Toxicity tests with priority substances in the Water Framework Directive

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Sponsor	Institute for Inland Water Management and Waste Water Treatment (RIZA)

Performed by	ing. L. van Bakkum, ing. M. Dubbeldam, ing. H. Kooman, ir. J.G.M. Derksen and Dr. J.F. Postma
Monitors	Mrs. M. Beek Mrs. J.L. Maas

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Reported by	Dr. J.F. Postma		5-20-2005
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Approved by	Dr. J.T. Meulemans		5-20-2005

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AquaSense
Kruislaan 411a
PO box 95125
NL-1090 HC Amsterdam
The Netherlands
Telephone+31 (0)20 592 22 44
Telefax +31 (0)20 592 22 49
Url=http://www.aquasense.com

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Summary

The work presented in this report is related to the process of setting environmental quality standards to define a “good chemical status”. Within the Water Framework Directive several substances have been indicated as ‘priority substances’. For these priority substances environmental quality standards (EQS) have been proposed by the Fraunhofer Institute in Germany and specify limits for surface water, transitional water, coastal waters and territorial waters.

In most cases the method, which has been used, requires the application of safety factors. The height of these safety factors depends on the amount of toxicity data available. Due to a shortage of toxicity data, rather high safety factors had to be applied for several substances, resulting in relatively low environmental quality standards. Since some of these standards will be hard to achieve within the water management, is seems advisable to increase the amount of available toxicity data. As a result improved environmental quality standards can be proposed in which lower safety factors might be applied.

Toxicity tests were carried out with two freshwater species (*Daphnia magna* and *Pseudokirchneriella subcapitata*) and with four marine species (*Phaeodactylum tricornutum, Psammechinus miliaris, Crassostrea gigas, Acartia tonsa*). Covering twelve different toxicants, a total number of 27 different tests have been performed, the results of which are presented in the table.

Table **Summary of results.**
Tested concentrations and toxicity data in µg/l, based on actual concentrations. A.1 to L.2 refers to the paragraph and the appendix number. Test results presented in *italic* should be considered preliminary due to severe decreases in the actual test concentrations during the toxicity test.

App.	Substance	Species	Duration	Parameters	NOEC (µg/l)	LOEC (µg/l)	EC_{10} (µg/l)	EC_{50} (µg/l)	EC_{90} (µg/l)
A.1	Alachlor	*Phaeodactylum tricornutum*	96 hrs	Growth rate (µ)	≥ 5 *	≥ 5	≥ 5	≥ 5	≥ 5
B.1	Anthracene	*Crassostrea gigas*	48 hrs	Larval development	≥ 2.8 *	≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8
B.2	Anthracene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 2.8 *	≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8
C.1	Benzo(a)pyrene	*Crassostrea gigas*	48 hrs	Larval development	≥ 1.6 *	≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6
C.2	Benzo(a)pyrene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 1.6 *	≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6
D.1	Benzo(k)fluoranthene	*Crassostrea gigas*	48 hrs	Larval development	≥ 0.62 *	≥ 0.62	≥ 0.62	≥ 0.62	≥ 0.62
D.2	Benzo(k)fluoranthene	*Daphnia magna*	21 days	Survival and reproduction	≥ 2.2 *	≥ 2.2	≥ 2.2	≥ 2.2	≥ 2.2
D.3	Benzo(k)fluoranthene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 2.6 *	≥ 2.6	≥ 2.6	≥ 2.6	≥ 2.6
E.1	C10-C13 chloroalkanes	*Psammechinus miliaris*	48 hrs	Larval development	≥ 21 *	≥ 21	≥ 21	≥ 21	≥ 21
F.1	Hexachlorobutadiene	*Crassostrea gigas*	48 hrs	Larval development	≥ 21 *	≥ 21	≥ 21	≥ 21	≥ 21
F.2	Hexachlorobutadiene	*Daphnia magna*	21 days	Survival reproduction	9.1 4.4	19 9.1	? 14	22 30	? 42
F.3	Hexachlorobutadiene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 21 *	≥ 21	≥ 21	≥ 21	≥ 21
G.1	Isoproturon	*Crassostrea gigas*	48 hrs	Larval development	98	150	≥ 520	≥ 520	≥ 520

*: Highest concentration tested
?: no estimation possible

Table *Continued*. Summary of results.
Tested concentrations and toxicity data in µg/l, based on actual concentrations. A.1 to L.2 refers to the paragraph and the appendix number. Test results presented in *italic* should be considered preliminary due to severe decreases in the actual test concentrations during the toxicity test.

App.	Substance	Species	Duration	Parameters	NOEC (µg/l)	LOEC (µg/l)	EC_{10} (µg/l)	EC_{50} (µg/l)	EC_{90} (µg/l)
G.2	Isoproturon	*Phaeodactylum tricornutum*	96 hrs	Growth rate (µ)	5.7	10	8.1 (6.6-10.3)	≥ 10	≥ 10
G.3	Isoproturon	*Psammechinus miliaris*	48 hrs	Larval development	310	555	502.7 (396.4–551.4)	> 555	≥ 555
H.1	Lindane	*Acartia tonsa*	48 hrs	Survival	<1.2	1.2	<1.2	1.5	≥ 5.1
H.2	Lindane	*Crassostrea gigas*	48 hrs	Larval development	≥ 450*	≥ 450	≥ 450	≥ 450	≥ 450
H.3	Lindane	*Psammechinus miliaris*	48 hrs	Larval development	≥ 680*	≥ 680*	≥ 680*	≥ 680*	≥ 680*
I.1	Naphtalene	*Crassostrea gigas*	48 hrs	Larval development	No parameters could be estimated, due to a strong decrease in actual test concentrations				
I.2	Naphtalene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 355*	≥ 355*	≥ 355*	≥ 355*	≥ 355*
I.3	Naphtalene	*Pseudokierchneriella subcapitata*	96 hrs	Growth rate (µ)	No parameters could be estimated, due to a strong decrease in actual test concentrations				
J.1	Nonylphenol	*Crassostrea gigas*	48 hrs	Larval development	No parameters could be estimated, due to a strong decrease in actual test concentrations				
J.2	Nonylphenol	*Psammechinus miliaris*	48 hrs	Larval development	12	17	5.4	> 28	> 28
K.1	*Pentachlorobenzene*	*Crassostrea gigas*	*48 hrs*	*Larval development*	≥ 49*	≥ 49*	≥ 49*	≥ 49*	≥ 49*
K.2	Pentachlorobenzene	*Psammechinus miliaris*	48 hrs	Larval development	≥ 19 *	≥ 19	≥ 19	≥ 19	≥ 19
L.1	Tetrachloroethene	*Crassostrea gigas*	48 hrs	Larval development	No parameters could be estimated, since no relation was found between nominal and actual concentrations				
L.2	Tetrachloroethene	*Psammechinus miliaris*	48 hrs	Larval development	No parameters could be estimated, since no relation was found between nominal and actual concentrations				

*: Highest concentration tested

1. Introduction

In 2000 the EU Water Framework Directive (2000/60/EC) has been adopted. The key objective of this Directive is to set up common goals for the state of the aquatic environment within the EU. The aim is that in 2015 a good ecological and chemical status of all groundwater and surface waters must have been achieved. Good ecological status is defined in terms of the quality of the biological community, the hydrological characteristics and the chemical characteristics. Good chemical status is defined in terms of compliance with the environmental quality standards established for chemical substances at European level.

The work presented in this report is related to the process of setting environmental quality standards to define the good chemical status. Within the Water Framework Directive several substances have been indicated as 'priority substances'. For these priority substances environmental quality standards (EQS) have been proposed by the Fraunhofer Institute in Germany and specify limits for surface water, transitional water, coastal waters and territorial waters.

In most cases the method, which has been used, requires the application of safety factors. The height of these safety factors depends on the amount of toxicity data available. For several substances, due to a shortage of toxicity data, rather high safety factors are applied resulting in very low environmental quality standards. Since some of these standards will be hard to achieve within the water management, is seems advisable to increase the amount of available toxicity data. As a result improved environmental quality standards can be proposed in which lower safety factors might be applied.

Three priority substances were tested with freshwater organisms and twelve priority substances with marine organisms. Table 1 provides an overview of the substances that have been tested, as well as the test organisms and test duration. Table 2 gives an overview of the guidelines that have been applied. More specific details on the methods are provided in chapter 2, while the results of the toxicity tests are presented for each 'substance – organism' combination in chapter 3.

Table 1 Overview of the substances that have been tested, the test organisms used as well as the test duration.

Substance	Group	Species	Test
Fresh water			
Benzo(k)fluoranthene	Crustaceans	*Daphnia magna*	21 days
Hexachlorobutadiene	Crustaceans	*Daphnia magna*	21 days
Naphtalene	Algae	*Pseudokirchneriella subcapitata*	96 hrs
Marine water			
Alachlor	Algae	*Phaeodactylum tricornutum*	96 hrs
Anthracene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Benzo(a)pyrene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Benzo(k)fluoroanthene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
C10-C13 chloroalkanes	Echinoderms	*Psammechinus miliaris*	48 hrs
Hexachlorobutadiene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Isoproturon	Molluscs Algae Echinoderms	*Crassostrea gigas* *Phaeodactylum tricornutum* *Psammechinus miliaris*	48 uur 96 hrs 48 hrs
Lindane	Crustaceans Molluscs Echinoderms	*Acartia tonsa* *Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs 48 hrs
Naphtalene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Nonylphenol	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Pentachlorobenzene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs
Tetrachloroethene	Molluscs Echinoderms	*Crassostrea gigas* *Psammechinus miliaris*	48 hrs 48 hrs

Table 2 Overview of the test guidelines.

Toxicity test	Specifications	Standard
Daphnia magna	21 days, 3 renewals/week	ISO 10706
Pseudokirchneriella subcapitata	96 hours [1]	ISO 8692
Phaeodactylum tricornutum	96 hours [1]	ISO 10253
Psammechinus miliaris	Embryonic development, 48 hours	ASTM E1563
Crassostrea gigas	Embryonic development, 48 hours	ASTM E724
Acartia tonsa	Acute immobility, 48 hours	ISO 14669

[1]: the guideline specifies a test duration of 72 hours. A duration of 96 hours was chosen to increase the sensitivity.

2. Methods

2.1. General test procedures

Beneath, a general outline of the test methods is provided (see also table 3). Additional methods are specified for each test organism in paragraphs 2.2 - 2.7. Details concerning the tested concentrations are given in chapter 3.

The test organisms are cultivated by AquaSense (*Daphnia magna*, algae), bought from commercial breeders (*Acartia tonsa*, adult oysters) or caught in the wild (adult sea urchins).

The Institute for Inland Water Management and Waste Water Treatment (RIZA) has provided all test substances and has given indications on the concentration series to be tested.

Note

Normally toxicity tests will start which a range-finding test after which a definitive test can be performed. For the present research it was decided to aim the concentration range especially on the NOEC values. If at the same time EC_{50}-values can be calculated, this is off course performed, but this was not considered to be necessary.

This approach was chosen since the procedure of setting environmental standards only use chronic NOEC values. Furthermore, this procedure of setting environmental standards is based on the lowest NOEC (or EC_{10}) value found, after which safety levels based on the amount of information are applied. For many substances and organisms is was therefore decided that an experimental prove that the NOEC value will be higher than the lowest value known so far, will be sufficient.

