spiking Procedure

The process of chemically spiking sediments described below has the primary objectives of ensuring that the test substance is evenly distributed throughout the test sediment, and that adequate contact between the substance and sediment is promoted.

The method does not purport to reproduce the adsorption characteristics which may obtain under equilibrium conditions or as a consequence of chronic exposure in the field.

### Identification of Chemical Property

In accordance with Annex 2, it is desirable to identify initially the partitioning and adsorptive properties of the material to be tested.

Material with a log $P_{o/w}$ of greater than 4 should be initially dissolved in an organic solvent of acceptably low toxicity (e.g., methanol or acetone), before addition to a small quantity of dried sediment. In general, all substances or preparations of low solubility should be coated initially onto dried sediment before introduction to the main mass of wet sediment.

Material which is known to be soluble or dispersible may be mixed with a small quantity of seawater before direct addition to the wet base sediment.

Powders should be dissolved or dispersed in an appropriate medium before addition to either dried sediment or wet sediment.

### Examination of Chemical Property

In cases where the properties of the chemical or product are not clearly identified by prior information, an examination of the behaviour of the material in seawater may provide a guide to the most appropriate preparation method.

The sample will be thoroughly homogenised before use. The original container will be placed on a roller or shaker table for one hour. If a shaker table is used, the speed or revolution should be set at approximately 150 rpm.

Add 1 g (±0,01g) of the homogenised sample to 1 litre (±0,01 litre) of 0,2 µm-filtered seawater in a clean conical flask or separating funnel of approximately 1,5 litre volume.

Stopper the vessel, and shake vigorously by hand, inverting the vessel at least ten times. Approximately five minutes treatment in a laboratory ultrasonic bath (power rating not constrained) is an acceptable alternative. In either case the choice of method must be documented.

Allow the contents of the vessel to settle for approximately 1 hour, and then observe the contents; record and classify the visible characteristics as follows:

1. No floating or settled materials, liquid or solid;
    a. Clear solution mixture - A;
    b. Homogeneous emulsion or fine/colloidal suspension - A;
    c. Neutrally buoyant droplets, particles or floc - B;
2. Floating, but no settled, liquids or solids - B;
3. Settled, but no floating, liquids or solids - C;
4. Floating and settled liquids or solids - D.

### Sediment Phase Preparation

### Source of sediment and preparation

Sediment should ideally be obtained from the same location from which the test population is obtained. This sediment should be initially characterised in terms of particle-size and organic content, which should lie within the limits specified in section 3.3.

Sediment should be collected and processed for testing as described in section 3.3.

**Chemical amendment of sediment**

The quantity of sediment prepared per test substance concentration will depend on the size of the test vessels, and on the depth of sediment required by the test procedure.

For tests conducted in accordance with the PARCOM Harmonised Offshore Chemical Notification Format (10 day test with *Corophium volutator*), a minimum depth of 15 mm of sediment is required in each replicate test vessel.

Immediately before the addition of a chemical or chemical solution, the base sediment must be thoroughly homogenised, and a sample of approximately 20 g wet weight removed and placed in an airtight container. This sample must be weighed (± 0,01 g) in a tarred container and then dried at approximately 60°C for about 24 hours.

The dried sample will be cooled to room temperature in a dessicator and re-weighed.

The ratio of the wet sample weight to dry sample weight (net of container weight in both cases) will be calculated and entered on the study record.

***Note: It is of primary importance that care be taken to avoid any alteration in the water content of the sediment between the time at which this determination is made and the time at which the test substance is added to the sediment.***

The preparation of spiked sediments for whole-sediment toxicity tests is carried out in a manner dependent on the properties of the test substance.

Substances which fall into category A above should be added to sediments as a solution or emulsion prepared in a small volume of seawater.

Substances which are powders, are described as insoluble, or which fall into categories B, C or D should be added initially to a small quantity of dried sediment and mixed thoroughly before mixing with a larger volume of wet sediment.

