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The toxicity and uptake of benzo(k)-
fluoranthene using *Brachydanio rerio* in an
early life stage test
(Draft OECD Guideline)

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SUMMARY AND CONCLUSIONS

At the request of RIZA and the Ministry of Housing, Physical Planning and the Environment, the influence of benzo(k)fluoranthene on hatching, mortality and the occurrence of egg and larval malformations in the fresh water fish species *Brachydanio rerio* was determined as laid down in the Draft OECD Guideline (ref. 1). For each test concentration, ca. 4 x 15 eggs were used. The exposure duration was 42 days. The test was carried out in an intermittent flow-through system. The nominal concentrations of benzo(k)fluoranthene tested were 0, 0.32, 0.56, 1.0, 1.8 and 3.2 $\mu\text{g.l}^{-1}$.

Hatching of the eggs after 6 days was not affected at any of the concentrations tested. At 1.0 $\mu\text{g.l}^{-1}$ and 1.8 $\mu\text{g.l}^{-1}$, the fish showed lesions along the sides of their bodies; deformed caudal fins and exhibited trembling movements. At all concentrations tested, the gills were red and swollen.

The lowest NOEC for the quantitatively investigated criteria (mortality and growth) was 0.32 $\mu\text{g.l}^{-1}$.

The results of the test were:

	in nominal conc.	in actual conc. ³⁾
42d LC50 (mortality)	: 0.95 $\mu\text{g.l}^{-1}$	0.68 $\mu\text{g.l}^{-1}$
42d NOEC ¹⁾ (mortality)	: 0.56 $\mu\text{g.l}^{-1}$	0.40 $\mu\text{g.l}^{-1}$
42d LOEC ²⁾ (mortality)	: 1.0 $\mu\text{g.l}^{-1}$	0.72 $\mu\text{g.l}^{-1}$
42d NOEC ¹⁾ (growth = length and weight)	: 0.32 $\mu\text{g.l}^{-1}$	0.23 $\mu\text{g.l}^{-1}$
42d LOEC ²⁾ (growth = length and weight)	: 0.56 $\mu\text{g.l}^{-1}$	0.40 $\mu\text{g.l}^{-1}$
42d NOEC ¹⁾ (condition)	: < 0.32 $\mu\text{g.l}^{-1}$	< 0.23 $\mu\text{g.l}^{-1}$
42d LOEC ²⁾ (condition)	: $\leq$ 0.32 $\mu\text{g.l}^{-1}$	$\leq$ 0.23 $\mu\text{g.l}^{-1}$

1) No observed effect concentration.

2) Lowest observed effect concentration.

3) Based on an average percentage of 48% present during the test.

72%



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The actual concentrations of benzo(k)fluoranthene in the test solutions were determined by HPLC and were between 36% and 109% of the nominal concentrations (average 72%).

The body burden of benzo(k)fluoranthene in the fish was determined for each concentration tested and the bioconcentration factor based on the total wet weight of the fish varied between < 1.1 and 1.5. This low bioconcentration of benzo(k)fluoranthene, compared to its log $K_{O/W}$ can most likely be attributed to metabolism of benzo(k)fluoranthene and depuration of its more polar metabolites or to specific working mechanisms.



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1. INTRODUCTION

At the request of RIZA and the Ministry of Housing, Physical Planning and the Environment, the possible effects of benzo(k)anthracene on the early life stages of the fresh water fish *Brachydanio rerio* were determined. The 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 objectives of the study were:

- to determine the Lowest Observed Effect Concentration (LOEC), i.e. the lowest tested concentration of benzo(k)anthracene at which it is observed to have a significant effect (at $p \leq 0.05$) on the development of early life stages of *Brachydanio rerio* when compared with the control;
- to determine the No Observed Effect Concentration (NOEC), i.e. the test concentration immediately below the LOEC.
- to determine the body burden and the bioconcentration factor of the test substance in the fish;

The test was carried out in the period March 18, 1992-April 29, 1992.

2. MATERIALS AND METHOD

2.1 Test substance

The test substance examined was benzo(k)fluoranthene. It was supplied by C.N. Schmidt B.V., Amsterdam and was labelled: "Promochem GmbH, D 4230, WS 350. According to the information given by the sponsor the purity was almost 100%. It was 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 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 and should be $25 \pm 1^\circ\text{C}$.

Appropriate concentrations were determined in a preliminary test and five concentrations of benzo(k)fluoranthene were tested with DSWL as a control. The test substance was dosed from a stock solution in tertiary butyl alcohol (= TBA) and therefore a solution of 84.2 μl TBA per litre dilution water was used as a solvent control.

A quantity of 10 mg benzo(k)fluoranthene was accurately weighed out and dissolved in 259 ml TBA resulting in a solution of 38.61 mg.l^{-1} . This solution was used as a stock solution for the dosage of the highest test concentration ($3.2 \mu\text{g.l}^{-1}$) and to prepare the other stock solutions. For the latter 15, 26, 47 and 84 ml were diluted to 150 ml resulting in stock solutions of 3.86 mg.l^{-1} , 6.69 mg.l^{-1} , 12.10 mg.l^{-1} and 21.62 mg.l^{-1} respectively. The dosed volume of the stock solution was 84.2 μl to one litre of dilution water, resulting in test solutions of 0.32, 0.56, 1.0, 1.8 and $3.2 \mu\text{g.l}^{-1}$.

An intermittent flow-through system (see section 2.5) was used. At intervals of 30 minutes, each of four replicate retention chambers was supplied with ca. 250 ml newly prepared solution. Each compartment contained about 15 fish (or eggs). The test solutions were not aerated and pH values and oxygen concentrations were measured weekly in all test solutions.

At the start of the test, about 42 potentially fertilized eggs were placed in each retention chambers. After the first 24 h of exposure, this number of eggs was reduced to a total of about 60 fertilized eggs per test concentration. 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 three batches were needed for the test and divided equally between all test solutions and control media.

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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 weekly, 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.

2.5 intermittent flow-through system

The intermittent flow-through system used was constructed according to Van Leeuwen et al. (ref. 3) and diagrams of it are given 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 2.6 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 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).

