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Early life stage tests with *Brachydanio rerio* and several polycyclic aromatic hydrocarbons using an intermittent flow-through system (Draft OECD Guideline)

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SUMMARY AND CONCLUSIONS

As part of a study carried out at the request of RIZA and the Ministry of Housing, Physical Planning and the Environment, the possible ecotoxicological effects of several Polycyclic Aromatic Hydrocarbons (PAHs) were determined in an Early Life Stage Test (ELS test). Effects on hatching, mortality, growth and the occurrence of egg and larval malformations in the fresh water species *Brachydanio rerio* were determined as laid down in the Draft OECD Guideline (ref. 1).

Because it was expected that some PAHs, in particular the poorly water soluble ones, would not cause observable adverse ecotoxicological effects in the ELS test, limit tests (test with only one "high" concentration), were carried out. Therefore one concentration of benzo(k)fluoroanthene, chrysene, benzo(a)pyrene, benzo(ghi)perylene and fluoranthene was tested simultaneously at their aqueous solubility level in one run of the intermittent flow-through system. For benzo(k)fluoranthene two concentrations were tested. The water solubility of the PAHs was determined in a preliminary experiment by chemically analysing saturated solutions generated by a column technique.

The test was carried out in an intermittent flow-through system, with dosing intervals of 60 minutes and the test substance was dosed from concentrated solutions in tertiary butyl alcohol. A quantity of 4 x 20 eggs were used for each test concentration. Chemical analysis of the test solutions were carried out weekly and at the end of the test all surviving fish were analysed to determine the bioconcentration factor (= BCF) of the PAH, to which they had been exposed. The exposure duration was 42 days.

There were no adverse effects with regard to hatching, mortality, growth or malformations observed after exposure to chrysene (nominally 1.8 $\mu g.l^{-1}$), benzo(a)pyrene or benzo(ghi)-perylene (nominally 0.34 $\mu g.l^{-1}$). After 7, 14 and 21 days of exposure to fluoranthene (nominally 320 $\mu g.l^{-1}$) 12.5%, 99% and 100% of the test fish died respectively. Benzo(k)fluoranthene was also toxic; 42% and 76% mortality were observed at the end of the test at the concentrations of nominally 1.8 $\mu g.l^{-1}$ and 3.2 $\mu g.l^{-1}$ respectively. Furthermore growth was significantly inhibited by benzo(k)fluoroanthene and red swollen gills were observed.

The bioconcentration factors based on the total wet weight of the fish were low, being 13 (and 7), 13, 3 and 60 for benzo(k)fluoranthene, chrysene, benzo(a)pyrene and benzo(ghi)-perylene respectively. These BCFs are far below the values that can be calculated on the basis of the log n-octanol/water partition coefficient. These low values can be attributed to the metabolization of the PAHs tested and the depuration of its more polar metabolites or to specific working mechanisms.

Only very low concentrations of benzo(a)anthracene (between 0.054 $\mu g.l^{-1}$ and 0.25 $\mu g.l^{-1}$) could be reached in the flow-through system and this PAH was therefore not tested in the ELS test. The summarized results of the ELS test were:

PAH	Aqueous solubility ($\mu g.l^{-1}$)	Dosage in flow-through ($\mu g.l^{-1}$)	Measured in flow-through (average) ($\mu g.l^{-1}$)	BCF (based on total wet weight)
Benzo(k)fluoranthene	0.64	1.8	0.52	13
		3.2	0.58	7
Chrysene	0.44	1.8	1.0	13
Benzo(a)pyrene	2.9	10	4.0	3
Benzo(ghi)perylene	0.10	0.32	0.16	60
Fluoroanthene	180	320	240	–
Benzo(a)anthracene	9	32	0.054-0.25	–

The PAHs fluoroanthene and benzo(k)fluoroanthene were chosen for further investigations in the ELS test with *Brachydanio rerio* because they induced toxic effects within their aqueous solubility.

1. INTRODUCTION

At the request of RIZA and the Ministry of Housing and Physical Planning, the possible ecotoxicological effects of various Polycyclic Aromatic Hydrocarbons (PAH) were investigated. Because it was expected that the poorly water soluble PAHs may not have adverse effects on the early life stages of *Brachydanio rerio*, limit tests (test with only one concentration) was carried out. Benzo(k)fluoranthene, benzo(ghi)perylene, benzo(a)pyrene, chrysene and fluoranthene were tested in an intermittent flow-through system during one run. Each PAH was dosed at its aqueous solubility level as determined by column technique. Based on the results of this test, PAHs were chosen for further investigations.

The toxicity test was carried out in conformity with the Draft OECD Guideline "Fish Early Life Stage Toxicity Test" (ref. 1). The duration of the test was 42 days.

The test was carried out in the period October 2, 1991-November 13, 1991.

2. MATERIALS AND METHOD

2.1 Test substance

The test substances examined in the ELS test were benzo(k)fluoranthene, chrysene, benzo(a)pyrene, benzo(ghi)perylene and fluoranthene. However, the water solubility was also examined for benzo(a)anthracene, anthracene, naphtalene and indeno[1,2,3-CD] pyrene. All PAHs were stored at room temperature in the dark.

2.2 Test organism

The organism used was the fresh water fish species *Brachydanio rerio*. Adult fish were obtained from the commercial tropical fish hatchery M.B. Ruijsbroek B.V. (Noordvliet 159, Maassluis), the Netherlands. They were acclimatized to the laboratory conditions (temp. 24°C, DSWL as medium) for at least three weeks. They were fed daily with *Artemia* nauplii (enriched with Selco) and fresh minced steak. Before the first controlled spawning in the laboratory, females and males were separated and placed in basins at 24°C. The females were kept under a 7 h light/17 h dark regime and held individually to reveal their spawning history (frequency of spawning, condition of eggs). The males were kept under natural daylight and dark conditions.

Around 6.30 h (about a week after a previous spawning in the laboratory), individual females (carrying eggs) were transferred to a basin at 26°C in which three males had been present since 15.00 h the previous day. As soon as the eggs were deposited they were collected and put into the test vessels. The stage of embryonic development at the start of the test was verified under a microscope to be the young blastula stage.

2.3 Dilution water

The dilution water used was DSWL, prepared from ground water. Its composition is given in Annex A. DSWL has proven to be suitable for the culturing of *Brachydanio rerio*.

2.4 Determination of aqueous solubility by column techique

Saturated solutions of each PAH were prepared by column technique. Chromosorb (Gas-Chrom), 60/80 mesh size was used as "high surface packing material" and coated with a PAH solution (about 0.1%) in acetone. The acetone was stripped by rotation evaporation. The coated carrier material (about 1% PAH w/w) was stored in a brown glass jar and kept in the dark.

Dilution water (DSWL, see section 2.3) was purged with the aid of a HPLC pump through a stainless steel column packed with PAH coated carrier material. The flow rate was about 3 $ml.min^{-1}$. The temperature of the generator column was kept at 25 ± 1°C in a water basin. The PAH content was determined by chemical analysis (see section 2.8).

2.5 Test method

The test was conducted in accordance with the Draft OECD Guideline (ref. 1). The exposure period was 42 days. The test was carried out under a 16 h light - 8 h dark regime (yellow light) in a temperature controlled room. The water supply was also temperature controlled. The temperature was measured daily in the control medium (25 ± 1°C).

One concentration of each PAH was tested in the region of its aqueous solubility limit as determined by column technique (see section 2.3).

Benzo(k)fluoranthene was the only substance tested at two concentrations. The stock solutions for the dosing of the various PAHs were prepared as follows:

- Benzo(k)fluoranthene: quantities of 11.1 mg and 10 mg were dissolved in 512.3 ml TBA and 259 ml TBA respectively, resulting in stock solutions of 21.6 $mg.l^{-1}$ and 38.6 $mg.l^{-1}$.
- Chrysene: a quantity of 10 mg was dissolved in 461.5 ml TBA, resulting in a stock solution of 21.7 $mg.l^{-1}$.
- Benzo(a)pyrene: a quantity of 14.7 mg was dissolved in 121.8 ml TBA, resulting in a stock solution of 120.7 $mg.l^{-1}$.
- Benzo(ghi)perylene: a quantity of 6.6 mg was dissolved in 500 ml TBA; 43.2 ml of it was diluted with TBA to 150 ml, resulting in a stock solution of 3.80 $mg.l^{-1}$.