Insoluble or poorly-soluble substances should be dissolved in a suitable organic solvent such as methanol or acetone before addition to dried sediment.

Where a solvent is used, additional control sediments must be prepared at least at the highest concentration of solvent used in the substance treatments.

Test chemical concentrations may be prepared either

a. as nominal concentrations per unit wet weight of the base sediment, and later corrected using the measured wet weight/dry weight ratio to units of mg/kg dry weight/l;

b. as nominal concentrations per unit dry weight, by calculating the appropriate addition rate per unit wet weight on the basis of the measured wet weight/dry weight ratio.

Calculation of the required quantity of test substance must take into account the weight of any dry sediment used in preliminary preparation.

Where the test substance is prepared as an aqueous suspension or emulsion, the volume of water used should be kept to a minimum.

**Mixing of test substance with sediment**

The test substance and carrier medium should be added to the appropriate weight of wet sediment in a suitable container (e.g., a polythene or polypropylene bottle) of a suitable volume (e.g., 1 or 2 litres for a *Corophium* test as referred to above; in general, a vessel volume of approximately twice the volume of sediment is acceptable. A clean spatula (e.g. stainless steel or polythene) should be used to initially disperse the test substance and carrier through the sediment.

Sufficient clean seawater is then added to create a freely-flowing slurry. Care must be taken to minimise the volume of water added, but the quantity must be sufficient to allow the mixture to flow freely when the container is inverted or shaken.

The container will be labelled with the study number, the test substance number and the nominal concentration, and placed horizontally on an orbital shaker (with a displacement of at least 30 mm) at about 150 rpm for approximately three hours. The purpose of this procedure is to ensure that the test substance is

evenly distributed throughout the sediment matrix; it is not intended to ensure any specified degree of partitioning of the test substance. A roller may also be used for mixing.

**Introduction of spiked sediment to test system**

When the sediment preparations have been shaken for the specified period, the containers will be removed from the shaking apparatus.

Each container will be finally shaken thoroughly by hand, with at least five inversions, and the test operator will establish that the preparation moves freely and that no unmixed residues remain adhered to the walls of the container. This procedure must be carried out for each container immediately before the contents are dispensed to the appropriate replicate vessels.

The test medium will be dispensed in small aliquots to each replicate test vessel in turn, to minimise bias in the chemical content or particle-size distribution of the medium between vessels. Any supernatant water present must be included in the test system and must be equally distributed between vessels before the solid material is dispensed.

If difficulty is encountered in dispensing the solid phase, a clean spatula may be used to manipulate the material; in this case, it is essential that a new spatula be used for each test substance and that each test substance be dispensed in order from lowest to highest nominal concentration.

Sediment preparation containers must be used only once and should be disposed of as soon as practicable after dispensing the solid phase to the test vessels.

Preparation of the test system will then proceed in accordance with the guideline.

# Part B: Protocol for a Fish Acute-Toxicity Test

## 1. Test Species

Three methods using two fish species were ring-tested:

a. Turbot (*Scophthalmus maximus*) larval test using a modified Shell procedure;

b. Turbot (*Scophthalmus maximus*) juvenile test using a modified OECD 203 guideline;

c. Sheepshead minnow (*Cyprinodon variegatus*) juvenile test using a modified OECD 203 guideline.

The turbot juvenile (96 hour) was the recommended species in the fish acute toxicity test for assessing the environmental hazards of offshore chemicals, but it was concluded that the juvenile sheepshead minnows could be used when the turbot juvenile was unavailable for practical reasons.

## 2. Test Chemicals

A total of four chemicals were used as test substances; these were the biocide Bioban P-1487, the corrosion inhibitor Servo CK 337, a linear alkyl benzene and 3,5 - dichlorophenol (DCP), which was used as a pure reference chemical. Two of these chemicals (Bioban P-1487 and DCP) were used in the previous PARCOM ring-tests of algal and invertebrate herbivore acute toxicity methods, so relative sensitivity could be assessed.

