Impulse Programme Marine Sciences 1992-1996

Project n° 4

Intercompartment distribution of monocyclic aromatic hydrocarbons and C_1-C_2 organochlorines in the North Sea environment

Part V
Bioavailibility of Volatile Organic Compounds to marine fish (in vitro tests)

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V. BIOAVAILABILITY OF VOLATILE ORGANIC COMPOUNDS TO MARINE FISH (IN VITRO TESTS)

V.1. INTRODUCTION

Halogenated aliphatic hydrocarbons (CHCs) and monoaromatic compounds (MAHs) have shown to affect the normal homeostasis of animals. Acute toxicity studies reported 96-h LC_{50} (lethal concentration for 50% of the population within 96 hours) values for benzene, toluene, ethylbenzene, p-,m- and o-xylene ranging from 2 to 11 ppm for striped bass (*Morone saxitilis*) and from 0.49 to 20 ppm for bay shrimp (*Crago franciscorum* ; Benville and Korn, 1977). Toluene was shown lethal to embryos, newly hatched protolarvae and 30-day old fathead minnow (*Pimephales promelas*) at concentrations of respectively 55-72, 25-36 and 18-30 ppm (Devlin, 1983) and m- and p-xylene reduced the fertilisation capacity of cod eggs at concentrations above 10 ppm at which o-xylene had no effect (Kjoersvik *et al.*, 1982). 50 % lethality of fathead minnow (*Pimephales promelas*) within 96 hr was reached with 40.7 mg/l trichloroethylene and between 18.4 and 21.4 mg/l tetrachloroethylene (Alexander *et al.*, 1978). 1,2-dichloroethylene is less acute toxic (fathead minnows : LC_{50} : 500 ppm).

In recent years studies of the chronic effects of contaminants gained considerable interest. The outcome of these studies may provide more accurate tools to determine "No Effect Concentrations" (NOEC) of contaminants. Example : o-xylene killed 50% of rainbow trout (*Salmo gairdneri*) in 96 hr at 13.5 mg/l but the lowest avoidance concentration was much lower : 0.01 mg/l (Walsh *et al.*, 1977). In fish sublethal effects of volatile organic and monoaromatic compounds were observed on hatching success, post-hatch survival, fry growth rates and survival (Moles *et al.*, 1981 ; Loekle *et al.*, 1983 ; Loekle, 1987 ; Smith *et al.*, 1991), total plasma protein changes (Pfeifer and Weber, 1979), histopathology, liver dysfunction and enhanced hepatocarcinogenesis (Casillas *et al.*, 1983 ; Loekle *et al.*, 1983 ; Casillas and Ames, 1986 ; Loekle, 1987 ; Kotsanis and Metcalfe, 1991), mitosis-related DNA synthesis (Simakhov, 1982 ; Droy *et al.*, 1988), genetic damage (Longwell, 1977), enzymatic changes (Statham *et al.*, 1978 ; Kleinow *et al.*, 1988 ; Folmar *et al.*, 1993), immunoresponse (Munson, 1987).

In general, for both acute and chronic toxicity tests of low molecular mass chlorinated organic compounds, the order of increasing relative toxicity based on the water-borne exposure concentrations is : chloroethanes, chloroethylenes, chlorobenzenes and chlorophenols. Sublethal toxicity of toluene was generally lower than of naphthalene (Moles *et al.*, 1981 ; Dange and Masurekar, 1985). More information on effects of chlorinated hydrocarbons and monoaromatic hydrocarbons can be found in section I.4.3.

In this chapter a laboratory study is described on the bioavailability (bioconcentration) of three water-borne chlorinated hydrocarbons (tetrachloroethylene, trichloroethylene and trichloroethane) and two substituted monoaromatic compounds (o-xylene and toluene) to fish and sublethal and chronic effects. Bioconcentration in the aquatic environment can be defined as the external availability of a chemical to an organism.

