CADASTER

CAse studies on the Development and Application of in-Silico Techniques for Environmental hazard and Risk assessment

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Collaborative Project

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WP 2: Database on experimental parameters and (Q)SARs for chemical and biological endpoints

Work Package Leader: Mojca Kos Durjava (Partner 2: Public Health Institute Maribor)

Task 2.3- Generation of new data (Deliverable 2.5)

Summary

This report provides an overview of the CADASTER testing of chemicals. New data were generated on endpoints and chemicals for which, as identified in WP3, insufficient data were available for model validation and proper hazard/risk assessment. Following evaluation of available experimental data and available (Q)SAR models within WP3, new experimental data on the most essential endpoints of assessment (i.e. new data for the endpoints that are responsible for most of the variance in the safety, hazard and risk assessment) were generated. The topic of study is emerging compounds that might develop into the future chemicals of concern for which typically limited data are available. Based on an initial evaluation of the availability of experimental data and QSAR models for various classes of the emerging compounds identified within NORMAN, four groups of compounds have been selected as the chemical classes of choice for CADASTER. Following toxicity and fate and behaviour testing was performed on the selected classes:

1 – Polybrominated diphenylethers (PBDE)

28-day sediment testing of PBDEs was performed on bioaccumulation with aquatic oligochaeta *Tubifex tubifex* by PHI.

2 - Perfluoroalkylated substances and their transformation products

Toxicity testing of fluorinated compounds was performed with lettuce (*Lactuca sativa*) and green algae (*Pseudokirchneriella subcapitata*) at the RIVM. Thereupon, testing was performed with two cladoceran species (*Daphnia magna* and *Chydorus sphaericus*), as well as with embryos of the zebrafish (*Danio rerio*), also at the RIVM.

3 – Substituted musks/fragrances

Toxicity testing of fragrances was performed with green algae (*Pseudokirchneriella subcapitata*) and with *Daphnia magna* at the PHI. Substituted musks/fragrances were tested also on ready biodegradability, at the PHI.

4 - Triazoles/benzotriazoles

Toxicity testing of substituted (benzo)triazoles was performed with *Daphnia magna* and with embryos of the zebrafish (*Danio rerio*) at the RIVM. Toxicity testing of substituted (benzo)triazoles was performed with green algae (*Pseudokirchneriella subcapitata*) at the PHI. Substituted (benzo)triazoles were tested also on ready biodegradability, at the PHI.

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WP 2: Database on experimental parameters and (Q)SARs for chemical and biological endpoints

Work Package Leader: Mojca Kos Durjava (Partner 2: Public Health Institute Maribor)

Task 2.3- Generation of new data (Deliverable 2.5)

General introduction

The chemicals were selected for the testing on the basis of existing toxicity data for both vertebrate and invertebrate species, as well as on principal component analysis aimed at selecting a minimum number of compounds to be tested in order to maximize the spanning of the chemical domain of both compound classes. In addition to the selection of the optimal set of chemicals to be tested, the criteria of availability of the chemicals in terms of possibilities of being able to purchase the chemicals was essential in the final selection of compounds to be tested.

Toxicity and fate and behaviour testing by PHI was performed on a pre-selected number of polybrominated diphenylethers, substituted musks/fragrances and substituted (benzo)triazoles. The testing performed includes testing of polybrominated diphenylethers on bioaccumulation with *Tubifex tubifex*. Ecotoxicity testing on substituted musks/fragrances includes testing with algae and daphnids. Fragrances were tested also on ready biodegradability. Substituted (benzo)triazoles were tested with algae and on ready biodegradability.

Toxicity testing by RIVM was performed on a pre-selected number of perfluorinated compounds and substituted (benzo)triazoles. The ecotoxicity testing performed includes testing of perfluorinated compounds with lettuce, algae, daphnids and zebrafish embryos. Substituted (benzo)triazoles were tested with daphnids and zebrafish embryos.

In this report an overview is given of the chemicals tested within each class of compounds, the materials and methods that were employed, the test setup, as well as the results obtained.

Report on testing of polybrominated diphenylethers (PBDEs)

The aim of CADASTER is to generate reliable data that can be used in the QSAR evaluation. From literature data and from PHI preliminary test results on bioaccumulation with *Tubifex tubifex* it was evident that testing of PBDEs should be performed in sediment if we want to achieve this goal (see Appendix 2). Following this choice we generated results that are useful for modelling. After reviewing available experimental sediment effect data it is obvious that effects in sediment occur at high concentrations (from 50 to 1500 mg/kg). Effect testing of PBDEs is mostly limited with availability and costs of individual congeners, since for effect testing of sediment we would have to have large amounts of pure congeners.

Therefore testing was focused on the sediment compartment with assessment of the bioaccumulation potential as endpoint. The BCF values obtained may subsequently be used to calculate toxicity endpoints, either using experimentally obtained critical body burdens (CBBs) for the various PBDE's, or by using QSAR approaches for predicting CBBs. The latter approach is advocated by for instance Hendriks et al, 2005.

Testing of polybrominated diphenylethers on bioaccumulation with *Tubifex tubifex*

28-day sediment testing on bioaccumulation with *Tubifex sp.* was performed at Public Health Institute Maribor (PHI) according to OECD guideline 315. In August 2009 PHI has started development of experimental test sets in laboratory. At the end of 2009 IDEA, University of Insubria, Linnaeus University and PHI prepared a list of chemicals relevant for the testing for PBDEs according to analytical possibilities, REACH relevance, structural representativeness and relevance according to toxicity and physicochemical properties. The list of relevant PBDEs is as follows: PBDE-002, PBDE-066, PBDE-077, PBDE-119, PBDE-126, PBDE-190, PBDE-197, PBDE-198, PBDE-203, PBDE-204 and PBDE-207. After consideration of availability of substances on the market and a contact with Prof. Bergman from Sweden, PHI decided to purchase 3 PBDE commercial mixtures (TBDE-71, TBDE-79 and TBDE-83R) and 5 BDE individual congeners (PBDE-002, PBDE-077, PBDE-126, PBDE-198 and PBDE-204) from Wellington Laboratories. In June 2010 the Testing strategy for PBDEs was presented (Appendix 2), following the discussion on Annual Meeting in Munich. PBDE testing was completed in January 2011.The results of the testing with *Tubifex tubifex* will be published in 2012.

Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in a wide number of synthetic applications such as building materials, furnishing textiles and electronic equipment, to reduce the risk of fire. Their occurrence in the environment is mainly the result of disposed products which contain them, losses at production sites and leaching from landfills. This has resulted in progressive contamination of the aquatic environment with predominant bioaccumulation of lower brominated congeners in aquatic biota (European Union Risk Assessment Report, 2000).

PBDEs are poorly soluble in water. Due to this property and due to their persistence they strongly accumulate in sediments. Benthic organisms that are living in or depending on the sediment may accumulate PBDEs and their life-functions may be threatened.

One of the aquatic oligochaeta species that is recommended for bioaccumulation testing is *Tubifex tubifex,* Müller (Tubificidae, Oligochaeta). *Tubifex* sp. inhabits the freshwater sediment and often represents the most abundant species especially in habitats with environmental conditions adverse to other animals. The following bioaccumulation study with *Tubifex tubifex* was conducted on 3 PBDE commercial mixtures and selected individual congeners.

Materials and methods

3 PBDE commercial mixtures (TBDE-71, TBDE-79 and TBDE-83R) and 5 BDE individual congeners (PBDE-002, PBDE-077, PBDE-126, PBDE-198 and PBDE-204) were purchased from Wellington Laboratories (Canada). Labelled internal standard solution (Catalogue number: EO-5277) and labelled Injection Internal standard solution (Catalogue number: EO-5275) were purchased from Cambridge Isotope Laboratories (Andover, USA).

Test was performed according to the Bioaccumulation in Sediment-dwelling Benthic Oligochaetes. OECD 315 (OECD 2008). Artificial sediment was developed based on the composition of artificial soil according to OECD Guideline 207 (OECD, 1984). It consisted of quartz sand, kaolinite clay and finely ground sphagnum peat. For determination of dry weight sediment was weighed after excess water has been removed. Sediment was dried at 105 °C for 2 hours and weighed again. The dry weight to wet ratio for sediment was 0.70.

The tubificid oligochaetes *Tubifex tubifex* were obtained from a fish food supplier and were cultured at 20 ± 2 °C and ~ 250 lx in permanent single species culture over several years. A system consisting of artificial sediment and tap water was used for the cultures. Each culture container was loaded with a layer of wet artificial sediment to a depth of approximately 2 cm, which should allow for natural burrowing behaviour of the worms. Tap water was added to form a layer of approximately 8 cm. The oligochaeta were fed a weekly diet consisting of 1.2 mg finely ground fish food per cm^2 of sediment surface. To avoid accumulation of ammonia, the overlying water was exchanged using a flow-through system.

For determination of dry weight the worms were weighed after excess water has been removed by gently touching the animals against the edge of the holding dish. They were dried at 105 °C for 2 hours and weighed again. The dry weight to wet ratio for *Tubifex tubifex* was 0.131.

The concentrations of the PBDEs in sediment, *Tubifex tubifex* and water were determined by high resolution gas chromatography coupled with high resolution mass spectrometry (HRGC/HRMS). GC-HRMS was performed with HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to a Finnigan MAT 95XP (Finnigan, Bremen, Germany) high resolution mass spectrometer. The GC separation was performed on a Zebron ZB-5HT INFERNO column (Phenomenex), 15 m x 0.25mm I.D. with a film thickness of 0.10 µm. An aliquot (2 µL) of sample extract was injected into the GC system in pulsed splitless mode at 250 °C. The mass spectrometer operates in the electron impact ionization mode using selected ion monitoring (SIM), at a minimum resolution of 8000. Samples were analyzed for the PBDEs concentrations using the isotope dilution or internal standard method based on US EPA 1614 protocol.

The artificial sediment was spiked with a PBDE mixture by coating the quartz sand fraction. A PBDE solution was prepared to give a final total concentration of 35-70 µg per gram of wet sediment. The quartz sand fraction of the sediment was soaked with this solution in a shallow glass vessel. After the solvent has evaporated, quartz sand was mixed with other constituents of sediment. The test substance concentrations in the whole sediment were more than 10 times below the LOECs for burrowing activity. The spiked sediment was then added to the test chamber.

Bioaccumulation experiments were carried out in an open system (see Figure 1). The replicate test chambers (700 mL glass cup) were incubated at 20 ± 2 °C. Each of the test chambers contained a 2 cm layer of spiked artificial sediment (150 g), 1.2 g of worms and 500 mL of tap water. Control chambers were loaded with clean sediment and 1.2 g of worms each. For each sampling two replicate test chambers were assembled. Before the test, adult worms of the same age class (10-12 weeks) were collected from the culture by sieving the sediment through a 1 mm mesh which retained adult individuals. The animals were weighed and transferred to the pre-weighed replicate test chambers. An acclimation period of 5 days was required to adopt oligochaeta to the test conditions.

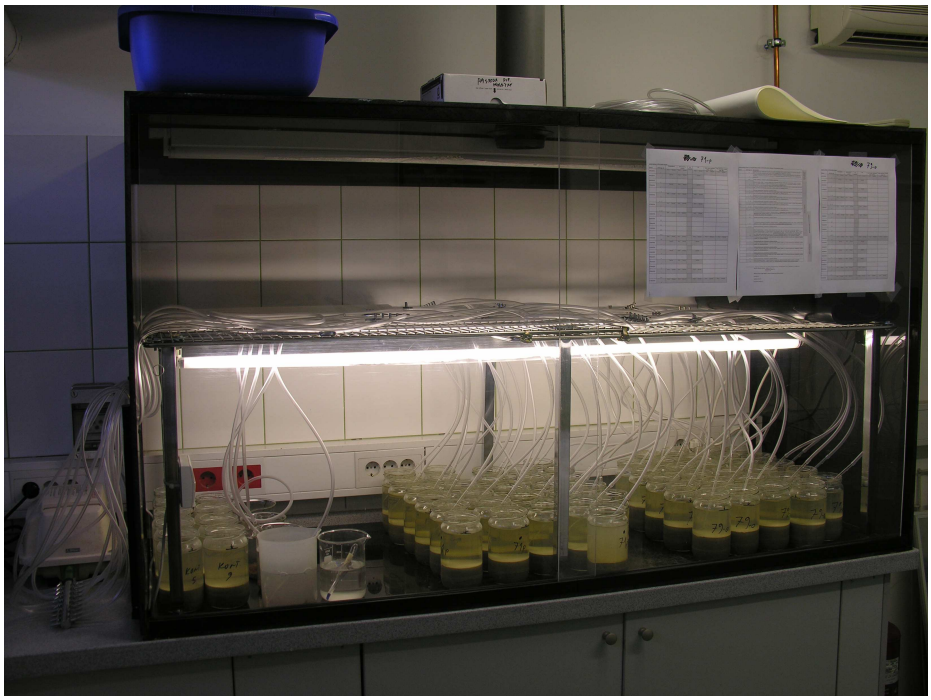

Figure 1: A photo of the test setup for bioaccumulation of PBDEs

For the determination of PBDE concentration in sediment, worms and in water, samples were taken after specified time intervals. At each sampling two replicate test chambers and the control chamber were removed from the incubation box. Temperature, dissolved oxygen and pH of the overlying water were measured in the controls, which were later re-incubated. 200 mL of overlying water, approximately 10 g of wet sediment and the oligochaeta were removed from each of the replicates for analytical purposes.

Experiments to determine the time course of the elimination kinetics were conducted immediately following the uptake phase. The remaining replicate test chambers were removed from the incubation box and processed as described above. After rinsing the worms were weighed and inserted into pre-weighed test chambers containing uncontaminated artificial sediment and tap water. The further procedures were performed according to the methods used during the uptake phase.

Results

The experimental procedure to determine uptake and elimination kinetics of PBDEs in *Tubifex tubifex* can be described with two characteristic examples of the time course of the uptake and elimination of BDE. (Figure 2). The bioaccumulation of lower PBDEs achieved the plateau within the 28 days of the uptake phase. A rapid increase of concentration in the oligochaeta occurred during first days of the test. The plateau or steady state was reached after a week of exposure. For some higher PBDEs plateau was not achieved during the test, probably due to their large size precluding crossing of cell walls in organisms (European Union Risk Assessment Report, 2002). The mean values of the concentrations measured in oligochaeta of each of two replicate test chambers were plotted; the error bars represent the corresponding standard deviation.

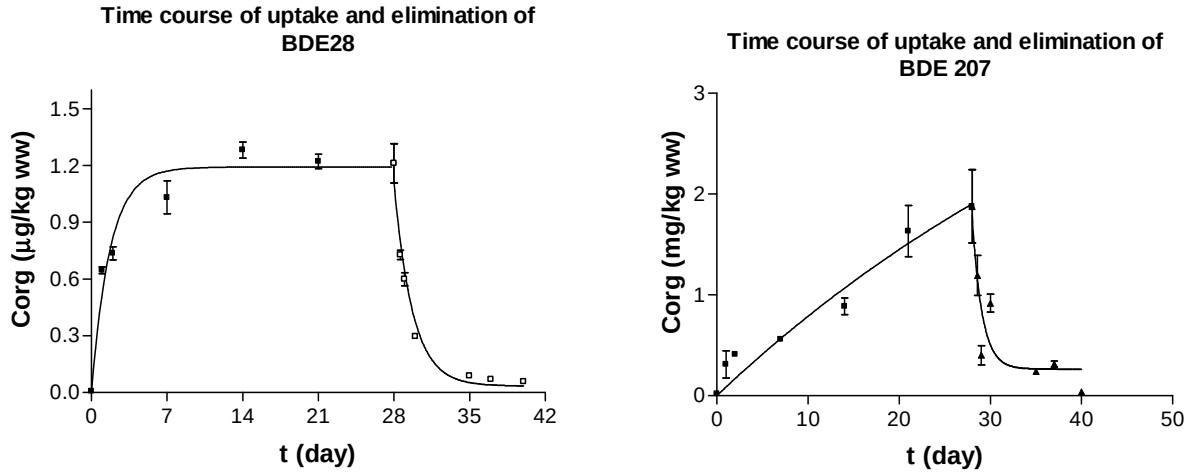

Figure 2: Time course of uptake and elimination of PBDE-28 and BDE 207in *Tubifex tubifex*

The endpoint of a bioaccumulation in benthic oligochaeta (OECD 315) is the bioaccumulation factor (BAF). The BAF was calculated as the ratio of concentration of the test substance in the test organism, C_{org}, and in the sediment, C_{sed}, at steady state. To describe the time course of uptake with one compartment model, equation 1 is used:

$$C_{org} = \frac{k_{up}}{k_{el}} * C_{sed}(1 - e^{-k_e * t}) \qquad (1)$$

k_{up} is uptake rate constant in tissue (g sediment kg^{-1} worm d^{-1}) and k_{el} is elimination rate constant (d^{-1}). When steady state is reached during the uptake phase, Equation 1 may be reduced to equation 2 and 3:

$$C_{org} = \frac{k_{up}}{k_{el}} * C_{sed} \qquad (2)$$

or

$$BAF = \frac{C_{org}}{C_{sed}} \quad (3)$$

If the steady state was not reached during the uptake phase, the BAF was calculated in the same manner for day 28. However, it is noted whether the BAF is based on steady state concentration or not. BAF was calculated also as a kinetic bioaccumulation factor, BAF_K as the ratio of the rate constant of uptake from sediment and the elimination rate constant assuming first order kinetics (Equation 4).

$$BAF_K = \frac{k_{up}}{k_{el}} \quad (4)$$

The biota-sediment accumulation factor (BSAF) is the lipid normalised concentration of test substance in the test organism divided by organic carbon normalised concentration of the substance in the sediment at steady state, calculated as follows:

$$BSAF = BAF_K * \frac{f_{oc}}{f_{lip}} \quad (5)$$

f_{oc} is the fraction of sediment organic carbon and f_{lip} is the fraction of organism lipid, both based either on dry weight, or on wet weight.

The bioaccumulation study with *Tubifex tubifex* was conducted on 3 PBDE commercial mixtures and selected individual congeners. Four tests were carried out with 4 PBDE mixtures, the results are presented in Table 1 and Table 2.

Table 1. Results of the bioaccumulation study for different PBDEs in *Tubifex tubifex*

	C_{org}[a] (µg/kg $_{ww}$)	**RSD_{Corg}** (%)	**C_{sed}**[b] (µg/kg $_{ww}$)	**RSD_{Csed}** (%)	**BAF**	**BAF_K**	**RSD_{BAFK}** (%)	**BASF**[f]
TEST 1								
PBDE-28	1.20	12.2	0.18	6.7	6.57	6.58	5.9	4.32
PBDE-47	77.0	12.0	11.6	3.8	6.64	5.55	9.8	3.64
PBDE-51	0.930	27.0	0.13	32.3	6.91	7.84	6.3	5.14
PBDE-66/42[e]	4.12	14.9	0.59	8.5	6.98	7.73	9.8	5.07
PBDE-99	87.9	0.5	12.0	7.6	7.34	5.34	10.9	3.50
PBDE-100	46.0	18.6	5.11	9.3	9.01*	10.47	19.2	6.87
PBDE-119	0.384	9.8	0.06	16.4	6.54*	9.16	14.7	6.01
PBDE-153	9.73	12.1	2.56	11.1	3.80*	4.64	12.5	3.04
PBDE-154	13.85	16.2	2.43	5.8	5.71*	8.01	20.0	5.25
PBDE-183	0.020	39.1	0.07	5.2	0.29	0.82	12.7	0.54
TEST 2								

	C_{org}[a] (µg/kg $_{ww}$)	RSD_{Corg} (%)	C_{sed}[b] (µg/kg $_{ww}$)	RSD_{Csed} (%)	BAF	BAF_K	RSD_{BAFK} (%)	BASF[f]
PBDE-153	15.0	1.3	4.60	21,2	3.26*	4.07	5.9	2.67
PBDE-154	3.36	3.6	0.73	29,6	4.61*	5.75	9.8	3.77
PBDE-180	0.21	5.6	0.53	8,0	0.39	0.46	6.3	0.30
PBDE-183	7.13	4.1	13.9	5,9	0.51	0.47	9.8	0.31
PBDE-197	7.93	11.8	16.4	10,9	0.49*	0.59	10.9	0.38
PBDE-203	2.68	21.0	3.41	6,3	0.78*	0.98	19.2	0.64
PBDE-207	1.88	38.7	12.5	1,3	0.15*	0.19	14.7	0.12
TEST 3								
BDE-2	[c]		[c]	[c]	[c]	[c]	[c]	[c]
PBDE-77	60.3	2.18	11.2	31.0	5.39	5.14	4,95	3.37
PBDE-126	45.3	1.86	7.95	31.9	5.69	5.75	10,5	3.77
TEST 4								
PBDE-198	19.4	1.7	10.1	9.6	1.91*	2.03	44	1.33
PBDE-204	12.5	5.2	9.68	8.5	1.29*	2.97	65	1.95
PBDE-206	0.73	1.7	1.01	9.7	0.72*	1.18	57	0.77
PBDE-207	0.89	9.3	0.91	15.2	0.97*	4.91	329	3.22
PBDE-208	0.56	4.3	0.33	26.0	1.67*	4.74	129	3.11
PBDE-209	16.9	4.1	24.8	2.9	0.68*	0.86	33	0.56
PBDE-209 36 day[d]	25.0	/	30.4	/	0.82*	1.53	/	1.00

[a] Concentration in *Tubifex tubifex* on day 28 of uptake phase, based on wet weight
[b] Concentration in sediment on day 28 of uptake phase, based on wet weight
[c] No data due to analytical difficulties
[d] Data for prolonged uptake phase, 36 days
[e] Results are sum of PBDE-66 and PBDE-42
[f] f_{oc} = 1.25 and f_{lip} = 1.9, both based on wet weight (Equation 5)
[g] The dry weight to wet ratio for Tubifex tubifex was 0.131.
[h] The dry weight to wet ratio for sediment was 0.70.
* Plateau was not achieved; BAF_K is more reliable endpoint than BAF

An absence of steady state is leading to apparently lower BAF. Bioaccumulation factors for PBDEs, for which plateau was not achieved in 28 days are marked with an asterisk.

Compared to threshold values indicating moderate to high bioaccumulation, the tubificid BAF are very low. This can be explained by the medium taken as a basis for the bioaccumulation, the sediment. The denominator of the quotient - the sediment concentration is high in compare to concentration in benthic animals (Equation 6). This means, that even when relatively high body concentration in benthic animals are reached, the bioaccumulation factor remains low.

At steady state accumulation from the sediment pore water and from the sediment organic carbon via ingestion is equal to accumulation from pore water exposure only. If it is assumed that the partition coefficient between biota and pore water is constant, the assumption can be written as:

$$BCF = \frac{C_{org}}{C_{wat}} \qquad (6)$$

C_{org} is the concentration in organism, C_{wat} is the concentration in pore water and BCF is the bioconcentration factor. In Table 2, concentrations in organism and in pore water together with calculated bioconcentration factors for different PBDEs are presented.