In all tests (with the exception of the liquid Tetrachloroethene) acetone was used to dissolve the substances. Concentrations of stock solutions were chosen in such a way that the maximum concentration acetone in the final test concentrations was normally less than 100 µl/l. There were however a few exceptions:

Oysterlarvae test with	lindane (183 µl/l);
Sea-urchin test with	chloroalkanes (280 µl/l), lindane (124 µl/l) nonylphenol (122 µl/l) and benzo(k)fluoranthene (158 µl/l)
Daphnia test with	hexachlorobutadiene (332 µl/l) and benzo(k)fluoranthene (173 µl/l)

Acetone concentrations differed for every substance and every test concentration (for details see chapter 3, paragraph A.1 to L.2). Additional solvent controls were performed for each test using the highest acetone concentration applied in that specific concentration series.

Toxicity tests with *Daphnia magna* were performed with so-called Elendt medium (M4), while toxicity tests with algae have been performed with the appropriate ISO-medium as defined in the guidelines. All other tests have been performed with natural sea water (± 32 ‰, filtered over 30µm) sampled from the Eastern Scheldt.

For each substance five different concentrations have been tested (with the exception of alachlor in the toxicity test with the marine algae *P. tricornutum*, and lindane in the toxicity test with *Acartia*), as well as a solvent control (CONtrol) and a medium control (BLank). In the algae tests additional controls without algae were used.

All toxicity tests were performed at the temperature specified in the guidelines, using a day / night interval of 16 hours light / 8 hours darkness. The algae tests were off course performed with a continuous light using a light intensity of 90 µE/m^2/s.
Glass test vessels were used for all toxicity tests. If volatile substances were tested, test vessels were closed with a lid (with the exception of algae tests).

As a control several physical and chemical parameters are measured at the beginning and the end of the tests, such as oxygen saturation, temperature, pH and conductivity.

Chemical analyses
Samples for chemical analyses were taken at the beginning and the end of every test. For several substances strong decreases in the actual concentrations are to be expected, for example already in the first few hours after addition of the stock solutions. It was therefore decided to start the actual tests by adding organisms 2 hours after addition of the stock solutions. As a consequence, also the samples taken for chemical analyses were taken after 2 hours. Depending on the volume needed to perform the chemical analyses (in relation to the required detection limit), samples were taken directly from the test solutions or from separate test vessels only meant for chemical analyses. These last replicates were off course incubated under the exact same conditions as the test replicates. For the tests with *Daphnia magna* additional samples have been taken in the third week. See table 4 for an overview.

The samples for chemical analyses were stored at 4°C in the dark and were all analysed within five working days. The laboratory of OMEGAM in Amsterdam performed the analyses.

Statistical analyses
Test results were evaluated with the statistical programme ToxCalc (Tidepool, 1995). Choices for the specific hypothesis test or point estimates were based on the flowcharts as provided by Toxcalc. Applied test are specified for each test in the appendices. Whenever possible, NOEC, LOEC, EC_{10}, EC_{50} and EC_{90} were calculated, based on actual concentrations.

Table 3 Summary of the test procedures.

Organism	Age at start	Duration (hrs)	No. replicas per conc.	Temp. (°C)	Medium	Test volume (ml)	Medium renewal	Food	Test parameters
Psammechinus miliaris	Embryos <4h after fertilization	48	3	20	Natural sea water	20	-	-	Larval development
Crassostrea gigas	Embryos <4h after fertilization	48	3	20	Natural sea water	20	-	-	Larval development
Daphnia magna	Juveniles <24 hours	21 days	10	20	Elendt M4	50	3 times / week	Daily algae suspension	Survival, reproduction
Pseudokirchneriella subcapitata	Exponential growing culture	96	3; all controls 6	23	ISO growth medium (fresh water)	20	-	-	Growth rate
Acartia tonsa	19 - 20 days	48	5	20	Natural sea water	20	-	-	Survival / immobility
Phaeodactylum tricornutum	Exponential growing culture	96	3; all controls 6	20	ISO growth medium (salt water)	20	-	-	Growth rate

Table 4 Overview of the samples taken for chemical analyses.

App	Substance	Species	Sampling at t= ... (hours)	Samples taken from test solution or from additional replicates	If additional replicates: which volume was used?	If additional replicates: where test organisms added?	Number of replicates sampled	Volume sampled (ml)
A.1	Alachlor	*Phaeodactylum tricornutum*	0 + 96 hrs	additional	500 ml	yes	1	130 ml
B.1	Anthracene	*Crassostrea gigas*	0 + 48 hrs	additional	250 ml	no	2	200 ml
B.2	Anthracene	*Psammechinus miliaris*	0 + 48 hrs	additional	250 ml	no	2	200 ml
C.1	Benzo(a)pyrene	*Crassostrea gigas*	0 + 48 hrs	additional	500 ml	no	2	500 ml
C.2	Benzo(a)pyrene	*Psammechinus miliaris*	0 + 48 hrs	additional	500 ml	no	2	500 ml
D.1	Benzo(k)fluoranthene	*Crassostrea gigas*	0 + 48 hrs	additional	250 ml	no	2	200 ml
D.2	Benzo(k)fluoranthene	*Daphnia magna*	0,2,19,21 days	additional	500 ml	no	1	250 ml
D.3	Benzo(k)fluoranthene	*Psammechinus miliaris*	0 + 48 hrs	additional	250 ml	no	2	200 ml

Table 4 CONTINUED: Overview of the samples taken for chemical analyses.

App	Substance	Species	Sampling at t= ... (hours)	Samples taken from test solution or from additional replicates	If additional replicates: which volume was used?	If additional replicates: where test organisms added?	Number of replicates sampled	Volume sampled (ml)
E.1	C10-C13 chloroalkanes	*Psammechinus miliaris*	0 + 48 hrs	additional	1000 ml	no	2	1000 ml
F.1	Hexachlorobutadiene	*Crassostrea gigas*	0 + 48 hrs	additional	25 ml	no	2	20 ml
F.2	Hexachlorobutadiene	*Daphnia magna*	0,2,19,21 days	test			10	50 ml
F.3	Hexachlorobutadiene	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml
G.1	Isoproturon	*Crassostrea gigas*	0 + 48 hrs	additional	25 ml	no	2	20 ml
G.2	Isoproturon	*Phaeodactylum tricornutum*	0 + 96 hrs	additional	500 ml	yes	1	130 ml
G.3	Isoproturon	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml
H.1	Lindane	*Acartia tonsa*	0 + 48 hrs	additional	1000 ml	no	1	20 ml
H.2	Lindane	*Crassostrea gigas*	0 + 48 hrs	additional	25 ml	no	2	20 ml
H.3	Lindane	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml
I.1	Naphtalene	*Crassostrea gigas*	0 + 48 hrs	additional	25 ml	no	2	20 ml
I.2	Naphtalene	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml
I.3	Naphtalene	*Pseudokierchneriella subcapitata*	0 + 96 hrs	additional	100 ml	yes	1	20 ml
J.1	Nonylphenol	*Crassostrea gigas*	0 + 48 hrs	additional	1000 ml	no	2	500 ml
J.2	Nonylphenol	*Psammechinus miliaris*	0 + 48 hrs	additional	1000 ml	no	2	500 ml
K.1	Pentachlorobenzene	Crassostrea gigas	0 + 48 hrs	additional	25 ml	no	2	20 ml
K.2	Pentachlorobenzene	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml
L.1	Tetrachloroethene	*Crassostrea gigas*	0 + 48 hrs	additional	25 ml	no	2	20 ml
L.2	Tetrachloroethene	*Psammechinus miliaris*	0 + 48 hrs	additional	25 ml	no	2	20 ml

2.2. Additional test procedures *Acartia tonsa*

Acute toxicity tests were performed according to ISO 14669, in which immobility of the organisms after 24 and 48 hours is assessed. Organisms were considered immobile, if they did not respond within 15 seconds to a gentle agitation of the test vial.

Test organisms were obtained from the Guernsey Sea Farms, Guernsey.

Natural seawater (± 32 ‰) is used as a blank and dilution medium. The test volume was 20 ml for each vial. Each concentration was tested in 5 replicates and with 5 individuals per replicate. The test temperature was 20 ± 1°C. Organisms were not fed during the exposure.

2.3. Additional test procedures *Crassostrea gigas*

Less than 4 hours after fertilization, toxicity tests are started when the embryos are in the 2-, 4- or 8-cell stages. Adult oysters were obtained from Guernsey Sea Farms, Guernsey. Normally 2-4 males and 2-4 females were selected. These conditioned oysters were stimulated to spawn by a temperature shock (20 – 25 – 20 – 25°C). If animals did not spawn, gametes were stripped from the gonads. Gametes (eggs and sperm cells) were collected in glass vessels. Solutions containing eggs were filtered and washed to remove excess of gonad tissue. After counting, 4 ml of sperm cells was added to a solution containing about 4000 eggs per ml. Every test vessel (20 ml) was inoculated with approximately 300 eggs. Three additional samples were directly fixated and used to assess the fertilization success. Tests are only considered valid if the fertilization is >70%.

In the beginning of the test fertilized eggs will settle down. Within a few hours however, free-swimming spherical shaped larvae (the so-called trochophore larvae) develop. After 12 to 48 hours veliger larvae develop from the trochophore larvae. In this larval stage an active swimming organ and a shell is developed. Normal developing veliger larvae form an oval shaped shell with a straight edge, the so-called D-larvae.

Natural sea water (32‰) was used as a control and dilution medium.

After 48 hours tests were ended by fixation using buffered formalin (37%, pH 7). The number of normally developed, malformed and retarded (i.e. delayed in their development) larvae were determined using a microscope.

The percentage of death, malformed and retarded larvae is corrected for the solvent control or the blank seawater by the formula of Abbott:

$$E = \frac{\frac{100 * (I - D_{test})}{I} - \frac{100 * (I - D_{ctr})}{I}}{100 - \frac{100 * (I - D_{ctr})}{I}}$$

In this formula E is the fraction of embryos that did not result in live larvae with completely developed shells adjusted for the controls, I is the number of embryos at the start of the test and D_{ctr} and D_{test} are the number of normally developed larvae in the control and the test concentrations. If E is 0, there is no effect compared to the control, if E is 100 there is a maximum affect, i.e. all larvae were death, malformed or retarded.
Statistical analyses were based on the percentage normal developed larvae as compared to the total number of larvae recovered at the end of the test. In toxicity tests with a low mortality (as was the case in all test performed here) this procedure increases the discriminatory power of the statistical analyses. Validity criteria are however specified (and checked) based on the percentage of death, malformed and retarded larvae as calculated using the Abbott formula.

2.4. Additional test procedures *Daphnia magna*

Daphnia magna was obtained from a laboratory culture (clone 4 as defined by Calow & Bradley, 1987), which has been maintained at the AquaSense laboratory for over 15 years. The test was performed with individuals less than 24 hours old and obtained from adults of approximately 3 weeks old.

During the test, all daphnids were fed daily with an increasing amount of algae suspension (*Scenedesmus* with yeast suspension[1] (10g / 75 mL) in response to the growth, i.e. 200 µl/test vessel from day 0 to 2, 300 µl/test vessel from day 3 to 5 and 400 µl/test vessel from day 6 and onwards.