An introduction on bioavailability was given in section III.4.2.5. Bioconcentration of the compounds in fish (*Solea solea*) was studied together with possible effects on several levels of the biological organisation : metabolism, physiology and diseases. As biochemical parameters were examined : ethoxyresorufin O-deethylase (EROD), glutathion S-transferase (GSH-T) and acetylcholinesterase. EROD specifically reflects the activity of the Cytochrome P4501A1 metabolism (Stegeman, 1989). The cytochrome P450 belong to a superfamily of structurally and functionally related haemoproteins. Cytochrome P450 is a central catalyst in the oxidative "phase I" biotransformation of endogenous and exogenous compounds. Extensive reviews are Payne *et al.*, 1987 ; Buhler and Williams, 1988,1989 ; Foureman, 1989 ; Stegeman, 1989 ; Stegeman and Kloepper-Sams, 1987 ; Stegeman *et al.*, 1990 ; Hansen and Addison, 1990 ; Jiminez and Stegeman, 1990 ; Duinker and Boon, 1986 ; De Voogt *et al.*, 1990 and Goksøyr and Förlin, 1992). Tetrachloromethane and aromatic compounds have shown to interact with the β-naphthoflavone-inducible cytochrome P450 (Anon., 1990 ; Kleinow *et al.*, 1988 ; Goksøyr *et al.*, 1991).
Glutathion S-transferase is a conjugating enzyme belonging to the family of the phase II detoxification enzymes. Conjugation reactions are a common pathway of metabolism for many endogenous compounds and have been shown to be of major importance in the biotransformation of xenobiotics. Oxidation reactions performed by the monooxygenase system are usually followed by phase II metabolism i.e. conjugations of the metabolite with activated forms of endogenous substances such as glucuronic acid, sulphate or glutathione (Sijm and Opperhuizen, 1989). Benzene has been shown to induce conjugating enzymes (Kalf *et al.*, 1987).
Acetylcholinesterase mediates the hydrolysis of acetylcholine at the nerve synapses. Inhibition of the enzyme causes an accumulation of acetylcholine and disruption of nerve function (Kemp and Wallace, 1990). Acute inhibition of 80% and chronic inhibition of 50% of acetylcholinesterase has been associated with direct mortality (Ludke *et al.*, 1975 ; Hill and Fleming, 1982).
The physiological indicators examined were swimming behaviour and respirometry. Respirometry is a means to assess the potential performance of fish in relation to environmental variables. Homeostatic costs are assumed to be higher in a stressed fish than in unstressed fish (Barton and Schreck, 1987). Effects of pollution can be assessed by simple counting the opercular beats, or, more precisely, by measuring respiratory gases. In these experiments, opercular beats were measured.
Gross pathology included the study of externally visible fish diseases. Certain lesions can be attributed to adverse environmental factors such as pollutants.