- Fluoranthene: a quantity of 0.6541 g was dissolved in 169.4 ml TBA resulting in a stock solution of 3.86 $g.l^{-1}$.
- Benzo(a)anthracene: a quantity of 52.2 mg was dissolved in 135.2 ml TBA, resulting in a stock solution of 386.09 $mg.l^{-1}$. this solution was not tested ultimately because it was not possible to reach an acceptable test concentration in practice. An extra concentration of 3.2 $\mu g.l^{-1}$ benzo(k)fluoranthene was then added to the run of the flow-through system. The dosed volume of each stock solution was 84.2 µl to one litre of dilution water, resulting in the following nominal concentrations of test solutions:

Benzo(k)fluoranthene	1.8	$\mu g.l^{-1}$
	3.2	$\mu g.l^{-1}$
Chrysene	1.8	$\mu g.l^{-1}$
Benzo(a)pyrene	10	$\mu g.l^{-1}$
Benzo(ghi)perylene	0.32	$\mu g.l^{-1}$
Fluoranthene	320	$\mu g.l^{-1}$
(Benzo(a)anthracene	32	$\mu g.l^{-1}$)

DSWL was used as a control, together with a solvent control (84.2 µl TBA in one litre dilution water.

An intermittent flow-through system (see section 2.5) was used. At intervals of 60 minutes each of four replicate compartments was supplied with ca. 250 ml newly prepared solution. Each compartment contained about 20 fish (or eggs). The test solutions were not aerated. pH values and oxygen concentrations were measured weekly in all test solutions.

At the start of the test, about 60 potentially fertilized eggs were placed in each test vessel. After the first 24 h of exposure, this number of eggs was reduced to about 20 fertilized eggs. This procedure allows exposure of the eggs to start in an early developmental stage in each test vessel. This is not possible if a time consuming examination for fertilization of each individual egg is carried out microscopically. Eggs from five batches were needed for the test and divided equally between all test solutions and control media.

The hatching of the eggs was followed daily. Immediately after hatching the fish were fed abundantly with rotifers, from mass laboratory cultures. From t = 9 days *Artemia nauplii* enriched with Selco were given as the main food in addition to the rotifers.

At least once per week, the dead eggs or larvae were counted and removed; the survivors were also counted and their size and condition (swimming behaviour, presence of malformations, or any other observable morphological or behavioural criterion) was visually compared with that of the control animals. At the end of the test, all individual fish were blotted dry with tissue paper and their total length and wet weight determined as quickly as possible.

2.6 intermittent flow-through system

The intermittent flow-through system used was constructed according to Van Leeuwen et al. (ref. 2) and diagrams of it are presented in Figures 1 and 2. Every 60 minutes, valve (1a) opened and dilution water was supplied from a reservoir tank (1) to a glass flow-splitter (2), consisting of eight one litre compartments. These compartments were successively filled (the first compartment filling and overflowing into the second compartment, etc.) until the water level reached a sensor (12). The water supply was then cut off and valves (3) under the compartments were activated and opened, causing the water to flow into the mixing bottles (4). At this same moment the test substance was dosed into the mixing bottle with the aid of a syringe pump (11) and mixed with the dilution water by magnetic stirrers (9). The one litre volume of each concentration then flowed over into the chamber (6) which had a total volume of 26 litre. The fish were confined in the test chambers in four cylindrical retention chambers with a volume of 220 ml each; these had nylon mesh screens covering the bottoms. Each of these chambers received about 250 ml test medium per cycle by means of a flow-splitting funnel (5). One litre of each concentration per cycle flowed out of the test chambers into an outlet (10) via outlet tubes (7).

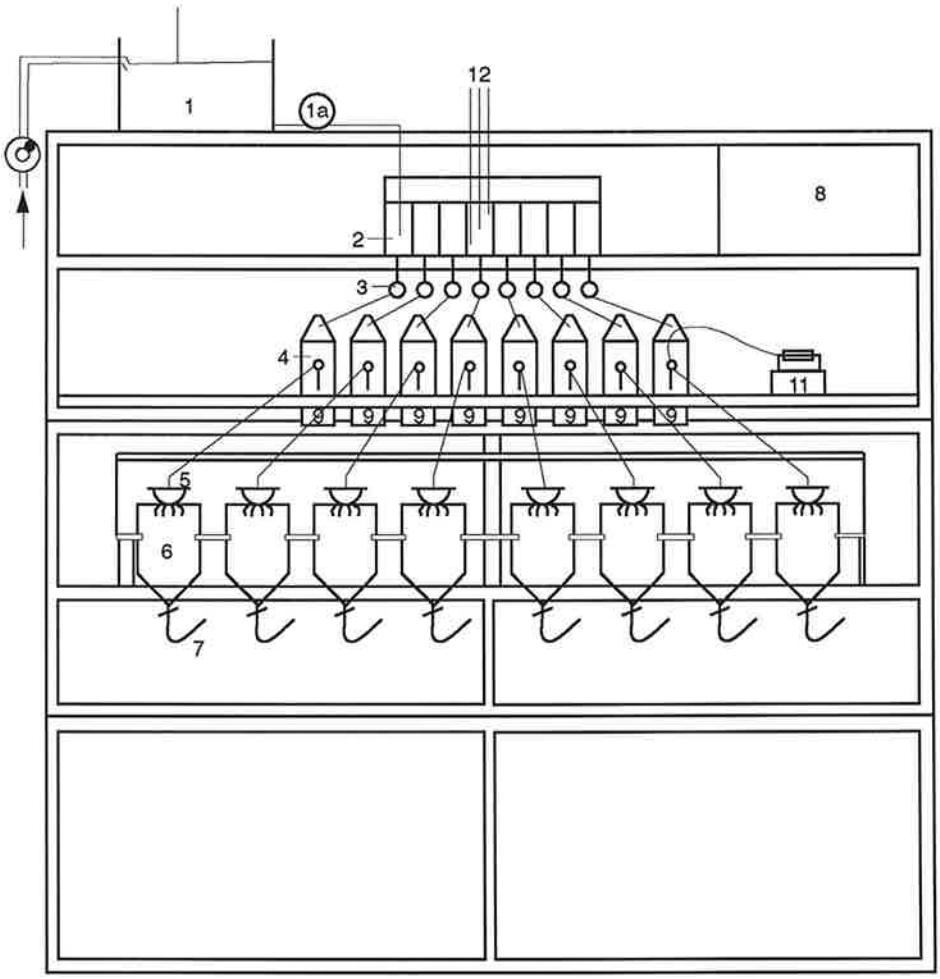

Figure 1 *Front view.*
1 = water supply
1a = valve
2 = one litre volume water dosing compartment
3 = valves (eightfold)
4 = mixture bottles (eightfold)
5 = flow splitters (eightfold)
6 = test chambers (eightfold)
7 = outlet tube
8 = control panel
9 = magnetic stirrers (eightfold)
11 = syringe pumps or peristaltic pumps
12 = sensor for water dosing compartment