Table 2. Bioconcentration factor BCF for different PBDEs in *Tubifex tubifex*

	C_{org}[a] (µg/kg_{ww})	**RSD_{Corg}** (%)	**C_{wat}**[b] (ng/L)	**RSD_{Cwat}** (%)	**BCF** (L/kg)	**BCF_K**[f] (L/kg)	**RSD_{BCFK}** (%)
TEST 1							
PBDE-28	1.20	12.2	0.094	10.4	12860	12380	3.3
PBDE-47	77.0	12.0	5.90	8.4	13060	12830	7.4
PBDE-51	0.930	27.0	0.065	13.4	14290	18250	10.2
PBDE-66/42[e]	4.12	14.9	0.195	22.6	21080	20810	5.6
PBDE-99	87.9	0.5	4.55	2.1	19320	15360	6.8
PBDE-100	46.0	18.6	1.04	5.9	44430*	43880	13.2
PBDE-119	0.384	9.8	0.014	50.2	28100*	36820	8.5
PBDE-153	9.73	12.1	0.394	4.4	24680*	39890	34.8
PBDE-154	13.85	16.2	0.405	3.0	34160*	52880	26.2
PBDE-183	0.020	39.1	0.157	6.8	128	340	19.7
TEST 2							
PBDE-153	15.0	1.3	1.03	28.6	14627*	16740	11.3
PBDE-154	3.36	3.6	0.29	30.5	11582*	13490	9.0
PBDE-180	0.21	5.6	0.14	51.1	1457	1541	13.1
PBDE-183	7.13	4.1	4.74	36.0	1505	1405	12.0
PBDE-197	7.93	11.8	2.23	38.4	3558*	3824	31
PBDE-203	2.68	21.0	0.38	38.5	7108*	16210	111
PBDE-207	1.88	38.7	0.75	41.3	2496*	6189	159

	C_{org} [a] (µg/kg$_{ww}$)	RSD_{Corg} (%)	C_{wat} [b] (ng/L)	RSD_{Cwat} (%)	BCF (L/kg)	BCF_K [f] (L/kg)	RSD_{BCFK} (%)
TEST 3							
BDE-2	[c]	[c]	[c]	[c]	[c]	[c]	[c]
PBDE-77	60.3	2.18	1.69	4.1	36760	34130	7.5
PBDE-126	45.3	1.86	0.89	0.06	50740	51270	15.3
TEST 4							
PBDE-198	19.4	1.7	2.14	52	9080[*]	9120	37
PBDE-204	12.5	5.2	2.60	54	4800[*]	10780	84
PBDE-206	0.73	1.7	.021	34	3500[*]	5240	47
PBDE-207	0.89	9.3	0.23	23	3860[*]	18200	380
PBDE-208	0.56	4.3	0.08	31	7130	20020	115
PBDE-209	16.9	4.1	5.8	50	2910	3550	46
PBDE-209 36 day[d]	25.0	/					

[a] Concentration in *Tubifex tubifex* on day 28 of uptake phase, based on wet weight
[b] Concentration in water on day 28 of uptake phase, based on wet weight
[c] No data due to analytical difficulties
[d] Data for prolonged uptake phase, 36 days
[e] Results are sum of PBDE-66 and PBDE-42
[f] f_{oc} = 1.25 and f_{lip} = 1.9, both based on wet weight
[g] The dry weight to wet ratio for *Tubifex tubifex* was 0.131.
[*] Plateau was not achieved; BAF_K is more reliable endpoint than BAF

The BCF values obtained may subsequently be used to calculate toxicity endpoints, either using experimentally obtained critical body burdens (CBBs) for the various PBDE's, or by using QSAR approaches for predicting CBBs.

Conclusion

The bioaccumulation of different polybrominated dipheylethers in sediment was investigated with *Tubifex tubifex*. Raw data of bioaccumulation test is available at Public Health Institute Maribor.

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Report on testing of poly- and perfluorinated compounds

Toxicity testing of fluorinated compounds was performed at RIVM. First of all it was performed with lettuce (*Lactuca sativa*) and green algae (*Pseudokirchneriella subcapitata*). In both cases the same set of test chemicals was used and in this report the results of both tests are therefore combined. Thereupon, testing was performed with two cladoceran species (*Daphnia magna* and *Chydorus sphaericus*), as well as with embryos of the zebrafish (*Danio rerio*). The results of the testing with lettuce and green algae as well as with the cladocerans species have been published[1] or will be published in 2012[2]. Thereupon, a review report was prepared summarizing available data and models on fate and effects of poly- and perfluorinated compounds[3].

Toxicity of poly- and perfluorinated compounds to lettuce (*Lactuca sativa*) and green algae (*Pseudokirchneriella subcapitata*)

Materials and methods

Test compounds

Seven PFCs were selected for the toxicity assessment on the basis of their chemical structural resemblance. The test set included: perfluorobutanoic acid (PFBA, CAS number: 375-22-4, 98%), 2,2,3,3,4,4,5,5-Octafluoro-1-pentanol (5H 4:1 FTOH, CAS number: 355-80-6, 98%), perfluorooctanoic acid (PFOA, CAS number: 335-67-1, 96%), perfluorononanoic acid (PFNA, CAS number: 375-95-1, 97%), perfluorodecanoic acid (PFDA, CAS number: 335-76-2, 98%), perfluoroundecanoic acid (PFUnA, CAS number: 2058-94-8, 95%) and perfluorododecanoic acid (PFDoA, CAS number: 307-55-1, 97%). All of these chemicals were purchased from Sigma-Aldrich. As the water solubility of some PFCs was very low, dimethyl sulfoxide (DMSO) was used for preparing stock solutions. However, the concentration of DMSO in test solutions did not exceed 0.2%, and a solvent control was tested simultaneously.

Root elongation test on lettuce (*Lactuca sativa*)

The 5-day root elongation test was performed according to the Ecological Effects Test Guidelines OPPTS 850.4200 (US EPA 1996) and the OECD Test Guidelines No. 208 (OECD 2006) with slight modifications. A 90×15 mm plastic Petri dish with an appropriate filter paper placed inside was used in order to avoid sorption of PFCs to the wall of the container. For each test, 6 exposure concentrations and a blank were conducted with three replicates. In those cases where it was necessary to use DSMO for the preparation of stock solutions, a solvent control was also tested simultaneously. To each Petri dish, 3 mL of either test solution, solvent

control, or just nutrient solution (blank control) was added. Five lettuce (*Lactuca sativa*) seeds were placed on the wet filter paper and then the Petri dishes were sealed with parafilm to prevent evaporation. Petri dishes containing lettuce seeds were placed in a plant test chamber with a constant room temperature of 18 ± 2 ℃. The photoperiod was set as 16 hour light / 8 hour darkness. After 5 days of incubation, the number of germinated seeds was determined and the length of the roots was measured by means of a ruler to the closest millimetre.

PAM test on algae (*Pseudokirchneriella subcapitata*)

The test follows the protocol of "PAM test: acute effects on photosynthesis in algae" developed in RIVM[4]. The unicellular green alga *Pseudokirchneriella subcapitata* was used for the PAM test. The alga was taken from a continuous culture. The algal culture was first diluted with Dutch standard water (DSW) to an initial cell density of 3×10^6 cells·mL^{-1}. Then the algal suspension was exposed to a concentration series of chemicals and placed in an incubator for 4.5 hours with 650 nm pulsed excitation light. Light energy was absorbed by the alga's photosystems and was then transformed into photoproducts, heat and fluorescence. Blocking of the photosynthesis by chemicals changes the intensity of the fluorescence signal. After 4.5 hours of incubation, the changes in the fluorescence signal were recorded and the effects of the toxicant on the photosynthetic efficiency were derived.

Data analysis

The concentrations that caused 50% inhibition effects (EC_{50}) with 95% confidence limits (CL) were calculated by a nonlinear curve-fitting procedure in GraphPad Prism v 4.0. The fitted model between the logarithm of nominal concentration *C* and the effect *E* is in the form of:

E= Bottom + (Top – Bottom)/(1 + 10^((Log EC_{50} – *C*) * HillSlope))

Here, it is hypothesized that the effect starts at the Bottom of the effect curve and goes to the Top with a sigmoid shape. HillSlope is the variable slope of the dose-response curve. The NOECs were determined by the Dunnett Software v 1.5 obtained from the US EPA.

Results and discussion

Toxicity to root elongation of lettuce (*Lactuca sativa*)

The EC_{50}s and NOECs that were derived for each of the PFCs studied, are summarized in Table 3. The endpoint of interest, root elongation of lettuce (*Lactuca sativa*) after a total exposure time of 5 days, includes possible effects of PFCs on germination. Limited by the solubility of PFUnA and PFDoA, the EC_{50} values for these chemicals could not be

experimentally determined. The measured EC_{50} values of the additional PFCs studied were in the range of 0.266 mM to 4.186 mM. Li (2009) evaluated 5-day EC_{50}s of APFO and PFOSK on root elongation of lettuce (*Lactuca sativa*) as 0.394 mM and 0.184 mM, respectively. The reported EC_{50} of APFO, the ammonium salt of PFOA, is about 4.57 times lower than the EC_{50} of PFOA determined in this study. PFOSK has a fluorinated carbon chain length of 8, which is the same as that of PFNA. The EC_{50} of PFOSK obtained by Li[5] is about 4.6 times lower than the value obtained for PFNA in our study. This deviation may be caused by different experimental conditions, such as temperature, photoperiod and the amount of test solution. In addition, different substituents also induce variation between the data, as different physicochemical properties of PFCs and their salts lead to different environmental behaviour and subsequent aquatic toxicity.

Table 3. Effects of seven PFCs on 5-day root elongation of lettuce (*Lactuca sativa*) and photosynthesis of green algae (*Pseudokirchneriella subcapitata*).

Chemicals	**CAS number**	**nC[a]**	**Lettuce (*Lactuca sativa*)**		**Green algae (*Pseudokirchneriella subcapitata*)**	
			EC_{50} (95% CL; mM)	**NOEC (mM)**	**EC_{50} (95% CL; mM)**	**NOEC (mM)**
PFBA	375-22-4	3	4.186 (3.937-4.450)	3	1.225 (1.002-1.497)	< 1
5H 4:1 FTOH	355-80-6	4	2.976 (2.539-3.489)	0.1	4.853 (4.058-5.804)	2
PFOA	335-67-1	7	1.801 (1.635-1.984)	1.5	1.807 (1.757-1.859)	1
PFNA	375-95-1	8	0.846 (0.558-1.281)	0.1	1.038 (0.975-1.104)	< 1
PFDA	335-76-2	9	0.266 (0.188-0.375)	0.1	0.851 (0.644-1.124)	< 1
PFUnA	2058-94-8	10	0.210[b]	-	0.565[b]	-
PFDoA	307-55-1	11	0.142[b]	-	0.394[b]	-

[a] The fluorinated carbon chain length.
[b] Predicted by the relationship found between log EC_{50} and nC.

Table 3 also lists the fluorinated carbon chain length of PFCs, which was represented as nC. It can be seen that the EC_{50}s decrease with increasing nC. This means that the toxicity increases with the fluorinated carbon chain length for the structurally similar compounds investigated here. A good relationship between log-transformed EC_{50} values and nC was obtained, as shown in Figure 3. From this relationship, the EC_{50} of PFUnA and PFDoA could be predicted as being 0.210 mM and 0.142 mM. This is indeed well above the water solubility of 3.19×10^{-5} mM and 8.87×10^{-7} mM of these two chemicals at 25℃, which was calcu lated by the online software of SPARC v4.5 as no experimental data on water solubility are available. From these data it can be concluded that acute toxicity of PFUnA and PFDoA to lettuce seeds will not be observed in the real environment as the water solubility is far lower than the predicted EC_{50} values.

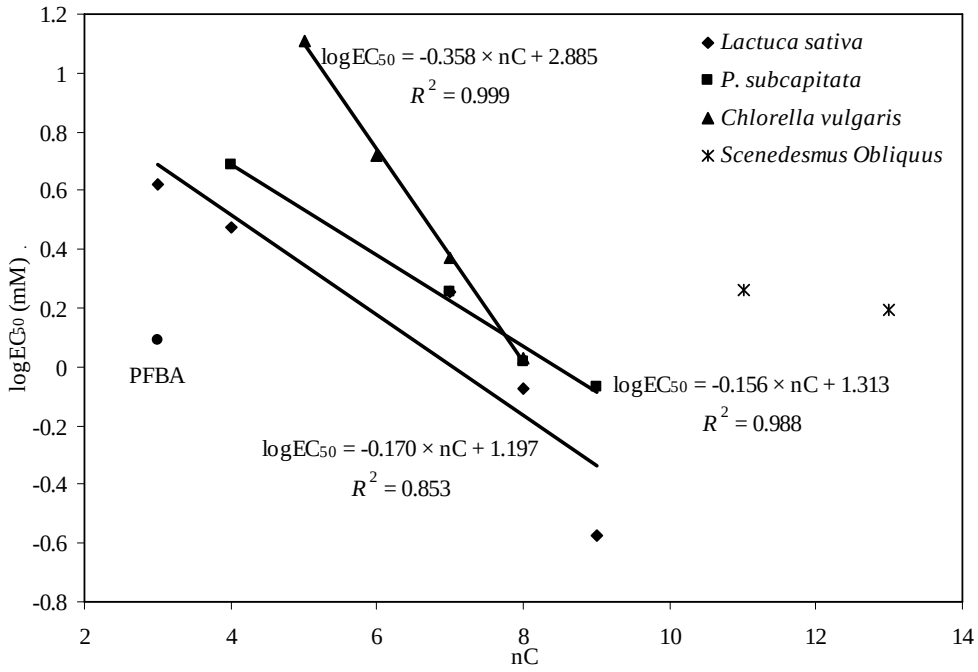

Figure 3. Relationships between log EC_{50} and the fluorinated carbon chain length nC.

In comparison with the EC_{50}s, the NOEC also decreased with nC, except that the NOEC of 5H 4:1 FTOH was an outlier as compared to the NOECs of PFCAs. The NOEC of 5H 4:1 FTOH was found to be just 0.1 mM. This is similar to the values obtained for PFNA and PFDA, both being PFCs with a higher fluorinated carbon chain length. This means that, possibly due to the presence of the OH-moiety, 5H 4:1 FTOH is more toxic than the corresponding perfluoroalkyl carboxylic acids of similar fluorinated carbon chain length. Thus it is recommended to pay more attention to the long-term chronic toxicity of 5H 4:1 FTOH in future research.

Toxicity effects on photosynthesis of algae (*Pseudokirchneriella subcapitata*)

The fluorescence signal was recorded by a PAM fluorometer after 4.5 hours of incubation and the EC_{50} for the toxic effect on the photosynthetic efficiency was calculated. The EC_{50} values of PFUnA and PFDoA could again not be experimentally determined due to the limitation of their water solubility. The toxicity data obtained for *P. subcapitata* are also listed in Table 1. Except for PFBA, the EC_{50} values decreased with increasing nC. Log-transformed EC_{50} values had a good linear relationship with nC, which is also shown in Figure 1. The relationship between logEC_{50} and nC could again be used to predict the EC_{50} of PFUnA and PFDoA. Similar to the observation in case of exposure of lettuce, the predicted EC_{50} of PFUnA and PFDoA far exceeded the water solubility of these compounds in the PAM test. Therefore, the chemicals will

not show acute harmful effects on the photosynthesis of *P. subcapitata* at realistic environmental concentrations. As expected on the basis of nC, PFDA has the lowest experimental EC_{50} (0.851 mM), whereas the largest EC_{50} was obtained for 5H 4:1 (4.853 mM). These EC_{50} values are far above concentrations typically found in surface water. Thus, acute harmful effects to the photosynthesis of the green algae are not expected.

It is interesting to observe that the log EC_{50} of PFBA deviates from the relationship obtained between log EC_{50} and nC. According to this relationship, the EC_{50} of PFBA should be about 7 mM. However, the actual EC_{50} of PFBA is 1.225 mM, which is 5.7 times lower than that predicted from the relationship obtained. It cannot be ruled out that, due to its reactivity under irradiation, PFBA reacted and was transferred to more toxic chemicals under the pulsed excitation light. Although PFBA did not exhibit a large acute toxicity, its long-term chronic toxicity and mixture toxicity with its derivates need additional evaluation. Furthermore, taking into account the current strict regulation on PFOS and related chemicals, the short-chained compounds such as PFBS and PFBA are becoming the predominant PFC pollutants in surface waters[6,7]. Therefore, the toxicity of the short-chained PFCs and their derivates should be paid more attention to as well. Similar to the observation with regard to the EC_{50} of PFBA, the NOEC of PFBA was lower than expected on the basis of nC: the NOEC was below 1 mM and lower than that of 5H 4:1 FTOH.

Variation in toxicity profiles across species

Figure 3 shows the relationships between the log EC_{50} and nC for lettuce and some algae species. For the toxic effect of PFCs on root elongation of lettuce (*Lactuca sativa*), a simple linear regression model was obtained:

$$\text{Log } EC_{50,\text{ lettuce}} = -0.170\ (\pm 0.0409) \times nC + 1.197\ (\pm 0.271) \qquad (7)$$

$n = 5$, $R^2 = 0.853$, $p = 0.0252$

Excluding the EC_{50} value of PFBA, the regression model for the acute toxic effects of PFCs on the photosynthesis of green algae (*Pseudokirchneriella subcapitata*) was:

$$\text{Log } EC_{50,\text{ algae}} = -0.156\ (\pm 0.0122) \times nC + 1.313\ (\pm 0.0881) \qquad (8)$$

$n = 4$, $R^2 = 0.988$, $p = 0.006$

The R^2 values of these two models are higher than 0.85 and the confidence level is above 95 %, so they can be used to reliably predict the corresponding toxicity effect of PFCs with similar structures. From parameters of these two models, it can be seen that the models are similar (parallel slopes), so a relationship for the toxicity effects across lettuce and green algae can be developed as:

$$\text{Log } EC_{50,\text{ lettuce}} = 1.196\ (\pm 0.437) \times \log EC_{50,\text{ algae}} - 0.245\ (\pm 0.161) \qquad (9)$$

$n = 4$, $R^2 = 0.789$, $p = 0.112$

As pointed out by Liu et al.[8], perfluoroalkyl acids are likely to be incorporated into the lipid bilayer of the cell membrane, thus increasing the membrane permeability, and subsequently causing toxic effects on *Scenedesmus obliquus*. The PFCs investigated here are likely to have similar modes of action for the lettuce seeds and green algae in the sense that they might disrupt the membrane properties first, and then induce harmful impacts. In an absolute sense, due to differences in inherent sensitivity of test species, and differences in test procedures and endpoints, the toxic effects will be different. For instance: the test on lettuce seeds took 5 days, while the PAM test just needs 4.5 hours. Given the shorter duration of the PAM test and the observed parallel response in this study, the PAM test can be used to replace the 5-day root elongation test on lettuce seeds for acute toxicity evaluation of corresponding PFCs.

Liu et al.[8] tested the 72-h toxicity of PFHxA, PFOA, PFDoA and PFTeA on growth of the freshwater green alga *Scenedesmus obliquus* using optical density and in vitro chlorophyll fluorescence. It was found that PFDoA and PFTeA inhibited algal growth in a concentration-dependent manner while PFHxA and PFOA did not inhibit algal growth within the tested concentration ranges. Based on *in vitro* chlorophyll fluorescence, IC_{50} values were 0.261 mM and 0.192 mM for PFDoA and PFTeA respectively. The log-transformed IC_{50} values are also shown in Figure 1, and were found to be above the regression line of the PAM test. The PAM test thus is more sensitive than the 72-h algal growth inhibition test on *Scenedesmus obliquus* based on these data. Latała et al.[9] tested the 72-h toxicity of PFHxA, PFHpA, PFOA and PFNA to the marine green alga *Chlorella vulgaris*. The EC_{50} values obtained range from 1.07 mM to 12.84 mM, which were also log-transformed and presented in Figure 1. It can be seen that in this case the log EC_{50} values were well correlated with nC. However the regression line is significantly steeper than that of the PAM test given the slope of -0.358 ± 0.008. These two lines intersect at a fluorinated carbon chain length of about 8. This implies that the freshwater algae *P. subcapitata* in the PAM test is more sensitive than the marine *algae C. vulgaris* in the 72-h algal growth inhibition test for PFCs with nC < 8. From this comparison it can be seen that the PAM test is not only time-efficient but also has a relatively high sensitivity. The PAM test can therefore be used to evaluate the acute toxicity effects of PFCs to algae and plant seeds.

Conclusion

The aquatic toxicity of seven poly- and perfluorinated compounds was investigated for lettuce (*Lactuca sativa*) and green algae (*Pseudokirchneriella subcapitata*). The toxic effects on lettuce and green algae were found to be similar in a relative sense and were shown to have a good relationship with the fluorinated carbon chain length. The toxicity of these chemicals increases along with increasing chain length. It is interesting to observe that PFBA is more toxic than expected, which may be caused by more toxic chemicals produced under the pulsed excitation light. As short-chained PFCs are becoming the predominant PFCs pollutants in the surface waters, long-term toxicity and mixture toxicity of PFBA with its derivates should be paid more

attention to. Compared to other tests, the PAM test is more time-efficient and relatively sensitive to the toxicity of PFCs, so it can be used for the acute toxicity evaluation of PFCs.

Acute toxicity of poly- and perfluorinated compounds to two cladocerans, *Daphnia magna* and *Chydorus sphaericus*

Materials and methods

Test compounds

Seven PFCs were selected for the toxicity assessment on the basis of their chemical structural resemblance. The test set included: perfluorobutanoic acid (PFBA, CAS number: 375-22-4, 98%), 2,2,3,3,4,4,5,5-octafluoro-1-pentanol (5H 4:1 FTOH, CAS number: 355-80-6, 98%), perfluorooctanoic acid (PFOA, CAS number: 335-67-1, 96%), perfluorononanoic acid (PFNA, CAS number: 375-95-1, 97%), perfluorodecanoic acid (PFDA, CAS number: 335-76-2, 98%), perfluoroundecanoic acid (PFUnA, CAS number: 2058-94-8, 95%) and perfluorododecanoic acid (PFDoA, CAS number: 307-55-1, 97%). All of these chemicals were purchased from Sigma-Aldrich. As the water solubility of PFNA, PFDA, PFUnA and PFDoA was very low, dimethyl sulfoxide (DMSO) was used for preparing stock solutions. However, the concentration of DMSO in test solutions did not exceed 0.2%, and a solvent control was tested simultaneously.