V.2. MATERIALS AND METHODS

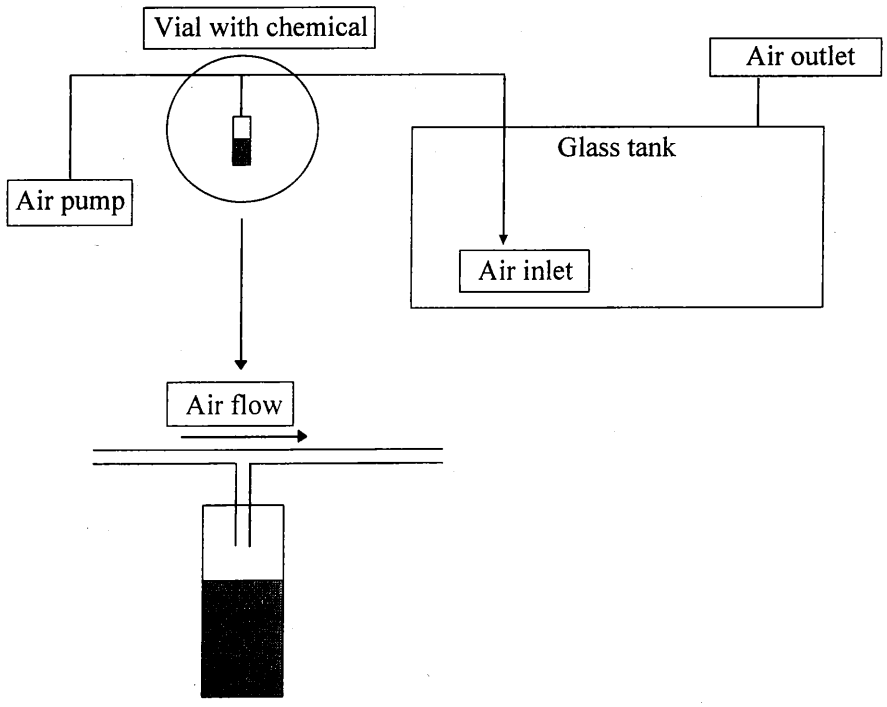
V.2.1 THE FISH

Soles (*Solea solea*, 15.2 ± 1.9cm ; 3.97 ± 1.02 g) were collected by beam trawling during cruises of the Belgian oceanographic research vessel Belgica. The animals were kept alive in 500 litres seawater tanks onboard and then transferred to the laboratory upon landing. Acclimation to laboratory conditions during several months took place in large tanks with a total volume of approx. 3 m^3 prior to the experiments.

V.2.2 THE EXPERIMENTAL CONDITIONS

The experiments were conducted in sealed mesocosms. The experimental hardware consisted of glass tanks containing an air inlet and outlet. The tanks were filled with 100 litres aerated synthetic sea water HW (Wiegandt, Germany ; see section IV.1.8.2 modified as follows : distilled water was replaced with tap water) and a 3 cm thick sand bed. The mesocosms were not filtered during the experiments and the temperature was kept at 16°C.
The air inflow rate was set at 400 ml/min. The chemicals were administered to the system via the air inlet. Figure V. 2.2-1 shows the schematic diagram of the experimental configuration that is used to expose the fish to the chemicals. Vials filled with tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene or toluene were connected to the air inlet tube so that evaporated chemical is transported to the system.
Each tank contained ten animals. The fish were transferred to the tanks two to three weeks prior to the start of the experiment and fed daily. Behavioural characteristics (respirometry) were regularly observed during the course of exposure.
After 7 days of exposure, sediment, water and fish were sampled and processed further as described in section V.2.3. The exposure experiments with tetrachloroethylene and 1,1,1-trichloroethane were also prolonged up to 29 days.

Figure V. 2.2-1 : Schematic diagram of the experimental configuration used to expose fish to volatile organics.



V.2.3 SAMPLE PREPARATION

Sediment and water were stored in sealed 20 ml glass vials prior to the chemical analysis.
The fish were examined for external visible disruptions and then killed with a blow on the head. Muscle tissue was filleted and stored at -20°C prior to the chemical analysis. The livers were excised and pieces of fresh liver tissues were submerged in 50 mM Tris-Cl, containing 150 mM NaCl and 1 mM EDTA, pH 8.0 and homogenised with a motor-driven Potter-Elvehjem homogeniser (1,000 rpm ; 0°C). The homogenate was then centrifuged at 10,000g during 10 min. at 4°C. These supernatants were used for protein and enzymatic analysis. The rests of the liver tissues are stored in liquid nitrogen to be used for additional analysis at a later stage.

V.2.4 DETERMINATION OF VOCS AND MAHS IN SEDIMENT, WATER AND FISH

The chemical analysis were conducted as described in chapter II. .

V.2.5 DETERMINATION OF BIOLOGICAL EFFECTS

V.2.5.1 CHEMICALS

7-ethoxyresorufin was prepared according to Klotz *et al.* (1984). Resorufin was obtained from Sigma Chemical Company and NADPH-Na_4 was from Boehringer Mannheim. Acetylthiocholine and Dithiobis 2-nitrobenzoic acid (DTNB) were from Merck and Aldrich, respectively. All other chemicals were of analytical grade.