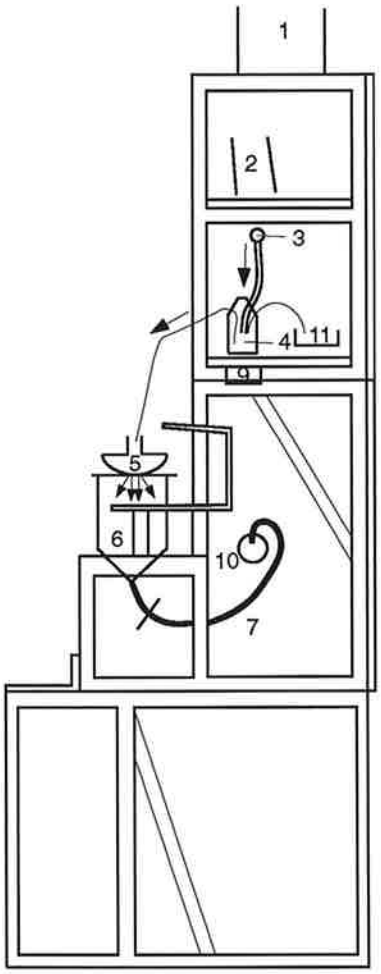

Figure 2 *Cross section.*

1 = water supply
2 = one litre volume water dosing compartment
3 = valves (eightfold)
4 = mixture bottles (eightfold)
5 = flow splitters (eightfold)
6 = test chambers (eightfold)
7 = outlet tube
9 = magnetic stirrers (eightfold)
10 = outlet
11 = syringe pumps or peristaltic pumps

2.7 Measurements of growth

From the measurements of the length and wet weight of the individual fish, the average length and weight and the standard deviation were calculated per test substance concentration and compared with those of the control fish. The two-tailed Dunnett-test ($p = 0.05$ and $p = 0.01$) was used to determine if the average length or weight were significantly different from those of the control fish held under the same circumstances.

2.8 Chemical analysis

2.8.1 Water samples

The actual concentrations of the various PAHs in the saturated solutions and in the test solutions were determined by chemical analysis. Every week samples of ca. 250 ml were analysed. The samples were taken in glass bottles and analysed the same day for their PAH content.

The samples of all PAHs except naphthalene were extracted with hexane and concentrated using a rotary evaporator, and then to nearly dryness under a gentle stream of nitrogen. The residue was taken up in methanol and analysed with Reversed Phase HPLC (with UV absorption and fluorescence detection). Quantification was based on external standards (NBS-PAK standard mix: Standard Reference Material 1647).

The concentration of naphthalene in the saturated solution prepared by column technique were determined gaschromatographically. A volume of 50 ml solution was extracted twice with 1 ml of CS_2. The combined extracts were analysed with the use of an apolair capillary column, followed by detection with a flame ionisation detector (FID). Identification was based on retention time and the ratio of FID signal; quantification was based on external standards (standard mix of aromatics and aliphatics in CS2).

2.8.2 Fish samples

At the end of the test all fish were frozen and kept in a freezer until analysis. The fish were weighed and homogenized with a glass pestle and transferred quantitatively to a 25 ml erlenmeyer flask; the internal standard(2-methyl-chrysene) was added and the homogenate was then hydrolysed for 3 hours with 4 M NaOH.
The hydrolysed homogenates were extracted with hexane followed by a clean up with an Al_2O_3 column. The extract was concentrated by evaporation to nearly dryness and to dryness with a gentle stream of nitrogen. The residue was dissolved in 0.5 ml acetone/methanol 1:1 (v;v). The extracts were analysed with Reversed Phase HPLC with UV absorbtion and fluorescence detection. The method was checked with a certified NBS-PAH Standard Reference Material 1647).

3. RESULTS

3.1 Determination of saturation values in DSWL

The result of the chemical analysis of the solutions prepared by column technique are given in Table 1.

Table 1 *Results of the chemical analysis of the saturated solutions prepared by column technique.*

PAH investigated	Samples taken after ca. (ml)	Concentration in $\mu g.l^{-1}$	Detection limit in $\mu g.l^{-1}$
Benzo(k)fluoranthene	250 - 500	0.680	0.0032
	500 - 750	0.640	
	750 - 1000	0.640	
Chrysene	250 - 500	0.41	0.036
	500 - 750	0.42	
	750 - 1000	0.51	
Benzo(a)pyrene	250 - 500	2.96	0.0064
	500 - 750	2.76	
	750 - 1000	2.96	
Benzo(ghi)perylene	250 - 500	0.104	0.028
	500 - 750	0.104	
Fluoranthene	500	180	0.4
	500 - 1000	170	
	1000 - 1233	190	
Naphthalene	250 - 500	< 4	4
	500 - 750	< 4	4
	750 - 1000	< 4	4
Anthracene	250 - 500	66	0.008
	500 - 750	52	
	750 - 1000	48	
Indeno[1,2,3-cd]pyrene	500 - 750	1.96	0.030
	1000 - 1250	1.28	
	1250 - 1500	0.60	
Benzo(a)anthracene	250 - 500	9.2	0.022
	500 - 750	9.2	
	750 - 1000	8.8	

The fact that the concentrations of naphthalene were below the detection limit cannot be explained. Based on these saturation values the concentrations of the PAHs to be tested in the limit tests were chosen. Due to the loss of test substance concentration in the flow-through system, the nominal concentrations were chosen somewhat above the water solubility.

3.2 pH

The pH values of the test solutions and control medium are recorded in Annex E, Table E1 and varied between 7.8 and 8.2. It is not likely that the pH affected the results of any of the PAHs tested.

3.3 Oxygen concentration

The oxygen concentrations of the test solutions and control medium are recorded in Annex E, Table E2. The lowest value measured was 7.6 $mg.l^{-1}$. It is not likely therefore that the oxygen concentration affected the results of any of the PAHs tested.

3.4 Temperature

The temperatures as measured in the control medium are recorded in Annex E, Table E3 and varied between 24.4°C and 25.1°C at the beginning and at the end of the test respectively. It is not likely that the temperature affected the results.

3.5 Effects

3.5.1 Hatching and mortality

The number of living eggs or larvae per test chamber are given in Annex B, Table B1; and are combined per test concentration for the replicate test compartments in Annex B, Table B2.

In the control medium and in the test substance concentrations (except one incidental case in the solvent control and in the fluoranthene solution) all eggs hatched into healthy larvae without visible malformations within 5 days. After 21 days of exposure, all fish had died in the fluoranthene solution. In the 1.8 $\mu g.l^{-1}$ and 3.2 $\mu g.l^{-1}$ benzo(k)fluoranthene solutions 48% and 5% died respectively. The fact that these effects are not concentration related is most likely caused by the initially low actual water concentrations and the slow building up of it at 3.2 $\mu g.l^{-1}$ (see Table 3). The percentage of surviving fish is given in Table 2 (see section 3.5.3).

3.5.2 Visual observations

In the control media and in the solutions of chrysene, benzo(a)pyrene (except for a few small animals) and benzo(ghi)perylene, all surviving larvae swam and fed actively during the exposure period and no malformations were noted during the test. At both concentration of benzo(k)fluoranthene, the test animals were smaller, the size was irregular and the swimming behaviour disturbed (trembling); the gills were red coloured and swollen.

3.5.3 Growth

The total lengths and the wet weights per fish are recorded for all PAHs tested in Annex C and in Annex D. A summary of the results is given in Table 2. Growth was significantly less at both benzo(k)fluoranthene concentrations.

Table 2 *Summary of results on hatching, mortality and growth of eggs/larvae of Brachydanio rerio exposed to several concentrations of benzo(k)fluoranthene, chrysene, benzo(a)pyrene, benzo-(ghi)perylene and fluoranthene.*

PAH	% of eggs hatched after 6 d	% mortality after 28 d	No. of fish	Length [1] (cm)	Wet weight [1] (mg)
0	100	0	82	1.55 ± 0.11	25.7 ± 5.8
o[TBA 2]	99	0	80	1.54 ± 0.11	25.6 ± 5.7
benzo(k)fluoranthene (1.8 µg.l^{-1})	100	48	42	1.19 ± 0.29 [1]	18.7 ± 14.1 [1]
benzo(k)fluoranthene (3.2 µg.l^{-1})	100	5.0	76	1.19 ± 0.18 [1]	18.2 ± 7.19 [1]
chrysene	100	2.5	78	1.57 ± 0.16	26.4 ± 7.33
benzo(a)pyrene	100	3.8	77	1.53 ± 0.15	26.5 ± 7.36
benzo(ghi)perylene	100	2.5	78	1.59 ± 0.13	27.9 ± 6.95
fluoranthene	99	100	0		

1) Significantly less than control (two-tailed Dunnett-test, p = 0.01).
2) Solvent control.