Test Organisms

Daphnia magna were purchased from local suppliers and cultured in the laboratory for more than two months prior to the experiments. Cultures of *D. magna* were maintained at 20 ± 1 ℃ in 6-L glass jars under a 16 : 8-h light : dark photoperiod. *D. magna* were fed green algae (*Pseudokirchneriella subcapitata*) every day and half of the medium was renewed with M4 solution once a week. The M4 solution was prepared following the OECD Guideline[10].

Chydorus sphaericus used in the present study was reared from one gravid female collected in the summer of 1998 from the Lake Drontermeer in The Netherlands. The animals were kept in plastic containers filled with 100 ml of Dutch standard water (DSW) and about 1 g of pre-combusted quartz sand (Sibelco M32, Antwerp, Belgium; grain size 100 ~ 400 μm). *C. sphaericus* were fed 2 ml of a food suspension consisting of dried ground nettle powder (*Urtica dioica*) (0.5% w/v) and 3×10^7 μm^3 *Nitzschia perminuta* ml^{-1} medium three times a week. Every week, around 70% of the culture medium was renewed by decanting most of the medium from

the container. Cultures of *C. sphaericus* were also maintained at 20 ± 1 ℃ and a 16 : 8-h li ght : dark photoperiod.

Acute immobilization tests on *Daphnia magna*

Daphnia acute immobilization tests were performed according to the OECD test guideline 202[10] with slight modifications. Fifty ml polypropylene disposable tubes (Fisher Scientific) were used as test vessels with 20 ml test solutions inside. Neonates of *D. magna* of less than 24 h were collected and then exposed to six concentrations of the tested chemicals and the control. Nominal concentrations of definitive test solutions are listed in Table 4. In those cases where it was necessary to use DMSO for the preparation of stock solutions, a solvent control was also tested simultaneously. Four replicate test vessels, each with five neonates, were used for each control or test concentration treatment. The animals were not fed during the test period and were inspected at 24 and 48 h. Temperature and photoperiod were kept the same during exposure and culturing. An organism was considered immobile when it was not able to swim within 15 s after gentle stirring. Testing and culturing conditions were similar with reconstituted M4 water used for culturing and testing. Preliminary range-finding tests were performed first to determine the definitive test concentrations for each compound. In the first test, it was found that only PFBA can markedly acidify the test solutions with pH as low as 2.35 for a 10 mM test solution. Thus, a second test was performed for PFBA with pH of the stock solution adjusted to 8.0 with sodium hydroxide solution and diluted hydrochloric acid solution.

Table 4. Bioassay methods and nominal concentrations of seven poly- and perfluorinated compounds used in the final toxicity tests.

Chemicals	**Species**	**Test Method**	**Nominal Concentration (mM)**
PFBA	*Daphnia magna*	OECD test guideline 202	0, 0.7, 0.75, 0.8, 0.83, 0.85 and 0.9
5H 4:1 FTOH			0, 0.8, 1, 1.1, 1.3, 1.5 and 1.8
PFOA			0, 0.35, 0.4, 0.45, 0.5, 0.55 and 0.6
PFNA			0, 0.1, 0.2, 0.3, 0.35, 0.5 and 0.6
PFDA			0, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4
PFUnA			0, 0.1, 0.2, 0.25, 0.3, 0.35 and 0.4
PFDoA			0, 0.08, 0.1, 0.12, 0.15, 0.18 and 0.2
PFBA	*Chydorus sphaericus*	Chydotox toxicity test [30]	0, 1.5, 2, 2.2, 2.5, 3 and 3.5
5H 4:1 FTOH			0, 0.5, 1, 1.5, 2, 2.5 and 3
PFOA			0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6
PFNA			0, 0.05, 0.07, 0.1, 0.15, 0.2 and 0.25
PFDA			0, 0.01, 0.03, 0.08, 0.1, 0.2 and 0.25

Chemicals	Species	Test Method	Nominal Concentration (mM)
PFUnA			0, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1
PFDoA			0, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06

Acute immobilization tests on *Chydorus sphaericus*

The 48-h acute immobilization tests with *Chydorus sphaericus* followed the protocol of the Chydotox toxicity test developed in the Dutch National Institute for Public Health and the Environment[11]. The tests were carried out in 2 ml HPLC vials to which 250 µl of test solution was added. Neonates (< 24 h) of *C. sphaericus* were collected by a mesh filter with a diameter of 250 µm and then exposed to six concentrations of the tested chemicals and the control. Nominal concentrations of definitive test solutions are also shown in Table 2. If DMSO was used in stock solutions, a solvent control was also tested simultaneously. Four replicate test vials, each with five neonates, were used for each control or test concentration. The vials were covered with a lid to prevent evaporation and incubated for 48 h under the same conditions as the culture. The animals were not fed during the test period and were checked under a reverse dissecting microscope at 24 and 48 h. Immobilization was determined based on inactivation of the animals after slightly tapping with a finger to the vial and monitoring them for 30 s. For PFBA, toxicity tests without and with pH adjustment were carried out as PFBA can acidify the test solutions at the test concentrations used in the present study.

Chemical analysis

Concentrations of PFBA, PFOA and PFNA in assays were partly confirmed by Liquid Chromatography – Electrospray Ionization – Tandem Mass Spectrometry (HPLC-ESI-MS/MS). Samples containing the highest and the lowest test concentrations were analyzed in all cases to confirm the nominal concentrations. In case of PFBA, all samples were analyzed. Samples were drawn right after the test and immediately diluted 1:10 (V:V) with methanol to prevent adsorption. For further measurement, these samples were subsequently diluted with water/methanol (1:1; V:V) to fit into the calibration curve. For measurement of PFOA and PFNA, the internal standard $^{13}C_2$-PFOA was added to the samples and used for quantification. As all concentrations in the tests that were verified by chemical analyses were very well in line with the nominal concentrations, the nominal concentrations were used in the present study.

The following devices were used: Applied Biosystems QTrap 3200, 2 x Perkin Elmer Series 200 Pump, Perkin Elmer Series 200 Degasser, Perkin Elmer Series 200 Autosampler. Column used: MZ Aqua C18 50 x 2.0 mm, 5 µm, 120 Å + MZ Aqua C18 10 x 2.0 mm, 5 µm 120 Å precolumn. Eluents: A: H_2O/MeOH (95/5; V/V) + 5 mM ammonium acetate, B: H_2O/MeOH (10/90; V/V) + 5

mM Ammonium acetate. Flow rate: 300 µL/min, gradient: 0 – 2 min: 95% A, 2 – 4 min: linear gradient to 0% A, 4 – 6 min: 0% A, 6 – 8 min: linear gradient to 95% A, 8 – 15 min: 95% A. Injection volume: 5 µL.

The MS was operated in the negative Electrospray Ionization mode and multiple reaction monitoring (MRM) of transition m/z 213 → 169. Linear calibration was performed at 0, 250, 500, 750, and 1000 ng.mL^{-1}. Repeatability at 250 ng.mL^{-1} was 4.4 % (n=4), LOD was estimated to be approximately 0.5 ng.mL^{-1} (for S/N =10).

One sample at each concentration was taken and analyzed. In order to assess the deviations, all samples at nominal concentration of 33 mM were analyzed. Relative standard deviation was 6.5 %. The measured concentrations were between 82 and 91 % of the nominal concentrations. This makes sense when taking into account the dilution by adding the animals to 250 µL of solution.

Data analysis

The concentrations that caused 50% inhibition effects (EC50) with 95% confidence limits (CL) were calculated by the Probit program v1.5 from the U.S. Environmental Protection Agency (U.S. EPA). The significance of differences in the average percentage immobility per concentration relative to the blank was assessed by the Dunnett program v 1.5 obtained from the U.S. EPA. No observed effect concentrations (NOECs) were determined based on results of the Dunnett's analyses.

Results and discussion

Toxicity to *Daphnia magna*

The acute effects of seven PFCs to *Daphnia magna* are shown in Table 3. As PFBA caused marked acidification of the test solutions, tests without and with pH adjustment to 8.0 were carried out, and the corresponding EC50 values were obtained. For PFBA, it was found that the EC50s changed greatly, which shows that the change of pH values had a significant effect on *D. magna*. The other PFCs did not show obvious acidification of test solutions in the experiments. Limited by the solubility and aggregation of PFUnA as concentrations approached the solubility limit, the 24-h EC50 and NOEC of PFUnA could not be obtained. The 24-h and 48-h immobility EC50-values of PFOA for *D. magna* were found to be 0.531 mM and 0.511 mM, respectively. These values agree well with values of 0.691 mM and 0.420 mM for the toxicity of the ammonium salt of PFOA on *D. magna* reported by Li[5]. Colombo et al.[12] gave 24-h and 48-h EC50s of APFO on *D. magna* as 1.389 mM and 1.113 mM, respectively, and Ji et al.[14] reported

similar values for PFOA of 1.630 and 1.151 mM. Different culture conditions and test procedure may partly account for the difference between the reported data.

Table 5. EC50 and no observed effect concentrations (NOECs) (mM) of seven poly- and perfluorinated compounds for *Daphnia magna* [a]

Chemicals	CAS number	nC	24 h		48 h	
			EC50 (95% CL)	NOEC	EC50 (95% CL)	NOEC
PFBA	375-22-4	3	0.865 (0.858-0.871) [b] > 20 [c]	0.85 [b] - [c]	0.848 (0.841-0.856) [b] > 20 [c]	0.83 [b] - [c]
5H 4:1 FTOH	355-80-6	4	1.332 (1.143-1.476)	1.10	1.222 (1.016-1.395)	1.00
PFOA	335-67-1	7	0.531 (0.506-0.555)	0.40	0.511 (0.446-0.617)	0.50
PFNA	375-95-1	8	0.481 (0.435-0.601)	0.35	0.326 (0.281-0.390)	0.20
PFDA	335-76-2	9	0.339 (0.310-0.366)	0.15	0.318 (0.278-0.345)	0.15
PFUnA	2058-94-8	10	0.238 [d]	-	0.236 (0.163-0.327)	0.10
PFDoA	307-55-1	11	0.162 (0.152-0.174)	0.15	0.129 (0.098-0.160)	0.12

[a] nC = the fluorinated carbon chain length; CL = confidence limit.
[b] Data for the test without pH adjustment.
[c] Data for the test with pH adjustment.
[d] Predicted by the relationship found between log EC50 and nC.

From Table 5 it can be seen that the EC50 and NOEC values of the PFCs studied decreased with increasing fluorinated carbon chain length (nC). The 24-h EC50 values were in the range of 0.162 to > 20 mM, while the 48-h EC50 values were in the range of 0.129 to > 20 mM. As the EC50 value of PFBA with pH adjustment was > 20 mM and could not be experimentally determined simply due to the high amounts of PFBA needed and subsequent impacts on the texture of the aquatic solutions, it was not used for development of the quantitative structure-activity relationships. For the toxicity of the other six PFCs on *D. magna*, good relationships between the experimental log EC50 and nC could be obtained:

$$\text{Log } EC50_{24h} = -0.127\ (\pm 0.009) \times nC + 0.646\ (\pm 0.071) \quad (10)$$

$n = 5$, $R^2 = 0.986$, $p = 7.090\times10^{-4}$

and

$$\text{Log } EC50_{48h} = -0.131\ (\pm 0.011) \times nC + 0.615\ (\pm 0.096) \quad (11)$$

$n = 6$, $R^2 = 0.971$, $p = 3.265\times10^{-4}$

The two equations are comparable and almost parallel. The statistics of equation 8 show that this is a significant relationship that can be used to reliably predict the toxicity of non-tested PFCs. Amongst others it is suited to predict the 24-h EC50 of PFUnA. The value was estimated to be 0.238 mM using equation 7, which is indeed well above its water solubility limit. In surface waters, the concentrations of the PFCs are usually found to be in the range of pM to nM. As the EC50 values obtained here are far above concentrations typically found in surface water, acute harmful effects of these chemicals to *D. magna* are not expected in the real environment.

From equation 7 and 8, the 24-h and 48-h EC50s of PFBA were predicted to be 1.84 mM and 1.66 mM. However, the experimental values obtained were just 0.865 mM and 0.848 mM without pH adjustment, which indicates that PFBA is more toxic than expected in this test. In another toxicity test on photosynthesis of green algae (*Pseudokirchneriella subcapitata*), PFBA also was found to be more toxic than expected[1]. At environmental conditions, PFBA will be present as the deprotonated species and will thus not have this high toxicity. However, environmental monitoring has recently shown that the short-chained compounds such as PFBA and Perfluorobutane sulfonate (PFBS) are becoming the predominant PFC pollutants in surface waters[6,7,14]. Thus more attention should be paid to assessment of mixture toxicity of PFBA with other chemicals or with its derivates in local surface waters.

Toxicity to Chydorus sphaericus

The acute effects of seven PFCs on *Chydorus sphaericus* are shown in Table 5. The 24-h EC_{50}s for the seven PFCs tested were in the range of 0.054 to > 20 mM, while the 48-h EC_{50}s were lower and ranged from 0.034 mM to > 20 mM. The 24-h and 48-h EC_{50}s of PFBA on *C. sphaericus* were determined to be 2.509 mM and 2.160 mM without pH adjustment. However, the EC_{50}s were > 20 mM after pH adjustment. This showed that the change of pH values of test solutions also had a significant effect on *C. sphaericus*. As the EC_{50} values obtained in the present study are again far above concentrations typically found in surface waters, the PFCs tested here are not expected to have acute harmful effects to *C. sphaericus* in the real environment.

In general, the EC_{50} and NOEC values decreased with increasing nC. However, PFNA with nC of eight performed differently from the other chemicals. The 24-h and 48-h EC_{50}s of PFNA were 0.121 mM and 0.060 mM, respectively. These values were slightly lower than those of PFDA (nC=9), which were 0.141 mM and 0.088 mM, respectively. Although 5H 4:1 FTOH has different functional groups as compared to the other chemicals investigated, it also conformed to the relationship between logEC50 and nC, which may implicate that the fluorinated chain length is the main factor for the toxicity of poly- and perfluorinated carboxylic acids and alcohols.

Therefore, the experimental EC_{50} values of six PFCs, except PFBA, were used for modelling the relationships with nC:

$$\text{Log } EC_{5024h} = -0.209\ (\pm 0.024) \times nC + 0.970\ (\pm 0.202) \quad (12)$$

$n = 6$, $R^2 = 0.950$, $p = 9.359\times10^{-4}$

and

$$\text{Log } EC_{5048h} = -0.201\ (\pm 0.039) \times nC + 0.689\ (\pm 0.327) \quad (13)$$

$n = 6$, $R^2 = 0.871$, $p = 6.48\times10^{-3}$

It can be seen that the equations have high statistical significance, so they can be used to reliably predict the adverse effects of similar, non-tested PFCs. For PFBA, the 24-h and 48-h EC_{50}s to *C. sphaericus* were predicted to be 2.20 mM and 1.222 mM using equation 9 and 10. These predicted values are lower than the experimental values obtained without pH adjustment and confirm the limited acute toxicity of short-chained PFCs.

Table 6. EC_{50} and no observed effect concentrations (NOECs) (mM) of seven poly- and perfluorinated compounds for *Chydorus sphaericus*[a].

Chemicals	CAS number	nC	24 h		48 h	
			EC50 (95% CL)	NOEC	EC50 (95% CL)	NOEC
PFBA	375-22-4	3	2.509 (2.409-2.607) [b]	2.2 [b]	2.160 (2.070-2.249) [b]	2.0 [b]
			> 20 [c]	- [c]	> 20 [c]	- [c]
5H 4:1 FTOH	355-80-6	4	1.393 (0.829-1.877)	1.0	0.842 (0.239-1.292)	< 0.5
PFOA	335-67-1	7	0.426 (0.223-0.537)	<0.2	0.282 (0.122-0.345)	<0.1
PFNA	375-95-1	8	0.121 (0.106-0.136)	0.07	0.060 (0.047-0.070)	< 0.05
PFDA	335-76-2	9	0.141 (0.110-0.173)	0.08	0.088 (0.051-0.124)	0.01
PFUnA	2058-94-8	10	0.069 (0.056-0.082)	0.04	0.034 (0.022-0.042)	0.01
PFDoA	307-55-1	11	0.054 (0.044-0.086)	0.02	0.046 (0.034-0.081)	0.02

[a] nC = the fluorinated carbon chain length; CL = confidence limit.
[b] Data for the test without pH adjustment.
[c] Data for the test with pH adjustment.

Interspecies relationships

The relationships between log EC_{50} and nC for the two cladocerans are shown in Figure 4. Interspecies analyses between these two cladocerans were performed based on 24-h and 48-h experimental EC50 values, and two relationships were obtained.

For 24-h toxicity:

$$\text{Log } EC_{50\textit{C. sphaericus}} = 1.6\ (\pm\ 0.3) \times \log EC_{50\textit{D. magna}} - 0.1\ (\pm\ 0.1) \quad (14)$$

$n = 5$, $R^2 = 0.888$, $p = 0.016$

For 48-h toxicity:

$$\text{Log } EC_{50\textit{C. sphaericus}} = 1.5\ (\pm\ 0.3) \times \log EC_{50\textit{D. magna}} - 0.3\ (\pm\ 0.17) \quad (15)$$

$n = 6$, $R^2 = 0.846$, $p = 0.009$

It can be seen that the relationships between the log-transformed EC_{50} values of the two cladocerans are significant, so the toxicity of a certain PFC for one cladocerans species can be used to predict the toxicity for the other using the equations. *D. magna* is a pelagic species that inhabits the upper water column, whereas *C. sphaericus* is a benthic species that lives on the sediments. Therefore their EC_{50}s represent aqueous and sediment toxicity of a chemical via exposure to the water phase, respectively. With these interspecies relationships, one could calculate aqueous or sediment toxicity of a similar PFC with known sediment or aqueous toxicity data. Furthermore, the Chydotox toxicity test needs less chemicals and materials, so it may be a promising test method for collection of toxicity data that are needed for environmental risk assessment.

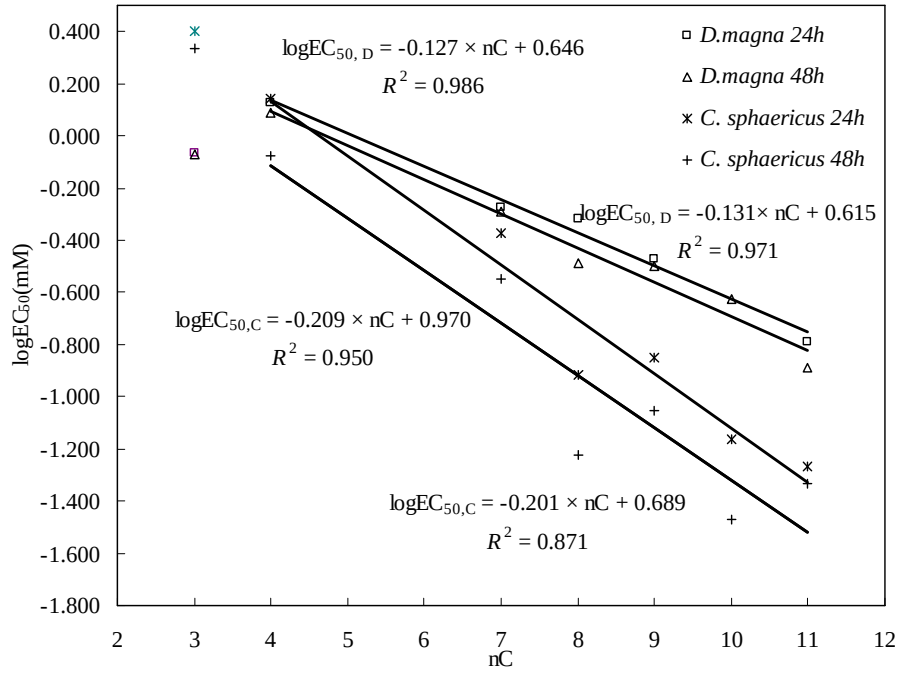

Figure 4. Relationships between log EC_{50} and the fluorinated carbon chain length (nC) for two cladocerans.

Conclusion

In the present study, the acute toxicity of seven PFCs to *D. magn*a and *C. sphaericus* was evaluated. In general, the measured EC50s and NOECs of the two cladocerans decreased with increasing fluorinated carbon chain length. As PFBA acidifies the test solutions, its toxicity was tested without and with pH adjustment. It was found that the EC50s changed greatly after pH adjustment, which may suggest that acidification has a significant effect on its toxicity. As the short-chained compounds such as PFBA and PFBS are becoming the predominant PFC pollutants in surface waters, their long-term toxicity and mixture toxicity with other pollutants in local aquatic environment needs more attention. The EC50 values obtained here are far above concentrations typically found in surface waters. Acute harmful effects of these PFCs to *D. magna* and *C. sphaericus* are therefore not expected in the real environment.

Assessment of the adverse effects of a diverse set of poly- and perfluorinated chemicals to zebrafish

Materials and methods

Test compounds

The poly- and perfluorinated compounds that were selected are summarized in Table 7. The interest of these compounds lies not only on the different length of their perfluorinated chain but also on the different functional groups, although most of them contain the carboxylic functionality.

Table 7. Summary of selected chemicals.

Functional group	*CAS*	*Name*	*nº C*
HO-C=O	375-22-4	PFBA	4
CH2-OH	355-80-6	FT-OH	5
HO-C=O	335-67-1	PFOA	8
HN4-C=O	3825-26-1	APFO	8
O-SO2-K	2795-39-3	PFOSK	8
HO-C=O	375-95-1	PFNA	9
HO-C=O	335-76-2	PFDA	10
HO-C=O	2058-94-8	PFUnA	11
HO-C=O	307-55-1	PFDoA	12

Nine PFCs were selected for the toxicity assessment on the basis of their chemical structural resemblance. The test set included: perfluorobutanoic acid (PFBA, CAS number: 375-22-4, 98%), 2,2,3,3,4,4,5,5-Octafluoro-1-pentanol (5H 4:1 FTOH, CAS number: 355-80-6, 98%), perfluorooctanoic acid (PFOA, CAS number: 335-67-1, 96%), perfluorononanoic acid (PFNA, CAS number: 375-95-1, 97%), perfluorodecanoic acid (PFDA, CAS number: 335-76-2, 98%), perfluoroundecanoic acid (PFUnA, CAS number: 2058-94-8, 95%) and perfluorododecanoic acid (PFDoA, CAS number: 307-55-1, 97%), perfluorooctane (PFOS) sulfonate potasium salt (PFOSK, CAS number: 2795-39-3), ammonium perfluorooctanoate (APFO, CAS number: 3825-26-1).

All chemicals were purchased from Sigma-Aldrich. As the water solubility of some of the PFCs was very low, dimethyl sulfoxide (DMSO) was used for preparing stock solutions. Seven PFCs were selected for the toxicity assessment on the basis of their chemical structural resemblance.

Fish Embryo Toxicity test with Zebrafish (*Danio rerio*)

The standard Operation Procedure describes a Fish Embryo Toxicity Test with the zebrafish *Danio rerio*. This test is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity test with juvenile and adult fish, i.e. the OECD Test Guideline 203[15], thus providing a reduction in fish usage.