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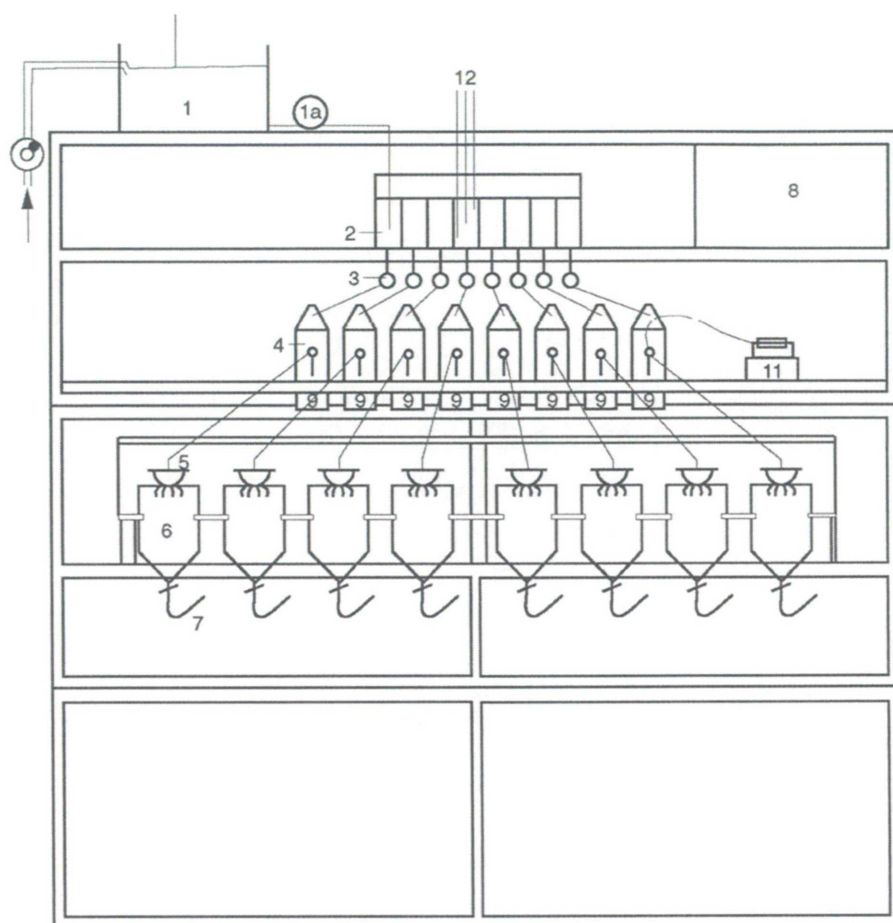


Figure 1 Front view.
 1 = water supply
 1a = valve
 2 = one litre volume water dosing compartment
 3 = valves (eightfold)
 4 = mixture bottles (eightfold)
 5 = flow splitters (eightfold)
 6 = test chambers (eightfold)
 7 = outlet tube
 8 = control panel
 9 = magnetic stirrers (eightfold)
 11 = syringe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

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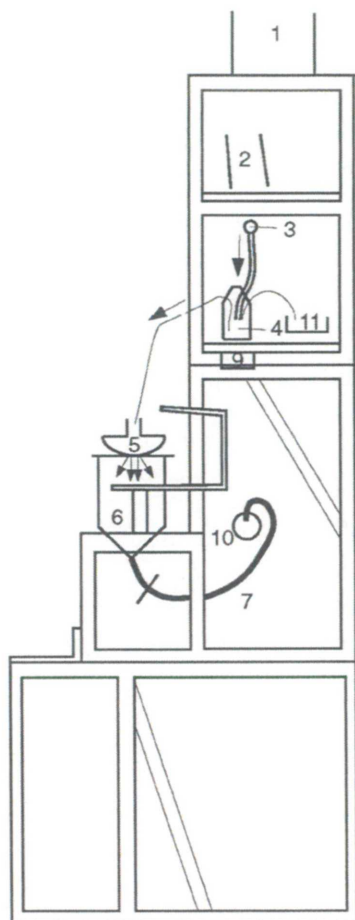


Figure 2 Cross section.

- 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

2.6 Treatment of the results

2.6.1 LC50 value

The effect of a test substance on the survival of animals is expressed by a quantity denoted as the LC50 (= Lethal Concentration, 50%), i.e. the exposure concentration of the substance which would prove lethal to 50% of an infinite population of the exposed animals. The LC50 is qualified according to duration of exposure.

The LC50 values and their confidence interval were calculated by means of a parametric model developed by Kooijman (ref. 2) A summary of this method is given in Annex F. For these calculations the combined mortality data per test concentration, as recorded in Annex C, Table C2 were used.

2.6.2 Growth

From the measurements of the length and wet weight of the individual fish, the average length or 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.

2.6.3 NOEC and LOEC values

The 'no observed effect concentrations' (NOEC values) are the highest concentrations tested showing no effects on the development of *Brachydanio rerio* throughout the exposure period. The NOEC values were estimated by comparing effects on mortality and growth of the exposed animals with those of the control animals. The 'lowest observed effect concentrations' (LOEC values) are the lowest concentrations tested with a significant effect on mortality or growth compared to the controls.

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2.6.4 Statistics

Statistical significance for mortality was determined with a binomial test at the 95% significance level, combining the results of the quadruplicates. Statistical significance for growth was determined with the two-tailed Dunnett -test with a 95% and 99% significance level. In both cases the observations at each concentration were compared with those of the control. In the case of significance at the 99% level only that significance is given.

2.7 Chemical analysis

The actual concentrations of benzo(k)fluoranthene in the test solutions and in the test fish were determined by chemical analysis using validated HPLC methods. Samples of ca. 250 ml were taken 5 days and 2 days before the start of the test and weekly during the test from the control solutions and from all test media. The samples were taken in glass bottles and analysed the same day. At the end of the test the fish of each concentration and the controls were blotted dry with tissue paper, sampled and stored frozen in a glass bottle until analysis.

2.7.1 Water samples

The samples were weighed and then extracted with 25 ml hexane. The hexane fraction was evaporated to nearly dryness, using a rotavapor and to dryness using 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 HPLC with UV absorption and fluorescence detection. Quantification was based on external standards (NBS-PAH Standard Reference Material 1647). The recovery of the method used varied between 86% and 109%.

2.7.2 Fish samples

The fish were weighed and then homogenized with the aid of a potter tube. The homogenate was transferred quantitatively to an erlenmeyer flask; the internal standard (2-methyl-chrysene) was added and then the homogenate was hydrolysed for 3 hours with 4 M NaOH.