The medium was renewed three times a week. During the test, several physical and chemical parameters were measured in both the old as well as the fresh medium.

In total, 4 series of samples have been taken for chemical analyses, i.e. fresh medium at T=0 and T=19 days and old medium at T=2 and 21 days. Separate vessels have been prepared for the chemical analyses of benzo(k)fluoranthene (500 ml). These vessels have been fed at the same rate as the test vessels.

[1] Ratio algae : yeastsuspension = 100 : 1

The survival and reproduction (qualitative, i.e. yes/no) is scored every day. At the renewals (three times a week) the number of juveniles is counted and the juveniles are removed.

With these data a ‘cohort life-table’ is prepared for every test concentration. This is a table with the number of juveniles per female during the test. From this ‘cohort life-table’ the average intrinsic population growth rate (r_m) is calculated for every test concentration as a measure for reproduction. Especially the moment of first reproduction (first brood) and the number of juveniles per brood are determinant for this parameter. The average r_m-values per concentration have been statistically evaluated in the way described in paragraph 2.1.

2.5. Additional test procedures *Phaeodactylum tricornutum* and *Pseudokirchneriella subcapitata*

An exponentially growing pre-culture was used as an inoculum for the test. This pre-culture was started 3 days before the beginning of the test. Growth medium was prepared by dissolving nutrient stocks in milli-Q or artificial sea water, as defined in the guidelines. The growth medium was inoculated with a small volume of algae suspension in order to maintain exponential growth until the start of the test.

In deviation from the guideline, the test duration was set at 96 hours instead of the usual 72 hours. With regard to this prolonged exposure the inoculation concentration at the start of the test was lowered to $5*10^3$ cells/ml (instead of 10^4 cells/ml) to prevent problems at the end of the test. For example, high algal densities might result in a lowered average growth rate or a too strong increase in pH.

The tests were performed in an incubator, continuously shaking at 200 rpm. Cell densities were measured by means of fluorescence at t=0, 48, 72 and 96 hours using a plate-reader (670 nm).

Based on the cell densities at the different time intervals, growth rates (μ) were calculated for each test concentration and replicate.

2.6. Additional test procedures *Psammechinus miliaris*

The test is started with embryos less than 4 hours after fertilization. These embryos are normally in the 2-, 4- and 8-cell stages.

Adult see urchins were obtained in the field, from an uncontaminated site in the Eastern Scheld, a tidal bay connected

with the North sea. In the laboratory they are conditioned to maturate by manipulating temperature, light and food regime.

Adult sea urchins were stimulated to spawn by injection of KCl (0.5 ml, 0.5 M). If spawning occurred the gametes (eggs and sperm cells) were collected in glass vessels. Sperm was collected ‘dry’ and activated by adding seawater. The eggs were rinsed three times with seawater before they were fertilized. Subsequently the number of fertilized eggs per ml was determined with a counting chamber. Every test vessel was inoculated with approximately 300 fertilized eggs.

Normal development of the fertilized eggs involves different stages. The egg cell starts dividing, and forms a free-swimming blastula in about 6 hours. In the blastula stage the cells are grouped at the outer side of the embyro, resulting in a hollow space, which is ciliated. In the next stage, the gastrula stage, a primitive intestinal canal is formed, as well as the beginning of skeleton parts. After 48 hours a pluteus larvae has been formed. This larval stage has a complete intestinal canal, skeleton and four fully-grown arms.

Natural seawater is used as a blank and dilution medium. In addition to the chemical analysis the Total Organic Carbon content of the seawater has been measured.

After 48 hours the test is ended by fixation of the test medium using 0.2 ml buffered formalin (37%, pH 7). The fixated larvae are examined under the microscope to determine the number of normal developed, malformed and retarded (i.e. delayed in their development) larvae. The percentage of death, malformed and retarded larvae is corrected for the solvent control or blank seawater by the formula of Abbott:

$$E = \frac{\frac{100 * (I - D_{test})}{I} - \frac{100 * (I - D_{ctr})}{I}}{100 - \frac{100 * (I - D_{ctr})}{I}}$$

In this formula E is the fraction of embryos that did not result in normally developed pluteus larvae adjusted for the controls, I is the number of embryos at the start of the test and D_{ctr} and D_{test} are the number of normally developed larvae in the control and the test concentrations. If E is 0, there is no effect compared to the control, if E is 100 there is a maximum effect, i.e. all larvae were death, malformed or retarded.
Statistical analyses were based on the percentage normal developed larvae as compared to the total number of larvae recovered at the end of the test. In toxicity tests with a low mortality (as was the case in all test performed here) this procedure increases the discriminatory power of the statistical analyses. Validity criteria are however specified (and checked) based on the percentage of death, malformed and retarded larvae as calculated using the Abbott formula.

3. Results

The results are discussed for each combination of ‘substance and organism in paragraphs A.1 - L.2. When applicable, the following aspects will be discussed:

- Any remarkable matters, such as deviations from the test method;
- An overview of nominal and actual test concentrations;
- Remarkable matters concerning the physical chemical parameters;
- Validity criteria;
- Test results
  A table with the results, i.e. NOEC, LOEC, EC_{10}, EC_{50} and EC_{90} values, including the 95% confidence limits, based on actual concentrations as well as dose - response graphs;

In the concomitant appendix the following information can be found:

- Results of the chemical analyses;
- The raw test data;
- Physical and chemical parameters;
- The elaboration of the test results in Toxcalc, i.e. NOEC, LOEC and EC values, based on actual concentrations.

Furthermore, the ASTM guidelines for the oyster larvae and the sea-urchin assay request that the Total Organic Carbon content of the seawater is measured during the experiments. This content has been measured three times with the following results: < 5, 8.5 and 11 mg C/l. Since both ASTM guidelines advise to keep the TOC content below 5 mg/l in the dilution water, this somewhat increased TOC content might have lowered the sensitivity of some of the toxicity tests. This variation in test result was however unexpected. TOC measurements were therefore not performed on each individual toxicity test with either sea-urchins or oyster larvae. If this research should be continued it is advised to perform TOC analyses for each individual toxicity test.

A.1 Alachlor, Phaeodactylum tricornutum

Nominal and actual test concentrations

An overview is presented in table A.1.1, illustrating that there was a good agreement between nominal and actual test concentrations. Furthermore, the decrease in test concentrations during the test was limited to a maximum of 14%.

Table A.1.1 Nominal and actual test concentrations of alachlor in the test with *Phaeodactylum tricornutum* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=96 hr.	Mean concentration (µg/l)	% loss of test compound T=0-T=96	µl/l acetone (nominal)
Blank	<0.02	<0.02	<0.02	--	0
Solvent Control	<0.02	<0.02	<0.02	--	89.2
0.18	0.12	0.17	0.15	-42	2.8
0.32	0.31	0.31	0.31	0	5.0
0.56	0.47	0.43	0.45	9	9.0
1.0	1.0	0.86	0.93	14	16.0
1.8	1.7	1.6	1.7	6	28.6
3.2	2.9	2.9	2.9	0	51.0
5.6	5.2	4.8	5.0	8	89.2

Validity criteria

The validity criteria applicable to the toxicity test with *Phaeodactylum tricornutum* are specified in table A.1.2 together with the actual values. As illustrated, the test is considered valid.

Table A.1.2 Validity criteria.

Parameters	Criteria	Actual values
Phaeodactylum tricornutum (ISO 10253)		
Increase in cell density after 72 hours	> 16	78
Variation in pH-value during the test	≤ 1.0	0.4

Test results

The average growth rate for each concentration together with the percentage inhibition (in relation to the solvent control) is presented in table A.1.3. Estimated parameters are presented in table A.1.4.

It is concluded that none of the tested concentration caused a significant effect on the growth rate of the marine algae *P. tricornutum*.

Table A.1.3 Results of the toxicity test after 4 days

Actual test concentration (µg/l)	Growth rate (mean)	(s.d.)	CV (%)	% inhibition on solvent control
Blank	1.38	0.01	1.0	
Solvent control	1.37	0.03	2.2	
0.15	1.40	0.02	1.4	-2.0
0.31	1.38	0.03	1.9	-1.0
0.45	1.37	0.04	3.1	0.1
0.93	1.40	0.00	0.2	-2.5
1.7	1.36	0.04	2.6	0.8
2.9	1.38	0.02	1.3	-0.9
5.0	1.40	0.03	2.3	-2.3

Table A.1.4 Estimated parameters (with 95% conf. limits if possible) for alachlor and *Phaeodactylum tricornutum* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 5	≥ 5	≥ 5	≥ 5	≥ 5

B.1 Anthracene, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table B.1.1, illustrating that there was a good agreement between nominal and actual test concentrations. Furthermore, the decrease in test concentrations during the test was limited to a maximum of 10%.

Note: these data are the same as presented for the test with *Psammechinus miliaris* (B.2), since both tests were performed simultaneously and with the same concentration series.

A strange result was noted for the measurements in the lowest test concentration. While the anthracene concentration was below detection limit at the beginning of the test (estimated concentration was 0.13 µg/l), a measurable concentration of 0.32 µg/l was reported at t=48 hours. According to the chemical laboratory OMEGAM, who carried out these analyses, this might be due to the small sample volumes, which were taken and as a consequence higher background signals. They for example also reported some other PAHs at t=0, while none of them were detected at the end of the experiments. Since even in the highest concentrations no significant effects were detected, these somewhat strange results did not influence the conclusion that the NOEC is higher than the highest test concentration (see below).

Table B.1.1 Nominal and actual test concentrations of anthracene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.01	< 0.01			
Solvent Control	< 0.02	< 0.02			63
0.32	< 0.15 (0.13)	0.32	0.23	< -146	6.3
0.56	0.22	0.60	0.41	-173	11
1.0	0.89	1.0	0.95	-12	20
1.8	1.6	1.8	1.7	-13	36
3.2	2.9	2.6	2.8	10	63

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table B.1.2 together with the actual values. As illustrated, the test is considered valid.

Table B.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	99 - 103

Test results
An overview of the percentage normal developed larvae in the different treatments is presented in table B.1.3. None of the tested concentrations did cause a significant effect on the larval development of the oyster *C. gigas*.

Table B.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	97.7	0.2	0.2	-2.8
Solvent control	98.4	1.6	1.6	
0.23	98.0	0.3	0.3	6.0
0.41	97.0	1.9	1.9	-4.1
0.95	98.3	0.8	0.8	-3.1
1.7	97.8	0.8	0.9	4.3
2.8	99.3	0.4	0.4	-2.4

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table B.1.4 Estimated parameters (with 95% conf. limits if possible) for anthracene and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8

B.2 Anthracene, *Psammechinus miliaris*

Nominal and actual test concentrations
An overview is presented in table B.2.1, illustrating that there was a good agreement between nominal and actual test concentrations. Furthermore, the decrease in test concentrations during the test was limited to a maximum of 10%.

Note: these data are the same as presented for the test with *Crassostrea gigas* (B.1), since both tests were performed simultaneously and with the same concentration series.