V.2.5.2 DETERMINATION OF THE PROTEIN CONTENT OF THE SUPERNATANTS

The protein content of the supernatants was measured with the method of Lowry (1951) modified according to Bensadoun and Weinstein (1976). Bovine serum albumin was used as standard protein.

V.2.5.3 DETERMINATION OF ETHOXYRESORUFIN O-DEETHYLASE

EROD activities were measured fluorimetrically according to a slightly modified procedure of Burke and Mayer (1974).

The incubation mixture (total volume : 2 ml) consisted of :

1 ml 0.2 M sodium phosphate pH 7.4,
400 µl water,
200 µl 25 µM 7-ethoxyresorufin in 20 % methanol,
200 µl protein in 50 mM Tris-Cl, 150 mM NaCl and 1 mM EDTA, pH 8.0,
200 µl 2 mM NADPH

The protein content in the reaction mixtures was maximally 204 µg.
The reaction was started with the addition of NADPH and the reaction was monitored during one minute.

The sodium phosphate buffer was prepared of dibasic sodium phosphate adjusted to pH 7.4 with HCl and 0.2 M end concentration.
The substrate stock solution, 125 µM 7-ethoxyresorufin in methanol was diluted five-fold with water immediately prior to use. 2 mM NADPH was freshly prepared before use.
The analysis were performed at room temperature on a Shimadzu RF-5001PC equipped with the data acquisition programme "labtime" (Shimadzu). The excitation and emission wave lengths were 510 and 585 nm respectively, and both slits were opened 5 nm.

V.2.5.4 DETERMINATION OF GLUTATHIONE S-TRANSFERASE

Glutathione S-transferase was measured fotometrically according to Warholm *et al.* (1985).
The method is based on the enzymatic formation of the conjugation product between glutathione and 1-chloro 2,4-dinitrobenzene is measured.

The incubation mixture (total volume 1 ml) consisted of :

500 µl 0.2 M sodium phosphate + 1 mM EDTA, pH 6.5
200 µl water
50 µl 20 mM glutathione
50 µl 20 mM CDNB in ethanol
200 µl protein in 50 mM Tris-Cl, 150 mM NaCl and 1 mM EDTA, pH 8.0,

The phosphate buffer was prepared as in section 2.5.3. The reaction was started with the addition of sample and fotometrically monitored at 340 nm during one minute. The protein content in the reaction mixture was maximally 51 µg.

V.2.5.5 DETERMINATION OF ACETYLCHOLINESTERASE

Acetylcholinesterase was also determined fotometrically according to a modified method of Ellman (1961).

The reaction mixture (total volume : 1 ml) consisted of :
450 ml 10 mM DTNB in 50 mM sodium phosphate, pH 7.4,
350 µl 50 mM sodium phosphate, pH 7.4,
100 µl 30 mM acetylthiocholine-iodide in 50 mM sodium phosphate, pH 7.4,
100 µl protein in 50 mM Tris-Cl, 150 mM NaCl and 1 mM EDTA, pH 8.0

The phosphate buffer was prepared as in section 2.5.3. The reaction was started with the addition of sample and was monitored at 412 nm and 30 °C during one minute. The protein content in the reaction mixture was maximally 102 µg.

V.2.5.6 DETERMINATION OF BEHAVIOUR

Swimming behaviour was observed and the opercular beats were measured.

V.3. RESULTS

V.3.1 CHEMICAL ANALYSIS

V.3.1.1 WATER, SEDIMENT AND MUSCLE TISSUE CONCENTRATIONS OF VOLATILE ORGANIC COMPOUNDS AFTER EXPOSURE

Fish were exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene as described in section 2.2. After 7 days of exposure water, sediment and fish were analysed. The experiments with tetrachloroethylene and 1,1,1-trichloroethane were prolonged up to 30 days.
Table V. 3.1.1-1contains the total amounts of the chemicals that were introduced in the tanks.