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The hydrolysed fish 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 gently stream of nitrogen. The residu 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 pH

The pH values of the test solutions and control medium are recorded in Annex E, Table E1 and varied between 7.9 and 8.2. It is not likely that the pH affected the results.

3.2 Oxygen concentration

The oxygen concentrations of the test solutions and control medium are recorded in Annex E, Table E2. The lowest value measured was 7.2 mg.l⁻¹. It is not likely therefore that the oxygen concentration affected the results.

3.3 Temperature

The temperatures as measured in the control medium are recorded in Annex E, Table E3 and varied between 24.6°C and 25.0°C at the beginning and at the end of the test respectively. It is not likely that the temperature affected the results.

3.4 Effects

3.4.1 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 vessels in Annex B, Table B2.

In the control media all eggs hatched within 6 days into healthy larvae without visible malformations; at 0.32, 0.56, 1.0, 1.8 and 3.2 µg.l⁻¹ 95%, 97%, 96%, 93% and 97% of the eggs hatched respectively. After 35 days of exposure all fish had died at the concentration of 3.2 µg.l⁻¹.

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There was 25% mortality in the control medium at the end of the test. The mortality during 42 d of exposure at the concentrations of $0.32 \mu\text{g.l}^{-1}$ and $0.56 \mu\text{g.l}^{-1}$ was not significantly different from the control mortality (binomial test; $p = 0.05$). At the concentrations of $1.0 \mu\text{g.l}^{-1}$ and higher the mortality was significantly different from the control animals (binomial test; $p = 0.05$). The NOEC for mortality was therefore $0.56 \mu\text{g.l}^{-1}$.

The LC50 values and their confidence intervals for eggs and larvae of *Brachydanio rerio* exposed to benzo(k)fluoranthene are presented in Table 1, along with the NOEC and the LOEC values for mortality. The slope of the concentration-effect curve was too steep to be calculated.

Table 1 Results of the early life stage test with benzo(k)fluoranthene.

Parameter (d = days)	Effect	Nominal concentration ($\mu\text{g.l}^{-1}$)
7d LC50	mortality	> 3.2
14d LC50	mortality	2.4
21d LC50	mortality	1.6
28d LC50	mortality	1.2
35d LC50	mortality	1.1
42d LC50	mortality	0.95
42d NOEC	mortality	0.56
42d LOEC	mortality	1.0
42d NOEC	condition	≤ 0.32
42d LOEC	condition	< 0.32
42d NOEC	growth	0.32
42d LOEC	growth	0.56

3.4.2 Visual observations

In the control media all surviving larvae swam and fed actively during the exposure period and no malformations were noted during the test. At $1.8 \mu\text{g.l}^{-1}$ and $1.0 \mu\text{g.l}^{-1}$ all surviving animals were deformed at the end of the test; haemorrhages at the side of the bodies, redly swollen gills and deformed caudal fins were observed. All fish made trembling movements. Fish with redly swollen gills were also observed at all other benzo(k)-fluoranthene concentrations tested.

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The NOEC and LOEC for condition were therefore $\leq 0.32 \mu\text{g.l}^{-1}$ and $< 0.32 \mu\text{g.l}^{-1}$. However, it should be noted that the criterion condition is a subjective one.

3.4.3 Growth

The total length and the wet weight per fish are recorded for each test solution and the control medium in Annex C and in Annex D. A summary of the results is given in Table 2. The lowest concentration tested with a significant effect on growth ($p = 0.05$ and measured as length) was $0.32 \mu\text{g.l}^{-1}$. However, at $p = 0.01$ this effect was not significant. At $0.56 \mu\text{g.l}^{-1}$ growth was less than in the control at $p = 0.01$. At $1.0 \mu\text{g.l}^{-1}$ growth (measured as weight) was significantly retarded ($p = 0.01$) for the surviving fish. Because length measurements are less accurate than weight measurements the NOEC and LOEC with respect to growth are estimated to be $0.32 \mu\text{g.l}^{-1}$ and $0.56 \mu\text{g.l}^{-1}$ respectively.

Table 2 Summary of results on hatching, mortality and growth of eggs/larvae of *Brachydanio rerio* exposed to several concentrations of benzo(k)anthracene.

Nominal conc. of benzo(k)-anthracene ($\mu\text{g.l}^{-1}$)	% of eggs hatched after 6 d ⁵⁾	% mortality after 28 d	No. of fish	Length ¹⁾ (cm)	Wet weight ¹⁾ (mg)
0	100	25	43	1.79 ± 0.33	42.6 ± 19.6
0TBA	100	25	45	1.79 ± 0.29	43.1 ± 19.0
0.32	95	15	49	1.63 ± 0.23 ³⁾	37.6 ± 13.2
0.56	97	21	45	1.50 ± 0.31 ⁴⁾	35.2 ± 18.6
1.0	96	78 ²⁾	13	0.89 ± 0.32 ⁴⁾	9.1 ± 12.2 ⁴⁾
1.8	93	78 ²⁾	1	0.6	0.8
3.2	97	100 ²⁾			

¹⁾ Mean per fish and standard deviation.

²⁾ Mortality significantly higher than that of the control animals (binomial test; $p = 0.05$).

³⁾ Significantly less than control (two-tailed Dunnett-test, $p = 0.05$).

⁴⁾ Significantly less than control (two-tailed Dunnett-test, $p = 0.01$).

⁵⁾ All hatched fish are included, also in cases where they died shortly after hatching (see Table B1).

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3.5 Chemical analysis

The results of the chemical analysis are given in Table 3. The test substance benzo(k)fluoranthene had a purity of ca. 100% according to the supplier. The actual concentrations of benzo(k)fluoranthene in the test solutions were determined weekly and appeared to be between 36% and 109% of the nominal concentrations (average 72%). It should be noted that calculating such 'averages' might be questionable.

Due to an erratic analysis at $t = 8$ days actual concentrations for that exposure time are not available.



Table 3 Results of the chemical analysis of the benzo(k)fluoranthene in the test solutions in the intermittent flow=through system.
The purity of the test substance was ca. 100%.