Zebrafish embryos are individually exposed in, e.g., 24-well microtiter plates with two millilitre of test solution. The test is initiated immediately after fertilization and is continued for 96 hours. Lethal effects, as described by four apical observations (coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heart beat), are determined by comparison with controls to identify the LC50 value. Additionally, the EC50 value was identified from the General Morphology score points (Figure 4), where each day the development of the embryo is scored giving points to each stage of the morphology development. For this measurement eleven end points were taken on account (detachment of tail, somite formation, eye development, movement, heartbeat, blood circulation, pigmentation of head-body, pigmentation of tail, pectoral fin, protruding mouth and hatching) being also compared with controls.

The test method is based on using a minimum of five test concentrations as well as appropriate negative and positive controls. Each chemical is tested with 20 embryos per test concentration and controls.

The dilution water used for this test, OECD water, is prepared the day before the beginning of each test, from a mixture of 10ml of each salt stock solution ($CaCl_2 \cdot 2\ H_2O$ 14.7 g per 500 ml, $MgSO_4 \cdot 7 \cdot H_2O$ 6.165 g per 500 ml, $NaHCO_3$ 3.325 g per 500ml and KCl 0.285 g per 500 ml) per litre of water, under continuous aeration.

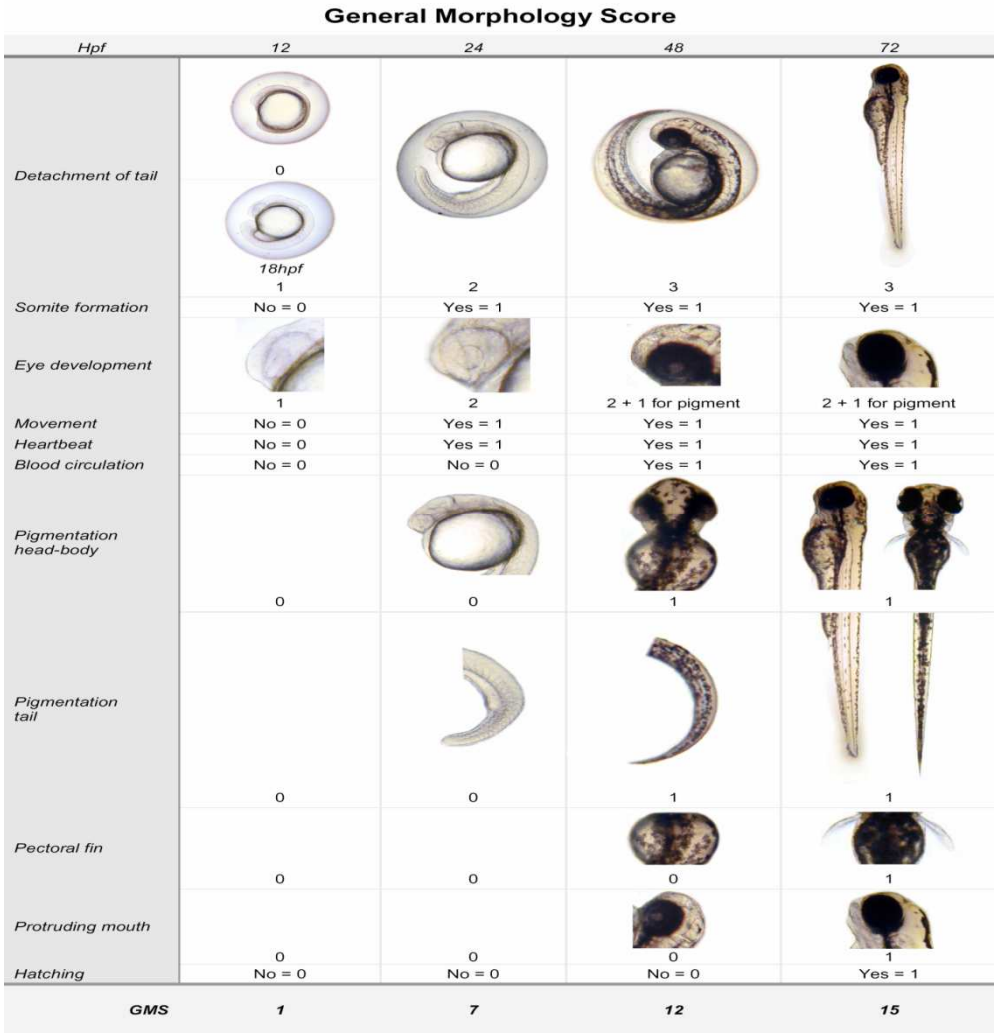

Figure 5. General morphology score of the zebrafish embryo.

For the substances difficult to be dissolved DMSO was used as solvent and therefore was tested in another plate. In most of the cases the final concentration exceeded the recommended 1000µl/L but it did not affect the results of the test. Pure dilution water was used as negative control and 3,4-dichloroaninile as positive control in a concentration of 4 mg/L.

In order to maintain the nominal concentration, all the solutions were refreshed after checking the embryos at 48 hours and not every day due to the high stability of the perfluorinated compounds.

Throughout the test, the incubation conditions were kept under control and checked at the beginning, after refreshing the solutions and at the end of each test. All the plates were covered with parafilm and kept at 26±1 ^{o}C in an incubator, with a light cycle of 14 hours light and 10 h dark, oxygen saturation > 80%, pH between 6.5-8.5 and total hardness between 10-250 mg/L.

In this case the battery of the selected perfluorinated chemicals were tested in order to make a predicted model based on the toxicity effects due to the perfluorinated carbon chain length.

Data analysis

On the one hand, for the FET test and the test with algae, the actual concentrations that caused 50 % inhibition effects (EC50) and the actual concentrations that caused 50 % lethality effects (LC_{50}) with 95% confidence limits (CL) were calculated by a nonlinear curve-fitting procedure in GraphPad Prism v4.0, for the Zebrafish and Algae. The fitted model between the logarithm of nominal concentration X and the response Y is in the form of:

$$Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\log EC_{50} - \text{hillsope})}}$$

In this equation, the Hill Slope was chosen as a variable slope of the dose-response curve. But on the other hand, the concentrations that caused 50% inhibition effects (EC_{50}) with 95% confidence limits (CL) were calculated by the Probit program v1.5 from US EPA.

Results and discussion

Figures 6 – 11 display the response curves obtained for all poly- and perfluorinated compounds studied in the FET test with zebrafish embryos. The Figures are arranged according to increasing number of carbon atoms of the compounds studied. In order to facilitate direct comparison across the chemicals studied, the x-scale of all curves shown in the Figures 6-11 is the same. The left hand side of each figure displays observed effects based on the morphology, the right hand side displays the observed lethal effects.

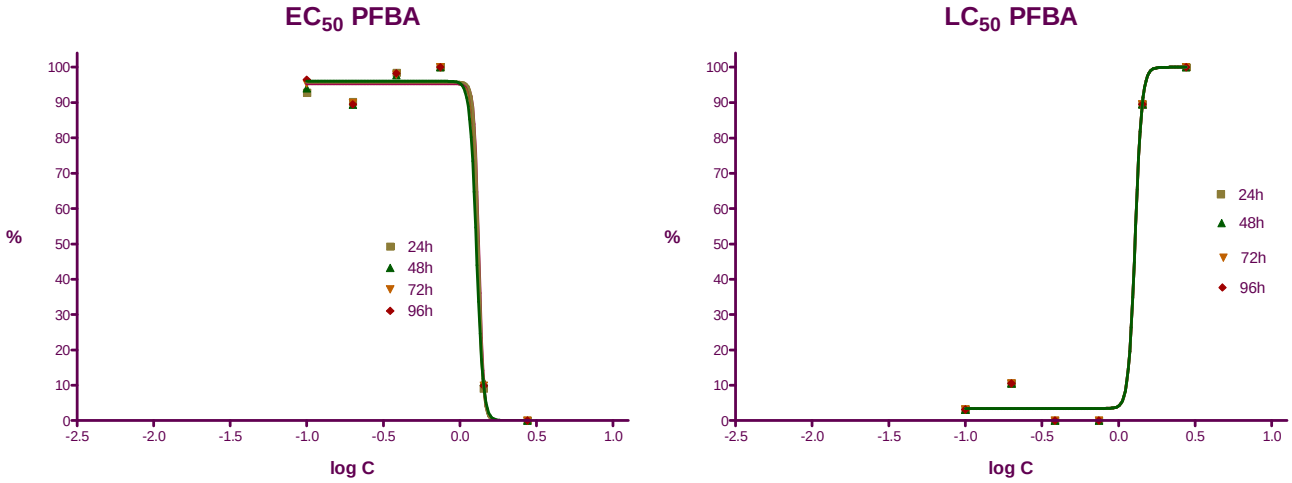

Figure 6. Results on zebrafish for fluorinated compounds containing 4 carbon atoms in the chain.

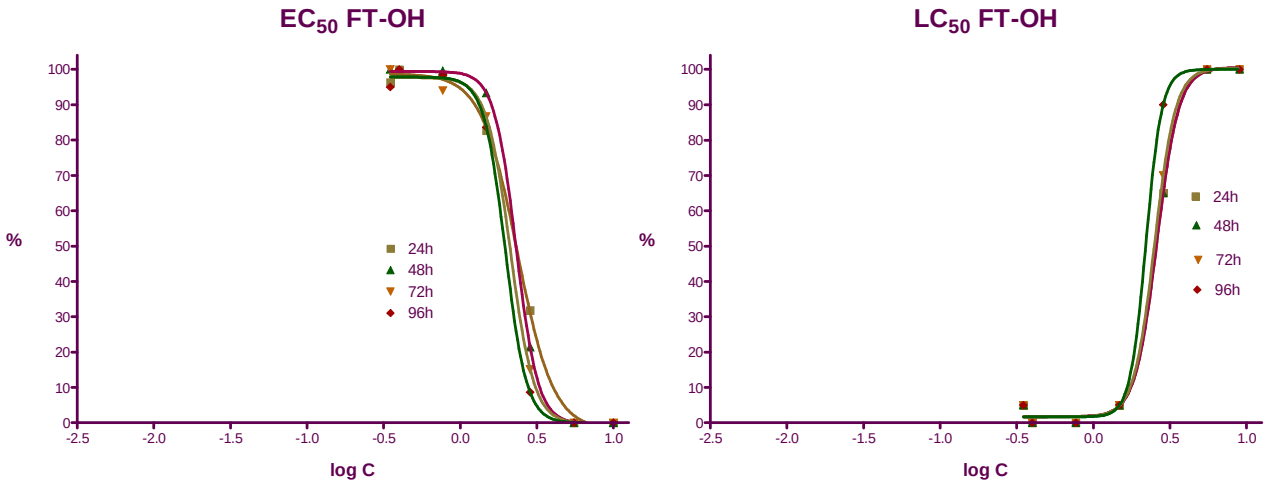

Figure 7. Results on zebrafish for fluorinated compounds containing 5 carbon atoms in the chain.

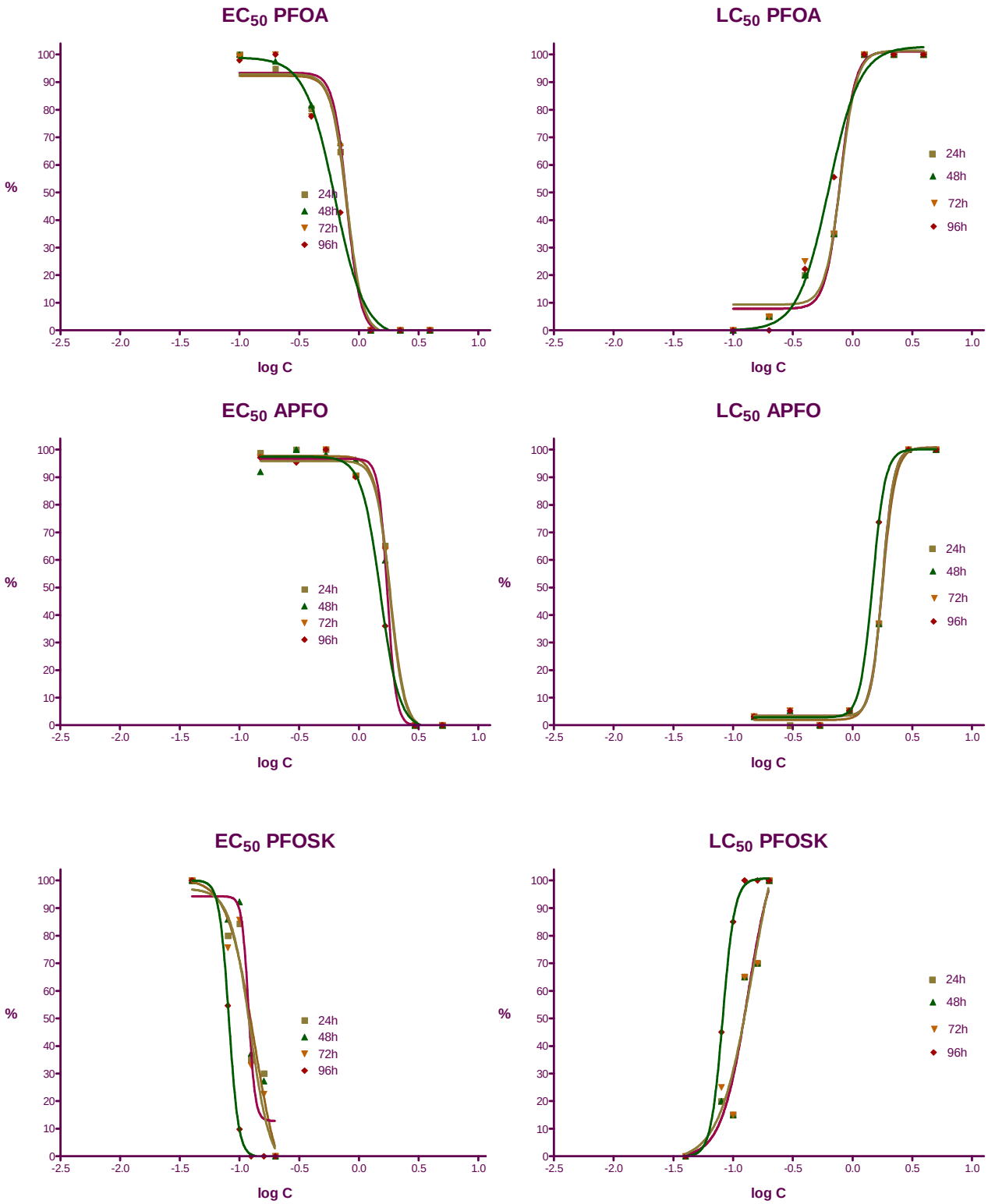

Figure 8. Results on zebrafish for fluorinated compounds containing 8 carbon atoms in the chain (carboxylic acid, ammonium salt of the carboxylic acid, potassium sulfonate salt).

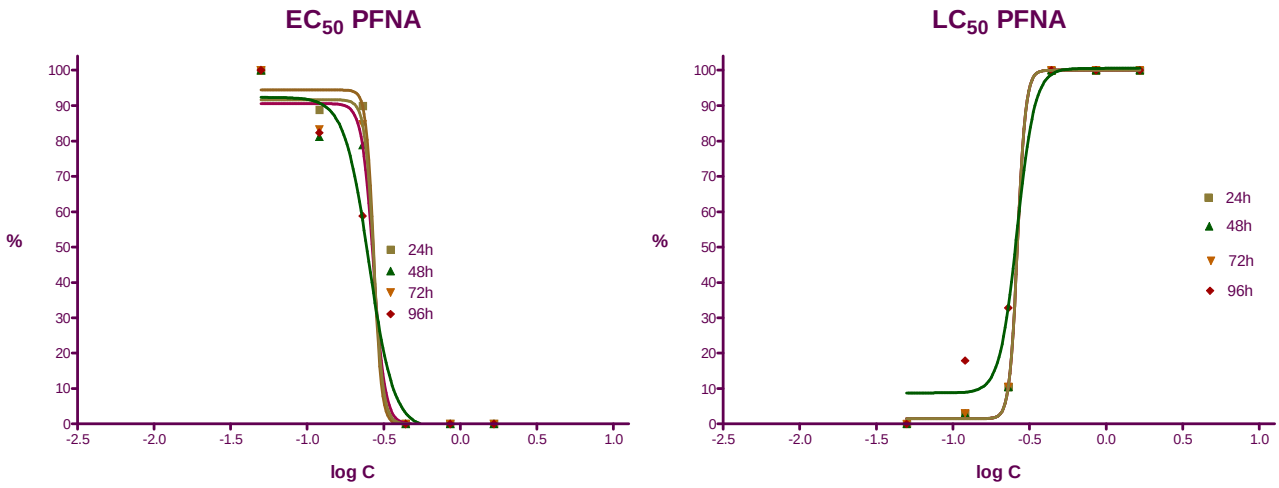

Figure 9. Results on zebrafish for fluorinated compounds containing 9 carbon atoms in the chain (carboxylic acid).

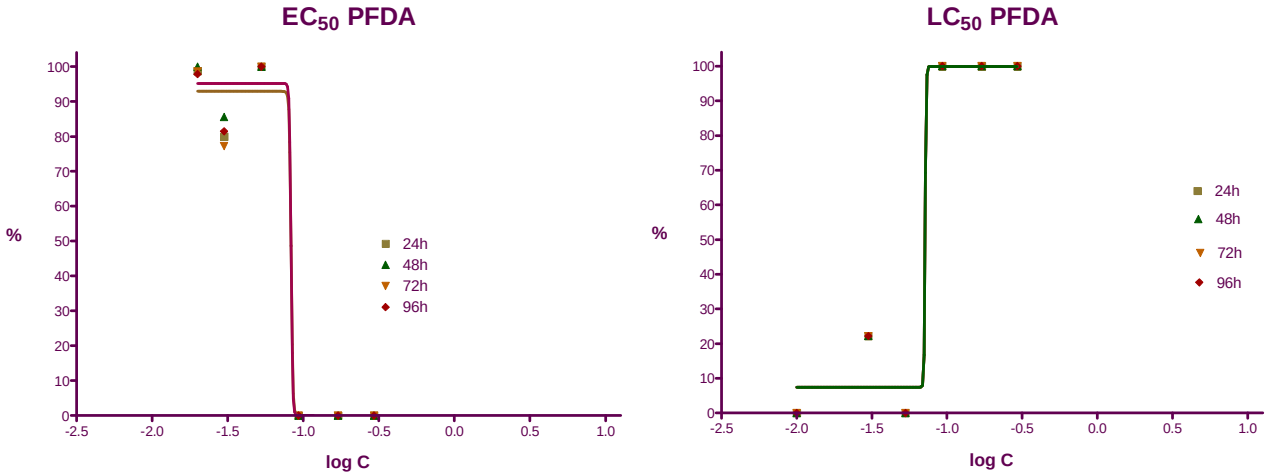

Figure 10. Results on zebrafish for fluorinated compounds containing 10 carbon atoms in the chain (carboxylic acid).

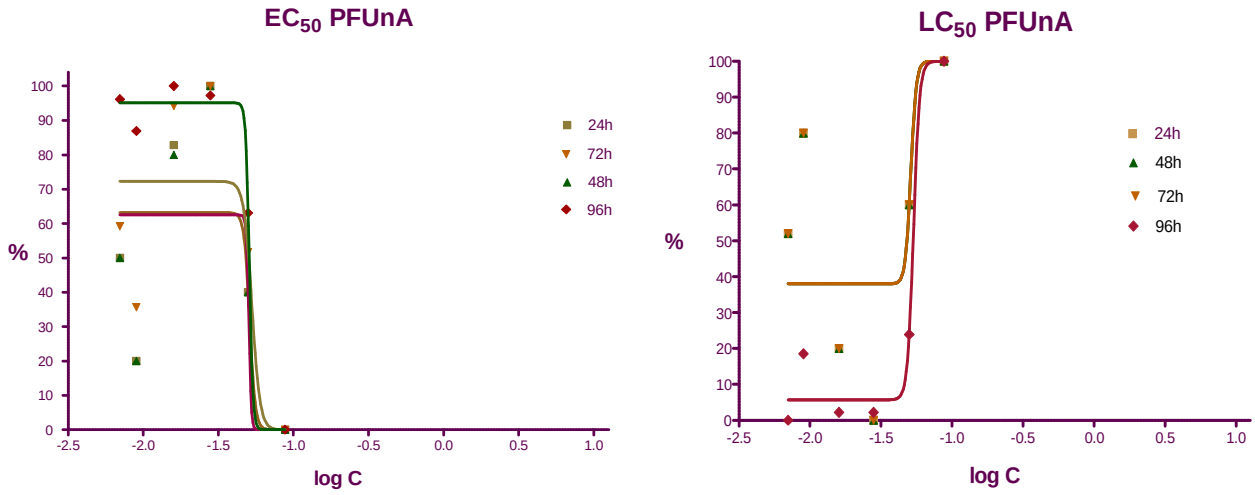

Figure 11. Results on zebrafish for fluorinated compounds containing 12 carbon atoms in the chain (carboxylic acid).

Table 8. Summary of all EC_{50}/LC_{50} obtained after each 24, 48, 72 and 96 hours on zebrafish embryos.

				FET (EC50) *(Mm)*				**FET (LC50) *(Mm)***			
Functional Group	***CAS***	***EC_{50}***	*nC*	*24h*	*48h*	*72h*	*96h*	*24h*	*48h*	*72h*	*96h*
HO-C=O	375-22-4	**PFBA**	4	1.31	1.33	1.32	1.28	1.28	1.28	1.28	1.28
CH_2-OH	355-80-6	**FTOH**	5	2.36	2.32	2.12	1.2	2.6	2.6	2.52	2.21
HO-C=O	335-67-1	**PFOA**	8	0.79	0.8	0.8	0.63	0.79	0.79	0.8	0.63
HN_4-C=O	3825-26-1	**APFO**	8	1.83	1.73	1.82	1.51	1.79	1.8	1.8	1.47
O-SO_2-K	2795-39-3	**PFOSK**	8	0.13	0.12	0.12	0.08	0.13	0.13	0.15	0.08
HO-C=O	375-95-1	**PFNA**	9	0.27	0.27	0.27	0.25	0.26	0.26	0.26	0.26
HO-C=O	335-76-2	**PFDA**	10	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.07
HO-C=O	2058-94-8	**PFUnA**	11	-	-	-	0.05	-	-	-	0.06
HO-C=O	307-55-1	**PFDoA**	12	-	-	-	≈□0.05	-	-	-	≈ 0.06

Comparison of the EC_{50} and LC_{50} values obtained shows that the toxicity of the fluorinated compounds studied is dependent on the length of the carbon chain, although the impact of chain length varies across the different functional groups.