Figure V. 3.1.1-1. Total amounts of analyte introduced into the tanks

Analyte	Period of exposure	
	7 days	30 days
Tetrachloroethylene	155.2 mg	955.8 mg
Trichloroethylene	611.2 mg	-
1,1,1-Trichloroethane	169.1 mg	1,868.8 mg
o-Xylene	45,5 mg	-
Toluene	155 mg	-

Figure V.3.1.1-2 shows the VOC and MAH concentrations measured in the water after 7 days exposure. None of the compounds were detectable in the control. In the other tanks the water concentrations of the contaminants were, when detectable, generally much higher than in the field (see chapter III). The administered compounds, except toluene, were up to 600 fold (in the case of 1,1,1-trichloroethane) higher than those observed in the field. The toluene concentration in the toluene-

exposed water did not exceed the lower detection limit. The 1,1,1-trichloroethane concentration had increased almost ten-fold between 7 days and 29 days of exposure.

Table V. 3.1.1-2 : Water concentrations (ng/ml) of volatile organic compounds exposed during 7 days.

Analytes	Not exposed	Exposed with :				
		Tetrachloro-ethylene	Trichloro-ethylene	1,1,1-Trichloro-ethane	o-Xylene	Toluene
Chloroform	nd[a]	0.09	nd	0.11	0.44	nd
1,1,1-Trichloro-ethane	nd	0.04	nd	**1.82**	0.33	nd
Carbontetra-chloride	nd	nd	nd	nd	nd	nd
Benzene	nd	0.3	nd	nd	0.4	nd
Trichloroethylene	nd	0.46	**0.09**	0.33	1.00	nd
Toluene	nd	0.33	nd	0.18	0.34	**nd**
Tetrachloro-ethylene	nd	**1.27**	nd	0.32	0.55	nd
Ethylbenzene	nd	0.13	nd	0.20	0.27	nd
m,p-Xylene	nd	0.3	nd	0.3	0.5	nd
o-Xylene	nd	0.13	nd	0.13	**0.16**	nd
1,2-Dichloro-ethane	nd	0.129	nd	0.185	0.064	nd
1,1-Dichloro-ethane	nd	nd	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-3: Water concentrations of volatile organic compounds (ng/ml) exposed during 29 days.

Analytes	Not exposed	Exposed with :	
		1,1,1-Trichloroethane	Tetrachloroethylene
Chloroform	nd[a]	nd[a]	nd
1,1,1-Trichloroethane	nd	**17.03**	4.45
Carbon tetrachloride	nd	nd	nd
Benzene	nd	nd	0.036
Trichloroethylene	nd	0.10	0.13
Toluene	nd	nd	0.04
Tetrachloroethylene	nd	nd	**0.42**
Ethylbenzene	nd	nd	nd
m-p Xylene	nd	nd	nd
o-Xylene	nd	nd	nd
1,2-Dichloroethane	nd	nd	nd
1.1-Dichloroethane	nd	nd	nd

[a] not detectable

Table V. 3.1.1-4 shows the VOC and MAH concentrations measured in the sediment after 7 days exposure. Most compounds were not detectable in the sediment. Similar results were obtained with sediments collected in the field (see section 3.3).

Table V. 3.1.1-4. Sediment concentrations (ng/g) of volatile organic compounds exposed during 7 days.

Analytes	Not exposed	Exposed with :				
		Tetrachloro-ethylene	Trichloro-ethylene	1,1,1-Trichloro-ethane	o-Xylene	Toluene
Chloroform	nd[a]	nd	nd	nd	nd	nd
1,1,1-Trichloroethane	nd	nd	nd	nd	nd	nd
Tetrachloromethane	nd	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	nd	nd
Trichloroethylene	nd	0.09	nd	nd	nd	nd
Toluene	nd	nd	nd	nd	nd	nd
Tetrachloroethylene	nd	0.08	nd	nd	0.05	nd
Ethylbenzene	nd	nd	nd	nd	0.15	nd
m,p-Xylene	nd	nd	nd	nd	nd	nd
o-Xylene	nd	nd	nd	nd	0.10	nd
1,2-Dichloroethane	nd	0.068	nd	nd	nd	nd
1.1-Dichloroethane	nd	0.012	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-5 to 10 contain the VOC and MAH concentrations measured in the sole muscle tissues after 7 days exposure. In non-exposed fish most compounds were not detectable. The values of the administered compounds were used to calculate the bioconcentration factors.