Nominal conc. of benzo(k) fluoranthene $\mu\text{g.l}^{-1}$	Samples at t = -5 days		Samples at t = -2 days		Samples at t = 2 days		Samples at t = 15 days		Samples at t = 22 days		Samples at t = 29 days		Samples at t = 35 days		Mean concentration during the test from t = 2 days	
	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.	$\mu\text{g.l}^{-1}$	% of nom.
0	<0.032		<0.032		<0.032		<0.032		<0.032		<0.032		<0.032			
0 TBA 2)	<0.032		<0.032		<0.032		<0.032		<0.032		<0.032		<0.032			
0.32	0.21	65	0.22	69	0.20	63	0.19	59	0.19	59	0.19	59	0.17	53	0.19 ± 0.01	58.6 ± 3.5
0.56	0.20	36	0.36	64	0.36	64	0.45	80	0.34	61	0.32	57	0.29	52	0.35 ± 0.06	62.8 ± 10.6
1.0 3)	0.69	69	0.66	66	0.66	66	0.87	87	0.61	61	0.61	61	0.61	61	0.67 ± 0.11	67.2 ± 11.2
1.8 3)	1.0	56	1.1	61	1.1	61	2.2	122	1.8	100	1.7	94	1.8	100	1.7 ± 0.4	95.4 ± 22.0
3.2 3)	1.4	44	1.6	50	1.6	50	3.2	100	3.5	109	2.6	72	1.8	56	2.5 ± 0.8	77 ± 26.2
Average of nominal at the sampling days		54		62		61		90		78		69		64		
Average % of nominal during the test: 72																

1) The detection limit was $0.0032 \mu\text{g.l}^{-1}$.

2) Solvent control.

3) Dosed above the water solubility (ca. $0.64 \mu\text{g.l}^{-1}$)

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Table 4 Results of benzo(k)fluoranthene analysis in fish.

Nominal water concentration ($\mu\text{g.l}^{-1}$)	Concentration in fish ¹⁾ (ng per g wet weight)	Detection limit (ng per g wet weight)	Recovery internal standard ³⁾ (%)	Wet weight of fish sample (g)
0	< 0.38	0.38	83	1.82
0 TBA ²⁾	< 0.40	0.40	75	1.75
0.32	< 0.40	0.40	88	1.75
0.56	0.58	0.47	90	1.50
1.0	7.8	7.8	97	0.090

1) Not corrected for the recovery percentage.

2) Solvent control (= TBA).

3) Recovery for the internal standard 2-methylchrysene.

3.6 Bioconcentration

The bioconcentration of benzo(k)fluoranthene in the fish after three week exposure was calculated on basis of the wet weight concentration in fish and the actual water concentration. The results are given in Table 5.

Table 5 Bioconcentration of benzo(k)fluoranthene after 42 days exposure.

Nominal water concentration ($\mu\text{g.l}^{-1}$)	Actual water concentration ($\mu\text{g.l}^{-1}$)	Concentration in fish (ng.g ⁻¹) wet weight	BCF ³⁾
0	0	< 0.38	
0 ¹⁾	0	< 0.40	
0.32	0.23	< 0.40	< 1.8
0.56	0.40	0.58	1.5
1.0	0.72	< 7.8 ²⁾	< 1.1

1) Solvent control (TBA).

2) Only 13 fishes survived and were analysed.

3) Based on the total wet weight.

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The bioconcentration factors were low. On basis of the log n-octanol/water partition coefficient ($= 6.0$) a BCF for benzo(k)fluoranthene in aquatic species of ca. 8.750 has been calculated (ref. 3). The most likely reason for this low bioconcentration factor is the metabolization of fluoranthene combined with depuration of the more polar metabolites. This is indicated by the presence of peaks, additional to the benzo(k)fluoranthene peak in the HPLC chromatogram.

The highest concentration measured in the fish was 7.8 ng.g^{-1} at a test concentration of $1 \text{ }\mu\text{g.l}^{-1}$. This corresponds to 0.03 nmol.g^{-1} . The lethal body burden of narcotic compounds, however, is ca. $2 \text{ }\mu\text{mol.g}^{-1}$ (ref. 5). Lethality observed at $1 \text{ }\mu\text{g.l}^{-1}$ can possibly also be attributed to the presence of more toxic metabolites or to specific working mechanisms.



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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 ($< 1 \text{ mg.l}^{-1}$). 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 CaCO₃, 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 batch used for this test was checked by chemical analysis and found to be:

	Period 18-03-1992 till 31-03-1992	31-03-1992 till 29-04-1992
Na ⁺	1.11 mmol.l ⁻¹	1.04 mmol.l ⁻¹
K ⁺	0.23 mmol.l ⁻¹	0.21 mmol.l ⁻¹
Ca ²⁺	1.39 mmol.l ⁻¹	1.33 mmol.l ⁻¹
Mg ²⁺	0.70 mmol.l ⁻¹	0.70 mmol.l ⁻¹
Cl ⁻	2.67 mmol.l ⁻¹	2.54 mmol.l ⁻¹
SO ₄ ²⁻	0.69 mmol.l ⁻¹	0.73 mmol.l ⁻¹

The hardnesses, expressed as CaCO₃, were 209 mg.l⁻¹ and 203 mg.l⁻¹ respectively. The total organic carbon contents were 1.5 mg.l⁻¹ and 1.4 mg.l⁻¹ respectively.



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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 at five concentrations of benzo(k)fluoranthene during the exposure period (a, b, c and d are the replicate retention chambers¹⁾).