For the carboxylic acids, especially for PFBA, PFOA, FPNA, PFDA, the toxicity was acute. After 24 hours, in the highest concentrations the embryos appeared coagulated/dead, and in the lowest concentrations there was not any effect. Along the experiments there was no more evidence of toxicity until after 72 or 96 hours of exposure, where the embryos started showing a disordered behaviour. This disorder, caused by the neurotoxic effects of these compounds is not recorded for the values of LC_{50} or EC_{50} until they are dead. In the first case because it is only taken on account the lethal endpoints and in the second one because even not having the ordinary movement they score the points for it. Instead of swimming as the control embryos do, the affected embryos moved spasmodically and shaking. The embryos that showed this disorder were dead in the next hours or almost dead. Upon exposing the embryos for more than the 96 hours prescribed in the standard test, the effects increased and more fish were found dead.

In the case of PFUnA and PFDoA, also being carboxylic acids, the behaviour of the fish towards these compounds was different. There was no acute toxicity at the 24 hours after exposure. First toxicity effects were observed after 72 hours. They show pericardial oedema and especially the disordered behaviour. Therefore after 96 hours the embryos in the highest concentrations were dead. But due to the highest concentration was not dead after 24 hours, these embryos were in a higher concentration of DMSO (1.6%) which could cause the pericardial oedema so it should not be taken on account. Nevertheless, not as in the other compounds, the toxicity was not increased between 11 and 12 carbons in the chain. Although the results after 96 hours of exposure to PFDoA were lost, the range of concentrations was almost the same and the embryos exposed to the higher concentration were dead the last day of the scoring. Therefore the EC/LC_{50} values for PFDoA are in the same order as for PFUnA.

FT-OH, which is an alcohol instead of a carboxylic acid, also showed acute toxicity at 24 hours after exposure but on the other hand the embryos exposed to it show more toxic effects before their death. For instance, they embryos suffer from pericardial and yolk oedema as well as a variety of malformations such as head tail and yolk.

Three different perfluorinated compounds with eight carbons in their chain were tested. PFOA (carboxylic acid), APFO (the ammonium salt of PFOA) and PFOSK (sulfonate potasium salt). PFOA followed the effects of the carboxylic groups as explained before, whereas APFO is less toxic and PFOSK was found to be much more toxic. In the case of PFOSK more cases of scoliosis were observed in fish than usual.

Conclusion

After analysis of all data it can be concluded that the fish embryo toxicity test works well. It is faster and also more reliable to derive LC_{50} values for the embryos than it is to derive EC_{50} values: in derivation of LC_{50}-values only four lethal endpoints are taken into account. The general trend observed is that the EC_{50}/LC_{50} values of the PFCs studied decrease with increasing fluorinated carbon chain length (nC). Besides the toxicity due to the fluorinated carbon chain length, there is clearly an additional effect on toxicity of extra functional groups present in the molecule. Finally, it is found that in general the zebrafish embryos have a similar sensitivity towards fluorinated compounds as daphnias. The general scale of sensitivity is: *Algae* < *Lettuce* < *Daphnia* ≈ *Zebrafish* < *Water flea.*

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Report on testing of substituted musks/fragrances

Toxicity test with algae and daphnids and ready biodegradability test was performed according to OECD guidelines 201, 202 and 301D at Public Health Institute Maribor (PHI). In 2010 PHI has started development of experimental test sets in laboratory. In 2010 University of Insubria, Linnaeus University and PHI prepared a list of chemicals relevant for the testing for substituted musks/fragrances on algae, daphnids and ready biodegradability according to analytical possibilities, REACH relevance, structural representativeness and relevance according to toxicity and physicochemical properties. The list of fragrances relevant for the testing on algae, daphnids and ready biodegradability is in Table 9.

Table 9. List of fragrances relevant for the testing on algae, daphnids and ready biodegradability

ID	Name	Functional Class	CAS Nr
FRA-001	Acetyl cedrene	Terpenes	032388-55-9
FRA-006	Benzyl cinnamate	Cynnamic acid der.	000103-41-3
FRA-022	Hexyl salycilate	Salycilic acid der.	006259-76-3
FRA-023	Hexylcinnamaldeyde	Cynnamic acid der.	000101-86-0
FRA-024	HHCB (Galaxolide)	Musks	001222-05-5
FRA-041	Methyl dihydrojasmonate	Terpenes	024851-98-7
FRA-049	Quinidine	Ref.Comp. (Drug)	000056-54-2
FRA-054	α-amylcinnamyl alcohol	Cynnamic acid der.	000101-85-9
FRA-056	Musk ambrette	Musks	000083-66-9
FRA-065	Cyclopentadecanolide	Musks	000106-02-5
FRA-069	Benzyl Benzoate	other compounds	000120-51-4

PHI has performed toxicity tests on freshwater alga *Pseudokirchneriella subcapitata* (OECD 201, Alga, Growth Inhibition Test) and on aquatic invertebrate *Daphnia magna* (OECD 202, *Daphnia sp*., Acute Immobilisation Test). PHI performed also ready biodegradability test (OECD 301D, Ready Biodegradability, Closed Bottle Test).

Introduction

Synthetic musks are used as base notes in fine fragrances and also in many other consumer products including laundry detergents, fabric softeners, air fresheners, cosmetics, hand soap, shampoo, and even toothpaste. There are four major classes that are structurally diverse: nitro, polycyclic, macrocyclic and alicyclic that has been in commerce for decades.

Most of the identified uses of synthetic musks lead to their release to municipal wastewater treatment and subsequently to the aquatic environment. The two most widely used, the

polycyclics Tonalide and Galaxolide have been found in wastewater, sewage sludge and aquatic biota in numerous monitoring studies and in human adipose tissue and breast milk. Musk xylene, a nitro musk is a candidate persistent, bioaccumulative and toxic (PBT) substance under the Oslo–Paris (OSPAR) Convention and is on the OSPAR List of Chemicals for Priority Action. Musk xylene has been designated very persistent and very bioaccumulative (vPvB) in a dossier prepared by the Netherlands under Annex XV of the REACH regulation, although at this time there is no production of musk xylene in Europe.

Studies have concluded that environmental risk from exposure to synthetic musks is generally low. However, several of the tested musks are quite toxic to fish, aquatic invertebrates and algae and sublethal effects at levels below 1 mg L-1 have been reported for copepods and early life stages of freshwater mussels.

Toxicity of substituted musks/fragrances to the green algae *Pseudokirchneriella subcapitata*

72 hours toxicity test on *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) on fragrances was performed at PHI according to OECD guideline 201. The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the ErCx. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

Materials and methods

Selected fragrances acetyl cedrene, benzyl cinnamate, hexyl salycilate, hexylcinnamaldeyde, HHCB (Galaxolide), methyl dihydrojasmonate, quinidine, α-amylcinnamyl alcohol, musk ambrette, cyclopentadecanolide and benzyl benzoate were purchased from SAFC Supply Solutions.

For the algae toxicity test the medium was prepared according to OECD 201, original medium of OECD TG 201, also according to ISO 8692. Reference substances 3,5-dichlorophenol and potassium dichromate were tested as a means of checking the test procedure.

Freshwater algae *Pseudokirchneriella subcapitata* were obtained from Culture collection, SAMS Research Services Ltd, Scotland. The initial cell concentration in the test cultures was approximately 10^4 cells/ml. The concentration range in which effects are likely to occur was determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series, was selected. The lowest selected concentration should not have an observed effect on the growth of the algae.

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum were prepared by diluting with filtered algal medium aliquots of stock solutions of the test substance and of algal suspension. The culture flasks were shaken and placed in the culturing apparatus. Because of the volatility of fragrances closed test flasks with increased head-space were used. The cultures were maintained at the temperature of 20 ℃ ± 2℃. The cell concentration in each flask was determined at 24, 48 and 72 hours after the start of the test by Perkin Elmer Victor 3, 1420 Multilabel Counter. The pH was measured at the beginning of the test and at 72 hours.

The concentrations of selected fragrances in test cultures were determined at the beginning and at the end of the test by:

- Gas chromatography with mass spectrometry (GC/MS). GC/MS was performed with HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to HP 5973 mass spectrometer. The GC separation was performed on a Zebron ZB-5MS column (Phenomenex), 30 m x 0.25mm I.D. with a film thickness of 0.25 µm. Test samples (2 mL) were transferred to glass tubes and dichloromethane was added (3 mL). Ultrasound assisted liquid/liquid extraction was performed (15 min). Phase separation was achieved by centrifugation at 4000 rpm for 5 min at room temperature. Water phase was removed by Pasteur pipette and dichloromethane phase was dried with anhydrous sodium sulphate. Aliquot of dichloromethane extracts were analyzed with GC/MS in full scan mode. Quantification of selected fragrances was performed according to external standard calibration.
- The concentrations of the quinidine were determined by liquid chromatography with mass spectrometry (LC/MS/MS). LC/MS/MS was performed with AT 1100 (Agilent Technologies) coupled to API 4000 mass spectrometer (AB Sciex). The LC separation was performed on a ZIC HILIC column (Merck), 100 x 2.1 mm with a 3.5 µm particles. For mobile phase formic acid and methanol were used. Sample preparation: Aliquot of the test water-samples were directly injected and analyzed with LC/MS/MS in MRM mode. Quantification of qinidine was performed according to external standard calibration.

The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves. To determine the concentration effect relationship comparison of growth rates can be used. The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_i - \ln X_i}{t_j - t_i} \qquad (16)$$

where μ_{i-j} is the average specific growth rate from time i to j, X_i is the biomass at time i and X_j is the biomass at time j.

Analysis of variance (ANOVA) techniques were employed for estimation of the LOEC and NOEC values. The mean for each concentration have been compared with the control mean using an appropriate multiple comparison or trend test method. ANOVA assumption of homogeneity of variance was assessed. Assessment was performed by using software TOXCALC – Toxicity Data Analysis Software (Version 5.0.32), Bonferroni t Test.

Results

The average specific growth rate and the percent inhibition of growth rate for each treatment replicate was calculated using software TOXCALC – Toxicity Data Analysis Software, Version 5.0.32.

The percentage reduction in average growth rate at each test substance concentration compared to the control value was plotted against the logarithm of the concentration. The E_rC_{50} may be read from the resulting graph. On Figure 12 the graph for benzyl cinnamate is presented.

Linear Interpolation (200 Resamples)					
Point	**mg/L**	**SD**	**95% CL(Exp)**		**Skew**
IC01*	0,0240	0,0217	0,0000	0,1950	0,2787
IC05	0,0766	0,0127	0,0000	0,1765	0,1090
IC10	0,1097	0,0157	0,0000	0,2331	0,2987
IC15	0,1418	0,0169	0,0088	0,2748	-0,1313
IC20	0,1735	0,0153	0,0531	0,2938	-0,1401
IC25	0,2051	0,0164	0,0759	0,3343	0,0355
IC40	0,2826	0,0122	0,1861	0,3791	0,0250
IC50	0,3237	0,0120	0,2293	0,4182	0,1499
IC60	0,3649	0,0130	0,2620	0,4678	0,1758
IC75	0,4266	0,0165	0,2963	0,5569	0,2277
IC80	0,4472	0,0180	0,3053	0,5891	0,2554
IC85	0,4678	0,0196	0,3135	0,6221	0,2842
IC90	0,4883	0,0754	0,0000	1,0827	1,1517
IC95	0,6512	0,1275	0,0000	1,6566	0,1394
IC99	0,9302	0,2073	0,0000	2,5655	-0,8130

* indicates IC estimate less than the lowest concentration

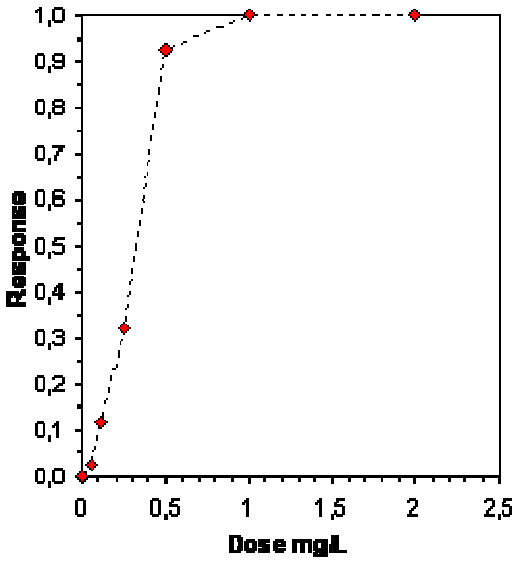

Figure 12. Dose response relationship with calculated data for toxicity test with freshwater alga *Pseudokirchneriella subcapitata* for benzyl cinnamate

Concentrations of test substances were measured and EC_{50} values are based on measured values. In Table 9 results of toxicity test on algae are presented – E_rC_{50}-72h.

Table 9. Toxicity testing with *Pseudokirchneriella subcapitata*, E_rC_{50}-72h (mg/L)

ID	**Substance**	**CAS No**	**E_rC_{50} – 72h**	**95 % interval**	**R^2**
FRA-001	Acetyl cedrene	375-22-4	>1.1	/	/
FRA-006	Benzyl cinnamate	355-80-6	0.32	0.23-0.42	0.948
FRA-022	Hexyl salycilate	335-67-1	0.97	0.93-1.02	0.944
FRA-023	Hexylcinnamaldeyde	375-95-1	1.14	0.55-1.73	0.892
FRA-024	HHCB (Galaxolide)	335-76-2	>0.7	/	/
FRA-041	Methyl dihydrojasmonate	2058-94-8	10.3	3.4-17.2	0.954
FRA-049	Quinidine	307-55-1	2.8	2.3-3.3	0.888
FRA-054	α-amylcinnamyl alcohol	101-85-9	3.8	3.4-4.2	0.872
FRA-056	Musk ambrette	83-66-9	0.98	0.71-1.3	0.861
FRA-065	Cyclopentadecanolide	106-02-5	>1.6	/	/
FRA-069	Benzyl Benzoate	120-51-4	0.24	0.17-0.31	0.895

Toxicity for the reference substances:

Pseudokirchneriella subcapitata, E_rC_{50}-72h, potassium dichromate: 0.72 – 0.96 mg/L

Pseudokirchneriella subcapitata, E_rC_{50}-72h, 3,5-dichlorophenol: 4.04 – 8.8 mg/L.

Toxicity for reference substance potassium dichromate and for 3,5-dichlorophenol was in the acceptability range according to ISO 8692.

In Table 10 results of toxicity test on algae are presented – NOEC-72h.

Table 10. Toxicity results on *Pseudokirchneriella subcapitata*, NOEC-72h (mg/L)

ID	Substance	CAS No	NOEC – 72h
FRA-001	Acetyl cedrene	375-22-4	0.55
FRA-006	Benzyl cinnamate	355-80-6	0.060
FRA-022	Hexyl salycilate	335-67-1	0.060
FRA-023	Hexylcinnamaldeyde	375-95-1	0.40
FRA-024	HHCB (Galaxolide)	335-76-2	0.23
FRA-041	Methyl dihydrojasmonate	2058-94-8	0.21
FRA-049	Quinidine	307-55-1	< 0.1
FRA-054	α-amylcinnamyl alcohol	101-85-9	0.22
FRA-056	Musk ambrette	83-66-9	0.29
FRA-065	Cyclopentadecanolide	106-02-5	0.53
FRA-069	Benzyl Benzoate	120-51-4	< 0.05

Conclusion

The aquatic toxicity of eleven selected musks/fragrances was investigated for green algae *Pseudokirchneriella subcapitata*. Raw data of algae toxicity test is available at Public Health Institute Maribor.

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Toxicity of substituted musks/fragrances to the aquatic invertebrate *Daphnia magna*

72 hours toxicity test on *Daphnia magna* on fragrances was performed at Public Health Institute Maribor (PHI) according to OECD guideline 202. The purpose of this test is to determine the effects of a substance on the mobility of daphnids. Juvenile daphnids, aged 8 to 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization was recorded at 48 hours and compared with control values. The results are analysed in order to calculate the EC_{50} at 48h.

Materials and methods

Selected fragrances acetyl cedrene, benzyl cinnamate, hexyl salycilate, hexylcinnamaldeyde, HHCB (Galaxolide), methyl dihydrojasmonate, quinidine, α-amylcinnamyl alcohol, musk ambrette, cyclopentadecanolide and benzyl benzoate were purchased from SAFC Supply Solutions, sold through Sigma Aldrich.

For the Daphnia magna toxicity test the dilution water was prepared according to according to ISO 6341, 1996, which is recommended by OECD 202. Reference substance Potassium dichromate was tested as a means of checking the test procedure.

Daphnia magna Strauss were obtained from MicroBioTests Inc., Belgium. At the start of the test, the animals were 8 to 24 hours old and, to reduce variability, they were not first brood progeny. The concentration range in which effects are likely to occur was determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series, were selected. The highest concentration tested resulted in 100 per cent immobilization and the lowest concentration gave no observable effect.

Test solutions of the chosen concentrations were prepared by dilution of a stock solution, which were prepared by dissolving the test substance in the dilution water. Because of the volatility of fragrances closed test tubes with increased head-space were used Test tubes were filled with appropriate volumes of dilution water and solutions of test substance. Ratio of air/water volume in the tubes was identical for test and control groups. Daphnids were then placed into test vessels. 20 animals were divided into four groups of five animals each, to be used at each test concentration and for the controls. Because of the volatility of fragrances closed test tubes with increased head-space were used. The temperature was 20 °C, and it was constant for each single test within 1 °C. A 16-hour light and 8-hour dark cycle was used. The test tubes were not aerated during the test. The tests were carried out without adjustment of pH. The daphnids were not fed during the test. The pH was measured at the beginning of The concentrations of selected fragrances in test samples were determined at the beginning and at the end of the test by:

- Gas chromatography with mass spectrometry (GC/MS). GC/MS was performed with HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to HP 5973 mass spectrometer. The GC separation was performed on a Zebron ZB-5MS column (Phenomenex), 30 m x 0.25mm I.D. with a film thickness of 0.25 µm. Test samples (2 mL) were transferred to glass tubes and dichloromethane was added (3 mL). Ultrasound assisted liquid/liquid extraction was performed (15 min). Phase separation was achieved by centrifugation at 4000 rpm for 5 min at room temperature. Water phase was removed by Pasteur pipette and dichloromethane phase was dried with anhydrous sodium sulphate. Aliquot of dichloromethane extracts were analyzed with GC/MS in full scan mode. Quantification of selected fragrances was performed according to external standard calibration.
- The concentrations of the quinidine were determined by liquid chromatography with mass spectrometry (LC/MS/MS). LC/MS/MS was performed with AT 1100 (Agilent Technologies) coupled to API 4000 mass spectrometer (AB Sciex). The LC separation was performed on a ZIC HILIC column (Merck), 100 x 2.1 mm with a 3.5 µm particles. For mobile phase formic acid and methanol were used. Sample preparation: Aliquot of the test water-samples were directly injected and analyzed with LC/MS/MS in MRM mode. Quantification of qinidine was performed according to external standard calibration.

Results

Data were summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation. The percentages immobilized 48 hours were plotted against test concentrations. Data were analysed by appropriate statistical methods (EPA Probit Analysis Program, Version 1.5) to calculate the slopes of the curves and the EC_{50} with 95% confidence limits (p = 0.95).

Concentrations of test substances were measured and EC_{50} values are based on measured values. In Table 11 results of toxicity test on *Daphnia magna* are presented, EC_{50}-48h.

Table 11. Toxicity results on *Daphnia magna*, EC_{50}-48h (mg/L)

ID	Substance	CAS No	EC_{50} – 48h	95 % interval
FRA-001	Acetyl cedrene	375-22-4	0.53	0.42-0.74
FRA-006	Benzyl cinnamate	355-80-6	1.5	1.19-1.92
FRA-022	Hexyl salycilate	335-67-1	0.42	0.34-0.53
FRA-023	Hexylcinnamaldeyde	375-95-1	0.24	0.17-0.29
FRA-024	HHCB (Galaxolide)	335-76-2	0.30	0.24-0.39
FRA-041	Methyl dihydrojasmonate	2058-94-8	20.2	15.1-26.9
FRA-049	Quinidine	307-55-1	5.6	4.0-9.1
FRA-054	α-amylcinnamyl alcohol	101-85-9	1.21	0.94-1.36

ID	Substance	CAS No	EC_{50} – 48h	95 % interval
FRA-056	Musk ambrette	83-66-9	2.1	1.5-2.5
FRA-065	Cyclopentadecanolide	106-02-5	0.45	0.35-0.59
FRA-069	Benzyl Benzoate	120-51-4	3.8	3.40-4.33

Toxicity for reference substance potassium dichromate was in the acceptability range according to ISO 6341: *Daphnia magna*, EC_{50}-48h, potassium dichromate: 0.6-2.1 mg/L.

Conclusion

The aquatic toxicity of eleven selected musks/fragrances was investigated for daphnids *Daphnia magna*. Raw data of daphnids toxicity test is available at Public Health Institute Maribor.

References

1. Boethling, R.S., 2011. Incorporating environmental attributes into musk design. Green Chemistry, DOI: 10.1039/c1gc15782e.
2. Environment Canada, 1996. Biological test method. Acute Lethality Test Using *Daphnia* sp. EPS 1/RM/11. Environment Canada, Ottawa, Ontario, Canada.
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4. ISO 6341, 1996. Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test. Third edition.
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Test for ready biodegradability on substituted musks/fragrances

28 days test for ready biodegradability in an aerobic aqueous medium was performed at PHI according to OECD guideline 301 D, closed bottle test. Substituted musks/fragrances are poorly soluble, volatile substances. To assess ready biodegradability of these substances, according to OECD 301, closed bottle test is a suitable method to produce reliable results.

A solution of the test substance in a mineral medium was inoculated and incubated under aerobic conditions in the dark at 20 ± 2 ℃. A test medium contained a relatively low concentration of biomass. A reference compound was run in parallel to check the operation of the procedures. The possible abiotic degradation was observed. Degradation for a reference substance was followed by the determination of DOC and measurements were taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. Degradation was followed by specific chemical analysis at frequent intervals over a 28-day period to assess degradation of substituted musks/fragrances. The degree of biodegradation was calculated by expressing the concentration of the test substance removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present.

Materials and methods

Selected fragrances acetyl cedrene, benzyl cinnamate, hexyl salycilate, hexylcinnamaldeyde, HHCB (Galaxolide), methyl dihydrojasmonate, quinidine, α-amylcinnamyl alcohol, musk ambrette, cyclopentadecanolide and benzyl benzoate were purchased from SAFC Supply Solutions, sold through Sigma Aldrich.

Mineral media was prepared according to OECD 301 D. In order to check the procedure, reference compound which meet the criteria for ready biodegradability was tested by setting up an appropriate vessel in parallel as part of normal test runs. The reference compound was sodium acetate and the degradation was tested by determining the removal of DOC.

The inoculum for the test was derived from aquarium water. Possible abiotic degradation was checked by determining the concentration of the substance with chemical analysis in controls which were inoculated and poisoned with mercury chloride.