A strange result was noted for the measurements in the lowest test concentration. While the anthracene concentration was

below detection limit at the beginning of the test (estimated concentration was 0.13 µg/l), a measurable concentration of 0.32 µg/l was reported at t=48 hours. According to the chemical laboratory OMEGAM, who carried out these analyses, this might be due to the small sample volumes, which were taken and as a consequence higher background signals. They for example also reported some other PAHs at t=0, while none of them were detected at the end of the experiments.

Table B.2.1 Nominal and actual test concentrations of anthracene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.01	< 0.01			0
Solvent Control	< 0.02	< 0.02			63
0.32	< 0.15 (0.13)	0.32	0.23	< -146	6.3
0.56	0.22	0.60	0.41	-173	11
1.0	0.89	1.0	0.95	-12	20
1.8	1.6	1.8	1.7	-13	36
3.2	2.9	2.6	2.8	10	63

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table B.2.2 together with the actual values. As illustrated, the test is considered valid.

Table B.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	72 - 78

Test results

The percentage of normal developed larvae was in most concentrations comparable to both blank and solvent control and in the range of 86 – 94%. The only exception is formed by the treatment with 1.7 µg/l, which showed an average percentage of only 79%. Although one of the replicates showed a normal development, the other two replicates showed an increased number of malformed larvae. This resulted in an increased standard deviation, but a statistical difference was still detected. Since this malformation was not observed in the highest

concentration tested (2.8 µg/l), an interrupted dose-response relationship is present. Based on the absence of any adverse effects in the highest concentration, the NOEC is set at a level of [3] 2.8 µg/l.

Table B.2.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	90.9	4.2	4.6	7.8
Solvent control	91.9	6.1	6.6	
0.23	87.9	4.2	4.8	12.4
0.41	89.6	4.2	4.7	3.3
0.95	85.9	4.0	4.7	22.4
1.7	78.8	11.0	13.9	26.6
2.8	93.5	1.5	1.6	16.6

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table B.2.4 Estimated parameters (with 95% conf. limits) for anthracene and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8	≥ 2.8

C.1 Benzo(a)pyrene, *Crassostrea gigas*

Nominal and actual test concentrations
An overview is presented in table C.1.1, illustrating that a further decrease in the actual concentrations during the test occurred as expected. However, it is felt that the mean actual concentrations can still be used to calculate NOEC values.

An increased detection limit was reported for the lowest test concentration, due to a small sample volume and, as a consequence, increased background noise. The estimated value is used for further calculations.

Note: these data are the same as presented for the test with *Psammechinus miliaris* (C.2), since both tests were performed simultaneously and with the same concentration series.

Table C.1.1 Nominal and actual test concentrations of benzo(a)pyrene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.01	< 0.01			0
Solvent Control	< 0.02	< 0.03			32
0.32	< 0.15 (0.14)	0.06	0.10	57	3.2
0.56	0.32	0.07	0.20	78	5.6
1.0	0.84	0.46	0.65	45	10
1.8	1.4	0.91	1.2	35	18
3.2	2.1	1.1	1.6	48	32

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table C.1.2 together with the actual values. As illustrated, the test is considered valid.

Table C.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	95 - 100

Test results

An overview of the percentage normal developed larvae in the different treatments is presented in table C.1.3. None of the tested concentrations did cause a significant effect on the larval development of the oyster *C. gigas*. The NOEC value is therefore set at [3] 1.6 µg/l, the highest concentration tested.

Table C.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	97.5	2.0	2.0	5.1
Solvent control	96.2	1.6	1.7	
0.10	97.9	0.9	1.0	-1.9
0.20	97.8	0.9	0.9	0.7
0.65	98.5	0.8	0.8	1.2
1.2	97.7	0.5	0.5	1.5
1.6	97.5	0.8	0.8	3.9

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table C.1.4 Estimated parameters (with 95% conf. limits) for benzo(a)pyrene and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6

C.2 Benzo(a)pyrene, *Psammechinus miliaris*

Nominal and actual test concentrations
An overview is presented in table C.2.1, illustrating that a further decrease in the actual concentrations during the test occurred as expected. However, it is felt that the mean actual concentrations can still be used to calculate NOEC and EC_{50}-values.

An increased detection limit was reported for the lowest test concentration, due to a small sample volume and, as a consequence, increased background noise. The estimated value is used for further calculations.

Note: these data are the same as presented for the test with *Crassostrea gigas* (C.1), since both tests were performed simultaneously and with the same concentration series.

Table C.2.1 Nominal and actual test concentrations of benzo(a)pyrene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.01	< 0.01			0
Solvent Control	< 0.02	< 0.03			32
0.32	< 0.15 (0.14)	0.06	0.10	57	3.2
0.56	0.32	0.07	0.20	78	5.6
1.0	0.84	0.46	0.65	45	10
1.8	1.4	0.91	1.2	35	18
3.2	2.1	1.1	1.6	48	32

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table C.2.2 together with the actual values. As illustrated, the test is considered valid.

Table C.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	71 - 72

Test results

An overview of the percentage normal developed larvae in the different treatments is presented in table C.2.3. None of the tested concentrations did cause a significant effect on the larval development of the marine sea-urchin *P. miliaris*. The NOEC value is therefore set at ^{3} 1.6 µg/l, the highest concentration tested.

Table C.2.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	90.9	4.2	4.6	-1.5
Solvent control	90.4	1.4	1.6	
0.10	85.7	5.6	6.5	-9.1
0.20	89.9	3.3	3.7	0.0
0.65	84.3	3.2	3.7	-24.2
1.2	82.8	4.1	4.9	-4.1
1.6	90.3	3.3	3.6	-19.2

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table C.1.4 Estimated parameters (with 95% conf. limits) for benzo(a)pyrene and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6	≥ 1.6

D.1 Benzo(k)fluoranthene, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table D.1.1, illustrating that at the start of the toxicity test (2 hours after preparation of test solutions) actual concentrations were already somewhat decreased. This reduction in the concentrations was highest in the highest test concentration (60%) and gradually decreased for the lower test concentrations (down to 6% in the lowest concentration). As expected, this decrease continued during the toxicity test. As a consequence actual concentrations at t=48 were on average 33% lower as compared to t=0, while no differences in the percentage were observed between the concentrations. However, it is felt that the mean actual concentrations can still be used to calculate NOEC and EC_{50}-values. As a consequence of the small sample volume taken, the analytical laboratory reported an increased background noise and problems with the matrix. Low concentrations of other PAHs were detected in some of the concentrations at t=0, while none of them were present at t=48 hours.

Table D.1.1 Nominal and actual test concentrations of benzo(k)fluoranthene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.02	< 0.01			0
Solvent Control	< 0.01	< 0.02			88.8
0.18	0.17	0.11	0.14	35	8.9
0.32	0.26	0.17	0.22	35	15.8
0.56	0.40	0.28	0.34	30	27.6
1.0	0.60	0.39	0.50	35	49.4
1.8	0.74	0.50	0.62	32	88.8

Validity criteria

The validity criteria applicable to toxicity test with *Crassostrea gigas* are specified in table D.1.2 together with the actual values. As illustrated, the test is considered valid.

Table D.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	97 – 107

Test results

An overview of the percentage normal developed larvae in the different treatments is presented in table D.1.3. None of the tested concentrations did cause a significant effect on the larval development of the oyster *C. gigas*. The NOEC value is therefore set at ³ 0.62 µg/l, the highest concentration tested.

Table D.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	99.0	1.4	1.4	-9.8
Solvent control	99.4	0.4	0.4	
0.14	97.1	2.4	2.4	-13.4
0.22	98.0	0.9	0.9	-15.8
0.34	98.7	0.9	1.0	-12.3
0.50	97.6	0.9	0.9	1.5
0.62	96.5	0.8	0.8	-13.8

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table D.1.4 Estimated parameters (with 95% conf. limits) for benzo(k)fluoranthene and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 0.62	≥ 0.62	≥ 0.62	≥ 0.62	≥ 0.62

D.2 Benzo(k)fluoranthene, *Daphnia magna*

Nominal and actual test concentrations

An overview is presented in table D.2.1, illustrating that at the start of the toxicity test (2 hours after preparation of test solutions) actual concentrations were already somewhat decreased. This reduction was highest in the lowest test concentration (34%) and gradually decreased for the higher test concentrations. As expected, this decrease continued during the toxicity test. As a consequence actual concentrations at t=2d and 21d were on average 37% lower as compared to t=0 and t=19. This loss of test compound during the test was somewhat higher at higher test concentrations. However, it is concluded that the mean actual concentrations can be used to calculate NOEC and EC_{50}-values.

Table D.2.1 Nominal and actual test concentrations of benzo(k)fluoranthene in the test with *Daphnia magna* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=2 d	Actual concentration (µg/l) T=19 d	Actual concentration (µg/l) T=21 d	Mean actual concentration (µg/l)	% loss of test-compound T=0/19-T=2/21	µl/l acetone (nominal)
Blank	<0.04	<0.04	<0.01	<0.01	<0.02		0
Solvent Control	<0.04	<0.03	<0.01	<0.01	<0.02		173
0.32	0.23	0.15	0.19	0.13	0.18	33	17.4
0.56	0.44	0.31	0.38	0.23	0.34	34	30.4
1.0	0.78	0.52	0.70	0.54	0.64	28	54.2
1.8	1.7	1.0	1.4	0.63	1.2	47	97.4
3.2	2.8	2.0	2.7	1.3	2.2	40	173

Validity criteria

The validity criteria applicable to toxicity test with *Daphnia magna* are specified in table D.2.2 together with the actual values. As illustrated, the test is considered valid.

Table D.2.2 Validity criteria.

Parameters	Criteria	Actual values
Daphnia magna (ISO 10706)		
Adult mortality and male development in blanc and control replicates	≤ 20%	0 – 10 %
Mean number of living offspring per living parent in blanc and control	≥ 60	73 – 74
Coefficient of variation (CV) based on the number of offspring per parent in blanc and control	≤ 20%	7 – 14 %

Test results

An overview of the average mortality, offspring and the average intrinsic population growth rate (r_m) in the different treatments is presented in table D.2.3. As illustrated, none of the tested concentrations caused a significant effect on *Daphnia magna*. The NOEC value is therefore set at ^{3} 2.2 µg/l, the highest concentration tested.

Remark

A strong decrease in oxygen concentration was observed in most test concentrations, especially after the first week. This decrease was also observed in the solvent control. Since there were no significant effects in the solvent control as compared to the blank, it can be concluded that the low oxygen concentration did not directly influence the test results.

Table D 2.3 Results of the toxicity test

Actual test concentration (µg/l)	Mortality (%) (mean)	Offspring (mean)	Offspring (s.d)	R_m (mean)	R_m (s.d.)	R_m CV (%)
Blank	0	73	5	0.393	0.022	7.3
Solvent control	10	74	11	0.393	0.014	14
0.18	0	80	11	0.389	0.019	13
0.34	0	73	15	0.387	0.011	21
0.64	20	68	17	0.405	0.021	25
1.2	10	67	6	0.358	0.013	8.7
2.2	0	67	15	0.382	0.009	22

Table D.2.4 Estimated parameters (with 95% conf. limits) for benzo(k)fluoranthene and *Daphnia magna* based on actual concentrations (average of four measurements).

	NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
Survival	≥ 2.2	≥ 2.2	≥ 2.2	≥ 2.2	≥ 2.2
Reproduction	≥ 2.2	≥ 2.2	≥ 2.2	≥ 2.2	≥ 2.2

D.3 Benzo(k)fluoroanthene, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table D.3.1, illustrating that there was in general a reasonable agreement between nominal and actual test concentrations. Furthermore, the losses during the toxicity test were limited to at maximum 21%.

Table D.3.1 Nominal and actual test concentrations of benzo(k)fluoranthene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.01	< 0.01			0
Solvent Control	< 0.01	< 0.01			158
0.32	0.28	0.22	0.25	21	16
0.56	0.47	0.43	0.45	9	28
1.0	0.82	0.72	0.77	12	49
1.8	1.5	1.2	1.4	20	89
3.2	2.7	2.5	2.6	7	158

Validity criteria

The validity criteria applicable to toxicity test with *Psammechinus miliaris* are specified in table D.3.2 together with the actual values. As illustrated, the test is considered valid.

Table D.3.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	80

Test results

The percentage of normal developed larvae was in most concentrations comparable to both blank and solvent control with a range of 87 - 89%. The only exception is formed by the lowest concentration of 0.25 µg/l, which showed an average percentage of only 72%. Although one of the replicates showed a rather normal development (82%), the other two replicates showed an increased number of malformed and retarded larvae. This resulted in an increased standard deviation, but a statistical difference was still detected. Based on the absence of any adverse effects in all other (higher) concentrations, the NOEC is set at a level of ³ 2.6 µg/l, the highest concentration tested.

Table D.3.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	87.8	0.4	0.4	-0.6
Solvent control	86.6	1.7	1.9	
0.25	72.4	8.6	11.9	22.5
0.45	89.2	3.6	4.0	-9.5
0.77	88.8	0.3	0.3	-1.0
1.4	87.3	0.8	0.9	-1.9
2.6	87.8	1.6	1.9	-0.9

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table D.3.4 Estimated parameters (with 95% conf. limits) for benzo(k)fluoranthene and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 2.6	≥ 2.6	≥ 2.6	≥ 2.6	≥ 2.6

E.1 C10-C13 chloroalkanes, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table E.1.1, illustrating that the actual concentrations were around 50% lower as compared to nominal values. However, losses during the toxicity test were minimal. NOEC values can therefore be based upon the mean actual concentration.

Note: The C10-C13 chloroalkanes used for the toxicity test were delivered solved in hexane. To prevent the use of the very toxic hexane as a solvent control, the chloroalkanes were (prior to the test) transferred into acetone. Simultaneously, the same procedure was applied to a sample of unpolluted, HPLC-grade hexane. This samples, transferred to acetone, was used as solvent control.

Table E.1.1 Nominal and actual test concentrations of C10-C13 chloroalkanes in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.5	< 0.5			
Solvent Control	< 0.5	< 0.5			280
5.6	2.4	2.2	2	8	28
10	5.3	4.6	5	13	50
18	7.3	7.9	8	-8	90
32	14	11	13	21	160
56	23	19	21	17	280

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table E.1.2 together with the actual values. As illustrated, the test is considered valid.

Table E.1.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	80

Test results

An overview of the percentage normal developed larvae in the different concentrations is presented in table E.1.3. None of the tested concentrations did cause a significant effect on the larval development of the marine sea-urchin *P. miliaris*. The NOEC value is therefore set at ³ 21 µg/l, the highest concentration tested.

Table E.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	87.5	1.6	1.8	0.0
Solvent control	86.6	1.7	13.9	
2.3	76.2	10.6	13.9	1.5
5.0	87.8	2.4	2.7	-10.2
7.6	89.4	4.1	4.6	-6.0
13	86.3	10.2	11.8	0.9
21	87.3	4.6	5.3	-8.1

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table E.1.4 Estimated parameters (with 95% conf. limits) for C10-C13 chloroalkanes and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 21	≥ 21	≥ 21	≥ 21	≥ 21

F.1 Hexachlorobutadiene, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table F.1.1. As expected, all actual concentrations at the beginning of the experiment were considerably lower compared to the nominal values. Starting the test two hours after preparation of the test solutions has therefore been an appropriate measure to limit the decrease in actual concentrations during the toxicity test. Still, actual concentrations showed a further decrease with percentages varying between 36 and 70%. This off course limits the ease at which test results can be interpreted. Since none of the tested concentrations did cause a significant effect (see table F.1.3), it is however felt that the NOEC value can still be considered trustworthy.

Note: these chemical data are the same as presented for the test with *Psammechinus miliaris* (F.3), since both tests were performed simultaneously and with the same concentration series.

Table F.1.1 Nominal and actual test concentrations of hexachlorobutadiene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.03	<0.06			0
Solvent Control	< 0.03	< 0.03			99
6	0.64	0.31	0.48	52	6.2
12	0.39	0.25	0.32	36	12
24	4.6	1.4	3.0	70	25
48	9.9	4.8	7.4	52	50
96	29	12	21	59	99

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table F.1.2 together with the actual values. As illustrated, the test is considered valid.

Table F.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	91 - 98

Test results

An overview of the percentage normal developed larvae in the different treatments is presented in table F.1.3. None of the tested concentrations did cause a significant effect on the larval development of the oyster *C. gigas*. The NOEC value is therefore set at ³ 21 µg/l, the highest concentration tested.

Table F.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	99.0	0.9	0.9	6.8
Solvent control	98.6	0.7	0.7	
0.48	99.1	0.6	0.6	-15.1
0.32	98.0	0.8	0.9	-0.8
3.0	98.3	0.4	0.4	-9.9
7.4	98.7	0.5	0.5	11.3
21	98.7	0.5	0.5	-4.3

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table F.1.4 Estimated parameters (with 95% conf. limits if possible) for hexachlorobutadiene and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 21	≥ 21	≥ 21	≥ 21	≥ 21

F.2 Hexachlorobutadiene, *Daphnia magna*

Nominal and actual test concentrations

An overview is presented in table F.2.1, illustrating that at the start of the toxicity test (2 hours after preparation of test solutions) actual concentrations were already strongly reduced (around 80%). This reduction occurred independent from the test concentrations. As expected, this decrease continued during the toxicity test. As a consequence actual concentrations at t=2d and 21d were on average 71% lower as compared to t=0 and t=19. This off course limits the easy at which test results can be interpreted.

Table F.2.1 Nominal and actual test concentrations of hexachlorobutadiene in the test with *Daphnia magna* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=2 d	Actual concentration (µg/l) T=19 d	Actual concentration (µg/l) T=21 d	Mean actual concentration (µg/l)	% loss of test-compound T=0/19-T=2/21	µl/l acetone (nominal)
Blank	< 0.02	< 0.06	< 0.02	< 0.02	< 0.03		0
Solvent Control	< 0.02	< 0.04	< 0.02	< 0.02	< 0.02		332
12	3.5	0.91	1.3	0.3	1.5	75	10
24	3.5	1.2	3.9	1.1	2.4	69	21
48	4.9	2.5	8.0	2.3	4.4	63	41
96	13	4.0	16	3.2	9.1	75	83
192	34	9.5	26	7.6	19	72	166
384	70	21	*)	*)	46	70	332

*) No surviving adults at this moment in time

Validity criteria

The validity criteria applicable to toxicity tests with *Daphnia magna* are specified in table F.2.2 together with the actual values. As illustrated, the test is considered valid.

Table F.2.2 Validity criteria.

Parameters	Criteria	Actual values
Daphnia magna (ISO 10706)		
Adult mortality and male development in blanc and control replicates	≤ 20%	0 – 10 %
Mean number of living offspring per living parent in blanc and control	≥ 60	72 - 78
Coefficient of variation (CV) based on the number of offspring per parent in blanc and control	≤ 20%	11 – 15 %

Test results

An overview of the average mortality, offspring and the intrinsic population growth rate (r_m) in the different concentrations is presented in table F.2.3. Increased mortality was observed in the two highest concentrations, 19.3 and 45.5 µg/l. Strong mortality was especially observed in the highest concentration, in which 100% mortality was reached within 3 days. Significant effects on the reproduction were also observed in one concentration step lower, being 9.1 µg/l. Estimated test parameters are presented in table F2.4, while in addition also the dosis effect curve is presented.

Remark

A strong decrease in oxygen concentration was observed in most test concentrations. This decrease was also observed in the

solvent control. Since there were no significant effects in the solvent control as compared to the blank, it can be concluded that the low oxygen concentration did not directly influence the test results. Furthermore, the strong mortality in the highest test concentration occurred within the first 3 days. At that time, a reduction in oxygen levels below critical limits was not yet detected. Oxygen levels started to fall after the first 7-10 days.

Table F 2.3 Results of the toxicity test

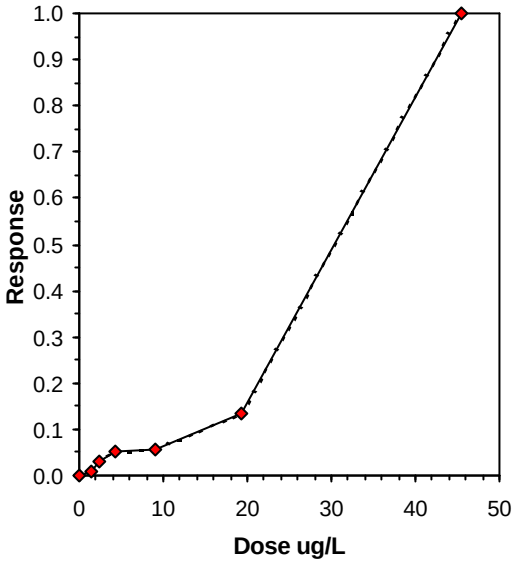
Actual test concentration (µg/l)	Mortality (%) (mean)	Offspring (mean)	Offspring (s.d)	R_m (mean)	R_m (s.d.)	R_m CV (%)
Blank	0	72	8	0.390	0.025	11
Solvent control	10	78	12	0.387	0.015	15
1.5	10	84	10	0.383	0.009	11
2.4	10	74	11	0.375	0.021	15
4.4	10	78	10	0.367	0.009	13
9.1	0	70	8	0.365	0.010	11
19.3	40	60	7	0.335	0.042	12
45.5	100*	0	-	0.000	-	-

*: 100% mortality was already observed within 3 days

Table F.2.4 Estimated parameters (with 95% conf. limits) for hexachlorobutadiene and *Daphnia magna* based on actual concentrations (average of four measurements).

	NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
Survival	9.1	19	*)	22	*)
Reproduction	4.4	9.1	14	30	42

*) not calculated due to the absence of more than one concentration with a partial response



F.3 Hexachlorobutadiene, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table F.3.1. As expected, all actual concentrations at the beginning of the experiment were considerably lower compared to the nominal values. Starting the test two hours after preparation of the test solutions has therefore been an appropriate measure to limit the decrease in actual concentrations during the toxicity test. Still, actual concentrations showed a further decrease with percentages varying between 36 and 70%. This off course limits the easy at which test results can be interpreted. However, since the NOEC is set at the highest concentration tested (see below), it is felt that this value can still be considered trustworthy.

Note: these chemical data are the same as presented for the test with *Crassostrea gigas* (F.1), since both tests were performed simultaneously and with the same concentration series.