## 3. Evaluation Criteria

The following criteria were used for discussion and evaluation of each of the test methods. They were considered to be important criteria, but not necessarily all of equal importance:

a. sensitivity of the species;

b. spectrum of response;

c. reproducibility;

d. repeatability;

e. organism availability;

f. costs, initial and running;

g. clarity of guidelines and ease of tests;

h. ecological relevance.

## 4. Summary of the Test Methods

4.1 A brief description of the test species and methods is found below:

| **Turbot (*Scophthalmus maximus*)** | **Juvenile Test** |
|---|---|
| Test Protocol: | Modified OECD 203, Fish Acute Toxicity Test |
| Test Design: | Acute, semi-static |
| Test Organism: | Juvenile turbot 4-6cm length |
| Measured Endpoint: | 96 h $LC_{50}$ and 24 h |
| Results Units: | mg/l |
| No. Fish/Test Concentration: | minimum 7; maximum loading 1g fish/litre |
| Exposure duration | 96 h |
| Test Temperature: | 15 ± 1,5 °C |
| Physical Measurements: | Temperature, $O_2$, pH and salinity. Aeration not recommended. |

# 5. Test Chemicals

The test chemicals chosen represent a broad spectrum of product categories commonly used in the offshore industry i.e. a biocide, a corrosion inhibitor and a detergent constituent. A pure reference chemical was also included for testing. The biocide (Bioban P-1487) and the corrosion inhibitor (Servo CK337) have previously been tested in the PARCOM Sediment Reworker Ring-test in 1993, and the reference chemical, 3,5 dichlorophenol, along with Bioban, were used in the PARCOM algal and invertebrate herbivore ring-tests in 1991.

The test compounds can briefly be described as follows:

**Bioban P-1487** (4-(2-nitrobutyl)-morpholin (70%) and 4,4'-(e-ethyl-2-nitrotrimethylene)) is a broad spectrum bactericide designed for application in oils and aqueous situations. According to the manufacturers Angus Chemie GmbH, Germany, it is moderately soluble in water (1100mg/l).

**Servo CK337** is an inhibitor of $CO_2$ and $H_2S$ corrosion, comprising an amine neutralised phosphoric acid ester that is described by the manufacturers Servo, Delden, the Netherlands, as a "cationic surface active agent in an aromatic hydrocarbon solvent".

**Linear Alkyl Benzene** (Petrelab 550), produced by Petroquimica, Spain, contains side alkyl chains of 10-13 carbon atoms. This high purity product is used primarily for the production of heavy duty powdered biodegradable detergents. It is described as insoluble in water, therefore is prepared as water accommodated fractions (WAF) in the tests.

**3,5- Dichlorophenol** is an organochlorine which is described as slightly soluble in water and is used in organic synthesis. It was used as a reference chemical for the purposes of this ring test and all participants obtained their own supplies.

# 6. Summary of the Ring-Test Results

## 6.1 Turbot juvenile

Seven laboratories carried out this test on at least two of the chemical compounds. All laboratories used one replicate per test concentration and between 7-10 fish in each replicate. One control replicate was used in each test with the exception of one laboratory which used two replicates. Control abnormality in all tests was within the acceptable limits for this test ie, less than 10% or one fish if less than 10 fish were used.

Test conditions were not fully reported for all tests. Where information was provided, test conditions were acceptable, but in one case a laboratory reported low dissolved oxygen concentrations at about 50% air saturated value; no explanation was evident.

## 6.2 Summary

Table 1 and Figure 1 summarise the data. No test was demonstrably or consistently more sensitive than the others and individual differences were small (less than half order of magnitude). The coefficient of variation and max:min ratios were consistently highest in the turbot larval test, but this may in part be a reflection of the greater number of tests carried out and in part by the inexperience of experimenters. It should be noted that standard deviations and coefficients of variation for each combination of test and substance should be used with caution, since some of the data distributions are bi-modal and thus not amenable to parametric statistics which assume normality of distribution.