Time (d)	Nominal concentration of test substance (μg.l ⁻¹)															
	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	44 ^a		42 ^a		39 ^a		44 ^a		44 ^a		41 ^a		42 ^a		43 ^a	
1	13 ^d		15 ^d		17 ^d		17 ^d		14 ^d		15 ^d		19 ^d		20 ^d	
	31 ^d ₃₎		27 ^d ₃₎		22 ^d ₃₎		27 ^d ₃₎		30 ^d ₃₎		26 ^d ₃₎		23 ^d ₃₎		23 ^d ₃₎	
2	15 ^a		15 ^a		15 ^a		15 ^a		15 ^b		15 ^b		15 ^b		15 ^b	
5	15 ^a		15 ^a		15 ^a		15 ^a		15 ^b		15 ^b		15 ^b		15 ^b	
6	1 ^a	14 ^a		15 ^a	15 ^a	15 ^a	15 ^a	15 ^a	15 ^b	15 ^b	15 ^b	15 ^b	16	13 ^b	1 ^b	14 ^b
		13 ^a		14 ^a										13 ^b		15 ^b
		2 ^d		1 ^d										14 ^b		12 ^b
7		13 ^a		13 ^a		13 ^a	12 ^a	13 ^b		14 ^b		12 ^b		1 ^d		3 ^d
				1 ^d		2 ^d	1 ^j	2 ^d		1 ^j		3 ^d				
8		12 ^a		11 ^a		13 ^a	10 ^a	13 ^b		12 ^b		12 ^b		12 ^b		11 ^b
		1 ^d		2 ^d			1 ^j			3 ^d		2 ^d		2 ^d		1 ^d
9		12 ^a		11 ^a		13 ^a	10 ^a	13 ^b		12 ^b		12 ^b		12 ^b		10 ^b
							1 ^d									1 ^d
12		12 ^{al}		11 ^{al}		13 ^{al}	9 ^{al}	13 ^{bl}		12 ^{bl}		12 ^{bl}		12 ^{bl}		10 ^{bl}
							1 ^d									1 ^d
14		12 ^{al}		11 ^{al}		13 ^{al}	9 ^{al}	13 ^{bl}		11 ^{bl}		12 ^{bl}		12 ^{bl}		9 ^{bl}
										1 ^d						1 ^d
16		12 ^{al}		11 ^{al}		13 ^{al}	9 ^a	13 ^{bl}		11 ^{bl}		12 ^{bl}		12 ^{bl}		9 ^{bl}
19		12 ^{al}		11 ^{al}		13 ^{al}	9 ^{al}	13 ^{bl}		11 ^{bl}		12 ^{bl}		12 ^{bl}		9 ^{bl}
21		12 ^{al}		11 ^{al}		13 ^{al}	8 ^{al}	13 ^{bl}		10 ^{bl}		12 ^{bl}		12 ^{bl}		9 ^{bl}
							1 ^c			1 ^{dq}						
28		12 ^{al}		11 ^a		13 ^{al}	8 ^a	13 ^{bl}		9 ^{bl}		12 ^{bl}		12 ^{bl}		8 ^b
							1 ^c			1 ^d						1 ^c
35		12 ^{al}		11 ^a		13 ^{al}	8 ^a	13 ^{bl}		9 ^b		12 ^{bl}		12 ^{bl}		8 ^b
							1 ^c									1 ^c
42		10 ^{al}		11 ^a		12 ^{al}	8 ^a	13 ^{bl}		9 ^b		10 ^{bl}		10 ^{bl}		8 ^b
		2 ^c				1 ^c	1 ^c					2 ^c		2 ^c		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 were slower.
 - f The quoted number of fish had a curvature of the spine.
 - g The quoted number of fish showed a disturbed swimming behaviour.
 - h The quoted number of fish had a swollen yolk sac.
 - i The quoted number of fish lay on the bottom of the test vessel.
 - j The quoted number of fish were bent.
 - k The quoted number of fish swam near the bottom of the test vessel.
 - l The fish had a irregular size.
 - m The quoted number of fish showed a trembling movement.
 - n The quoted number of fish had swollen bellies.
 - o The quoted number of fish had redly swollen gills.
 - p The quoted number of fish had haemorrhages on the sides of their bodies.
 - q The fish died accidentally.
 - t The quoted number of fish had a small caudal fin.
 - u The quoted number of fish had a small deformed caudal fin.
- 2) Solvent control (TBA).
- 3) Selection of fertilized eggs.

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Time (d)	Nominal concentration of test substance (μg.l ⁻¹)															
	0.32								0.56							
	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	43 ^a		41 ^a		40 ^a		42 ^a		43 ^a		43 ^a		43 ^a		43 ^a	
1	20		15		12		19		13		16		16		12	
	23 ^d ₃₎		26 ^d ₃₎		28 ^d ₃₎		23 ^d ₃₎		30 ^d ₃₎		27 ^d ₃₎		27 ^d ₃₎		31 ^d ₃₎	
	15 ^b		15 ^d		15 ^b		15 ^b		15 ^b		15 ^b		15 ^b		12 ^b	
2	15 ^b		15 ^b		15 ^b		15 ^b		15 ^b		15 ^b		15 ^b		12 ^b	
5		14 ^b _{1^d}	4 ^b	11 ^b		15 ^b		14 ^b _{1^f}	1 ^b	14 ^b		15 ^b	1 ^b	14 ^b		12 ^b
6		14 ^b	3 ^b	12 ^b		15 ^b		14 ^b _{1^{fi}}	1 ^b	14 ^b		15 ^b	1 ^b	14 ^b		12 ^b
7		14 ^b	2 ^b	12 ^b _{1^d}		13 ^b _{1^j} _{1^d}		14 ^b _{1^h}		15 ^b		15 ^b	1 ^b	13 ^b _{1^d}		11 ^b _{1^j}
8		14 ^b		12 ^b _{1^j} _{1^d}		12 ^b _{2^d}		13 ^b _{1^b} _{1^d}		15 ^b		15 ^b		14 ^b		11 ^b _{1^d}
9		14 ^b		13 ^b		12 ^b		13 ^b _{1^d}		14 ^b _{1^d}		15 ^b		14 ^b		11 ^b
12		14 ^{bl}		13 ^{bl}		12 ^{bl}		13 ^{bl}		13 ^{bl} _{1^d}		14 ^b _{1^j}		13 ^{bl} _{1^d}		11 ^b
14		14 ^b		12 ^{bl} _{1^d}		12 ^b		13 ^b		12 ^{bl} _{1^d}		14 ^b _{1^d}		11 ^b _{2^d}		11 ^{bl}
16		14 ^b		12 ^b		12 ^b		13 ^b		12 ^{bl}		14 ^b		10 ^b _{1^d}		11 ^b
19		14 ^b		12 ^{bl}		12 ^b		13 ^b		11 ^b _{1^d}		14 ^b		10 ^b		11 ^b
21		13 ^b _{1^d}		12 ^{bl}		12 ^b		13 ^b		11 ^c		14 ^c		10 ^c		11 ^c
28		12 ^b _{1^{dq}}		12 ^{bl}		12 ^b		13 ^b		11 ^c		14 ^c		10 ^c		11 ^c
35		12 ^{blo}		12 ^{blo}		12 ^{bo}		13 ^{bo}		11 ^{clom}		13 ^{clom} _{1^d}		10 ^{clom}		11 ^{clom}
42		12 ^{clo}		12 ^{clo}		12 ^{clo}		13 ^{co}		11 ^{colst}		13 ^{colst}		10 ^{colst}		10 ^{colst} _{1^{clst}}

- 1) The following codes are used to denote condition:
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 - 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 were slower.
 - f The quoted number of fish had a curvature of the spine.
 - g The quoted number of fish showed a disturbed swimming behaviour.
 - h The quoted number of fish had a swollen yolk sac.
 - i The quoted number of fish lay on the bottom of the test vessel.
 - j The quoted number of fish were bent.
 - k The quoted number of fish swam near the bottom of the test vessel.
 - l The fish had an irregular size.
 - m The quoted number of fish showed a trembling movement.
 - n The quoted number of fish had swollen bellies.
 - o The quoted number of fish had redly swollen gills.
 - p The quoted number of fish had haemorrhages on the sides of their bodies.
 - q The fish died accidentally.
 - t The quoted number of fish had a small caudal fin.
 - u The quoted number of fish had a small deformed caudal fin.
- 2) Solvent control (TBA).
- 3) Selection of fertilized eggs.