Table F.3.1 Nominal and actual test concentrations of hexachlorobutadiene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.03	<0.06			0
Solvent Control	< 0.03	< 0.03			99
6	0.64	0.31	0.48	52	6.2
12	0.39	0.25	0.32	36	12
24	4.6	1.4	3.0	70	25
48	9.9	4.8	7.4	52	50
96	29	12	21	59	99

Validity criteria

The validity criteria applicable to toxicity test with *Psammechinus miliaris* are specified in table F.3.2 together with the actual values. As illustrated, the test is considered valid.

Table F.3.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	72 - 74

Test results

The results of the toxicity test are presented in table F.3.3 and illustrate that the percentage normal developed larvae were in general somewhat lower as compared to both the blank and the solvent control. Significant differences were however only found for the lowest concentration tested with an actual concentration of 0.48 µg/l. The second concentration (nominal concentration of 12) turned out to have a somewhat lower mean actual concentration (0.32, see table F.3.1). This treatment did not cause significant effects on the larval development. Furthermore, also in the highest concentration tested (21 µg/l) no adverse effects were found. The NOEC value is therefore set at ^{3} 21 µg/l, the highest concentration tested.

Table F.3.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	90.9	4.2	4.6	2.1
Solvent control	86.6	7.1	8.2	
0.48	75.5	0.7	1.0	7.9
0.32	77.3	5.7	7.4	0.4
3.0	78.3	4.8	6.2	11.9
7.4	81.8	3.2	3.9	7.5
21	87.5	4.9	5.6	-7.0

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table F.3.4 Estimated parameters (with 95% conf. limits) for hexachlorobutadiene and *Psammechinus miliaris* based on actual concentrations (average of two measurements). ≥

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 21	≥ 21	≥ 21	≥ 21	≥ 21

G.1 Isoproturon, *Crassostrea gigas*

Nominal and actual test concentrations
An overview is presented in table G.1.1, illustrating that there was in general a good agreement between nominal and actual test concentrations. Furthermore, there was no decrease in test concentrations during the test.

Table G.1.1 Nominal and actual test concentrations of isoproturon in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.2	< 0.15			0
Solvent Control	< 0.04	< 0.14			50.2
56	34	38	36	-12	5.0
100	96	99	98	-3	9.0
180	140	160	150	-14	16.1
320	280	310	295	-11	28.7
560	500	540	520	-8	50.2

Validity criteria

The validity criteria applicable to toxicity test with *Crassostrea gigas* are specified in table G.1.2 together with the actual values. As illustrated, the test is considered valid.

Table G.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	108 - 114

Test results

In all concentrations, the percentage of normal developed larvae was higher than 90% (see table G.1.3). Still there was a slight effect in the three highest concentrations, in which the larval development was inhibited with (at most) 5% as compared to the solvent control. Due to a low variability within the replicates, these effects were statistical significant. The estimated EC_{10}-value is however 3730 µg/l and as such much higher than the highest concentration tested (520 µg/l).

Estimated parameters are presented in table G.1.4.

Table G.1.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	99.4	0.3	0.3	5.0
Solvent control	99.3	0.5	0.5	
36	96.9	3.0	3.1	8.7
98	98.3	1.3	1.3	3.2
150	96.6	1.4	1.4	5.6
295	95.4	1.2	1.3	0.9
520	94.1	1.2	1.2	10.4

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table G.1.4 Estimated parameters (with 95% conf. limits) for isoproturon and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
98	150	≥ 520	≥ 520	≥ 520

G.2 Isoproturon, *Phaeodactylum tricornutum*

Nominal and actual test concentrations

An overview is presented in table G.2.1, illustrating that there was a good agreement between nominal and actual test concentrations. Furthermore, the decrease in test concentrations during the test was limited to a maximum of only 6%.

Table G.2.1 Nominal and actual test concentrations of isoproturon in the test with *Phaeodactylum tricornutum* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=96 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=96	µl/l acetone (nominal)
Blank	<0.01	<0.01	<0.01	-	0
Solvent Control	<0.01	<0.01	<0.01	-	71.4
1	0.89	0.89	0.89	0	7.2
1.8	1.8	1.7	1.8	6	12.8
3.2	2.9	2.9	2.9	0	22.8
5.6	5.6	5.7	5.7	-2	40.0
10	10	10	10	0	71.4

Validity criteria

The validity criteria applicable to the toxicity test with *Phaeodactylum tricornutum* are specified in table G.2.2 together with the actual values. As illustrated, the test is considered valid.

Table G.2.2 Validity criteria.

Parameters	Criteria	Actual values
Phaeodactylum tricornutum (ISO 10253)		
Increase in cell density after 72 hours	> 16	79
Variation in pH-value during the test	≤ 1.0	0.4

Test results

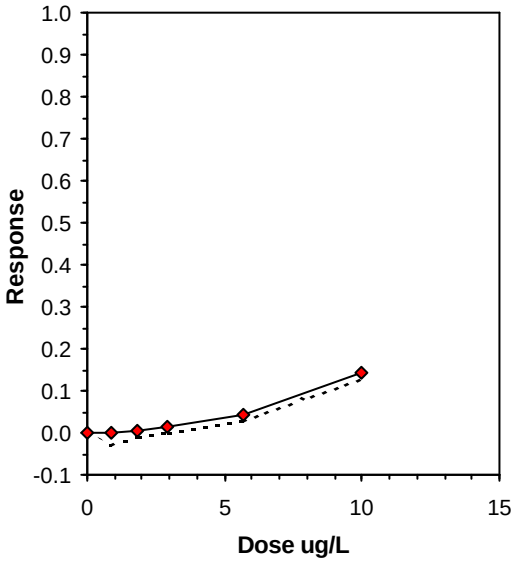
The average growth rate for each concentration together with the percentage inhibition (in relation to the solvent control) is presented in table G.2.3. Significant effects on the growth rate of the algae were only observed in the highest concentration tested (10 µg/l), where the growth was reduced by on average 13%. Estimated parameters are presented in table G.2.4.

Table G.2.3 Results of the toxicity test after 4 days.

Actual test concentration (µg/l)	Growth rate (mean)	(s.d.)	CV (%)	% inhibition on solvent control
Blank	1.35	0.04	2.9	
Solvent control	1.37	0.04	2.8	
0.89	1.41	0.01	0.7	-3.0
1.8	1.38	0.02	1.8	-1.0
2.9	1.37	0.02	1.3	0.0
5.7	1.33	0.03	2.4	3.0
10	1.19	0.06	5.3	12.9

Table G.2.4 Estimated parameters (with 95% conf. limits) for isoproturon and *Phaeodactylum tricornutum* based on actual concentrations (average of two measurements), together with the dose response curve.

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
5.7	10	8.1 (6.6 – 10.3)	≥ 10	≥ 10



G.3 Isoproturon, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table G.3.1, illustrating that there was a good agreement between nominal and actual test concentrations. Furthermore, the decrease in test concentrations during the test was limited to a maximum of only 6%.

Table G.3.1 Nominal and actual test concentrations of isoproturon in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.10	< 0.41			
Solvent Control	< 0.10	< 0.10			50
56	52	52	52	0	5.0
100	99	99	99	0	9.0
180	170	180	175	-6	16
320	320	300	310	6	29
560	540	570	555	-6	50

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table G.3.2 together with the actual values. As illustrated, the test is considered valid.

Table G.3.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	84 - 95

Test results

A significant increased number of retarded larvae was observed in the highest concentration tested (555 µg/l). The NOEC value is therefore 310 and the estimated EC_{10}-value 502.7 µg/l. No EC_{50}-values could be estimated.

Table G.3.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	85.0	2.2	2.5	11.3
Solvent control	86.0	2.0	2.3	
52	84.8	1.8	2.1	14.9
99	86.1	0.9	1.0	7.4
175	88.3	2.2	2.4	13.9
310	84.4	2.0	2.4	7.4
555	74.8	2.6	3.5	26.5

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table G.3.4 Estimated parameters (with 95% conf. limits) for isoproturon and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
310	555	502.7 (396.4 – 551.4)	≥ 555	≥ 555

H.1 Lindane, *Acartia tonsa*

Nominal and actual test concentrations

An overview is presented in table H.1.1, illustrating that the actual concentrations at t=0 were in general around 35-40% lower as compared to the nominal values. However, there was no further decrease in the test concentrations during the toxicity test. Therefore the mean actual concentrations are considered to be a good indication of the exposure level during the test.

Table H.1.1 Nominal and actual test concentrations of lindane in the acute test with *Acartia tonsa* as well as the nominal concentrations acetone in the testsolutions.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	<0.03	<0.04			0
Solvent Control	<0.04	<0.05			54.0[1]
1.8	1.4	1.0	1.2	29	3.0
3.2	2.1	2.0	2.1	5	5.4
5.6	3.1	3.7	3.4	-19	9.4
10	5.2	4.9	5.1	6	16.7
18	14	13	14	7	30.1
32	15	20	18	-33	54.0

[1]: Due to toxicity in the highest acetone concentrations each acetone concentration used for the test solutions was also tested as solvent control.

Validity criteria

The validity criteria applicable to acute toxicity tests with *Acartia tonsa* are specified in table H.1.2 together with the actual values. As illustrated, the test is considered valid.

Table H.1.2 Validity criteria.

Parameters	Criteria	Actual values
Acartia tonsa (ISO 14669)		
Immobile organisms in the control at the end of the test (%)	≤ 10	8

Test results

An overview of the percentage mobility in the different treatments is presented in table H.1.3. Since a significant toxicity was observed in the highest acetone concentrations tested, mobility in the test concentrations as well as in the solvent controls is presented.

Due to the significant effects of acetone, statistical analyses were also performed on the concentration series of acetone. The NOEC value for acetone was 16.7, with a LC_{50}-value of 56.5 µl/l. As significant effects of acetone alone were observed in the two highest concentrations, these concentrations were not taken into consideration for the statistical analyses on the effects of lindane.

Strong effects of lindane were already observed in the lowest concentration tested, due to which a NOEC value could not be estimated. The LC_{50}-value is 1.5 µg/l. It should however been noted, that even in these rather low concentrations the acetone

control exceeded the validity criteria for control mortality (<10%, while a mortality of 11-14% was observed). This might have increased the sensitivity of the organisms.

Table H.1.3 Results of the toxicity test.

	Mobility at the end of the test (%)			
	Lindane			Acetone
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**(mean)**
Blank	92	11.0	11.9	-
1.2	48	14.5	30.1	86
2.1	42	4.5	10.6	89
3.4	41	17.9	43.3	82
5.1	32	11.0	34.2	80
14	3	7.5	223.6	57
18	0	0	-	45

Table H.2.4 Estimated parameters (with 95% conf. limits) for lindane and *Acartia tonsa* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≤ 1.2	1.2	≤ 1.2	1.5	≥ 5.1

H.2 Lindane, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table H.2.1, illustrating that the actual concentrations were in general around 40% lower as compared to the nominal values. However, there was no further decrease in the test concentrations during the toxicity test. Therefore the mean actual concentrations are considered to be a good indication of the exposure level for the oyster larvae.