3.6 Chemical analysis of water samples

The results of the chemical analysis of the PAH solutions are given in Table 3. For benzo(a)anthracene it appeared not to be possible to reach an acceptable actual concentration. Therefore the dosing of this substance was not continued and an extra benzo(k)-fluoranthene concentration of nominal 3.2 µg.l^{-1} was added in its place in the run. The latter water concentration continued to build up during the test and possibly had not reached its equilibrium at t = 36 days., the latest sampling time during the test. The actual concentrations of the other PAHs were between 40% and 75% values of the nominal dosed amount.

Table 3 *Results of the chemical analysis of the fluoranthene, test solutions in the intermittent flow-through system (concentrations in µg.l^{-1}). The purity of the test substance was 96%.*

PAH	Nominal concentration of PAH	Solubility [1]	Samples at								Mean concentration during the test ± s.d.	% of nominal
			t = -9d	t = -7d	t = 2d	t = 8d	t = 16d	t = 22d	t = 29d	t = 36d		
benzo(k)fluoranthene	1.8	0.64	0.30	0.37	0.64	0.57	0.55	0.63	0.53	0.58	0.58 ± 0.04	32
benzo(k)fluoranthene	3.2	0.64	–	–	0.02	0.67	0.65	0.48	1.2	1.5	0.75 ± 0.53	23
chrysene	1.8	0.44	1.2	1.3	1.2	1.2	0.90	0.62	0.74	0.80	0.91 ± 0.24	51
benzo(a)pyrene	10	2.9	4.2	4.3	4.4	4.2	4.0	4.0	3.4	3.2	4.0 ± 0.4	40
benzo(ghi)perylene	0.32	0.10	0.18	0.19	0.17	0.15	0.16	0.14	0.10	0.17	0.16 ± 0.02	50
fluoranthene	320	180	210	230	250	260	220	230	– [3]	– [3]	240 ± 18	75
benzo(a)anthracene	32	2.2	0.24	0.048	0.25[4]							

1) As determined in DSWL solutions by column technique.
2) Dosing not continued because of low actual concentrations. Extra benzo(k)fluoranthene solution of 3.2 µg.l^{-1} added to the run of the system.
3) All test animals were dead.
4) Analysis at t = 0 days.

3.7 Chemical analysis of the test fish

The results of the chemical analysis of the various PAHs in the test fish are given in Table 3.

Table 4 *Results of PAH analysis in the intermittent flow-through system.*

PAH tested (nominal water concentration in $\mu g.l^{-1}$)	Concentration in test fish [1] (ng per g wet weight)	Dection limit (ng per g wet weight)	Recovery internal standard [3] (%)	Wet weight of fish sample (g)
benzo(k)fluoranthene (1.8 $\mu g.l^{-1}$)	7.5	1.1	89	0.8
benzo(k)fluoranthene (3.2 $\mu g.l^{-1}$)	5.1	0.64	87	1.4
chrysene	12	4.8	84	2.1
benzo(a)pyrene	12	0.60	92	2.0
benzo(ghi)perylene	9.6	2.8	87	2.2

The concentrations of benzo(k)fluoranthene, chrysene, benzo(a)pyrene en benzo(ghi)-perylene in the control and the solvent control were below 4.8, 0.45, 0.60 and 2.8 $ng.g^{-1}$ wet weight respectively.

3.8 Bioconcentration

The bioconcentration of the various PAHs in the test fish after 42 days exposure was calculated on the basis of the wet weight concentration in the fish and the actual water concentration.

Table 5 *Bioconcentration of PAHs in Brachydanio rerio exposed for 42 days in an intermittent flow-through system.*

PAH tested	Nominal water concentration ($\mu g.l^{-1}$)	Actual water concentration ($\mu g.l^{-1}$)	Dection limit ($\mu g.l^{-1}$)	Concentration in the test fish ($ng.g^{-1}$)	BCF [1)]	log n-octanol water
benzo(k)fluoranthene	1.8	0.58	0.0032	7.5	13	6.0
benzo(k)fluoranthene	3.2	0.75	0.0032	5.1	7	6.0
chrysene	1.8	0.91	0.036	12	13	5.6
benzo(a)pyrene	10	4.0	0.0042	12	3	6.0
benzo(ghi)perylene	0.32	0.16	0.020	9.6	60	6.6

1) Based on the total wet weight of the fish.

The bioconcentration factors were low. On basis of the log n-octanol/water partition coefficient (5.6 and higher) for the PAHs investigated bioconcentration factors above 4000 have been calculated (ref. 3). The reason for the low bioconcentration factors is most likely the metabolization of the PAHs combined with depuration of their more polar metabolites or to specific working mechaninisms. The first mentioned reason is indicated by extra peaks in addition to these PAHs investigated, as observed in the HPLC chromatogram.

3.9 Choice of PAHs for further investigations

The results obtained in the 42 days limit tests show that that chrysene, benzo(a)pyrene and benzo(ghi)perylene induced no adverse effects with regard to survival or condition (visually observed) to the zebra fish *Brachydanio rerio* in the ELS test. Fluoranthene and benzo(k)fluoranthene induced adverse effects in this test and were therefore chosen for further investigations in full tests.

4. REFERENCES

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Organization for Economic Co-operation and Development, Paris (1988).

2) Van Leeuwen, Büchner, J.L. and Van Dijk, M. (1988).
Intermittent flow system for population toxicity studies demonstrated with daphnias and copper.
Bull. Environm. Contam. Toxicol. 40, 496-502.

3) Integrated Criteria Document (PAHs) (1989).
National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, Report no. 758474011.

ANNEX A COMPOSITION OF THE SYNTHETIC MEDIUM (DSWL) USED IN THE TEST

The nominal composition is as described below:

Na^+	1.19	$mmol.l^{-1}$
K^+	0.20	$mmol.l^{-1}$
Ca^{2+}	1.36	$mmol.l^{-1}$
Mg^{2+}	0.73	$mmol.l^{-1}$
Cl^-	2.72	$mmol.l^{-1}$
SO_4^{2-}	0.73	$mmol.l^{-1}$

This medium is prepared by addition of several salts to groundwater from a locality near Linschoten (the Netherlands). The groundwater contains several other trace elements (<< 1 $mg.l^{-1}$). Media prepared from it have proved to be suitable for growing several species of water organisms. The equilibrium pH of the medium, after aeration, should be 8.3-8.5, but usually is slightly less, namely 8.0-8.2. The hardness, expressed as $CaCO_3$, is about 210 $mg.l^{-1}$.

The medium is prepared in large amounts (10,000 l) and the concentration of the following components of the batches used for this test was checked by chemical analysis and found to be:

	Period 21-09-1992 till 11-10-1992		11-10-1992 till 13-11-1992	
Na^+	1.20	$mmol.l^{-1}$	1.17	$mmol.l^{-1}$
K^+	0.22	$mmol.l^{-1}$	0.20	$mmol.l^{-1}$
Ca^{2+}	1.30	$mmol.l^{-1}$	1.32	$mmol.l^{-1}$
Mg^{2+}	0.74	$mmol.l^{-1}$	0.72	$mmol.l^{-1}$
Cl^-	2.71	$mmol.l^{-1}$	2.67	$mmol.l^{-1}$
SO_4^{2-}	0.69	$mmol.l^{-1}$	0.69	$mmol.l^{-1}$

The hardnesses, expressed as $CaCO_3$, were 204 $mg.l^{-1}$ in both batches used.
The total organic carbon contents were 1.5 $mg.l^{-1}$ and 1.7 $mg.l^{-1}$ respectively.