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Time (d)	Nominal concentration of test substance ($\mu\text{g.l}^{-1}$)															
	1.0								1.8							
	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	42 ^a		43 ^a		42 ^a		42 ^a		42 ^a		40 ^a		41 ^a		43 ^a	
1	15		10		22		13		13		13		13		16	
	27 ^d 3)		33 ^d 3)		20 ^d 3)		29 ^d 3)		29 ^d 3)		27 ^d 3)		28 ^d 3)		27 ^d 3)	
	15 ^b		15 ^b		15 ^b		15 ^b		13 ^b		13 ^b		14 ^b		15 ^b	
2	15 ^b		15 ^b		15 ^b		15 ^b		13 ^b		13 ^b		14 ^b		15 ^b	
5	1 ^b	14 ^b	1 ^b	14 ^b		15 ^b		15 ^b		13 ^e		13 ^e		13 ^e		12 ^b
6	1 ^b	14 ^b	1 ^b	14 ^b		15 ^b		15 ^b		13 ^b		13 ^b		12 ^b 1 ^d		12 ^b 1 ^f 1 ^d
7	16	14 ^b	1 ^b	14 ^b		15 ^b		15 ^b		13 ^e		13 ^e		12 ^e	1 ^b	12 ^e 1 ^f
8	1 ^b	14 ^c	1 ^b	14 ^c		14 ^c 1 ^d		15 ^c		13 ^{ce}		13 ^{ce}		12 ^{ce}	1 ^b	12 ^{ce} 1 ^b
9	1 ^b	14 ^b		15 ^b		14 ^b		15 ^b		13 ^{ce}		13 ^{ce}		12 ^{ce}		13 ^{ce} 1 ^h
12		14 ^{bl} 1 ^d		14 ^b 1 ⁱ		13 ^b 1 ^d		12 ^b 3 ^d		5 ^c 2 ^{cl} 6 ^d		7 ^c 3 ^{cl} 3 ^d		9 ^c 1 ^{cl} 2 ^d		3 ^c 4 ^{cl} 7 ^d
14		11 ^c 3 ^d		13 ^c 2 ^d		12 ^c 1 ^d		9 ^c 3 ^d		5 ^c 2 ^d		6 ^c 4 ^c		6 ^c 4 ^d		4 ^c 3 ^d
16		10 ^c 1 ^d		13 ^c		11 ^c 1 ^d		9 ^c		5 ^c		6 ^c		5 ^c 1 ^d		3 ^c 1 ^d
19		10 ^c		13 ^c		11 ^c		9 ^c		5 ^c		6 ^c		5 ^c		3 ^c
21		10 ^{cm}		11 ^{cm}		11 ^{cm}		8 ^{cm} 1 ^d		4 ^{cm} 1 ^d		5 ^{cm} 1 ^d		5 ^{cm}		3 ^{cm}
28		4 ^{cn} 6 ^c		8 ^{cn} 3 ^c		3 ^{cn} 3 ^c 5 ^d		4 ^{cn} 4 ^c		2 ^{cn} 1 ^c 1 ^d		5 ^c		4 ^c 1 ^d		2 ^c 1 ^d
35		4 ^{cmno} 6 ^{cm}		3 ^{cm} 3 ^{cmn} 5 ^d		1 ^{cmnp} 2 ^{cmp} 3 ^d		3 ^{cmp} 2 ^{cmnp}		1 ^{cmpr} 2 ^d		2 ^{cmr} 3 ^d		4 ^{cmr}		1 ^c 1 ^d
42		3 ^{omu} 3 ^{mpru} 2 ^{cmu} 2 ^d		2 ^{pomu} 4 ^d		2 ^{cmu} 1 ^d		1 ^{cmu} 4 ^d		0 1 ^d		0 2 ^d		1 ^{cmu} 3 ^d		0 1 ^d

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

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

i The quoted number of fish lay on the bottom of the test vessel.

j The quoted number of fish were bent.

k The quoted number of fish swam near the bottom of the test vessel.

l The fish had a irregular size.

m The quoted number of fish showed a trembling movement.

n The quoted number of fish had swollen bellies.

o The quoted number of fish had redly swollen gills.

p The quoted number of fish had haemorrhages on the sides of their bodies.

q The fish died accidentally.

t The quoted number of fish had a small caudal fin.

u The quoted number of fish had a small deformed caudal fin.

2) Solvent control (TBA).

3) Selection of fertilized eggs.



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Time (d)	Nominal concentration of test substance ($\mu\text{g.l}^{-1}$)							
	3.2							
	a		b		c		d	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	42 ^a		43 ^a		40 ^a		43 ^a	
1	19 23 ^d 3)		19 24 ^d 3)		19 21 ^d 3)		17 26 ^d 3)	
2	15 ^b		15 ^b		15 ^b		15 ^b	
5	1 ^b	14 ^e	1 ^b	13 ^e		15 ^e		15 ^e
6	1 ^b	14 ^e	1 ^b	13 ^e		15 ^e		15 ^e
7	1 ^b	14 ^e	1 ^b	13 ^e		15 ^e		15 ^e
8		15 ^{ce}		13 ^{ce}		13 ^{ce}		15 ^{ce}
9		15 ^{ce}		13 ^{ce}		2 ^d 12 ^{ce}		15 ^{ce}
12		3 ^{cei} 12 ^d		3 ^{ce} 2 ^{ci} 1 ^{ch} 7 ^d		1 ^{cef} 1 ^{cei} 1 ^{ceij} 11 ^d		1 ^{cei} 1 ^{ceij} 13 ^d
14		1 ^{cei} 2 ^d		3 ^c 3 ^d		1 ^{ci} 1 ^d		0 2 ^d
16		0 1 ^d		3 ^c		0 1 ^d		0
19		0		3 ^c		0		0
21				3 ^c				
28				2 ^c 1 ^d				
35				0 2 ^d				