Table H.2.1 Nominal and actual test concentrations of lindane in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.1	< 0.1			
Solvent Control	< 0.1	< 0.1			182.6
56	11	11	11	0	18.3
100	62	61	62	2	32.6
180	120	120	120	0	58.7
320	200	240	220	-20	104.4
560	480	420	450	13	182.6

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table H.2.2 together with the actual values. As illustrated, the test is considered valid.

Table H.2.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	116 - 122

Test results

An overview of the percentage normal developed larvae in the different treatments is presented in table H.2.3. None of the tested concentrations did cause a significant effect on the larval development of the oyster *C. gigas*. The NOEC value is therefore set at ³ 450 µg/l, the highest concentration tested.

Table H.2.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	91.0	2.3	2.5	-4.8
Solvent control	96.6	0.3	0.3	
11	97.6	0.4	0.4	-4.4
62	97.6	1.3	1.3	-10.6
120	99.4	0.7	0.7	1.9
220	96.5	1.5	1.6	-9.0
450	97.6	0.7	0.7	1.1

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table H.2.4 Estimated parameters (with 95% conf. limits) for lindane and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 450	≥ 450	≥ 450	≥ 450	≥ 450

H.3 Lindane, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table H.3.1, illustrating that actual concentrations were in general around 50% lower as compared to nominal values. However, there was no further decrease in the test concentrations during the toxicity test, even an increase was noted. Therefore the mean actual concentrations are considered to be a good indication of the exposure level.

Table H.3.1 Nominal and actual test concentrations of lindane in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.04	0.18			0
Solvent Control	< 0.05	0.05			124.3
100	41	64	53	-56	12.4
180	50	120	85	-140	22.4
320	180	250	215	-39	39.8
560	260	370	315	-42	69.6
1000	580	780	680	-34	124.3

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table H.3.2 together with the actual values. As illustrated, the test is considered valid.

Table H.3.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	79 - 80

Test results

The percentage normal developed pluteus larvae was in all test concentrations above 80% (rane: 83 – 89). No statistical significant differences were observed between the blank and the solvent control as well as with any of the test concentrations. NOEC value is therefore set at the highest concentration tested.

Table H.3.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	84.0	3.5	4.2	0.6
Solvent control	84.3	1.7	2.0	
53	87.8	0.4	0.4	-12.7
85	84.6	1.0	1.2	3.5
215	88.2	1.5	1.7	-1.1
315	88.9	3.3	3.7	-2.2
680	83.4	3.0	3.6	6.3

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table H.3.4 Estimated parameters (with 95% conf. limits) for lindane and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 680	≥ 680	≥ 680	≥ 680	≥ 680

I.1 Naphtalene, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table I.1.1, illustrating that at the start of the toxicity test (2 hours after preparation of test solutions) actual concentrations of naphtalene have already decreased by on average 40%. As expected, this decrease continued during the toxicity test and all values were below detection levels after 48 hours. This strongly limits the applicability of the test results.

Note: As a consequence of the small sample volume taken, the analytical laboratory reported an increased background noise and problems with the matrix. An increased detection limit was therefore reported for the lowest test concentrations and the blanks. Furthermore, low concentrations of other PAHs were detected in some of the concentrations at t=48, while none of them were present at t=0 hours.

Table I.1.1 Nominal and actual test concentrations of naphtalene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 10.8	< 10.4			0
Solvent Control	< 11.3	< 9.3			95
30	< 10.6	< 10.4	< 10.5	> 1	6.0
60	33	< 14.9	< 24	> 54.7	12
120	88	< 0.05	< 44	> 99.9	24
240	140	< 0.05	< 70	100	48
480	310	< 0.05	< 155	100	95

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table I.1.2 together with the actual values. As illustrated, the test is considered valid.

Table I.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	111 - 114

Test results

In all test concentrations the percentage of normal developed larvae was higher than 90% (see table I.1.3) and no clear differences with the development in either the blank or the solvent control were observed. The interpretation of these results is however strongly hampered by the strong reduction in the actual concentrations during the test. It can only be concluded that an initial concentration up to 310 µg/l did not cause any negative effects.

Table I.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Nominal test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	94.9	1.6	1.7	2.5
Solvent Control	95.7	1.3	1.3	
30	92.8	2.5	2.7	0.2
60	95.2	0.7	0.7	-19.8
120	97.0	1.0	1.1	-10.8
240	97.5	1.1	1.1	-9.3
480	95.3	3.8	4.0	-20.5

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

I.2 Naphtalene, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table I.2.1, illustrating that all actual concentrations at the start of the experiment were already around 20-25% decreased compared to nominal values. As expected, this decrease continued during the toxicity test and losses were generally above the 50%. However, the decrease in actual concentration in the highest concentration tested (nominal value of 480 µg/l) was limited to 18% only. Since the larval development in this concentration did not differ significantly from the controls, it is concluded that this actual concentration can be used to set NOEC values (see below).

Note: As a consequence of the small sample volume taken, the analytical laboratory reported an increased background noise and problems with the matrix. An increased detection limit was therefore reported for the lowest test concentrations and the blanks.

Table I.2.1 Nominal and actual test concentrations of naphtalene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 5.7	< 5.3			0
Solvent Control	< 5.4	< 5.9			95
30	22	< 5.9	< 14	> 73	6.0
60	43	< 15	< 29	> 65	12
120	93	51	72	45	24
240	190	76	133	60	48
480	390	320	355	18	95

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table I.2.2 together with the actual values. As illustrated, the test is considered valid.

Table I.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	86 - 89

Test results

An increased number of retarded larvae were observed in the lowest concentration tested (nominal: 30 µg/l) and significant differences were observed with the solvent control. However, in all other concentrations no such adverse effects were detected. As a consequence, the EC_{10}-value is estimated as "≥ 355 µg/l". It is therefore decided to set the NOEC-value at ≥ 355 µg/l, the highest concentration tested.

Table I.2.3 Results of the toxicity test.

Actual test concentration (µg/l)	Normal developed larvae at the end of the test (%) (mean)	(s.d.)	CV (%)	% effect on solvent control[1]
Blank	89.6	0.7	0.8	3.4
Solvent control	91.5	0.8	0.8	
< 14	76.4	6.9	9.1	23.7
< 29	85.1	1.2	1.4	2.6
72	85.6	8.2	9.6	9.0
133	86.4	2.1	2.5	3.2
355	84.4	4.3	5.0	29.5

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table I.2.4 Estimated parameters (with 95% conf. limits) for naphtalene and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	LOEC µg/l	EC_{10} µg/l	EC_{50} µg/l	EC_{90} µg/l
≥ 355	≥ 355	≥ 355	≥ 355	≥ 355

I.3 Naphtalene, *Pseudokirchneriella subcapitata*

Nominal and actual test concentrations

An overview is presented in table I.3.1, illustrating that at the start of the toxicity test (2 hours after preparation of test solutions) actual concentrations of naphtalene have already decreased by on average 40-50%. As expected, this decrease continued during the toxicity test and all values were below detection levels after 96 hours. Furthermore, a rather strange concentration was found for the highest test concentration at t=0. Together, this strongly limits the applicability of the test results.

Table I.3.1 Nominal and actual test concentrations of naphtalene in the test with *Pseudokirchneriella subcapitata* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=96 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=96	µl/l acetone (nominal)
Blank	< 2.2	< 1.5			
Solvent Control	< 1.7	< 2.7			21.9
180	110	< 1.0	< 56	> 99	2.2
320	160	< 2.9	< 81	> 98	3.9
560	310	< 1.0	< 156	100	6.8
1000	490	< 1.2	< 246	100	12.2
1800	<103 (100)	< 2.2	51	98	21.9

Validity criteria

The validity criteria applicable to the toxicity test with *Pseudokirchneriella subcapitata* are specified in table I.3.2 together with the actual values. As illustrated, the test is considered valid.

Table I.3.2 Validity criteria.

Parameters	Criteria	Actual values
Pseudokirchneriella subcapitata (ISO 8692)		
Increase in cell density after 72 hours	> 16	150
Variation in pH-value during the test	≤ 1.5	0.8

Test results

The average growth rate for each concentration together with the percentage inhibition (in relation to the solvent control) is presented in table I.3.3. Estimated parameters are presented in table I.3.4. It is concluded that none of the tested concentration caused a significant effect on the growth rate of the freshwater algae *P. subcapitata*. The interpretation of these results is however strongly hampered by the strong reduction in the actual concentrations during the test and the strange test concentration in the highest test concentration at t=0. No conclusions are therefore drawn. It can only be noted that an initial concentration of 490 µg/l did not affect the growth rate.

Table I.3.3 Results of the toxicity test after 4 days

Nominal test concentration (µg/l)	Growth rate (mean)	(s.d.)	CV (%)	% inhibition on solvent control
Blank	1.52	0.01	0.6	
Solvent control	1.52	0.01	0.8	
180	1.51	0.02	1.1	0.7
320	1.52	0.01	0.5	0.2
560	1.52	0.02	1.5	0.4
1000	1.52	0.05	3.3	0.5
1800	1.55	0.01	0.6	-1.4

J.1 Nonylphenol, *Crassostrea gigas*

Nominal and actual test concentrations
An overview is presented in table J.1.1, illustrating that the actual concentrations at the start of the experiment were on average 25 – 50% lower as compared to the nominal values (with the exception of 3.2 µg/l). More severe differences were however observed at the end of the experiment, since nonylphenol was not detected any more. This strongly limits the applicability of the test results.

Table J.1.1 Nominal and actual test concentrations of nonylphenol in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	< 0.5	< 0.5			0
Solvent Control	< 0.5	< 0.5			35.6
1.8	0.73	< 0.5	< 0.6	> 32	3.6
3.2	< 0.5	< 0.5	< 0.5	?	6.3
5.6	7.8	< 0.5	< 4.1	> 94	11.1
10	7.6	< 0.5	< 4.1	>93	19.8
18	11.0	< 0.5	< 5.7	>95	35.6

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table J.1.2 together with the actual values. As illustrated, the test is considered valid.

Table J.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	120 - 122

Test results

The interpretation of the test results is hampered by the fact that no nonylphenol was detected at the end of the test. Statistical analyses indicated an interrupted dose-response, since the decreased number of normal developed larvae in the 3.2 µg/l treatment (nominal) was significant different from the solvent control as well as from the blank, due to an increased number of malformations. However no adverse effects were observed in the two highest test concentrations. Furthermore, the treatment with 3.2 µg/l nominal was also in other respect remarkable, as no nonylphenol was detected at the beginning of the experiment.

The interpretation of these results is however hampered by the strong reduction in the actual concentrations during the test. No conclusions are therefore drawn. It can only be noted that an initial concentration of 11 µg/l did not affect the larvae.

Table J.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Nominal test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	91.0	2.3	2.5	-0.9
Solvent control	94.0	3.1	3.3	
1.8	96.4	1.5	1.5	-12.1
3.2	76.8	3.3	4.3	18.9
5.6	90.9	4.5	5.0	2.6
10	94.6	1.9	2.0	-9.8
18	92.9	1.1	1.2	-7.8

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

J.2 Nonylphenol, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table J.2.1, illustrating that the difference between the nominal and actual concentrations at t=0 decreased with increasing concentrations. Furthermore, the decrease in test concentration during the toxicity test was on average 60%, which limits the applicability of the test results.