A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the end of the test is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14.

The concentrations of selected fragrances in test samples were determined at the beginning of the test, at day 14 and at day 28 by:

- Gas chromatography with mass spectrometry (GC/MS). GC/MS was performed with HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to HP 5973 mass spectrometer. The GC separation was performed on a Zebron ZB-5MS column (Phenomenex), 30 m x 0.25mm I.D. with a film thickness of 0.25 µm. Test samples (2 mL) were transferred to glass tubes and dichloromethane was added (3 mL). Ultrasound assisted liquid/liquid extraction was performed (15 min). Phase separation was achieved by centrifugation at 4000 rpm for 5 min at room temperature. Water phase was removed by Pasteur pipette and dichloromethane phase was dried with anhydrous sodium sulphate. Aliquot of dichloromethane extracts were analyzed with GC/MS in full scan mode. Quantification of selected fragrances was performed according to external standard calibration.
- Liquid chromatography with mass spectrometry (LC/MS/MS) for determination of the quinidine. LC/MS/MS was performed with AT 1100 (Agilent Technologies) coupled to API 4000 mass spectrometer (AB Sciex). The LC separation was performed on a ZIC HILIC column (Merck), 100 x 2.1 mm with a 3.5 µm particles. For mobile phase formic acid and methanol were used. Sample preparation: Aliquot of the test water-samples were directly injected and analyzed with LC/MS/MS in MRM mode. Quantification of quinidine was performed according to external standard calibration.

Results

In the calculation of D_t, percentage degradation, the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. With specific chemical analytical data available, degradation at day t of the test is calculated from:

$$D_t = \left[1 - \frac{C_t - C_{blt}}{C_0 - C_{bl0}}\right] * 100 \qquad (17)$$

where

D_t = % degradation at day t of the test,

C_0 = mean starting concentration of the test substance in the inoculated culture medium containing the test substance in mg/L,

C_t = mean concentration of the test substance in the inoculated culture medium containing test substance at day t of the test in mg/L,

C_{bl0} = mean starting concentration of the test substance in blank inoculated mineral medium in mg/L,

C_{blt} = mean concentration of the test substance blank inoculated mineral medium at day t of the test in mg/L.

In table 12 measured concentrations in µg/L of tested substituted musks/fragrances are presented at day 0, at day 14 and at day 28.

Table 12. Mean value of measured concentrations (µg/L) for tested substituted musks/fragrances at day 0, day 14 and day 28

Fragrance ID	**C_0**	**C_{14}**	**C_{28}**	**C_{bl0}**	**C_{blt}[a]**
FRA-001	5	4.24	3.15	0.1	0.1
FRA-006	5	0.15	0.13	0.1	0.1
FRA-022	12.5	0.1	0.1	0.1	0.1
FRA-023	5	0.1	0.1	0.1	0.1
FRA-024	0.5	0.35	0.30	0.1	0.1
FRA-041	25	25	12.0	0.1	0.1
FRA-049	100	34.5	7.85	0.1	0.1
FRA-054	5	1.44	0.63	0.1	0.1
FRA-056	0.5	0.14	0.1	0.1	0.1
FRA-065	0.5	0.1	0.1	0.1	0.1
FRA-069	2.5	0.1	0.1	0.1	0.1

[a] C_{blt} represents concentration in blank at day 0, at day 14 and at day 28. All concentrations were below the detection limit of the method (< LOD) and are calculated as LOD/2

In table 13 biodegradation at day 0, at day 14 and at day 28 is presented, calculated from the data presented in table 12 with the use of equation 14.

Table 13. Biodegradation, D_t (%) at day 0, at day 14 and at day 28 for selected substituted musks/fragrances

Fragrance ID	D_0	D_{14}	**D_{28}**	**D_{28} 95 % interval**
FRA-001	0	15.6	37.8	±4.8
FRA-006	0	99.0	99.4	±12.2
FRA-022[a]	0	100.0	100.0	±26.5
FRA-023	0	100.0	100.0	±12.1
FRA-024	0	38.8	51.3	±1.3
FRA-041	0	16.7	60.1	±22.7
FRA-049[a]	0	65.6	92.2	±4.3
FRA-054	0	72.8	89.2	±9.7
FRA-056	0	90	100	±32
FRA-065[a]	0	100	100	±13.9
FRA-069	0	100	100	±13.9

[a] Significant abiotic degradation occurred during the test

The percentage of degradation of the test substance can be plotted against time. On figure 13 the graph with data for biodegradation test is presented for substituted musks/fragrances and reference substance sodium acetate.

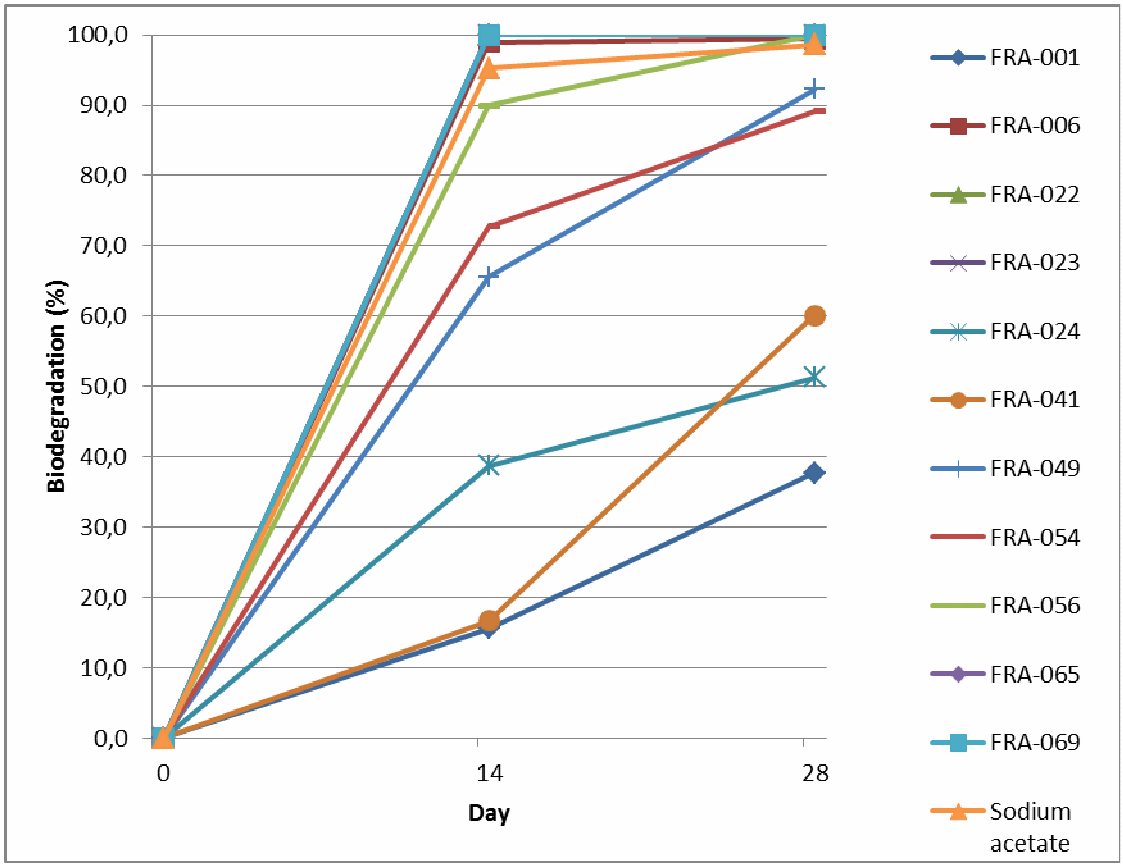

Figure 13. The percentage of biodegradation of substituted musks/fragrances and reference substance sodium acetate

Conclusion

The ready biodegradability of eleven selected musks/fragrances was investigated. As expected, the majority of the substances are readily biodegradable. Significant abiotic degradation was observed in three tested substances. Raw data of the test is available at Public Health Institute Maribor.

References

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Report on testing of (benzo)triazoles (B)TAZ

Toxicity testing of substituted (benzo)triazoles was performed at the RIVM with *Daphnia magna* and with embryos of the zebrafish (*Danio rerio*). Toxicity testing of substituted (benzo)triazoles was performed also at PHI with green algae *Pseudokirchneriella subcapitata* according to OECD 201. At PHI also ready biodegradability test was performed according to OECD 301D.

The chemicals to be tested, were selected on the basis of a Principal Component Analysis performed by Linnaeus University (partner 5 of the CADASTER project), supplemented with data on vertebrate and invertebrate toxicity collected by the University of Insubria (partner 3 in the CADASTER project). Thereupon, a further selection was applied based upon the possibility of purchasing the chemicals to be tested. In this section, an overview of the methods applied will be given, as well as the testing results obtained. The list of (B)TAZ relevant for the testing on daphnids is presented in Table 14.

Table 14. The list of (B)TAZ relevant for the testing on daphnids

	Name	**CAS No**
1	Benzotriazole	95-14-7
2	Cyproconazole	94361-06-5
3	Diclobutrazol	75736-33-3
4	Fenchlorazol-ethyl	103112-35-2
5	Flusilazole	85509-19-9
6	Guanazole	1455-77-2
7	Hexaconazole	79983-71-4
8	Myclobutanil	88671-89-0
9	Paclobutrazol	76738-62-0
10	Ribavirin	36791-04-5
11	Triadimefon	43121-43-3
12	Triticonazole	131983-72-7

The list of (B)TAZ relevant for the testing on algae and ready biodegradability is presented in Table 15.

Table 15. The list of (B)TAZ relevant for the testing on algae and ready biodegradability

	Name	**CAS No**
1	Cyproconazole	94361-06-5
2	Diclobutrazol	075736-33-3
3	Difenoconazole	119446-68-3
4	Diniconazole	083657-24-3

	Name	CAS No
1	Cyproconazole	94361-06-5
5	Epoxiconazole	106325-08-0
6	Hexaconazole	79983-71-4
7	Myclobutanil	88671-89-0
8	Paclobutrazol	076738-62-0
9	Penconazole	066246-88-6
10	Propiconazole	060207-90-1
11	Triadimefon	43121-43-3
12	Triazophos	024017-47-8
13	Uniconazole-P	083657-17-4

Introduction

Benzotriazoles are used as a restrainer in photographic emulsions and as a reagent for the analytical determination of silver. More importantly, they have been extensively used as a corrosion inhibitor in the atmosphere and underwater. Besides all the applications mentioned above, the benzotriazoles can be used as antifreezes, in heating and cooling systems, hydraulic fluids and vapour phase inhibitors as well.

Triazoles are used in plant protection as fungicides and are used on many different types of plants including field crops, fruit trees, small fruit, vegetables, and turf. Fungicides are highly effective against many different fungal diseases, especially powdery mildews, rusts, and many leaf-spotting fungi. As biocides they are some of the most widely used active substances on the global market. Triazoles are toxic to fish and other aquatic organisms and practically nontoxic to birds and bees.

Benzotriazoles and triazoles are generally fairly water-soluble, not readily degradable and have a limited sorption tendency. Hence, they are only partly removed in wastewater treatment plants and a substantial fraction reaches surface water such as rivers and lakes.

Toxicity of substituted (benzo)triazoles to the freshwater alga *Pseudokirchneriella subcapitata*

72 hours toxicity test with *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) on substituted (benzo)triazoles was performed at PHI according to OECD guideline 201. The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae. Exponentially growing test organisms are exposed to the

test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the ErCx. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

Materials and methods

Selected substituted (benzo)triazoles cyproconazole, diclobutrazol, difenoconazole, diniconazole, epoxiconazole, hexaconazole, myclobutanil, penconazole, propiconazole, paclobutrazol, triadimefon, triazophos, uniconazole-P were purchased from Dr. Ehrenstorfer, Germany.

For the algae toxicity test the medium was prepared according to OECD 201, original medium of OECD TG 201, also according to ISO 8692. Reference substances 3,5-dichlorophenol and potassium dichromate were tested as a means of checking the test procedure.

Freshwater algae *Pseudokirchneriella subcapitata* were obtained from Culture collection, SAMS Research Services Ltd, Scotland. The initial cell concentration in the test cultures was approximately 10^4 cells/ml. The concentration range in which effects are likely to occur was determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series, were selected. The lowest selected concentration should not have an observed effect on the growth of the algae.

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum were prepared by diluting with filtered algal medium aliquots of stock solutions of the test substance and of algal suspension. The culture flasks were shaken and placed in the culturing apparatus. The cultures were maintained at the temperature of 20 ℃ ± 2℃. The cell concentration in each flask was deter mined at 24, 48 and 72 hours after the start of the test by Perkin Elmer Victor 3, 1420 Multilabel Counter. The pH was measured at the beginning of the test and at 72 hours.

The concentrations of selected substituted (benzo)triazoles in test cultures were determined at the beginning and at the end of the test by gas chromatography with mass spectrometry (GC/MS). GC/MS was performed with HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to HP 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). The GC separation was performed on a DB-5MS column (Agilent Technologies) 40 m x 0.18mm I.D. with a film thickness of 0.18 µm.

Sample preparation: Test water-samples (2 mL) were transferred to glass tubes and hexane was added (3 mL). Ultrasound assisted liquid/liquid extraction was performed (15 min). Phase separation was achieved by centrifugation at 4000 rpm for 5 min at room temperature. Hexane phase was removed by Pasteur pipette in to a vial and diluted with ethyl acetate. Extracts were analyzed with GC/MS in selected ion monitoring (SIM) mode. Quantification of selected pesticides was performed according to external standard calibration.

The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves. To determine the concentration effect relationship comparison of growth rates can be used. The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_i - \ln X_j}{t_j - t_i} \qquad (18)$$

where μ_{i-j} is the average specific growth rate from time i to j, X_i is the biomass at time i and X_j is the biomass at time j.

Analysis of variance (ANOVA) techniques were employed for estimation of the LOEC and NOEC values. The mean for each concentration have been compared with the control mean using an appropriate multiple comparison or trend test method. ANOVA assumption of homogeneity of variance was assessed. Assessment was performed by using software TOXCALC – Toxicity Data Analysis Software (Version 5.0.32), Bonferroni t Test.

Results

The average specific growth rate and the percent inhibition of growth rate for each treatment replicate was calculated using software TOXCALC – Toxicity Data Analysis Software, Version 5.0.32.

The percentage reduction in average growth rate at each test substance concentration compared to the control value was plotted against the logarithm of the concentration. The E_rC_{50} may be read from the resulting graph, On Figure 14 the graph for difenoconazole is presented.

Linear Interpolation (200 Resamples)					
Point	**mg/L**	**SD**	**95% CL(Exp)**		**Skew**
IC01	0,1345	0,0229	0,0398	0,2292	-0,1485
IC05	0,3929	0,0223	0,3005	0,4853	-0,7815
IC10	0,5185	0,0150	0,4565	0,5805	-0,1046
IC15	0,6441	0,0133	0,5888	0,6993	-0,1058
IC20	0,7696	0,0125	0,7180	0,8212	-0,0882
IC25	0,8952	0,0125	0,8434	0,9470	-0,0652
IC40	1,3184	0,0254	1,2130	1,4238	-0,1947
IC50	1,7320	0,0212	1,6442	1,8198	-0,1947
IC60	2,1456	0,0170	2,0753	2,2158	-0,1947
IC75	2,7660	0,0106	2,7221	2,8099	-0,1947
IC80	2,9728	0,0085	2,9377	3,0079	-0,1947
IC85	3,1796	0,0064	3,1533	3,2059	-0,1947
IC90	3,3864	0,0042	3,3688	3,4040	-0,1947
IC95	3,5932	0,0021	3,5844	3,6020	-0,1947
IC99	3,7586	0,0004	3,7569	3,7604	-0,1947

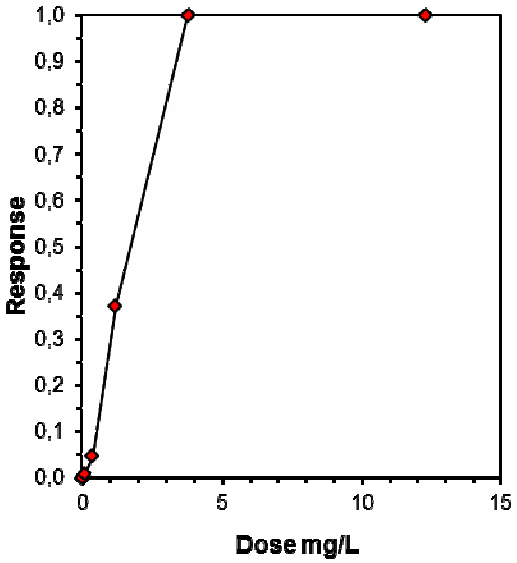

Figure 14. Dose response relationship with calculated data for toxicity test with green algae *Pseudokirchneriella subcapitata* for difenoconazole

Concentrations of test substances were measured. Calculated E_rC_{50}-72h results are based on measured values. In Table 16 results of toxicity test on algae are presented – E_rC_{50}-72h.

Table 16. Toxicity testing with *Pseudokirchneriella subcapitata*, E_rC_{50}-72h (mg/L)

Substance	**CAS No**	**E_rC_{50} – 72h**	**95 % interval**	**R^2**
Cyproconazole	94361-06-5	9.62	5.62-14.0	0.981
Diclobutrazol	075736-33-3	5.55	5.32-5.78	0.916
Difenoconazole	119446-68-3	1.73	1.64-1.82	0.908
Diniconazole	083657-24-3	1.88	1.81-1.95	0.952
Epoxiconazole	106325-08-0	10.5	/	0.974
Hexaconazole	79983-71-4	4.01	3.54-4.49	0.906
Myclobutanil	88671-89-0	15.3	14.3-16.3	0.896
Paclobutrazol	076738-62-0	12.3	9.19-15.4	0.960
Penconazole	066246-88-6	3.62	3.40-3.85	0.829
Propiconazole	060207-90-1	4.50	4.03-4.98	0.964
Triadimefon	43121-43-3	8.12	5.71-10.5	0.947
Triazophos	024017-47-8	10.6	9.24-11.9	0.946
Uniconazole-P	083657-17-4	>7.5[b]	/	/

Toxicity for the reference substances:

Pseudokirchneriella subcapitata, E_rC_{50}-72h, potassium dichromate: 0.72 – 0.96 mg/L

Pseudokirchneriella subcapitata, E_rC_{50}-72h, 3,5-dichlorophenol: 4.04 – 8.8 mg/L.

Toxicity for reference substance potassium dichromate and for 3,5-dichlorophenol was in the acceptability range according to ISO 8692.

In Table 17 results of toxicity test on algae are presented – NOEC-72h.

Table 17. Toxicity testing with *Pseudokirchneriella subcapitata*, NOEC-72h (mg/L)

Substance	**CAS No**	**NOEC – 72h**
Cyproconazole	94361-06-5	0.19
Diclobutrazol	075736-33-3	0.24
Difenoconazole	119446-68-3	0.12
Diniconazole	083657-24-3	0.59
Epoxiconazole	106325-08-0	< 0.12
Hexaconazole	79983-71-4	< 0.20
Myclobutanil	88671-89-0	< 0.26
Paclobutrazol	076738-62-0	< 0.64
Penconazole	066246-88-6	0.20
Propiconazole	060207-90-1	0.070
Triadimefon	43121-43-3	2.0
Triazophos	024017-47-8	2.4
Uniconazole-P	083657-17-4	< 0.90

Conclusion

The aquatic toxicity of thirteen selected (benzo)triazoles was investigated for green algae *Pseudokirchneriella subcapitata*. Raw data of algae toxicity test is available at Public Health Institute Maribor.

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Toxicity of substituted (benzo)triazoles to the aquatic invertebrate *Daphnia magna*

72 hours toxicity test on *Daphnia magna* on fragrances was performed at RIVM according to OECD guideline 202. The purpose of this test is to determine the effects of a substance on the mobility of daphnids. Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization was recorded at 48 hours and compared with control values. The results are analysed in order to calculate the EC_{50} at 48h.

Materials and methods

Test compounds

Twelve substituted (benzo)triazoles were selected for the toxicity assessment on the basis of the general approach to the experimental design. The chemicals tested are given in Table 14.

Testing Methods - Acute immobilization tests on *Daphnia magna*

Determining EC50 values for chemicals with the Daphnia magna acute immobilization test (48 hours) was performed according to OECD guideline 202[10]. The test duration was 48 hours, and the culture conditions of the Daphnids were: 25-30 adults in 4 liter M4-medium, aerated with slow bubbling air (few bubbles/min), fed with algae (*Pseudokierchneriëlla subcapitata*) and yeast extract (20 mg/l) daily, except during weekends. Juveniles were taken from adults that are 2-4 weeks old.

Juveniles were in between 0-24 H of age, 5 juveniles per 100 ml glass beaker were used with 20 ml of test solution per beaker. Four beakers were used of each test concentration, with a minimum of 5 test concentrations per chemical excluding the controls/blanks. The test medium used was M4, which was prepared according to the OECD Guideline[11]. The culture and test conditions were similar: 16H light, 8H dark, temperature 19 ℃ +/- 1 ℃.

Most substances were dissolved in DMSO, 100, 500, 750 or 1000 µl stock into M4 medium up to 100 ml. Dissolved oxygen levels did not change significantly, nor did pH during the experiments.

GraphPad Prism 4.01 was used to calculate EC50 values, applying the method of nonlinear regression curve fitting, assuming a sigmoid dose-response curve with top of 100 % and bottom 0 %.

Results

A systematic overview of the EC50-values obtained is given in Table 18. Apart from the EC50-values, the 95 % confidence intervals and the values of R^2 are given. R^2-values are indicative of the goodness-of-fit of the dose-response curve fitted to the data.

Table 18. Overview of EC50-values (mg/L) obtained for a set of 12 substituted (benzo)triazoles. The endpoint of toxicity assessment was immobility of *Daphnia magna*.

Substance	EC_{50}	95% interval	R^2
Benzotriazole	155.4	154.4 - 156.5	1.000
Cyproconazole	30.92	21.36 - 44.77	0.955
Diclobutrazol	12.93	10.38 -16.10	0.857
Fenchlorazol-ethyl	>2.50	/	/
Flusilazole	3.17	1.66 - 6.05	0.902
Guanazole	4.13	3.53 - 4.84	0.990
Hexaconazole	4.9	4.43 - 5.41	0.990
Myclobutanil	12.36	10.28 -14.87	0.984
Paclobutrazol	45.2	35.38 - 57.74	0.717
Ribavirin	684	588 - 796	0.928
Triadimefon	29.06	28.34 - 29.80	0.998
Triticonazole	9.562	5.703 -16.03	0.930

The details on the main findings for each of the chemicals tested (including the reference compound), are given below.