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 - d The quoted number of fish (eggs) were dead.
 - e The quoted number of fish were slower.
 - f The quoted number of fish had a curvature of the spine.
 - g The quoted number of fish showed a disturbed swimming behaviour.
 - h The quoted number of fish had a swollen yolk sac.
 - i The quoted number of fish lay on the bottom of the test vessel.
 - j The quoted number of fish were bent.
 - k The quoted number of fish swam near the bottom of the test vessel.
 - l The fish had a irregular size.
 - m The quoted number of fish showed a trembling movement.
 - n The quoted number of fish had swollen bellies.
 - o The quoted number of fish had redly swollen gills.
 - p The quoted number of fish had haemorrhages on the sides of their bodies.
 - q The fish died accidentally.
 - t The quoted number of fish had a small caudal fin.
 - u The quoted number of fish had a small deformed caudal fin.
- 2) Solvent control (TBA).
- 3) Selection of fertilized eggs.

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Table B2 Total number of surviving eggs and larvae in the control media and at five concentrations of benzo(k)fluoranthene during the exposure time (combined for the four replicate retention chambers).

Time (d)	Nominal concentration of test substance ($\mu\text{g.l}^{-1}$)													
	0		0 TBA 1)		0.32		0.56		1.0		1.8		3.2	
	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae	eggs	larvae
0	169	0	170	0	166	0	172	0	169	0	166	0	168	0
1	62	0	66	0	66	0	57	0	60	0	55	0	74	0
	2)	2)	2)	2)	2)	2)	2)	2)	2)	2)	2)	2)	2)	2)
1	60	0	60	0	60	0	57	0	60	0	55	0	60	0
2	60	0	60	0	60	0	57	0	60	0	55	0	60	0
5	1	59	3	57	4	55	2	55	2	58	2	52	2	57
6	0	57	1	59	3	56	2	55	2	58	1	52	2	57
7	0	52	0	54	2	54	1	54	2	58	1	51	1	57
8	0	47	0	48	0	52	0	55	2	58	1	51	0	56
9	0	46	0	47	0	52	0	54	1	58	0	52	0	56
12	0	45	0	47	0	52	0	52	0	54	0	34	0	13
14	0	45	0	45	0	51	0	48	0	45	0	21	0	5
16	0	45	0	45	0	51	0	47	0	43	0	19	0	3
19	0	45	0	45	0	51	0	46	0	43	0	19	0	3
21	0	45	0	45	0	50	0	46	0	40	0	17	0	3
28	0	45	0	44	0	49	0	46	0	35	0	14	0	2
35	0	45	0	43	0	49	0	45	0	24	0	8	0	0
42	0	45	0	43	0	49	0	45	0	13	0	1	0	0

1) Solvent control (= TBA)

2) Selection of fertilized eggs.

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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 fish 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; DSWL control is coded as 0.0500).

Conc.	raw data												
0.0000	2.00	2.00	2.00	1.60	2.10	1.90	1.90	2.00	1.70	1.60	1.60	1.40	1.30
0.0000	2.10	2.10	1.90	2.00	2.00	1.70	1.90	1.70	1.70				
0.0000	2.10	1.80	1.40	2.10	1.10	2.00	1.00	2.00	2.00	1.60	1.70	1.40	
0.0000	2.20	2.10	2.00	1.30	1.90	2.00	1.80	1.80	1.60				
0.0500	2.00	2.00	1.00	2.00	1.70	1.10	1.90	2.20	1.40	2.20	1.60	1.40	
0.0500	1.90	1.80	2.20	2.00	1.90	1.60	1.80	1.70	1.70	1.90	1.60		
0.0500	1.60	2.10	2.30	1.30	2.00	1.60	1.70	1.90	1.90	0.900	1.70	1.50	1.70
0.0500	2.00	2.00	2.00	1.70	2.20	2.00	1.00	2.00	1.90				
0.320	1.80	1.90	1.70	1.80	1.80	1.20	1.60	1.80	1.20	1.30	1.80	1.50	
0.320	1.80	1.90	1.40	1.90	1.80	1.80	1.80	1.50	1.80	1.50	1.00	1.20	
0.320	1.80	1.50	1.80	1.70	1.30	1.70	1.20	1.90	1.90	1.80	1.70	1.80	
0.320	1.40	1.50	1.70	1.80	1.60	1.70	1.60	1.60	1.60	1.80	1.60	1.80	1.50
0.560	1.90	1.70	1.70	1.80	1.50	1.60	1.30	1.80	1.00	1.30	1.30		
0.560	1.80	0.900	1.70	1.60	1.50	0.900	1.80	1.40	1.40	1.30	1.30	0.800	1.60
0.560	2.00	2.00	1.50	1.60	1.40	1.70	1.10	1.60	1.70	1.40			
0.560	1.90	2.10	1.70	1.60	1.50	1.50	1.40	1.40	0.800	1.20	1.30		
1.00	1.70	1.30	0.700	0.900	1.00	1.10	0.700	0.700					
1.00	0.800	0.600											
1.00	0.700	0.700											
1.00	0.700												
1.80	0.600												

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; weight in mg; DSWL control is coded as 0.0500).