**Table 1: Mean $LC_{50}$ values (mg/l) for each test substance and method**

| | Turbot juveniles (96 hour) |
|---|---|
| Bioban P1487 | mean = 1,08<br>n = 7<br>sd = 0,48<br>max:min = 2,8<br>CV 44% |
| Servo CK337 | mean = 21,31<br>n = 5<br>sd = 13,80<br>max:min = 6,7<br>CV 65% |
| 3,5-Dichlorophenol | mean = 0,66<br>n = 6<br>sd = 0,10<br>max:min = 1,4<br>CV 15% |
| Linear alkylbenzene | mean > 1 000<br>n = 3 |

# 7. Avalability of the Test Animals

It is essential for any test method that the organism is available throughout the year. This can usually be achieved by collection of the test species from wild populations or by laboratory culture.

Turbot larvae and juveniles for the ring test were obtained from turbot hatcheries, one in the UK and one in Norway (see Appendix 1). There are also hatcheries in Denmark, France and Spain although the year round availability of supply from these hatcheries is not known. Turbot larvae and juveniles cannot be collected from wild populations nor can they be easily cultured in the laboratory.

# 8. Bibliography

1. Kooijman, S. A. L. M. (1981). Parametric analyses of mortality rates in bioassays. Water Res. 15 pp. 108-119.

2. United States Environmental Protection Agency (1985). Methods of measuring the toxicity of effluents to fresh water and marine organisms (Third Edition). EPA/600/4-8/013

# Annex 1: Guideline for measuring the acute toxicity of offshore chemicals to juvenile marine fish, the turbot *Scophthalmus maximus* and the sheepshead minnow *Cyprinodon variegatus*.[4]

**Based on OECD Guideline Number 203 and Ring-tested by the Paris Commission 1994.**

## Introduction

1. The amended version of OECD Guideline 203 was successfully ring-tested in 1994 by an international group of laboratories under the leadership of the MAFF Fisheries Laboratory, Burnham-on-Crouch, UK. The main difference from the version of OECD 203 which was adopted by OECD on 17/7/92 is that the guideline permits the testing of offshore chemical products which may be a complex mixture of substances, and it uses one of two marine test species. The recommended species are turbot juveniles (Scophthalmus maximus - minimum of 7 per group) or sheepshead minnow juveniles (Cyprinodon variegatus - minimum of 10 per group). A maximum spacing factor of 3,2 between test concentrations is acceptable.

## Principle of the Test

2. The fish are exposed to the test substance for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours. If a full toxicity test is undertaken, the concentrations which kill 50 per cent of the fish ($LC_{50}$) are determined where possible.

3. Useful information on the test substance includes its structural formula, purity, stability in water and light, pKa,$P_{ow}$, vapour pressure and results of a test for ready biodegradability (see OECD Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

## Validity of the Test

4. For a test to be valid the following conditions should be fulfilled:

- the mortality in the control(s) should not exceed 10 % (or one fish if less than ten are used) at the end of the test;
- constant conditions should be maintained as far as possible throughout the test and, semi-static procedures should be used[5];
- the dissolved oxygen concentration must have been at least 60 % of the air saturation value throughout the test.

## Description of the Method

## Apparatus

5. Normal laboratory equipment and especially the following are necessary:

   a. oxygen meter;

   b. adequate apparatus for temperature control;

   c. tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading.

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[4] The heading and paragraphs 1, 2, 11, 12, 13, 14, 15, 18, 19 of this Annex were amended by OSPAR in 2005 following the advice of its Offshore Industry Committee. OSPAR 2006 agreed on an amended paragraph 14, and to include a new paragraph after paragraph 14.