ANNEX B DETAILS ON HATCHING, SURVIVAL, CONDITION AND MALFORMATIONS OF THE TEST FISH

Table B1 *Number of eggs and larvae and their condition in the control medium and the solutions of benzo(k)fluoroanthene, chrysene, benzo(a)pyrene, benzo(ghi)perylene and fluoranthene during the exposure period (a, b, c and d are the replicate retention chambers.*

Time (d)	Test substance															
	0								0 TBA 2)							
	a		b		c		d		a		b		c		d	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	58[a]		62[a]		62[a]		56[a]		62[a]		62[a]		60[a]		60[a]	
1	46		50		47		45		52		45		43		47	
	12[d]		12[d]		15[d]		11[d]		10[d]		17[d]		17[d]		13[d]	
	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)
	20[a]		20[a]		20[a]		20[a]		20[b]		20[b]		19[b]		20[d]	
5	0	22[a]	0	20[a]	0	20[a]	0	20[a]	0[b]	20[b]	1[b]	19[b]	0[b]	19[b]	0[b]	20[b]
7	0	22[a]	0	20[a]	0	20[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]
14	0	22[a]	0	20[a]	0	22[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]
21	0	22[a]	0	20[a]	0	20[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]
28	0	22[a]	0	20[a]	0	20[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]
35	0	22[a]	0	20[a]	0	20[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]
42	0	22[a]	0	20[a]	0	20[a]	0	20[a]		20[b]		20[b]		19[b]		20[b]

1) The following codes are used to denote condition:
a Condition of the quoted number of fish (eggs), visually assessed, normal (= good).
b Condition of the quoted number of fish (eggs), visually assessed, equal to that of the control animals.
c The quoted number of fish were smaller.
d The quoted number of fish (eggs) were dead.
e The quoted number of fish had a swollen yolk sac.
f The quoted number of fish had a curvature of the spine.
g The quoted number of eggs was covered with fungi.
h The quoted number of fish (just hatched) was covered with fungi.
l The quoted numbers of fish showed irregular growth.
k The quoted number of fish were dark coloured.
m The quoted number of fish died accidentally.
n The quoted number of fish showed disturbed swimming behaviour (trembling) and had red coloured gills.
z The quoted number of fish showed a disturbed swimming behaviour.
2) Solvent control (= TBA).
3) Selection of fertilized eggs.

Time (d)	Test substance															
	benzo(k)fluoranthene (1.8 µg.l^{-1})								benzo(k)fluoranthene (3.2 µg.l^{-1})							
	a		b		c		d		a		b		c		d	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	62[a]		61[a]		60[a]		60[a]		60[a]		58[a]		60[a]		57[a]	
1	43		49		47		43		48		42		44		43	
	19[d]		12[d]		13[d]		17[d]		12[b]		16[d]		16[d]		14[d]	
	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)
	20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]	
5	0	20[b]	0	20[b]	0	20[b]	0	20	0	20[b]	0	20[b]	0	20[b]	0	20[b]
								18[b]								
								2[f]								
7		18		18		19[g]		18		20[b]		20[b]		20[b]		20[b]
		2[d]		2[d]		16[c]		2[d]								
		15[c]		15[c]		1[f]		12[c]								
		1[f]		1[f]		2[ez]		2[f]								
		2[ez]		2[ez]				4[ez]								
14		19		16		18		14.4[d]		20[b]		20[b]		20[b]		20[b]
		16[b]		2[d]		1[d]		13[b]								
		3[c]		15[b]		14[b]		1[c]								
				1[c]		4[c]										
21		19[c]		16[c]		16		12		19[c]		20[c]		19[c]		19[c]
						2[d]		2[d]		1[d]				1[d]		
						c		c								
28		18		16[l]		16[l]		10		19[c]		20[c]		19[c]		19[c]
		1[d]						1[d]								
		11[c]						1[m]								
		7[l]						9[l]								
35		14[ln]		14[ln]		15[ln]		10[ln]		19[cn]		20[cn]		19[cn]		18[cn]
		4[d]		2[d]												1[d]
42		9[ln]		12[ln]		11[ln]		10[ln]		19[cln]		20[cln]		19[cln]		18[cln]
		5[d]		2[d]		4[d]										

1) The following codes are used to denote condition:
a Condition of the quoted number of fish (eggs), visually assessed, normal (= good).
b Condition of the quoted number of fish (eggs), visually assessed, equal to that of the control animals.
c The quoted number of fish were smaller.
d The quoted number of fish (eggs) were dead.
e The quoted number of fish had a swollen yolk sac.
f The quoted number of fish had a curvature of the spine.
g The quoted number of eggs was covered with fungi.
h The quoted number of fish (just hatched) was covered with fungi.
l The quoted numbers of fish showed irregular growth.
k The quoted number of fish were dark coloured.
m The quoted number of fish died accidentally.
n The quoted number of fish showed disturbed swimming behaviour (trembling) and had red coloured gills.
z The quoted number of fish showed a disturbed swimming behaviour.
2) Solvent control (= TBA).
3) Selection of fertilized eggs.

Time (d)	Test substance															
	chrysene (1.8 µg.l^{-1})								benzo(a)pyrene (10 µg.l^{-1})							
	a		b		c		d		a		b		c		d	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	61[a]		61[a]		61[a]		61[a]		59[a]		60		61[a]		59[a]	
1	44		43		37		47		46		48		34		45	
	17[d]		18[d]		24[d]		14[d]		13[d]		12[d]		27[d]		14[d]	
	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)
	20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]	
5	0	20[b]	0	20[b]	0	20[b]	0	20[b]	0	20[b]	0	20[b]	0	20[b]	0	20[b]
7		20		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]
		18[b]														
		1[ce]														
		1[c]														
14		18[b]		20[b]		20[b]		20[b]		19[b]		20[b]		20[b]		20
		2[d]								1[d]						17[b]
																3[c]
21		18[b]		20[b]		20		20[b]		19		20[b]		20[b]		19
						19[b]				16[b]						18[b]
						1[c]				3[c]						1[c]
																1[d]
28		18[b]		20[b]		20		20[b]		19[b]		20[b]		20[b]		19
						1[c]										18[b]
						19[b]										1[c]
35		18[b]		20[b]		20		20[b]		19[b]		19[b]		20[b]		19
						19[b]						1[d]				18[b]
						1[c]										1[c]
42		18[b]		20[b]		20		20[b]		19[b]		19[b]		20[b]		19
						1[c]										18[b]
						19[b]										1[c]

1) The following codes are used to denote condition:

a Condition of the quoted number of fish (eggs), visually assessed, normal (= good).

b Condition of the quoted number of fish (eggs), visually assessed, equal to that of the control animals.

c The quoted number of fish were smaller.

d The quoted number of fish (eggs) were dead.

e The quoted number of fish had a swollen yolk sac.

f The quoted number of fish had a curvature of the spine.

g The quoted number of eggs was covered with fungi.

h The quoted number of fish (just hatched) was covered with fungi.

l The quoted numbers of fish showed irregular growth.

k The quoted number of fish were dark coloured.

m The quoted number of fish died accidentally.

n The quoted number of fish showed disturbed swimming behaviour (trembling) and had red coloured gills.

z The quoted number of fish showed a disturbed swimming behaviour.

2) Solvent control (= TBA).

3) Selection of fertilized eggs.