Table V. 3.1.1-5. Concentrations (ng/g) of volatile organic compounds in muscle tissue of non-exposed sole.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4
Chloroform	2.59	5.04	0.97	nd[a]
1,1,1-Trichloorethane	nd	nd	nd	nd
Tetrachloromethane	nd	nd	nd	nd
Benzene	nd	nd	nd	nd
Trichloroethylene	nd	nd	nd	nd
Toluene	nd	nd	0.4	0.3
Tetrachloroethylene	nd	nd	0.07	0.07
Ethylbenzene	nd	nd	nd	nd
m,p-Xylene	nd	nd	0.3	0.3
o-Xylene	nd	nd	nd	0.16
1,2-Dichloroethane	nd	nd	nd	nd
1,1-Dichloroethane	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-6. Concentrations (ng/g) of volatile organic compounds in muscle tissue of sole exposed to tetrachloroethylene during 7 days.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5
Chloroform	0.06	0.05	0.06	0.04	0.04
1,1,1-Trichloroethane	nd[a]	nd	nd	nd	nd
Carbon tetrachloride	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	nd
Trichloroethylene	nd	nd	nd	nd	nd
Toluene	nd	nd	nd	nd	nd
Tetrachloroethylene	0.31	0.32	0.41	0.39	0.14
Ethylbenzene	nd	nd	nd	nd	0.17
m,p-Xylene	nd	nd	nd	nd	nd
o-Xylene	nd	nd	nd	nd	nd
1,2-Dichloroethane	0.007	nd	nd	0.064	0.014
1,1-Dichloroethane	nd	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-7. Concentrations (ng/g) of volatile organic compounds in muscle tissue of sole exposed to trichloroethylene during 7 days.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6
Chloroform	0.08	0.07	0.20	0.15	0.05	0.04
1,1,1-Trichloro-ethane	nd[a]	0.01	nd	nd	nd	nd
Tetrachloromethane	nd	nd	nd	nd	nd	nd
Benzene	nd	2.0	2.2	1.0	nd	nd
Trichloroethylene	**0.40**	**0.45**	**0.78**	**0.30**	**0.03**	**0.01**
Toluene	nd	0.4	nd	0.3	nd	nd
Tetrachloroethylene	0.24	nd	nd	nd	1.27	0.11
Ethylbenzene	0.26	2.86	nd	0.85	nd	nd
m,p-Xylene	0.6	nd	3.8	nd	nd	nd
o-Xylene	0.34	0.16	0.17	0.25	0.04	nd
1,2-Dichloorethane	nd	nd	0.139	0.070	nd	0.007
1,1-Dichloorethane	nd	nd	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-8. Concentrations (ng/g) of volatile organic compounds in muscle tissue of sole exposed to 1,1,1-trichloroethane during 7 days.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Fish 7
Chloroform	0.15	0.14	0.20	0.14	0.16	0.19	0.25
1,1,1-Trichloroethane	**0.22**	**0.59**	**0.35**	**0.38**	**0.28**	**0.25**	**0.03**
Tetrachloromethane	nd[a]	nd	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	0.3	0.3	nd
Trichloroethylene	0.01	0.02	0.25	0.02	0.01	0.02	nd
Toluene	0.1	0.1	0.3	0.2	0.2	0.3	nd
Tetrachloroethylene	0.02	0.02	0.59	0.22	0.11	0.11	nd
Ethylbenzene	nd	nd	0.45	0.13	0.34	0.35	nd
m,p-Xylene	nd	nd	1.0	nd	0.1	0.2	nd
o-Xylene	nd	0.05	0.53	0.07	0.09	0.12	nd
1,2-Dichloroethane	0.016	0.013	0.170	0.016	0.023	0.025	nd
1,1-Dichloroethane	nd	nd	0.109	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-9. Concentrations (ng/g) of volatile organic compounds in muscle tissue of sole exposed to toluene during 7 days.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5
Chloroform	0.07	0.10	0.07	0.06	0.10
1,1,1-Trichloorethaan	0.03	0.01	nd	nd	nd
Tetrachloromethane	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	nd
Trichloroethylene	0.13	0.03	0.03	0.02	0.04
Toluene	**0.25**	**0.173**	**0.10**	**0.09**	**0.16**
Tetrachloroethylene	0.61	0.18	nd	nd	nd
Ethylbenzene	0.49	0.15	nd	nd	nd
m,p-Xylene	1.2	nd	nd	nd	nd
o-Xylene	0.64	nd	nd	nd	nd
1,2-Dichloroethane	0.065	0.017	0.011	0.009	0.019
1.1 Dichloroethane	0.011	nd	nd	nd	nd