<u>Control substance potassium dichromate, EC50-24H:</u>

0.70 (0.53 – 0.91) mg/l, R^2: 0.962

0.51 (0.50 – 0.51) mg/l, R^2: 0.999

0.71 (0.46 – 1.10) mg/l, R^2: 0.998

<u>Dissolving medium DMSO,</u>

EC_{50}-48H: 1.961% in M4 (1.960 – 1.962 mg/l); R^2 1.000; effects observable on *D. magna* at > 1% DMSO.

Paclobutrazol [76738-62-0], Fluka Pestanal, analytical standard, lot SZE8204X

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range: up to 50 mg/l, dilution step 2.2

EC50: 45.20 mg/l (35.38 to 57.74 mg/l), R^2 0.7174

Concentration range: 0 to 70 mg/l, 1% DMSO in M4 medium, dilution step 10 mg/l. Maybe floating material in the higher concentrations, although not noticed.

EC50: 130.9 mg/l (24.67 to 694.2 mg/l), R^2 0.6354

Concentration range: 0 to 99 mg/l, 7 concentrations, 1% DMSO in M4 medium.

Observation at 24H: floating material from 60 mg/l and higher concentrations.
EC50: 115.6 mg/l (33.96 – 393.8 mg/l), R^2 0.4970

2-(2-Hydroxy-5-methylphenyl)benzotriazole [95-14-7], Aldrich, lot #MKBF-1054V
Dissolved in DMSO; all concentrations in 0.1% DMSO in 100 ml M4 medium. Concentration range: up to 440 mg/l, dilution step 2.2
EC50: 155.4 mg/l (154.4 – 156.5 mg/l), R^2 1.000

Guanazole / 3,5-Diamino-1,2,4-triazol [1455-77-2], Aldrich lot STBB3150
Dissolved in M4 medium
Concentration range up to 1308 mg/l, dilution step 10 and 5 concentrations
0% effect at 1.3 mg/l, 100% effect at 13 mg/l
Tested again; concentration range up to 15 mg/l, 6 concentrations.
EC50: 4.131 mg/l (3.525 - 4.841 mg/l), R^2 0.9898

Triadimefon **[43121-43-3],** Fluka Pestanal, Lot SZB8042XV
Dissolved in DMSO; all concentrations in 0.1% DMSO in 100 ml M4 medium. Concentration range up to 18.22 mg/l, dilution step 2.2
After 48 hours Daphnia moved very slowly, but still could move in highest concentrations. The animals are affected already in low concentration.
EC50: > 18.22 mg/l
Tested again; concentration range up to 440 mg/l, 6 concentrations; the highest 2 concentrations appeared to be insoluble, so only 4 concentrations left for calculation. The 100% immobility was at 56 mg/l, at 28 mg/l a few Daphnia immobile, so EC50 value must be in between 28 and 56 mg/l. Graphpad Prism could not make this graph.
These 2 ranges together gave a calculated EC50 value of 34.30 mg/l (27.9 – 42.1 mg/l) with R^2 0.9916.
Tested with concentration range 17 – 70 mg/l gives an EC50-48H: 24.40 mg/l (15.29 – 38.94mg/l), R^2 0.7448, leaving out an unreliable figure it gives an **EC50-48H of 29.06 mg/l (28.34 – 29.80 mg/l), R^2 0.9978**

Fenchlorazol-ethyl [103112-35-2], Fluka Pestanal, lot SZE7312X

Dissolved in DMSO; all concentrations in 1% DMSO in 100 ml M4 medium. Concentration range up to 10 mg/l, dilution step 2. This concentration became opalescent in one hour. Precipitation occurred in highest 2 concentrations, 5 and 10 mg/l after 24 hour.

EC50: >2.50 mg/l. No effect found in the soluble range with 1% DMSO, also not in the opalescent concentrations 5 and 10.

Diclobutrazol [075736-33-3], Fluka Pestanal lot SZE6306X

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 100 mg/l, dilution step 10. Floating substance at 100 mg/l, so not completely dissolved in 0.5% DMSO in M4.

Next concentration range from 15 to 40 mg/l with 1% DMSO; Clear solutions until 20 mg/l and 100% effect at 15 mg/l.

EC50: 11.13 mg/l (0.1977 – 626.1 mg/l), R^2 0.9874

To find an EC50 value with a smaller interval, a concentration range 0 to 20 mg/l, with max. 0.4% DMSO.

EC50: 12.93 mg/l (10.38 – 16.10 mg/l), R^2 0.8527

Hexaconazole [79983-71-4], Fluka Pestanal, lot SZB8275XV

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium.

Concentration range up to 100 mg/l, dilution step 10.

EC50: 2.75 mg/l (2.708 to 2.801 mg/l) R^2 1.000

Again in a smaller range, concentration 0.4 to 12.5 mg/l, dilution step 2

EC50: 8.76 mg/l (5.10 to 15.03 mg/l) R^2 0.9576

With a concentration range 1.25 to 50 mg/l, with 0.75% DMSO in M4 medium: At 24H Daphnia moved slower in 2.5 mg/l and in higher concentrations, in 10 mg/l and higher (almost) no Daphnia moving anymore.

EC50: 4.90 mg/l (4.43 – 5.41 mg/l) R^2 0.9901

Triticonazole [131983-72-7], Fluka Pestanal, lot SZBA197X

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 50 mg/l, dilution step 2

EC50: 9.56 (5.70 - 16.03 mg/l), R^2 0.9304

Flusilazole [85509-19-9], Fluka Pestanal, lot SZB8294XV

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 25 mg/l, dilution step 2

EC50: 3.17 mg/l (1.66 – 6.05 mg/l)

Myclobutanil [88671-89-0], Fluka Pestanal, lot SZBA028X

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 52 mg/l, dilution step 2

EC50: 11.54 mg/l (9.48 - 14.04 mg/l), R^2 0.9837

From 6 mg/l on the Daphnia moved very slowly.

Cyproconazole [94361-06-5], Fluka Pestanal, batch SZB7283XV

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 92 mg/l, dilution step 2.2

EC50: 26.51 mg/l (19.93 - 35.26 mg/l), R^2 0.9548

Ribavirin [36791-04-5], Sigma lot 061M4129V

Dissolved in DMSO; all concentrations in 0.5% DMSO in 100 ml M4 medium. Concentration range up to 94 mg/l, dilution step 2.2

EC50: > 94 mg/l

Dissolved in DMSO; maximum concentration DMSO 0.55% in 100 ml M4 medium. Maximum concentration: 357.5 mg/l.

EC50: > 360 mg/l

EC50: 684.2 mg/l (587.9 – 796.2 mg/l), R^2 0.9284

Toxicity of substituted (benzo)triazoles to embryos of *Danio rerio*

Materials and methods

Test compounds

The set of chemicals selected for performing the 72 hrs fish embryo test, as well as some additional information, is given in Table 19. A total of eleven substituted (benzo)triazoles were selected, all compounds tested were obtained from Fluka unless stated otherwise.

Table 19. Overview of the substituted (benzo)triazoles tested with the *Danio rerio* - 72 hrs fish embryo test.

Compound	Cas Nr	Supplier	Standard	Lot nr
Benzotriazole	95-14-7	Aldrich	Pestanal	MKBF1054V
Cyproconazole	94361-06-5	Fluka	Pestanal	SZB7283XV
Fenchlorazol-ethyl	103112-35-2	Fluka	Pestanal	SZE7312X
Flusilazole	85509-19-9	Fluka	Pestanal	SZB8294XV
Guanazole	1455-77-2	Fluka	Pestanal	STBB3150
Hexaconazole	79983-71-4	Fluka	Pestanal	SZB8275XV
Myclobutanil	88671-89-0	Fluka	Pestanal	SZBA028X
Paclobutrazol	76738-62-0	Fluka	Pestanal	SZE8204X
Ribavirin	36791-04-5	Fluka	Pestanal	061M4129V
Triadimefon	43121-43-3	Fluka	Pestanal	SZB8042XV
Triticonazole	131983-72-7	Fluka	Pestanal	SZBA197X

Maintenance of fish and egg spawning

For details on fish maintenance and egg production, see Hermsen et.al.[16]

Testing Methods - Acute immobilization tests on *Daphnia magna*

Stock solutions were prepared in DMSO, with final test concentration of 0.2 % carrier (2ml/l) in dilution series. Six test concentrations per compound were prepared in Dutch Standard Water (DSW: demineralised water supplemented with $NHCO_3$ (100mg/l), $KHCO_3$ (20 mg/l), $CaCl_2.2H_2O$ (200 mg/l), and $MgSO_4.7H_2O$ (180 mg/l) and then aerated for 24 h at 27 ℃. For Fenchlorazole-ethyl, 1H-Benzotriazole and Paclobutrasol maximum solubility was reached below the highest nominal test concentration. In these cases crystals were observed on the bottom of the well, or floating on the surface of the test medium. The observed effects in these over-saturated solution were not taken into account during the final calculations. A negative and positive control (3,4-dichloroaniline (CAS nr.: 85-76-1, Sigma-Aldrich)) was used in concentration of 1 and 8 mg/L respectively. Results showed that background sensitivity of used egg clutches were within expected ranges.

Toxicity exposure protocol

The test method followed the draft OECD protocol “Fish Embryo Toxicity” (FET) test (OECD[15]). Each test was repeated three times. If necessary, concentration ranges were adjusted in between different experiments. To simultaneously start the exposure, 40 fertilized eggs were brought in 50 ml test solution. Viable eggs were selected and incubated in 24-well plates (Flat bottom Falcon, Omnilabo, Breda). Eggs were individually incubated in 2 ml medium. Per plate, four wells were used for the control and 10 wells for a test concentration. Temperature during exposure was kept at 26.5±0.5℃. The pH of the dilution medium (DSW) was on average 8.11 (range 7.4-8.3). Oxygen during exposure was ≥ 6.6 mg/l. Water hardness was 214 mg/l $CaCO_3$ which was according the given range (10-250 mg/l). Embryos were exposed under static conditions and microscopic observations were done at 24, 48 and 72 hours after the start of the experiments under a Wild –binocular microscope 10-60x. Lethal endpoints were: coagulation of embryo, no tail detachment, absence of heartbeat, no somite formation.

Statistical analyses

All LC_{50} values were calculated using the trimmed Spearman-Kärber model (Hamilton *et al*.[17]). Since all eggs were obtained from the same batch, the controls for all compounds were pooled.

Results

The calculated LC_{50} data are reported in Table 20.

Table 20. LC_{50} values (mg/l) after 72 hrs of exposure of substituted (benzo)triazoles in a Fish embryo toxicity test with lethal endpoints: mortality/ no heartbeat/ no somite formation/ no detachment of tail.

Compound	**Cas Nr**	**Test 1**		**Test 2**		**Test 3**	
		LC_{50}	**95% BTI**	**LC_{50}**	**95% BTI**	**LC_{50}**	**95% BTI**
Guanazole	1455772	≥29.7	-	≥79.3	-	17320	-
Fenchlorazole	103112-35-2	2.52	1.31-4.85	3.33	2.9-3.81	1.78	-
Ribavirin	36791-04-5	≥73.3	-	≥147	-	≥1000	-
1H-benzotriazole	95147	15.98	8.61-29.66	2.63	1.68-4.11	0.67	-
Paclobutrazol	76738-62-0	14.54	3.69-57.31	≥88.1	-	54.26	44.94-65.51
Flusilazole	85509-19-9	6.12	4.92-7.62	5.46	-	8.1	5.72-12.38
Hexaconazole	79983-71-4	9.68	6.72-13.93	5.3	4.02-7	7.6	5.31-10.87
Cyproconazole	94361-06-5	40.16	29.36-54.91	45.04	36.21-56.04	40.73	28.63-57.94
Triadimefon	43121-43-3	36.03	25.04-54.85	50.89	-	45.36	36.24-56.77
Mycobutanil	88671-89-0	14.1	11.26-17.65	14.1	11.33-17.54	15.82	11.55-21.67
Triticonazole	131983-72-7	≥31.8	-	≥95.3	-	≥95.3	-

LC_{50} (mg/l) calculated with the trimmed Spearman-Kärber method.

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6. Hamilton, M.A., Russo, R.C., Thurston, R.V. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. Environmental Science and Technology 11(7): 714-719; Correction (1978) 12(4): 417, 1977.

Supplement A: data used for calculation of LC50

Fish embryo toxicity test

lethal endpoints taken into account

coagulation
no detachment of tail
no formation of somites
no heartbeat

no 100% solubility reached, crystals were observed

General morphology endpoints
GM additional general morphology endpoints were:
eye development, movement, blood circulation, pigmentation head-body, visability pectoral fins, protruding mouth and hatching

Test performed by Evert-Jan van den Brandhof - RIVM-Bilthoven-Netherlands

4-10-2011 FIRST RUN

Guanazole (M= 99.09)

72hrs

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	16	1	6.25	13.25	
0,2%DMSO	0	0	10	1	10	13.5	100
	0.00951	0.096	10	0	0	14.14	104.7407
	0.04756	0.48	10	2	20	12	88.88889
	0.23782	2.4	10	0	0	14.7	108.8889
	1.18908	12	10	2	20	11.4	84.44444
	5.9454	60	10	2	20	11.5	85.18519
	29.727	300	10	0	0	14.7	108.8889

Fenchlorazole-ethyl (M= 403.48)

control	0	0	16	0	0	14.875	
0,2%DMSO	0	0	10	1	10	12.9	100
	0.00646	0.016	10	0	0	15	116.2791
	0.03228	0.08	10	2	20	11.8	91.47287
	0.16139	0.4	10	0	0	15	116.2791
	0.80696	2	10	0	0	13.5	104.6512
	4.0348	10	10	6	60	6.1	47.28682
	20.174	50	10	10	100	0	0

Ribacirin (M= 244.24)

control	0	0	16	0	0	14.75	
0,2%DMSO	0	0	10	0	0	15	100
	0.02345	0.096	10	0	0	14.44	96.26667
	0.11724	0.48	10	0	0	15	100
	0.58618	2.4	10	2	20	12	80
	2.93088	12	10	0	0	14.8	98.66667
	14.6544	60	10	1	10	13.3	88.66667
	73.272	300	10	0	0	15	100

1H-benzotriazole (M= 119.12)

control	0	0	16	1	6.25	13.938	
0,2%DMSO	0	0	10	1	10	13.3	100
	0.01144	0.096	10	1	10	14.3	107.5188
	0.05718	0.48	10	1	10	12.9	96.99248
	0.28589	2.4	10	3	30	10.1	75.93985
	1.42944	12	10	1	10	12.5	93.98496
	7.1472	60	10	3	30	10.5	78.94737
	35.736	300	10	8	80	3	22.55639

Paclobutrazol (M= 293.8)

control	0	0	16	0	0	14.75	
0,2%DMSO	0	0	10	0	0	15	100
	0.0282	0.096	10	3	30	10.1	67.33333
	0.14102	0.48	10	1	10	10.75	71.66667
	0.70512	2.4	10	4	40	9	60
	3.5256	12	10	4	40	8	53.33333
	17.628	60	10	1	10	11.7	78
	88.14	300	10	10	100	3.6	24

3,4, DCA

0	4	0	0	15	
1	10	0	0	14.8	
8	10	10	100	0	

25-10-2011 SECOND RUN

Guanazole (M= 99.09)

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	12	0	0	14.08	
0,2%DMSO	0	0	10	0	0	14.1	100
	2.47725	25	10	1	10	12.6	89.3617
	4.9545	50	10	0	0	14.2	100.7092
	9.909	100	10	0	0	14.1	100
	19.818	200	10	0	0	14.4	102.1277
	39.636	400	10	0	0	14.1	100
	79.272	800	10	0	0	14.4	102.1277

Fenchlorazole-ethyl (M= 403.48)

control	0	0	12	0	0	14.167	
0,2%DMSO	0	0	10	0	0	14.1	100
	0.62943	1.56	10	0	0	14	99.29078
	1.26088	3.125	10	1	10	12.6	89.3617
	2.52175	6.25	10	0	0	12	85.10638
	5.0435	12.5	10	10	100	0	0
	10.087	25	10	10	100	0	0
	20.174	50	10	10	100	0	0

Ribacirin (M= 244.24)

control	0	0	12	0	0	14.083	
0,2%DMSO	0	0	10	0	0	14.1	100
	4.5795	18.75	10	0	0	14.2	100.7092
	9.159	37.5	10	0	0	14.1	100
	18.318	75	10	1	10	12.7	90.07092
	36.636	150	10	0	0	14.1	100
	73.272	300	10	0	0	14.1	100
	146.544	600	10	0	0	14	99.29078

1H-benzotriazole (M= 119.12)

control	0	0	12	0	0	14.083	
0,2%DMSO	0	0	10	0	0	14.1	100
	0.22395	1.88	10	0	0	14.4	102.1277
	0.4467	3.75	10	0	0	14.9	105.6738
	0.8934	7.5	10	0	0	14.5	102.8369
	1.7868	15	10	3	30	10.2	72.34043
	3.5736	30	10	7	70	2.9	20.56738
	7.1472	60	10	8	80	2.7	19.14894

Paclobutrazol (M= 293.8)

control	0	0	12	0	0	14.083	
0,2%DMSO	0	0	10	0	0	14.1	100
	2.76172	9.4	10	0	0	14	99.29078
	5.52344	18.8	10	0	0	14	99.29078
	11.0175	37.5	10	0	0	14	99.29078
	22.035	75	10	0	0	14	99.29078
	44.07	150	10	0	0	11.1	78.7234
	88.14	300	10	2	20	8.8	62.41135

3,4, DCA

0	4	0	0	15	
1	10	0	0	14.3	
8	10	10	100	0	

6-12-2011 THIRD RUN

Guanazole (M= 99.09)

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	14	100
	100	1mM	10	0	0	14.8	105.714
	300	3.02 mM	10	0	0	15	107.143
	1000	10.09 mM	10	0	0	15	107.143
	3000	30.27 mM	10	0	0	14.9	106.429
	10000	100.9 mM	10	0	0	14.1	100.714
	30000	302mM	10	10	100	0	0

Fenchlorazole-ethyl (M= 403.48)

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.629429	1.56	10	0	0	15	100
	1.260875	3.125	10	0	0	12.7	84.6667
	2.52175	6.25	10	10	100	0.8	5.33333
	5.0435	12.5	10	10	100	0	0
	10.087	25	10	10	100	0	0
	20.174	50	10	10	100	1	6.66667

Ribacirin (M= 244.24)

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	31.25	127.94	10	0	0	15	100
	62.5	255.89	10	1	10	15	100
	125	511.79	10	1	10	13.5	90
	250	1023.58	10	1	10	13.5	100
	500	2047.16	10	0	0	14.4	106.667
	1000	4094.3	10	0	0	14.1	97.9167

1H-benzotriazole (M= 119.12)

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.223946	1.88	10	0	0	14.4	96
	0.4467	3.75	10	0	0	14.3	95.3333
	0.8934	7.5	10	10	100	1.1	7.33333
	1.7868	15	10	10	100	0.6	4
	3.5736	30	10	10	100	0	0
	7.1472	60	10	10	100	0	0

Paclobutrazol (M= 293.8)

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	2.76172	9.4	10	0	0	15	100
	5.52344	18.8	10	0	0	14.9	99.3333
	11.0175	37.5	10	1	10	13.3	88.6667
	22.035	75	10	0	0	14.4	96
	44.07	150	10	1	10	10.9	72.6667
	88.14	300	10	10	100	4	26.6667

3,4, DCA

0	4	0	0	15	
1	10	0	0	14.4	
8	10	10	100	0	

Test performed by Sanne Hermsen- RIVM-Bilthoven-Netherlands

72hrs

27-7-2009 FIRST RUN

Flusilazole M= 315.4

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	16	1	6.25	14.063	
0,2%DMSO	0	0	10	0	0	15	100
	0.3154	1	10	0	0	15	100
	0.9462	3	10	0	0	14.9	99.33333
	3.154	10	10	0	0	13.7	91.33333
	9.462	30	10	9	90	8.1	54
	31.54	100	10	10	100	0	0
	94.62	300	10	10	100	0	0

Hexaconazole M= 314.24

control	0	0	16	0	0	14.875	
0,2%DMSO	0	0	10	0	0	15	100
	0.31424	1	10	0	0	14.8	98.66667
	0.94272	3	10	0	0	14.7	98
	3.1424	10	10	0	0	14.2	94.66667
	9.4272	30	10	5	50	7.7	51.33333
	31.424	100	10	10	100	0	0
	94.272	300	10	10	100	0	0

Cyproconazole M= 291.8

control	0	0	16	0	0	15	
0,2%DMSO	0	0	10	0	0	14.8	100
	0.2918	1	10	0	0	14.4	97.2973
	0.8754	3	10	0	0	14.7	5.037697
	2.918	10	10	1	10	13.1	4.489376
	8.754	30	10	0	0	13.8	4.729267
	29.18	100	10	1	10	11.1	3.803975
	87.54	300	10	10	100	7	2.398903

Triadimefon M= 293.7

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	14.8	100
	0.2937	1	10	0	0	14.8	100
	0.8811	3	10	0	0	14.9	100.6757
	2.937	10	10	0	0	13.8	93.24324
	8.811	30	10	1	10	12.7	85.81081
	29.37	100	10	2	20	9.1	61.48649
	88.11	300	10	10	100	3	20.27027

Mycobutanil M= 288.8

control	0	0	16	0	0	14.938	
0,2%DMSO	0	0	10	0	0	15	100
	0.2888	1	10	0	0	14.9	99.33333
	0.8664	3	10	0	0	15	100
	2.888	10	10	1	10	13.1	87.33333
	8.664	30	10	0	0	14	93.33333
	28.88	100	10	10	100	6	40
	86.64	300	10	10	100	0	0

Triticonazole M= 317.81

control	0	0	12	0	0	14.917	
0,2%DMSO	0	0	9	1	11.111111	15	100
	0.31781	1	10	1	10	13.4	89.33333
	0.95343	3	10	0	0	14.9	99.33333
	3.1781	10	10	1	10	13.3	88.66667
	9.5343	30	10	0	0	14.6	97.33333
	31.781	100	10	0	0	13.6	90.66667

3,4, DCA

control	0		4	0	0	11.75	
1 mg/l	1		10	0	0	12	
8 mg/l	8		10	10	100	0	

24-8-2009 SECOND RUN

Flusilazole M= 315.4

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	12	1	8.3333333	13.75	
0,2%DMSO	0	0	12	0	0	15	100
	0.3154	1	10	0	0	14.7	98
	0.9462	3	10	0	0	15	100
	3.154	10	10	0	0	12.1	80.66667
	9.462	30	10	10	100	8.1	54
	31.54	100	10	10	100	2.1	14
	94.62	300	10	10	100	0	0

Hexaconazole M= 314.24

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.31424	1	10	0	0	14.9	99.33333
	0.94272	3	10	0	0	14.7	98
	3.1424	10	10	1	10	13.6	90.66667
	9.4272	30	10	9	90	8.4	56
	18.8544	60	10	10	100	0	0
	31.424	100	10	10	100	0	0