Time (d)	Test substance															
	benzo(ghi)perylene (0.32 µg.l^{-1})								fluoranthene (320 µg.l^{-1})							
	a		b		c		d		a		b		c		d	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	59[a]		60[a]		61[a]		63[a]		59[a]		59[a]		62[a]		60[a]	
1	43		40		45		48		38		44		42		42	
	16[d]		20[d]		16[d]		15[d]		21[d]		15[d]		20[d]		18[d]	
	20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]		20[b]	
	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)	3)
5	0	20	0	20	0	0	20	0	1[g]	19	0	19	1[h]	17	0	20
		19[b]								2		1[def]		2[def]		2
		1[efz]								2[e]		2		2		2[e]
		2[e]										2[e]		2[e]		
7		20[b]		20[b]		20		20[b]		19		18		15		18
		1[d]				1[f]				19[c]		1[d]		2[d]		2[d]
										16		18[c]		15[c]		18[c]
										2[e]		4[k]		15		5[f]
										1[f]		18		2[e]		2[k]
										4[k]		2[e]		6[f]		15
												5[f]		2[k]		2[e]
14		20[b]		20[b]		19[b]		20[b]		0		0		0		
						1[d]				19[d]		18[d]		15[d]		1
																17[d]
																c
21		20[b]		20[b]		19[b]		20[b]		0		0		0		0
																1[d]
28		20[b]		20[b]		19[b]		20[b]								
35		20[b]		20[b]		19[b]		20[b]								
42		20[b]		19[b]		19[b]		20[b]								
				1[d]												

1) The following codes are used to denote condition:
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- e The quoted number of fish had a swollen yolk sac.
- f The quoted number of fish had a curvature of the spine.
- g The quoted number of eggs was covered with fungi.
- h The quoted number of fish (just hatched) was covered with fungi.
- l The quoted numbers of fish showed irregular growth.
- k The quoted number of fish were dark coloured.
- m The quoted number of fish died accidentally.
- n The quoted number of fish showed disturbed swimming behaviour (trembling) and had red coloured gills.
- z The quoted number of fish showed a disturbed swimming behaviour.

2) Solvent control (= TBA).

3) Selection of fertilized eggs.

Table B2 *Total number of surviving eggs and larvae in the control media and PAH solutions of benzo(k)fluoroanthene, chrysene, benzo(a)pyrene, benzo(ghi)perylene and fluoranthene during the exposure time (combined for the four replicate retenention chambers).*

Time (d)	PAH tested															
	0		0[TBA 2)]		B(k)F (1.8)		B(a)F (3.2)		Chr		B(a)P		B(ghi)P		Flu	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	238	0	244	0	243	0	235	0	244	0	239	0	243	0	241	0
1	188	0	187	0	182	0	177	0	171	0	173	0	178	0	166	0
	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)	1)
1	82	0	80	0	80	0	80	0	80	0	80	0	80	0	80	0
5	0	82	1	79	0	80	0	80	0	80	0	80	0	80	1	75
7	0	82	0	80	0	74	0	80	0	80	0	80	0	79	0	70
14	0	82	0	80	0	69	0	80	0	78	0	79	0	79	0	1
21	0	82	0	80	0	63	0	78	0	78	0	78	0	80	0	0
28	0	82	0	80	0	60	0	77	0	78	0	78	0	79		
35	0	82	0	80	0	53	0	76	0	78	0	77	0	79		
42	0	82	0	80	0	42	0	76	0	78	0	77	0	78		

1) Selection of fertilized eggs.
2) Solvent control (= TBA).

ANNEX C DETAILS ON LENGTH AND WET WEIGHT OF THE FISH PER TEST SUBSTANCE CONCENTRATION (in fourfold, a, b, c and d)

Table C1 *Total length of the test fishes per test substance concentration (the nominal concentrations for the replicate retention chambers a, b, c and d are given in vertical sequence; length in cm)*[1)].

Conc.	raw data																	
0.00	1.40	1.50	1.60	1.50	1.50	1.50	1.50	1.30	1.50	1.50	1.70	1.50	1.40	1.60	1.60	1.60	1.60	1.
	50	1.40	1.60															
0.00	1.50	1.50	1.50	1.40	1.50	1.50	1.50	1.50	1.60	1.60	1.40	1.70	1.60	1.40	1.50	1.60	1.40	1.
	50	1.60	1.70															
0.00	1.60	1.40	1.70	1.70	1.40	1.50	1.60	1.70	1.70	1.40	1.60	1.80	1.60	1.80	1.40	1.30	1.40	1.
	50	1.40																
0.00	1.40	1.60	1.60	1.70	1.50	1.70	1.60	1.50	1.40	1.50	1.50	1.60	1.60	1.50	1.50	1.60	1.50	1.
	60	1.60	1.70															
1.00	1.70	1.50	1.20	1.30	1.60	1.40	1.60	1.70	1.60	1.40	1.60	1.50	1.50	1.50	1.70	1.40	1.70	1.
	80	1.50	1.50	1.60	1.40													
1.00	1.60	1.50	1.60	1.60	1.60	1.50	1.60	1.70	1.50	1.70	1.50	1.70	1.50	1.40	1.70	1.60	1.70	1.
	60	1.50	1.50															
1.00	1.50	1.60	1.60	1.70	1.60	1.60	1.60	1.40	1.50	1.40	1.60	1.30	1.60	1.70	1.60	1.40	1.60	1.
	50	1.50	1.60															
1.00	1.60	1.50	1.70	1.50	1.60	1.70	1.50	1.40	1.60	1.70	1.50	1.40	1.50	1.70	1.50	1.40	1.50	1.
	70	1.60	1.50															
2.00	1.70	1.10	0.900	1.00	1.00	1.60	1.30	0.900	1.40									
2.00	0.700	0.800	0.800	1.40	1.60	1.60	1.20	1.10	1.10	1.10	1.40	1.60						
2.00	0.800	1.00	1.10	1.80	1.50	1.70	1.40	1.00	1.30	1.40	0.900							
2.00	0.900	0.700	1.20	1.20	1.20	1.10	1.30	1.20	1.00	1.00								
3.00	1.00	1.00	1.40	1.20	1.00	1.10	1.10	1.20	1.10	1.10	1.40	1.50	1.30	1.30	1.40	1.40	1.30	1.
	10	1.20																
3.00	0.900	1.30	1.40	1.00	1.50	1.00	1.10	1.20	1.20	1.10	1.60	1.20	1.40	0.900	1.20	1.20	1.10	1.
	20	1.00	1.10															
3.00	1.10	1.40	1.40	0.900	1.30	1.10	1.40	1.00	1.10	1.00	1.40	1.50	1.10	1.30	1.30	1.20	1.00	1.
	30	1.30																
3.00	1.10	0.900	1.10	1.40	1.40	1.00	1.00	1.10	1.30	1.00	1.50	1.30	1.30	1.30	1.40	0.900	0.900	1.
	10																	
4.00	1.70	1.40	1.50	1.70	1.50	1.60	1.50	1.70	1.60	1.90	1.80	1.70	1.80	1.50	1.60	1.60	1.70	1.
	50																	
4.00	1.50	1.60	1.60	1.80	1.50	1.60	1.50	1.80	1.60	1.60	1.40	1.40	1.60	1.50	1.60	1.80	1.70	1.
	50	1.20	1.60															
4.00	0.800	1.60	1.50	1.60	1.50	1.50	1.70	1.30	1.30	1.90	1.80	1.50	1.70	1.60	1.60	1.60	1.50	1.
	60	1.70	1.40															
4.00	1.60	1.60	1.50	1.70	1.50	1.70	1.60	1.60	1.50	1.60	1.60	1.70	1.30	1.60	1.40	1.50	1.70	1.
	40	1.60	1.50															
5.00	1.30	1.60	1.50	1.50	1.40	1.60	1.70	1.50	1.70	1.60	1.50	1.50	1.70	1.60	1.50	1.40	1.60	1.
	70	1.70																
5.00	1.60	1.50	1.50	1.50	1.70	1.50	1.70	1.60	1.50	1.60	1.60	1.30	1.60	1.70	1.50	1.30	1.70	1.
	60	1.50																
5.00	1.40	1.60	1.80	1.70	1.60	1.30	1.60	1.60	1.50	1.50	1.50	1.50	1.60	1.40	1.70	1.60	1.40	1.
	20	1.30	1.40															
5.00	0.800	1.60	1.50	1.70	1.50	1.50	1.60	1.30	1.60	1.30	1.70	1.70	1.50	1.30	1.50	1.50	1.60	1.
	40	1.60																
6.00	1.50	1.50	1.60	1.50	1.60	1.80	1.50	1.60	1.30	1.60	1.60	1.70	1.50	1.50	1.70	1.30	1.50	1.
	80	1.60	1.50															
6.00	1.50	1.70	1.50	1.80	1.60	1.80	1.60	1.50	1.50	1.40	1.70	1.60	1.60	1.50	1.50	1.50	1.70	1.
	70	1.50																
6.00	1.90	1.80	1.50	1.30	1.50	1.70	1.70	1.80	1.50	1.70	1.80	1.70	1.30	1.50	1.80	1.70	1.60	1.
	70	1.60																
6.00	1.60	1.70	1.70	1.50	1.50	1.50	1.60	1.40	1.50	1.60	1.60	1.70	1.30	1.60	1.50	1.60	1.50	1.
	70	1.70	1.60															