Table J.2.1 Nominal and actual test concentrations of nonylphenol in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0 -T=48	µl/l acetone (nominal)
Blank	< 0.5	< 0.5			0
Solvent Control	< 0.5	< 0.5			121.7
5.6	1.4	< 0.5	< 1	> 64	12.2
10	4.8	1.1	3	77	21.7
18	15	8.5	12	43	39.1
32	24	9.7	17	60	69.5
56	40	16	28	60	121.7

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table J.2.2 together with the actual values. As illustrated, the test is considered valid.

Table J.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	79 - 95

Test results

At first, the percentage normal developed larvae was calculated using the total number of larvae recovered at the end of the test (as was done for all other tests). Results are presented in table J.2.3. These results indicated that there were no significant differences found between any of the tested concentrations. However, the percentages showed different results after the

correction using the Abbott's formula and a clear increase in effect was noted with increased test concentrations. These differences are caused by differences in the mortality rate. As discussed in §2.6, the Abbott's formula is calculating the percentages normal developed larvae based on the number of fertilized eggs, used to start the toxicity tests. When differences in the mortality rate are observed between the concentrations, this is the preferred method. Statistical analyses were therefore based on the results after the correction with the Abbott's formula (both dataset are presented in the appendices). Furthermore, also a statistical difference between the blank and the solvent control was noted, with a somewhat decreased survival rate in the blank. The blank was based on six different replicates (as compared to three for the solvent controls) and might therefore represent less variable results. Parameters were therefore estimated using the blank instead off the solvent control (see parameters in table J.2.4).

Table J.2.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	84.2	3.5	4.2	17.0
Solvent control	89.3	1.4	1.6	
< 1	85.5	4.6	5.4	16.3
3	81.8	10.4	12.7	28.4
12	88.9	1.6	1.8	26.8
17	85.0	3.8	4.4	36.3
28[2]	77.7	12.0	15.4	40.1

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

[2]: Only two replicates could be counted

Table J.2.4 Estimated parameters (with 95% conf. limits) for nonylphenol and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
12	17	5.4	> 28	> 28

K.1 Pentachlorobenzene, *Crassostrea gigas*

Nominal and actual test concentrations

An overview is presented in table K.1.1, illustrating that (for the three lowest test concentrations) actual concentrations at t=0 were in close agreement with the nominal concentrations. Deviations were however observed in the two highest concentrations. Comparable differences between the three lowest and the two highest concentrations were found at the end of the test (t=48 hours). A marked decrease in the actual concentration around the 50% was observed for the concentrations 10, 18 and 32 µg/l, while almost no decrease was observed in the 56 and 100 µg/l. It was therefore hypothesized that the measurements of the actual concentrations in the 56 and 100 µg/l treatment at t=0 somehow underestimated the actual values. However, no prove could be found in the chemical analysis to support this hypothesis.

Table K.1.1 Nominal and actual test concentrations of pentachlorobenzene in the test with *Crassostrea gigas* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	0.57	<0.6			0
Solvent Control	<0.2	<0.1			100
10	8.8	3.5	6.2	60	10
18	17	8.6	13	49	18
32	28	13	21	54	32
56	27	25	26	7	56
100	53	44	49	17	100

Validity criteria

The validity criteria applicable to toxicity tests with *Crassostrea gigas* are specified in table K.1.2 together with the actual values. As illustrated, the test is considered valid from the biological point of view.

Table K.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	70 - 100

Test results

The percentage of normal developed larvae was in most test concentrations higher than the solvent control (see table K.1.3) and no significant differences were detected. The NOEC-value is therefore set at the highest concentration tested, 49 µg/l.

Estimated parameters are presented in table K.1.4.

Table K.1.3 Results of the toxicity test.

		Normal developed larvae at the end of the test (%)			
Nominal test concentration (µg/l)	**Actual test concentration (µg/l)**	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank		90.1	1.2	1.3	-55.7
Solvent control		64.4	5.5	8.6	
10	6.2	70.7	1.8	2.6	-23.8
18	13	62.0	3.5	5.7	0.6
32	21	77.4	2.0	2.6	-30.8
56	26	75.1	1.2	1.6	-36.5
100	49	74.8	1.9	2.5	-38.0

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table K.1.4 Estimated parameters (with 95% conf. limits) for pentachlorobenzene and *Crassostrea gigas* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 49	≥ 49	≥ 49	≥ 49	≥ 49

K.2 Pentachlorobenzene, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table K.2.1, illustrating that the actual concentrations at the beginning of the tests were on average 40% lower as compared to the intended nominal values. The decrease in concentration during the toxicity test was limited to (on average) 25%.

Table K.2.1 Nominal and actual test concentrations of pentachlorobenzene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48	µl/l acetone (nominal)
Blank	<0.04	<0.04			
Solvent Control	<0.04	<0.04			79.8
3.2	1.3	1.2	1.3	8	8.0
5.6	3.2	2.3	2.8	28	14.0
10	6.4	3.8	5.1	41	25.0
18	11	7.5	9.3	32	44.9
32	21	16	19	24	79.8

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table K.2.2 together with the actual values. As illustrated, the test is considered valid.

Table K.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	74 - 90

Test results
An overview of the percentage normal developed larvae in the different treatments is presented in table K.2.3. None of the tested concentrations did cause a significant effect on the larval development of the marine sea-urchin *P. miliaris*. The NOEC value is therefore set at ³ 19 µg/l, the highest concentration tested.

Table K.2.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Actual test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	93.4	2.0	2.1	-21.1
Solvent control	85.6	2.2	2.6	
1.3	83.0	6.5	7.8	-3.3
2.8	88.9	2.1	2.4	2.6
5.1	78.9	5.1	6.5	2.5
9.3	87.1	1.7	2.0	-16.2
19	85.1	2.4	2.8	-6.9

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

Table K.2.4 Estimated parameters (with 95% conf. limits) for pentachlorobenzene and *Psammechinus miliaris* based on actual concentrations (average of two measurements).

NOEC µg/l	**LOEC** µg/l	**EC_{10}** µg/l	**EC_{50}** µg/l	**EC_{90}** µg/l
≥ 19	≥ 19	≥ 19	≥ 19	≥ 19

L.1 Tetrachloroethene, *Crassostrea gigas*

Nominal and actual test concentrations
An overview is presented in table L.1.1, clearly illustrating that chemical analysis were in no relation to the intended, nominal concentrations whatsoever. There was even no relation with the increasing concentrations. The cause of these results is still unknown. It might have partly been caused by the fact that tetrachloroethene is a liquid with a mass 1.6 times water. Small (unnoticed) droplets might have been present, preventing a representative sample from a homogeneous mixture. No

conclusions can therefore be drawn based on these test results. Chemical analysis should first been further clarified, after which toxicity tests can be repeated.

Note: these data are the same as presented for the test with *Psammechinus miliaris* (L.2), since both tests were performed simultaneously and with the same concentration series.

Table L.1.1 Nominal and actual test concentrations of tetrachloroethene in the test with *Crassostrea gigas*.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48
Blank	< 0.2	< 0.2		
180	19000	14000	16500	26
320	5300	13000	9150	-145
560	11000	17000	14000	-55
1000	41000	36000	38500	12
1800	8000	33000	20500	-313

Validity criteria

The validity criteria applicable to toxicity test with *Crassostrea gigas* are specified in table L.1.2 together with the actual values. As illustrated, the test is considered valid.

Table L.1.2 Validity criteria.

Parameters	Criteria	Actual values
Crassostrea gigas (ASTM 724)		
Normal developed larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	99

Test results

Significant effects of tetrachloroethene on the larval development were noted in all concentrations, except for the lowest concentration of 180 µg/l nominal. No dose response was noted. As noted for the chemical analyses, these test results cannot be used to draw any conclusions on the toxicity of tetrachloroethene.

Table L.1.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Nominal test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on control[1]**
Blank	99.2	0.7	0.7	
180	94.0	2.1	2.3	-0.8
320	80.8	8.6	10.6	17.3
560	46.7	5.9	12.7	60.9
1000	37.3	31.0	83.1	68.8
1800	82.2	8.6	10.4	12.2

[1]: Percentages are corrected for the average development in the controls using the Abbott's formula according to the guideline.

L.2 Tetrachloroethene, *Psammechinus miliaris*

Nominal and actual test concentrations

An overview is presented in table L.2.1, clearly illustrating that chemical analysis were in no relation to the intended, nominal concentrations whatsoever. There was even no relation with the increasing concentrations. The cause of these results is still unknown. It might have partly been caused by the fact that tetrachloroethene is a liquid with a mass 1.6 times water. Small (unnoticed) droplets might have been present, preventing a representative sample from a homogeneous mixture. No conclusions can therefore be drawn based on these test results. Chemical analysis should first been further clarified, after which toxicity tests can be repeated.

Note: these data are the same as presented for the test with *Crassostrea gigas* (L.1), since both tests were performed simultaneously and with the same concentration series.

Table L.2.1 Nominal and actual test concentrations of tetrachloroethene in the test with *Psammechinus miliaris* as well as the nominal concentrations acetone in the solution.

Nominal concentration (µg/l)	Actual concentration (µg/l) T=0	Actual concentration (µg/l) T=48 hr.	Mean concentration (µg/l)	% loss of test-compound T=0-T=48
Blank	< 0.2	< 0.2		
180	19000	14000	16500	26
320	5300	13000	9150	-145
560	11000	17000	14000	-55
1000	41000	36000	38500	12
1800	8000	33000	20500	-313

Validity criteria

The validity criteria applicable to toxicity tests with *Psammechinus miliaris* are specified in table L.2.2 together with the actual values. As illustrated, the test is considered valid.

Table L.2.2 Validity criteria.

Parameters	Criteria	Actual values
Psammechinus miliaris (ASTM 1563)		
Normal developed pluteus larvae relative to the number of fertilized embryos introduced at t_0 (%)	≥ 70	72

Test results

Strong effects of tetrachloroethene on the larval development were noted in all concentrations. No dose response was however found. As noted for the chemical analyses, these test results cannot be used to draw any conclusions on the toxicity of tetrachloroethene.

Table L.2.3 Results of the toxicity test.

	Normal developed larvae at the end of the test (%)			
Nominal test concentration (µg/l)	**(mean)**	**(s.d.)**	**CV (%)**	**% effect on solvent control[1]**
Blank	90.9	4.2	4.6	
180	0			100
320	3.0	5.2	173	99.6
560	0			100
1000	3.3	5.8	173	99.6
1800	3.3	5.8	173	99.6

[1]: Percentages are corrected for the average development in the solvent controls using the Abbott's formula according to the guideline.

4. Literature

ISO 10706 (2000). Water quality - Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea).

ISO 8692 (2002). Water quality - Fresh water algal growth inhibition test with unicellar green algae.

ISO 10253 (1995). Water quality - Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*.

ASTM (1998). Designation E1563-95. Standard guide for conducting static acute toxicity tests with Echinoid embryos. Annual Book of ASTM Standards, volume 11.05. American Society for Testing and Materials (ASTM), West Conshohocken, PA, USA.

ASTM (1998). Designation E724-98. Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve mollucs. Annual Book of ASTM Standards, volume 11.05. American Society for Testing and Materials (ASTM), West Conshohocken, PA, USA.

Appendices