[a] not detectable

Table V. 3.1.1-10. Concentrations (ng/g) of volatile organic compounds in muscle tissue of sole exposed to o-xylene during 7 days.

Analytes	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5
Chloroform	0.07	0.06	0.06	0.07	0.11
1,1,1-Trichloroethane	nd[a]	nd	nd	nd	nd
Tetrachloromethane	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	nd
Trichloroethylene	nd	nd	nd	0.01	nd
Toluene	nd	nd	nd	nd	nd
Tetrachloroethylene	0.15	0.10	0.06	nd	nd
Ethylbenzene	0.15	0.11	nd	nd	nd
m-p Xylene	nd	nd	nd	nd	nd
o-Xylene	**0.06**	**0.07**	**0.08**	**nd**	**0.07**
1,2-Dichloroethane	0.013	0.012	0.011	nd	0.014
1,1-Dichloroethane	nd	nd	nd	0.010	nd

[a] not detectable

V.3.1.2 CALCULATION OF THE BIOCONCENTRATION FACTORS

The chemical results of the exposure experiments were used to calculate bioconcentration factors (BCF). Table V. 3.1.2-1 contains the results.

Table V. 3.1.2-1 : Bioconcentration factors of tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, toluene and o-xylene at 16 °C.

Analytes	Bioconcentration factor Mean ± SD
Tetrachloroethylene	0.2 ± 0.1
Trichloroethylene	3.8 ± 3.3
1,1,1-Trichloroethane	0.2 ± 0.1
Toluene	13 ± 6
o-Xylene	0.4 ± 0.1

The BCF values obtained in this experiment indicate that these compounds do not or only moderately accumulate in fish. The experimental BCFs are much lower than the values calculated from the field data shown in table III.4.39 but these field BCFs are in most cases higher than reported values (table III.4.39).
The BCFs of trichloroethylene and 1,1,1-trichloroethane, obtained in the 7 day experiment under the described conditions with sole (*Solea solea*), agree well with some reported values. The BCF of 1,1,1-trichloroethane in bluegill sunfish (*Lepomis macrochirus*) in a 28 day test was 8.9 (Davies and Dobbs, 1984). Moderate bioconcentration was also suggested for trichloroethylene (BCF between 2 and 25 ; Barkley *et al.*, 1980). All these results indicate that trichloroethylene and 1,1,1-trichloroethane have little tendency to concentrate in fish despite their log K_{ow} = 2.42 and 2.49, respectively. Our results indicate that tetrachloroethylene did also not accumulate in the fish although reported BCF values in flathead minnow (*Pimephales promelas*) and bluegill sunfish (*Lepomis macrochirus*) are 38.9 and 49, respectively (Neely *et al.*, 1974 ; Barrows *et al.*, 1980). However, based on the reported log K_{ow} (3.40), a BCF of 226 was estimated for tetrachloroethylene (Lyman *et al.*, 1981). This theoretical value agrees well with the BCFs calculated from the marine data (table III.4.39).
Reported BCFs of toluene vary between 1.67 in Manila clam (*Tapes semidecussata* ; Nunes and Benville, 1979) and 380 in algae (*Chlorella fusca* ; Freitag *et al.*, 1985). The experimental BCF value (13 ± 6) obtained in this study is similar to that reported for eel (*Anguilla japonica* ; Ogata and Miyake, 1978) but the BCFs calculated from the field data in Whiting (*Merlangius merlangus*) and dab (*Limanda limanda*) are higher (table III.4.39). Similarly, our experimental findings that o-xylene also has no tendency to accumulate in fish contradicts the field data in table III.4.39. The mean field BCFs of toluene and o-xylene in fish do not show important differences and this is in agreement with their almost similar log K_{ow}.
One explanation for the differing experimental and field BCFs might be that the experimental values are underestimated due to the continuous administration of product during the experiment combined with a high air/water and low water/fish exchange rate or high depuration rate so that a condition of non-equilibrium is established which may result in too high water and too low tissue concentrations of the compounds. A low water/fish exchange rate of the compounds should however not be expected from their log K_{ow} values.
In the case of toluene high depuration rates have been reported (Korn *et al., 1977*) and differences in metabolism of toluene with temperature have been observed. Metabolism was greater at 12 than 4°C (Thomas and Rice, 1986). Differences in metabolic rates may thus also contribute to the differences between the "*in vitro*" and field BCFs of toluene. The tank experiments were performed at 16 °C and this temperature is usually much higher than the bottom water temperatures on the Belgian continental shelf. In early spring bottom water temperatures on the Belgian continental shelf usually decrease to values between 2 and 4 °C (RVZ, unpublished results).