1) 0.00 = TBA control
1.00 = DSWL control
2.00 = Benzo(k)fluoranthene (1.8 $\mu g.l^{-1}$)
3.00 = Benzo(k)fluoranthene (3.2 $\mu g.l^{-1}$)
4.00 = Chrysene
5.00 = Benzo(a)pyrene
6.00 = Benzo(ghi)perylene

Table C2 *Wet weight of the test fish per test substance concentration (the nominal concentrations for the replicate retention chambers a, b, c and d are given in vertical sequence; wet weight in mg)[1].*

Conc.	raw data																
0.00	16.4	19.4	27.3	18.8	24.4	21.9	22.7	16.9	21.6	22.2	33.3	21.2	20.0	28.1	31.4	26.1	26.5
	25.5	18.8	25.3														
0.00	22.3	24.5	22.9	15.8	24.2	26.3	23.7	20.9	30.7	25.3	19.9	31.8	29.6	18.2	25.3	33.9	17.8
	26.0	27.1	37.3														
0.00	26.4	23.1	39.6	34.9	16.6	22.4	26.7	36.9	32.3	22.8	30.2	36.1	34.1	36.8	19.8	15.6	23.2
	26.2	16.2															
0.00	16.8	26.0	28.8	31.0	26.7	30.3	27.3	24.3	17.1	24.5	24.7	28.7	29.3	26.7	28.8	29.0	24.5
	28.3	24.5	35.3														
1.00	27.2	23.4	12.2	12.2	31.1	16.5	25.9	31.3	29.3	17.9	24.6	26.9	27.1	25.5	27.8	20.0	33.2
	38.1	20.9	19.6	24.1	15.4												
1.00	32.3	24.2	28.1	25.3	30.3	26.6	27.0	34.7	25.0	33.8	23.7	30.9	23.8	17.3	35.6	23.8	33.9
	27.4	25.8	22.8														
1.00	24.6	27.0	25.4	36.4	26.6	27.1	23.2	19.4	20.0	20.2	25.9	17.8	33.0	39.5	24.3	19.9	26.9
	26.4	23.4	36.6														
1.00	28.3	24.6	32.2	20.6	27.5	34.5	21.1	18.1	23.9	31.0	25.1	20.2	21.8	31.4	17.7	19.6	21.0
	32.0	24.7	23.8														
2.00	44.0	12.2	6.20	5.70	8.10	40.5	22.8	5.10	28.0								
2.00	1.30	3.60	4.50	25.5	40.4	46.1	15.8	9.50	16.0	12.1	20.6	32.6					
2.00	3.20	9.90	15.5	56.7	24.7	52.9	17.4	7.80	21.1	25.9	6.30						
2.00	4.10	1.60	19.0	22.0	19.1	15.8	23.2	16.4	13.4	10.3							
3.00	8.80	10.8	24.2	20.5	9.90	13.5	16.0	17.9	12.7	11.7	27.8	29.2	23.4	23.9	23.3	24.0	18.8
	14.1	22.0															
3.00	8.20	24.6	27.5	11.4	28.8	13.1	12.0	18.2	16.5	13.0	34.6	16.1	29.1	5.70	18.2	19.1	16.0
	17.2	8.20	14.4														
3.00	13.6	27.4	28.4	7.90	20.3	13.3	23.6	11.9	13.2	11.2	24.8	31.6	15.2	23.3	20.3	17.2	13.1
	24.6	21.5															
3.00	14.2	6.50	14.7	26.2	25.7	11.4	11.0	13.4	20.1	9.90	31.1	25.5	21.7	31.2	26.3	8.20	7.00
	15.2																
4.00	34.1	17.1	17.3	29.9	22.9	27.2	25.1	37.6	23.3	43.7	34.1	35.2	43.8	24.2	26.9	32.3	32.3
	24.5																
4.00	23.6	29.2	25.9	33.6	23.5	28.7	23.2	36.8	33.7	25.1	16.9	18.1	28.8	23.1	23.2	40.1	27.3
	21.6	12.8	21.3														
4.00	2.50	25.5	22.2	31.6	21.2	18.6	36.8	13.8	16.6	45.1	33.7	23.0	30.4	30.3	27.1	28.8	24.5
	27.1	29.7	19.6														
4.00	29.9	26.1	25.8	27.2	19.1	31.7	23.3	27.3	23.2	26.1	24.3	34.3	16.2	26.3	18.5	21.1	34.3
	22.0	25.6	21.6														
5.00	17.3	32.5	29.8	20.6	15.5	26.8	26.6	27.3	40.0	29.3	20.9	22.2	29.5	32.5	23.1	16.4	24.6
	32.5	32.2															
5.00	32.2	23.2	22.9	25.7	28.1	22.3	33.6	23.8	21.3	34.5	28.1	16.1	29.8	36.5	27.2	19.8	34.4
	26.6	24.7															
5.00	23.7	31.3	43.6	37.1	33.0	19.3	25.7	28.4	24.4	27.0	24.7	26.3	26.2	23.1	37.1	25.7	17.9
	12.2	14.8	20.8														
5.00	1.90	32.7	25.3	37.9	23.7	26.0	30.6	17.7	31.9	39.0	45.1	31.8	23.6	14.0	25.2	29.0	29.0
	18.0	27.3															
6.00	23.0	23.2	29.1	23.3	30.3	37.2	22.7	30.4	15.0	27.1	29.1	31.8	20.7	21.0	32.3	14.6	22.8
	48.7	23.7	22.5														
6.00	24.9	35.9	24.1	35.9	27.3	39.8	27.9	24.6	22.2	17.3	33.4	30.6	27.2	25.7	25.9	22.9	34.4
	35.2	24.2															
6.00	43.4	42.6	24.2	14.7	23.1	32.8	37.3	37.7	24.5	32.8	36.9	35.0	12.8	24.2	33.8	28.7	2 5
	27.1	32.7															
6.00	31.1	29.2	31.6	26.2	21.7	24.2	27.5	20.6	24.0	32.1	26.5	32.0	14.5	30.1	21.9	33.4	20.9
	35.3	30.4	27.1														

[1] 0.00 = TBA control
1.00 = DSWL control
2.00 = Benzo(k)fluoranthene (1.8 $\mu g.l^{-1}$)
3.00 = Benzo(k)fluoranthene (3.2 $\mu g.l^{-1}$)
4.00 = Chrysene
5.00 = Benzo(a)pyrene
6.00 = Benzo(ghi)perylene

ANNEX D DETAILS ON WET WEIGHT AND LENGTH OF THE FISH PER TEST SUBSTANCE CONCENTRATION

Table D1 *Wet weight (the nominal concentrations for the replicate retention chambers a, b, c and d are given in vertical sequence weight in mg)*[1)].