[5] Definitions: $LC_{50}$ in this Test Guideline is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 % of a test batch of fish within a particular period of exposure (which must be stated).

**Selection of species**

6. Either species of *S. maximus* juvenile or C. variegatus juvenile may be used, the choice being at the discretion of the testing laboratory. It is suggested that the species used be selected on the basis of such important practical criteria as, for example, their ready availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological factors. The fish should be in good health and free from any apparent malformation.

**Holding of fish**

7. All fish must be obtained and held in the laboratory for at least 12 days before they are used for testing. They must be held in water of the quality to be used in the test for at least four days immediately before testing and under the following conditions:

| | |
|---|---|
| Light: | 12 to 16 hours photoperiod daily |
| Temperature: | *S. maximus* 13,5-16,5°C; *C. variegatus* 18-22°C |
| Oxygen concentration: | at least 60 % of air saturation value |
| Feeding: | according to supplier's recommendations until 24 hours before the test is started. |

8. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10 % of population in seven days: rejection of entire batch
- mortalities of between 5 and 10 % of population: acclimatisation continued for seven additional days
- mortalities of less than 5 % of population: acceptance of batch.

9. Reference seawater: natural seawater is preferred, but artificial (tropic marin or equivalent) is acceptable. It should have a salinity of 30 to 36 ‰ and it should be free from contamination.

**Test solutions**

10. Test solutions/dispersions of the chosen concentrations are prepared in accordance with PARCOM guidelines.

11. If there is a marked change in the pH of the test water following addition of the test substance, pH adjustment should be undertaken (see algae and crustacea test protocols, ISO/DIS 10253 and ISO TC 147/SC5/WG2 respectively).

**Procedure**

**Conditions of exposure**

12.

| | |
|---|---|
| Duration: | 96 hours |
| Loading: | maximum loading of 1,0g fish/litre |
| Light: | 12 to 16 hours photoperiod daily |
| Temperature: | *S. maximus* 13,5-16,5°C; *C. variegatus* 18-22°C |
| Oxygen concentration: | not less than 60 % of the air saturation value. |
| Feeding: | none |
| Solution replacement: | test media must be replaced after 48h (see 14 below). |
| Disturbance: | disturbances that may change the behaviour of the fish should be avoided |

**Number of fish**

13. At least 7 turbot juveniles must be used or 10 sheepshead minnows at each test concentration and in the controls. Sheepshead minnows should be immature juveniles, 1,5-2,5 cm in length; ditto for turbot juveniles except 4-6 cm in length.

**Test concentrations**

14. At least five concentrations in a geometric series with a factor preferably not exceeding 3,2. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range. Where there are no existing fish toxicity test data, it is recommended that a limit test is conducted using the LC50 or EC50 of the most sensitive test species of the other taxonomic groups tested. If no significant mortality occurs in this limit test (when compared with the control), it is unnecessary to undertake a full toxicity test and the end point of the fish test should be reported as greater than the concentration tested (> limit concentration)

15. Most substances exhibit a fairly similar degree of toxicity to both algae and crustacea but there are occasions where a substance appears to be very much more toxic to one class of organism than the other. Should the apparent toxicity be found to be due to a physical effect, such as chelation and not toxicity, the limit test should be conducted at the LC50 or EC50 of the next most sensitive species where no physical effect was evident.

16. If significant mortality occurs in the first limit test (when compared with the control), it is recommended that a further limit test is conducted using a concentration that is one order of magnitude lower than the concentration used in the initial limit test. If no significant mortality occurs in this limit test (when compared with the control), it is unnecessary to undertake a full toxicity test and the end point of the fish test should be reported as greater than the concentration tested (> limit concentration). If significant mortality occurs in this limit test (when compared with the control), a full toxicity test must be conducted to establish $LC_{50}$ values.