Cyproconazole M= 291.8

control	0	0	12	0	0	13.636	
0,2%DMSO	0	0	10	0	0	15	100
	0.2918	1	10	0	0	14.8	98.66667
	2.918	10	10	0	0	14.9	99.33333
	8.754	30	10	0	0	14.1	94
	29.18	100	10	1	10	8.9	59.33333
	87.54	300	10	10	100	5.8	38.66667
	145.9	500	10	10	100	0	0

Triadimefon M= 293.7

control	0	0	12	1	8.3333333	13.75	
0,2%DMSO	0	0	10	0	0	15	100
	0.2937	1	10	0	0	15	100
	0.8811	3	10	0	0	15	100
	2.937	10	10	0	0	14.9	99.33333
	8.811	30	10	0	0	14.1	94
	29.37	100	10	0	0	9.9	66
	88.11	300	10	10	100	3	20

Mycobutanil M= 288.8

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	14.6	100
	0.2888	1	10	0	0	14.5	99.31507
	2.888	10	10	0	0	14.3	97.94521
	8.664	30	10	0	0	14	95.89041
	17.328	60	10	1	10	10.3	70.54795
	28.88	100	10	10	100	6.7	45.89041
	86.64	300	10	10	100	0	0

Triticonazole M= 317.81

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	14.6	100
	0.31781	1	10	0	0	14.6	100
	0.95343	3	10	0	0	14.9	102.0548
	3.1781	10	10	0	0	14.9	102.0548
	9.5343	30	10	0	0	15	102.7397
	31.781	100	10	0	0	14.9	102.0548
	95.343	300	10	0	0	14	95.89041

3,4, DCA

control	0		4	0	0	15	
1 mg/l	1		10	0	0	14.889	
8 mg/l	8		10	10	100	0	

31-8-2009 THIRD RUN

Flusilazole M= 315.4

	mg/l	µM	n	Lethal	%	GM	%
control	0	0	12	0	0	15	
0,2%DMSO	0	0	12	0	0	15	100
	0.3154	1	10	1	10	13.4	89.3333
	0.9462	3	10	0	0	14.9	99.3333
	3.154	10	10	0	0	12.7	84.6667
	9.462	30	10	6	60	7.5	50
	31.54	100	10	10	100	5	33.3333
	94.62	300	10	10	100	0	0

Hexaconazole M= 314.24

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.31424	1	10	0	0	15	100
	0.94272	3	10	0	0	14.9	99.3333
	3.1424	10	10	1	10	11.7	78
	9.4272	30	10	5	50	7.7	51.3333
	18.8544	60	10	10	100	0	0
	31.424	100	10	10	100	0	0

Cyproconazole M= 291.8

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.2918	1	10	1	10	13.5	90
	2.918	10	10	1	10	13.5	90
	8.754	30	10	0	0	14.2	94.6667
	29.18	100	10	2	20	6.4	42.6667
	87.54	300	10	10	100	7	46.6667
	145.9	500	10	10	100	0.9	6

Triadimefon M= 293.7

control	0	0	12	1	8.3333333	13.667	
0,2%DMSO	0	0	10	0	0	15	100
	0.2937	1	10	0	0	15	100
	0.8811	3	10	0	0	15	100
	2.937	10	10	0	0	14.8	98.6667
	8.811	30	10	1	10	12.9	86
	29.37	100	10	0	0	9	60
	88.11	300	10	10	100	0.4	2.66667

Mycobutanil M= 288.8

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	15	100
	0.2888	1	10	0	0	15	100
	2.888	10	10	1	10	13.4	89.3333
	8.664	30	10	0	0	14	93.3333
	17.328	60	10	0	0	11.9	79.3333
	28.88	100	10	9	90	6.8	45.3333
	86.64	300	10	10	100	0	0

Triticonazole M= 317.81

control	0	0	12	0	0	15	
0,2%DMSO	0	0	10	0	0	14.9	100
	0.31781	1	10	0	0	14.8	99.3289
	0.95343	3	10	0	0	15	100.671
	3.1781	10	10	0	0	14.8	99.3289
	9.5343	30	10	1	10	13.1	87.9195
	31.781	100	10	0	0	14.5	97.3154

3,4, DCA

control	0		4	0	0	15	
1 mg/l	1		10	1	10	13.5	
8 mg/l	8		10	10	100	0	

Ready biodegradability test on substituted (benzo)triazoles

28 days test for ready biodegradability in an aerobic aqueous medium was performed at PHI according to OECD guideline 301 D, closed bottle test. Benzotriazoles and triazoles as a chemical group are known to be fairly water-soluble, not readily degradable and of a limited sorption tendency.

A solution of the test substance in a mineral medium was inoculated and incubated under aerobic conditions in the dark at 20 ± 2 ℃. A test medium contained a relatively low concentration of biomass. A reference compound was run in parallel to check the operation of the procedures. The possible abiotic degradation was observed. Degradation for a reference substance was followed by the determination of DOC and measurements were taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. Degradation was followed by specific chemical analysis at frequent intervals over a 28-day period to assess degradation of substituted (benzo)triazoles. The degree of biodegradation was calculated by expressing the concentration of the test substance removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present.

Materials and methods

Selected substituted (benzo)triazoles cyproconazole, diclobutrazol, difenoconazole, diniconazole, epoxiconazole, hexaconazole, myclobutanil, penconazole, propiconazole, paclobutrazol, triadimefon, triazophos, uniconazole-P were purchased from Dr. Ehrenstorfer, Germany.

Mineral media was prepared according to OECD 301 D. In order to check the procedure, reference compound which meet the criteria for ready biodegradability was tested by setting up an appropriate vessel in parallel as part of normal test runs. The reference compound was sodium acetate and the degradation was tested by determining the removal of DOC.

The inoculum for the test was obtained from aquarium water. Possible abiotic degradation was checked by determining the concentration of the substance with chemical analysis in controls which were inoculated and poisoned with mercury chloride.

A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the end of the test is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14.

The concentrations of selected (benzo)triazoles in test samples were determined at the beginning of the test, at day 1, at day 14 and at day 28 by on-line solid phase extraction - liquid chromatography – electrospray - tandem mass spectrometry (on-line SPE Spark, LC-ESI-MS/MS API 2000). On-line SPE preconcentration was performed by loading 20 ml of the sample onto the cartridges PLRP-s (crosslinked styrene-divinylbenzene polymer, 15-25 µm particle size). Analytes were eluted trough LC Zorbax SB-C18 column (Agilent Technologies, 3.0 x 150mm particle size 3.5 µm) with the chromatographic mobile phase using gradient 5mM NH_4OH buffer/acetonitrile at the flow rate 0.25 ml/min. Further LC-MS/MS determination was performed in the multiple reaction monitoring (MRM) mode by recording two MRM

transitions per compounds. Quantification of selected pesticides was performed according to external standard calibration.

Results

In the calculation of D_t, percentage degradation, the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. With specific chemical analytical data available, degradation at day t of the test is calculated from:

$$D_t = \left[1 - \frac{C_t - C_{blt}}{C_0 - C_{bl0}}\right] * 100 \qquad (16)$$

where

D_t = % degradation at day t of the test,

C_0 = mean starting concentration of the test substance in the inoculated culture medium containing the test substance in mg/L,

C_t = mean concentration of the test substance in the inoculated culture medium containing test substance at day t of the test in mg/L,

C_{bl0} = mean starting concentration of the test substance in blank inoculated mineral medium in mg/L,

C_{blt} = mean concentration of the test substance blank inoculated mineral medium at day t of the test in mg/L.

In table 21 measured concentrations in µg/L of tested substituted (benzo)triazoles are presented at day 0, at day 1, at day 14 and at day 28.

Table 21. Mean value of measured concentrations (µg/L) for tested substituted musks/fragrances at day 0, at day 1, at day 14 and day 28

Name	**C_0**	**C_1**	**C_{14}**	**C_{28}**	**C_{blt}[a]**
Cyproconazole	1	1	0.8	0.95	0.025
Diclobutrazol	5	5	4.9	4.85	0.025
Difenoconazole	5	5	4.8	4.65	0.025
Diniconazole	10	10	9.05	9	0.025
Epoxiconazole	5	5	4.65	4.9	0.025
Hexaconazole	10	10	10.0	9.3	0.025
Myclobutanil	10	10	9.95	8.35	0.025
Paclobutrazol	10	10	8.85	8.65	0.025
Penconazole	5	5	4.9	4.85	0.025
Propiconazole	1	1	0.9	1	0.025
Triadimefon	10	10	10	8.85	0.025

Name	**C_0**	**C_1**	**C_{14}**	**C_{28}**	**C_{blt}** [a]
Triazophos	0.1	0.1	0.09	0.085	0.025
Uniconazole-P	50	50	50	43.9	0.025

[a] C_{blt} represents concentration in blank at day 0, at day 1, at day 14 and at day 28. All concentrations were below the detection limit of the method (< LOD) and are calculated as LOD/2

In table 22 biodegradation at day 0, at day 1, at day 14 and at day 28 is presented, calculated from the data presented in table 21 with the use of equation 16.

Table 22. Biodegradation, D_t (%) at day 0, at day 1, at day 14 and at day 28 for selected substituted (benzo)triazoles

Fragrance ID	D_0	D_1	D_{14}	**D_{28}**	**95 % interval D_{28}**
Cyproconazole	0.0	0.0	20.5	5.1	0.36
Diclobutrazol	0.0	0.0	2.0	3.0	0.08
Difenoconazole	0.0	0.0	4.0	7.0	0.31
Diniconazole	0.0	0.0	9.5	10.0	0.36
Epoxiconazole	0.0	0.0	7.0	2.0	0.04
Hexaconazole	0.0	0.0	0.0	7.0	0.10
Myclobutanil	0.0	0.0	0.5	16.5	1.32
Paclobutrazol	0.0	0.0	11.5	13.5	0.28
Penconazole	0.0	0.0	2.0	3.0	0.12
Propiconazole	0.0	0.0	10.3	0.0	/
Triadimefon	0.0	0.0	0.0	11.5	0.63
Triazophos	0.0	0	13.3	20	3.67
Uniconazole-P	0.0	0	0	12.2	0.73

The percentage of degradation of the test substance can be plotted against time. On figure 15 the graph with data for biodegradation test is presented for substituted (benzo)triazoles and reference substance sodium acetate. Abiotic degradation did not occur during the test.

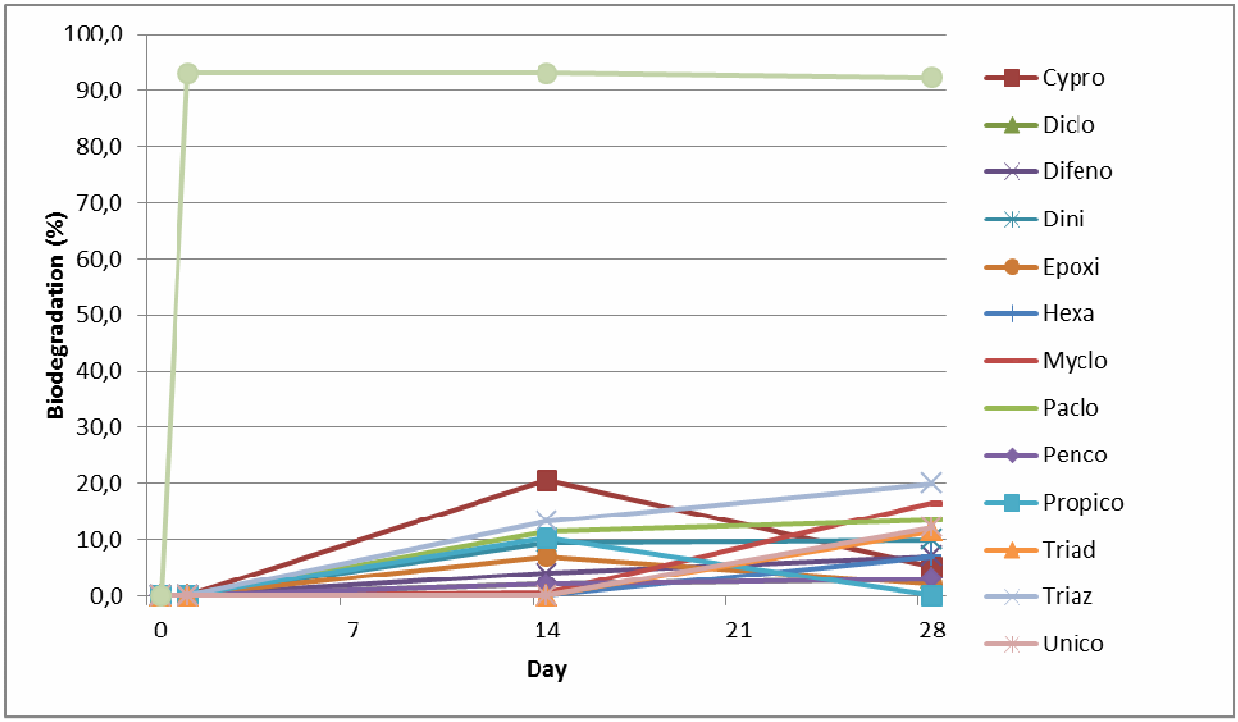

Figure 15. The percentage of biodegradation of substituted (benzo)triazoles and reference substance sodium acetate

Conclusion

The ready biodegradability of thirteen selected (benzo)triazoles was investigated. As expected, substances are not readily biodegradable. Raw data of the test is available at Public Health Institute Maribor.

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Appendix 1: List of Abbreviations and Glossary

BCF	Bioconcentration factor is the concentration of a particular chemical in a biological tissue per concentration of that chemical in water surrounding that tissue.
(B)TAZ	(Benzo)triazoles
C&L	Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures.
PBDEs	Polybrominated diphenylethers
PFCs	Perfluorinated chemicals
Pseudokirchneriella subcapitata	*Selenastrum capricornutum*
REACH	Regulation (EC) No 1907/2006 on the Registration, evaluation, authorisation and restriction of chemicals.

Appendix 2: Justification for the testing program for sediment testing of PBDEs

Based on an initial evaluation of the availability of experimental data and QSAR models for various classes of the emerging compounds identified within NORMAN, one of the compounds that have been selected as the chemical classes of choice for CADASTER are polybrominated diphenylethers (PBDE). PBDE are typical class of hydrophobic chemicals that pose a threat to man and the environment.

Selection of PBDEs relevant for the testing

Selection of PBDEs relevant for the testing was performed by Partner 2 - PHI, Partner 3 - UI and Partner 5 – HIK and is based on:

- analytical limitation and availability of standards (P2-PHI)
- ranking according to their toxic (fate) characteristics (P2-UI)
- assessment of the chemical domain (P5-HIK)

A list of selected PBDEs was finalized after Interim meeting in Rome (September 2009). The selected PBDEs are BDE-002, BDE-066, BDE-077, BDE-119, BDE-126, BDE-180, BDE-197, BDE-203, BDE-204 and BDE-207.

Selection and development of test sets for PBDEs

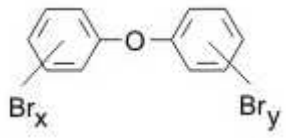
Selection and development of test sets was performed by PHI in consultation with other partners (e-mail communication, Interim meeting in Rome) and is based on:

- physical-chemical properties
- environmental fate and behaviour
- existing ecotoxic effect data

<u>Structure, physical-chemical properties and the use of PBDEs</u>

PBDEs are used as flame retardants in a wide numbers of synthetic applications. Losses at the production sites and leaching from the landfills resulted in progressive contamination of the aquatic environment with predominant bioaccumulation of lower brominated congeners in aquatic biota[1].

Polybrominated diphenylethers are a class of structurally similar brominated hydrocarbons, in which 2-10 bromine atoms are attached to the diphenyl ether molecule. The general chemical structure of PBDE is shown below:



It can be seen from the structure that the brominated compounds can be formed in several compounds called congers. The 209 congeners are based on a number of bromine substituents. There are 10 homologous groups of PBDEs (monobrominated through decabrominated). Only about 42 congeners have been synthesized in pure form or on a laboratory scale.

Some of the physical-chemical properties for selected PBDEs are listed in Table 1.

Table 1: Physical-chemical properties for hexabromo-, octabromo- and decabromodiphenyl ether[2]

Property	**Hexabromodiphenyl ether**	**Octabromodiphenyl ether**	**Decabromodiphenyl ether**
Solubility in water	11 µg/L ; 3 µg/L	20-30 µg/L	11 µg/L
Log Kow	6.39	5.53	8.58
Log Koc	3.33-3.87	No data	No data
Vapour pressure	$5.2x10^{-8}$ mmHg	$7x10^{-11}$ mmHg	No data
Henry's law constant	$3.9x10^{-6}$ atm-m^3/mol $1.38x10^{-6}$ atm-m^3/mol		

From Table 1 we can see that PBDEs have hydrophobic nature and limited solubility in water. In water PBDEs will tend to accumulate in sediment or suspended solids.

Ecotoxicological data and data on fate and behaviour for PBDEs

Due to the hydrophobic nature and subsequent limited solubility, no direct effects are expected following exposure via the water phase. Some ecotoxicological data and data on fate and behaviour from the literature[3] are presented in Table 2, Table 3 and Table 4.

Table 2: Summarized ecotoxicological and fate data for DBDE[3]

Test	**Result**
Ready biodegradation	Not readily biodegradable
Anaerobic sediment degradation	Not degraded after 32 weeks or 2 years
Aqueous photodegradation	Half-life >> 90 days
Algae, EC_{50}, marine, 96h	> 1 mg/L
Fish, LC50, 48h	> 500 mg/L

Test	Result
Fish bioconcentration	Not bioconcentrating, BCF < 5 and < 50
Sediment organism chronic, 28d	*Lumbriculus variegates*
(2% and 5% organic carbon)	EC_{50} > 5000 mg/kg dwt of sediment
	NOEL ≥ 5000 mg/kg dwt sediment

From Table 2 it is evident that decabromodiphenylether (DBDE) is not acutely toxic to fish[4] or marine algae[5] and is not expected to be chronically toxic in aquatic species due to its large molecular weight, negligible water solubility, and the lack of toxicity exhibited by OBDE[6]. DBDE is not toxic to the sediment oligochaete, *Lumbriculus variegatus* (≥ 5000 mg/kg dwt), when tested over a 28-day period in sediments with either 2% or 5% organic carbon[7].

Table 3: Summarized ecotoxicological and fate data for OBDE[3]

Test	Result
Ready biodegradation	Not readily biodegradable
Daphnid, EC_{50}	EC_{50} > water solubility
Daphnid, chronic, 21d	NOEC > water solubility
Fish, LC50, 48h	> 500 mg/L
Fish bioconcentration	Not bioconcentrating, BCF < 4 at 8 wk

From Table 3 it is evident that octabromodiphenylether (OBDE) is not acutely toxic to fish or daphnia at the limit of its water solubility[8]. It is not chronically toxic to *Daphnia magna* in a 21-day life-cycle study at the limit of its water solubility[9].

Table 4: Summarized ecotoxicological and fate data for PeBDE[3]

Test	Result
Ready biodegradation	Not readily biodegradable
Algae, EC_{50}, 96h, freshwater	NOEC > water solubility
Daphnid, EC_{50}	EC_{50} = 14 µg/L, NOEC = 4.9 µg/L
Daphnid, chronic, 21d	EC_{50} = 9.8 µg/L, NOEC = 5.2 µg/L
Fish, LC_{50}, 48h	≥ 500 mg/L (*Orzyas latipes*)
Fish, LC_{50}, 96h	≥ water solubility (*Onchorhynchus mykiss*)
Fish bioconcentration	Bioconcentrating: BCF 10000-14000
Sediment organism chronic, 28d	*Hyalella, Chironomus, Lumbriculus sp.*

Test	Result
(2% and 5% organic carbon)	EC_{50} > 50 mg/kg dwt of sediment
Soil nitrification organisms, 28d	NOEC > 1 mg/kg soil dwt
Terrestrial plant, 21d	NOEC > 1000 mg/kg dwt
Earthworm, 14d	NOEC > 500 mg/kg soil dwt

From Table 3 it is evident that pentabromodipheylether (PeBDE) is not acutely toxic to fish[10] or algae[11] at the limit of its water solubility. Effects can be seen on *Daphnia magna* bellow the limits of its water solubility[12], but it may have been due to physical impairment rather than a direct toxic effect. The 28-day EC_{50} after chronic exposure to the sediment organisms *Hyalella azteca*[13], *Chironomus riparius*[14] and *Lumbriculus variegatus*[15] to the PeBDE commercial product was > 50 mg/kg dry sediment. PeBDE was not toxic to earthworms in 14-day study[16] or to soil nitrification organisms in a 28-day study[17].

The preliminary bioaccumulation test results for PBDEs

Partner 2, PHI, is at the moment performing a bioaccumulation test with *Tubifex tubifex*[18] on PBDEs. Preliminary data on bioaccumulation for some of the congeners are in the Table 5.

Table 5: Preliminary data from bioaccumulation test performed at PHI

	BAF	Log BCF_{est}	Log $K_{SED\ inf}$ [a]
BDE-28	1.9	4.15	3.67
BDE-47	1.7	4.14	3.66
BDE-51	3.4	4.16	3.66
BDE-66/42	4.4	4.32	3.83
BDE-99	3.2	4.29	3.77
BDE-100	8.1	4.67	4.06
BDE-119	3.9	4.48	4.01
BDE-153	8.9	4.48	4.25
BDE-154	10.8	4.57	4.16
BDE-183	0.69	2.76	3.62

[a] The values are informative, estimated as concentration in sediment versus concentration in water (at day 28)

Preliminary test results presented in Table 5 are in line with other experimental data in this report. Estimated BCF and Ksed are high for all congeners. Results confirm that after BDEs will enter water compartment, substances tend to accumulate in sediment and in organisms. Thus it is evident that testing in sediment is preferred.

Conclusion

The aim of CADASTER is to generate reliable data that can be used in the QSAR evaluation. From literature data and from PHI preliminary test results on bioaccumulation with *Tubifex tubifex* it is evident that testing of PBDEs should be performed in sediment if we want to achieve this goal. Following this choice we will generate results that are useful for modelling. Oligochaeta are proven to be a good model organism to replace aquatic vertebrate such as fish in assessing bioaccumulative properties of substances.

After reviewing available experimental sediment effect data it is obvious that effects in sediment occur at high concentrations (from 50 to 1500 mg/kg). Effect testing of PBDEs is mostly limited with availability and costs of individual congeners, since for effect testing of sediment we would have to have large amounts of pure congeners.

Therefore PHI proposes that testing is focussed on the sediment compartment with assessment of the bioaccumulation potential as endpoint. The BCF values obtained may subsequently be used to calculate toxicity endpoints, either using experimentally obtained critical body burdens (CBBs) for the various PBDE's, or by using QSAR approaches for predicting CBBs. The latter approach is advocated by for instance Hendriks et al.

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