Conc.	raw data												
0.0000	54.9	55.5	45.7	24.4	55.3	49.4	45.4	55.9	29.2	30.4	26.7	18.1	11.1
0.0000	68.3	74.1	54.6	53.2	52.5	33.8	47.1	31.4	31.0				
0.0000	63.1	38.2	20.1	69.7	9.30	55.2	7.30	60.5	58.8	27.5	34.3	17.7	
0.0000	86.3	68.1	53.9	13.6	50.7	58.4	39.9	36.3	34.3				
0.0500	50.7	55.6	8.30	56.7	32.1	8.00	40.8	75.2	18.7	79.6	25.9	19.1	
0.0500	56.8	42.4	65.9	55.5	48.9	31.9	40.9	38.1	38.8	50.3	27.7		
0.0500	25.5	55.3	76.3	17.5	51.1	25.9	37.6	46.5	46.2	4.80	40.3	20.9	39.0
0.0500	60.1	50.6	60.1	37.5	83.5	55.7	5.20	60.5	46.0				
0.320	46.2	56.8	46.9	44.9	45.9	11.7	33.6	45.7	12.8	18.3	47.1	25.8	
0.320	44.3	53.3	19.0	52.7	53.3	44.3	48.1	32.2	49.4	21.6	10.8	16.4	
0.320	46.5	27.4	43.3	47.8	23.8	42.5	11.7	54.2	58.2	45.9	37.3	51.4	
0.320	20.0	27.5	42.0	46.5	34.3	35.7	34.4	37.6	34.1	48.9	34.2	44.5	32.2
0.560	65.1	45.6	41.3	52.9	32.8	37.3	22.5	47.9	10.9	20.5	15.3		
0.560	54.4	8.30	44.4	39.8	35.3	8.00	57.8	25.8	25.3	20.1	21.9	4.80	48.2
0.560	77.9	72.9	36.5	36.3	28.9	41.8	12.4	34.8	42.6	28.7			
0.560	67.8	69.5	45.4	38.3	31.2	35.0	36.2	28.7	3.10	15.1	16.8		
1.00	45.9	16.5	2.00	8.20	12.8	12.6	2.20	2.10					
1.00	8.10	1.20											
1.00	2.50	2.30											
1.00	1.90												
1.80	0.800												

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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; DSWL control is coded as 0.0500).

Conc.	mean	s.d.	N	MEAN	S.D.	test stat.	
0.0000	38.6	15.9	13.0	43.1	19.0		
0.0000	49.6	15.5	9.00				
0.0000	38.5	22.3	12.0				
0.0000	49.1	21.2	9.00				
0.0500	39.2	24.5	12.0	42.5	19.6	-0.138	
0.0500	45.2	11.4	11.0				
0.0500	37.5	18.7	13.0				
0.0500	51.0	21.3	9.00				
0.320	36.3	15.4	12.0	37.6	13.2	-1.48	
0.320	37.1	16.1	12.0				
0.320	40.8	13.6	12.0				
0.320	36.3	7.86	13.0				
0.560	35.6	17.0	11.0	35.2	18.6	-2.08	
0.560	30.3	17.8	13.0				
0.560	41.3	19.9	10.0				
0.560	35.2	20.5	11.0				
1.00	12.8	14.5	8.00	9.10	12.2	-6.11	**
1.00	4.65	4.88	2.00				
1.00	2.40	0.141	2.00				
1.00	1.90	1.00	1.00				
1.80	0.800	1.00	1.00	0.800	1.00	-2.38	

* level of significance: 95 %.

** level of significance: 99 %.

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Table D2 Length (the nominal concentrations for the replicate retention chambers a, b, c and d are given in vertical sequence, length in cm; DSWL control is coded as 0.0500).

Conc.	mean	s.d.	N	MEAN	S.D.	test stat.	
0.0000	1.78	0.259	13.0	1.79	0.291		
0.0000	1.90	0.166	9.00				
0.0000	1.68	0.386	12.0				
0.0000	1.86	0.274	9.00				
0.0500	1.71	0.410	12.0	1.77	0.334	-0.382	
0.0500	1.83	0.179	11.0				
0.0500	1.71	0.359	13.0				
0.0500	1.87	0.350	9.00				
0.320	1.62	0.255	12.0	1.63	0.226	-2.56	*
0.320	1.62	0.295	12.0				
0.320	1.67	0.226	12.0				
0.320	1.63	0.125	13.0				
0.560	1.54	0.280	11.0	1.50	0.314	-4.71	**
0.560	1.38	0.339	13.0				
0.560	1.60	0.275	10.0				
0.560	1.49	0.348	11.0				
1.00	1.01	0.352	8.00	0.892	0.315	-9.62	**
1.00	0.700	0.141	2.00				
1.00	0.700	0.0000	2.00				
1.00	0.700	1.00	1.00				
1.80	0.600	1.00	1.00	0.600	1.00	-3.99	**

* level of significance: 95 %.

** level of significance: 99 %.

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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)fluoranthene (a, b, c and d are the replicate retention chambers) during the early life stage test.*

Time (d)	Nominal concentration of benzo (k)fluoranthene ($\mu\text{g.l}^{-1}$)						
	0	0 TBA 1)	0.32	0.56	1.0	1.8	3.2
0	8.2	8.2	8.2	8.2	8.2	8.2	8.2
7	8.1	8.1	8.1	8.1	8.1	8.1	8.1
14	8.1	8.1	8.1	8.2	8.1	8.2	8.2
21	7.9	8.0	7.9	8.0	7.9	8.1	8.1
28	8.1	8.1	8.1	8.1	8.1	8.2	8.2
35	8.1	8.1	8.1	8.1	8.1	8.2	8.2
42	8.2	8.2	8.2	8.2	8.2	8.2	

1) Solvent control.

Table E2 *Oxygen concentrations (mg.l^{-1}) in the control medium and in the test solutions of benzo(k)fluoranthene (a, b, c and d are the replicate retention chambers) during the early life stage test.*

Time (d)	Nominal concentration of benzo(k)fluoranthene ($\mu\text{g.l}^{-1}$)						
	0	0 TBA 1)	0.32	0.56	1.0	1.8	3.2
0	8.8	8.8	8.7	8.9	8.8	8.9	8.9
7	8.4	8.4	8.4	8.6	8.5	8.6	8.6
14	8.6	8.6	8.3	8.2	8.1	8.1	8.2
21	7.3	7.4	7.3	7.8	7.3	8.3	8.3
28	7.7	7.5	7.4	7.2	7.6	7.8	7.9
35	8.2	7.9	8.2	8.0	8.3	8.8	7.8
42	9.1	8.8	8.7	8.7	8.6	8.5	

1) Solvent control.



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Table E3 *Temperature (°C) in the control medium of benzo(k)fluoranthene during the early life stage test.*

Time (d)	Temperature (°C)
1	24.9
2	25.0
3	24.9
4	24.9
5	24.9
6	24.8
7	24.6
8	24.9
9	25.0
10	24.9
11	24.9
12	24.9
13	24.9
14	24.9
15	25.0
16	24.9
17	24.8
18	24.9
19	24.9
20	24.8
21	24.9
22	24.9
23	24.9
24	24.9
25	25.0
26	24.8
27	25.0
28	24.9
29	25.1
30	25.0
31	25.0
32	25.0
33	24.9
34	24.8
35	24.9
36	24.8
37	24.9
38	24.9
39	25.0
40	24.8
41	25.0
42	25.0

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

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