17. If a full toxicity test is conducted, the test should consist of at least five concentrations in a geometric series with a factor preferably not exceeding 3,2. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

18. If the test material is expected to be unstable, then more frequent solution changes should be considered. In addition, if suitable analytical methods exist then verification of the concentrations in the test solution is desirable.

***Controls***

19. A control consisting of one blank is run in addition to the test series.

***Observations***

20. The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation). Measurement of pH, dissolved oxygen, salinity and temperature should be carried out at least daily in the control and at least at the highest concentration.

**Data and Reporting**

**Treatment of results**

21. For a valid test the control mortality should not exceed 10% (or one fish if less than ten are used) at the end of the test.

22. Results of limit tests are expressed as greater than the limit concentration.

23. Results of full toxicity tests are expressed as 24, 48, 72 and 96h $LC_{50}$ values. $LC_{50}$ values with 95% confidence limits can be calculated using an appropriate statistical method (Ref. 1-5).

**Test report**

24. The test report should include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties
- identification data

Test fish:

- scientific name, strain (if appropriate) , size, supplier, any pre-treatment

Test conditions:

- test procedure used (e.g. semi static, fish loading)
- water quality characteristics (pH, temperature and salinity)
- dissolved oxygen concentration, pH values, salinity and temperature of the test solutions at 24 hour intervals
- methods of preparation of stock and test solutions
- concentrations used
- number of fish in each test solution

Results:

- maximum concentration causing no mortality within the period of the test (if appropriate)
- minimum concentration causing 100 % mortality within the period of the test (if appropriate)
- cumulative mortality at each concentration at the recommended observation times
- statistical procedures used for determining the $LC_{50}$ values
- mortality in the controls
- incidents in the course of the test which might have influenced the results
- abnormal responses of the fish
- limit test results should be reported as greater than the limit concentration
- full toxicity test results should be reported as $LC_{50}$ values, with 95 % confidence limits, for each of the recommended observation times (if possible), and be accompanied by details of the statistical procedures used for determining the $LC_{50}$ values.

Discussion of the results.

**Bibliography**

1. Litchfield J.T. and Wilcoxon F. (1949). A simplified method of evaluating dose-effect experiments. J. Pharmacol and Exper. Tber., 26; pp 99-113.
2. Sprague J.B. (1969). Measurement of pollutant toxicity to fish. I Bioassay methods for acute toxicity. Water Res. I, 793-821.
3. Sprague J.B. (1970). Measurement of pollutant toxicity to fish. II Utilising and applying bioassay results. Water Res. 4, 3-32.
4. Stephan C.E. (1977). Methods for calculating an $LC_{50}$. In Aquatic Toxicology and Hazard Evaluation (edited by Mayer F.I. and Hamelink J.L.). ASTM STP 634. pp 65-84, American Society for Testing and Materials.
5. Finney D.J. (1978). Statistical Methods in Biological Assay. Griffin, Weycombe, UK.

## Appendix 1

### Suppliers of Juvenile Turbot

As of November 1994, turbot hatcheries exist at the following European locations:

| **NAME** | **LOCATION** | **FAX NO.** (Where known**)** |
|---|---|---|
| Mannin Seafarms | Isle of Man | +44 1624 823134 |
| Tinfos Aqua | Norway | +47 383 51051 |
| Maximus | Denmark | |
| Lars Bach | Denmark | |
| France Turbot I and II | France | +33 51 39 54 39 |
| Sepia | France | |
| Ferme Marine de Doubet | France | |
| Tinamenor | Spain | |
| Prodemar | Spain | +34 81 74 22 03 |
| Cultipec | Spain | |
| Insuamar | Spain | |
| Insuina | Spain | |

***Note:***

***An important point is that consideration has to be given to the EC Fish Health Zone regulations controlling the movement of any live fish. Fish may be transported between zones of equal status or from a high status zone to a zone with lower status. Information on this can be obtained directly from the supplier, or appropriate regulatory authority.***