	Conc.	mean	s.d.	N	MEAN	S.D.	test stat.	
TBA blanco	0.00	23.4	4.58	20.0	25.6	5.74		
	0.00	25.2	5.51	20.0				
	0.00	27.4	7.68	19.0				
	0.00	26.6	4.28	20.0				
blanco	1.00	24.1	6.74	22.0	25.7	5.80	0.0460	
	1.00	27.6	4.78	20.0				
	1.00	26.2	6.04	20.0				
	1.00	25.0	5.14	20.0				
Benzo (k) Fluoranthee 1,8	2.00	19.2	15.3	9.00	18.7	14.1	-4.71	**
	2.00	19.0	14.6	12.0				
	2.00	21.9	17.9	11.0				
	2.00	14.5	7.24	10.0				
Benzo[k] Fluoranthen 3,2	3.00	18.6	6.30	19.0	18.2	7.19	-6.01	**
	3.00	17.6	7.75	20.0				
	3.00	19.1	6.73	19.0				
	3.00	17.7	8.32	18.0				
Chrysen	4.00	29.5	7.79	18.0	26.4	7.33	0.649	
	4.00	25.8	6.72	20.0				
	4.00	25.4	9.06	20.0				
	4.00	25.2	4.88	20.0				
Benzo pyreen	5.00	26.3	6.49	19.0	26.5	7.36	0.742	
	5.00	26.9	5.56	19.0				
	5.00	26.1	7.65	20.0				
	5.00	26.8	9.68	19.0				
Benzo ghi pryleen	6.00	26.4	7.73	20.0	27.9	6.95	1.87	
	6.00	28.4	5.90	19.0				
	6.00	29.9	8.46	19.0				
	6.00	27.0	5.29	20.0				

* level of significance: 95 %.
** level of significance: 99 %.

1) 0.00 = TBA control
1.00 = DSWL control
2.00 = Benzo(k)fluoranthene (1.8 µg.l^{-1})
3.00 = Benzo(k)fluoranthene (3.2 µg.l^{-1})
4.00 = Chrysene
5.00 = Benzo(a)pyrene
6.00 = Benzo(ghi)perylene

Table D2 *Length (the nominal concentrations for the replicate retention chamber a, b, c and d are given in vertical sequence; length in mm)*[1].

	Conc.	mean	s.d.	N	MEAN	S.D.	test stat.	
TBA bianco	0.0000	1.51	0.0933	20.0	1.54	0.109		
	0.0000	1.52	0.0910	20.0				
	0.0000	1.55	0.154	19.0				
	0.0000	1.56	0.0883	20.0				
bianco	1.00	1.53	0.146	22.0	1.55	0.114	0.573	
	1.00	1.58	0.0894	20.0				
	1.00	1.54	0.105	20.0				
	1.00	1.55	0.105	20.0				
Benzo (k) Fluoranthene 1,8	2.00	1.21	0.302	9.00	1.19	0.290	-11.4	**
	2.00	1.20	0.325	12.0				
	2.00	1.26	0.329	11.0				
	2.00	1.08	0.181	10.0				
Benzo (k) Fluoranthene 3,2	3.00	1.22	0.157	19.0	1.19	0.177	-13.3	**
	3.00	1.18	0.188	20.0				
	3.00	1.22	0.174	19.0				
	3.00	1.17	0.197	18.0				
chrysen	4.00	1.63	0.132	18.0	1.57	0.161	1.32	
	4.00	1.57	0.145	20.0				
	4.00	1.53	0.228	20.0				
	4.00	1.56	0.110	20.0				
Benzo a) pyren	5.00	1.56	0.117	19.0	1.53	0.153	-0.468	
	5.00	1.55	0.117	19.0				
	5.00	1.51	0.152	20.0				
	5.00	1.48	0.209	19.0				
Benzo (ghi) perylen	6.00	1.56	0.131	20.0	1.59	0.133	1.97	
	6.00	1.59	0.115	19.0				
	6.00	1.64	0.167	19.0				
	6.00	1.57	0.108	20.0				

* level of significance: 95 %.
** level of significance: 99 %.

1) 0.00 = TBA control
1.00 = DSWL control
2.00 = Benzo(k)fluoranthene (1.8 µg.l^{-1})
3.00 = Benzo(k)fluoranthene (3.2 µg.l^{-1})
4.00 = Chrysene
5.00 = Benzo(a)pyrene
6.00 = Benzo(ghi)perylene

ANNEX E DETAILS ON pH, OXYGEN VALUES AND TEMPERATURES MEASURED DURING THE TEST

Table E1 *pH values in the control medium and in the test solutions of benzo(k)fluoroanthene, benzo(ghi)perylene, benzo(a)pyrene, chrysene and fluoranthene during the early life stage test.*

Time (d)	Nominal concentration of fluoranthene (µg.l^{-1})							
	0	0 TBA 1)	B(k)F (1.8 µg.l^{-1})	B(k)F (3.2 µg.l^{-1})	B(ghi)P	B(a)P	Chrysene	Fluor-anthene
0	8.1	8.1	8.1	8.2	8.1	8.2	8.2	8.2
7	8.0	8.1	8.1	8.1	8.1	8.1	8.1	8.1
14	8.1	8.0	8.0	8.0	8.0	8.1	8.1	8.1
21	7.9	7.9	7.9	7.9	7.8	7.9	7.9	8.0
28	8.0	8.0	8.0	8.0	7.9	8.0	8.0	8.0
35	7.9	8.0	8.0	8.0	8.0	8.0	8.0	–
42	8.1	8.1	8.1	8.1	8.1	8.1	8.1	–

1) Solvent control (= TBA).

Table E2 *Oxygen concentrations ($mg.l^{-1}$.) in the control medium and in the test solutions of benzo(k)fluoroanthene, benzo(ghi)perylene, benzo(a)pyrene, chrysene and fluoranthene during the early life stage test.*

Time (d)	Nominal concentration of fluoranthene ($\mu g.l^{-1}$)							
	0	0 TBA 1)	B(k)F (1.8 $\mu g.l^{-1}$)	B(k)F (3.2 $\mu g.l^{-1}$)	B(ghi)P	B(a)P	Chrysene	Fluor-anthene
0	8.3	8.3	8.3	8.9	8.4	8.7	8.8	9.0
7	9.0	8.7	8.7	8.9	8.8	8.9	8.8	8.8
14	8.4	8.0	8.0	8.0	8.1	8.0	7.7	8.0
21	7.6	7.8	8.1	8.3	8.3	8.1	8.2	8.3
28	9.4	8.1	8.2	7.6	7.7	7.5	7.6	–
35	8.3	7.8	8.1	7.6	8.0	7.6	7.7	–
42	8.4	8.3	8.0	8.3	8.1	8.1	8.3	–

1) Solvent control (= TBA).

Table E3 *Temperature (°C) in the control medium of benzo(k)fluoroanthene, chrysene, benzo(a)pyrene, benzo(ghi)perylene and fluoranthene during the early life stage test.*

Time (d)	Temperature (°C)
0	24.9
1	24.8
2	24.9
5	24.9
6	25.1
7	25.0
8	25.0
9	25.0
10	25.0
11	25.0
12	24.8
13	24.9
14	24.8
15	24.9
16	24.9
17	24.9
18	24.9
19	24.9
20	24.8
21	24.7
22	24.8
23	24.9
24	24.9
25	25.0
26	25.0
27	24.8
28	24.4
29	24.7
30	24.8
31	24.7
32	24.8
33	24.7
34	24.7
35	24.6
36	24.8
37	24.8
38	24.6
39	24.8
40	24.6
41	24.9
42	24.9

ANNEX F ESTIMATION OF THE LC50 AND ITS CONFIDENCE INTERVAL

At a given time, the mortality probability of an individual is assumed to be logistically related to the logarithm of the test substance concentration, i.e.

$$p_i = \frac{e_i + p_0}{1 + e_i}, \text{ where } e_i = (c_i/\alpha)^{1/\beta} \text{ and}$$

p_i is the mortality probability in the i^{th} concentration

p_0 is the mortality probability in concentration 0

α is the LC50

β is a parameter inversely proportional to the maximum gradient of the dose response function

c_i is the i^{th} concentration.

The parameters p_0, α and β are estimated from the counts by means of the maximum likelihood method; i.e. the parameter values to be selected maximize the probability of the counts as a function of the three parameters. Since the distribution of α will not be symmetrical the variance-covariance matrix is not estimated for the parameters p_0, α and β themselves, but for p_0, $\gamma = \ln \alpha$ and β. The variance-covariance matrix is estimated by the inverse of the information matrix.
The 95% confidence limits of the LC50 are now given by

$$\alpha \,.\, \exp (\pm 2\, [\text{var } (\gamma)]^{1/2}) = \alpha \,.\, \exp (\pm 2\, [\text{var } (\ln \alpha)]^{1/2}).$$