V.3.2 BIOLOGICAL EFFECTS

V.3.2.1 BIOCHEMICAL ANALYSIS

V.3.2.1.1 EROD

Hepatic EROD activities were measured in sole exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene during 7 days. In all cases the EROD remained undetectable although the lower detection limit is approx. 10 pmoles resorufin produced per min. and per mg protein. Since basal activities were undetectable, no inhibitory effects of the contaminants could be examined. Inhibitory interactions of tetrachloromethane with β-naphthoflavone-mediated P450 induction have been demonstrated in winter flounder (*Pseudopleuronectes americanus*) (Kleinow *et al.*, 1988).
The examined contaminants did not exert any inducing effects neither. Polyaromatic hydrocarbons are well known inducers of hepatic EROD (Goksøyr *et al.*, 1991 ; Addison *et al.*, 1994 ; Monosson and Stegeman, 1994) and benzene is converted via a cytochrome P450-mediated pathway to benzene oxide which is transformed by epoxide hydatase to the 1,2 dihydrodiol (Gonasun *et al.*, 1973). Benzene induces benzene hydroxylase and the inductive pattern of the enzyme is influenced by the addition of methyl groups to benzene (Pathiratne *et al.*, 1986). Benzene did not induce aniline hydroxylase or aminopyrine N-demethylase, whereas toluene and xylene did (Post and Snyder, 1983). Our results now indicate that EROD in sole is also not induced by toluene and xylene although that xylene has been shown to be an inducer of EROD, n-hexane 2-oxidase and benzo[a]pyrene 4,5-hydroxylase in rats when administered by inhalation (Toftgård and Nilsen, 1982).

V.3.2.1.2 GLUTATHIONE S-TRANSFERASE

shows the raw data of the specific hepatic glutathione S-transferase activities in sole exposed to low doses of tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene during 17 days, compared to non-exposed animals.
The results in figure V.3.2.1.2.-1 clearly show that tetrachloroethylene, trichloroethylene and 1,1,1-trichloroethane did not affect the hepatic glutathione S-transferase activities. This was confirmed by Kruskal-Wallis nonparametric ANOVA and convenient multiple comparison tests. A similar observation was obtained with chloroform that did not influence UDP-glucuronosyltransferase (Castren and Oikari, 1987). Although it was also expected that both o-xylene and toluene exerted inducing effects on the detoxification enzyme, statistical analysis did not reveal any significant differences between the data sets from exposed and non-exposed fish. It is clear that the enlarged standard deviations in the latter data sets mainly influenced the statistical analysis. Unequivocal conclusions imply that more long-term studies are needed on

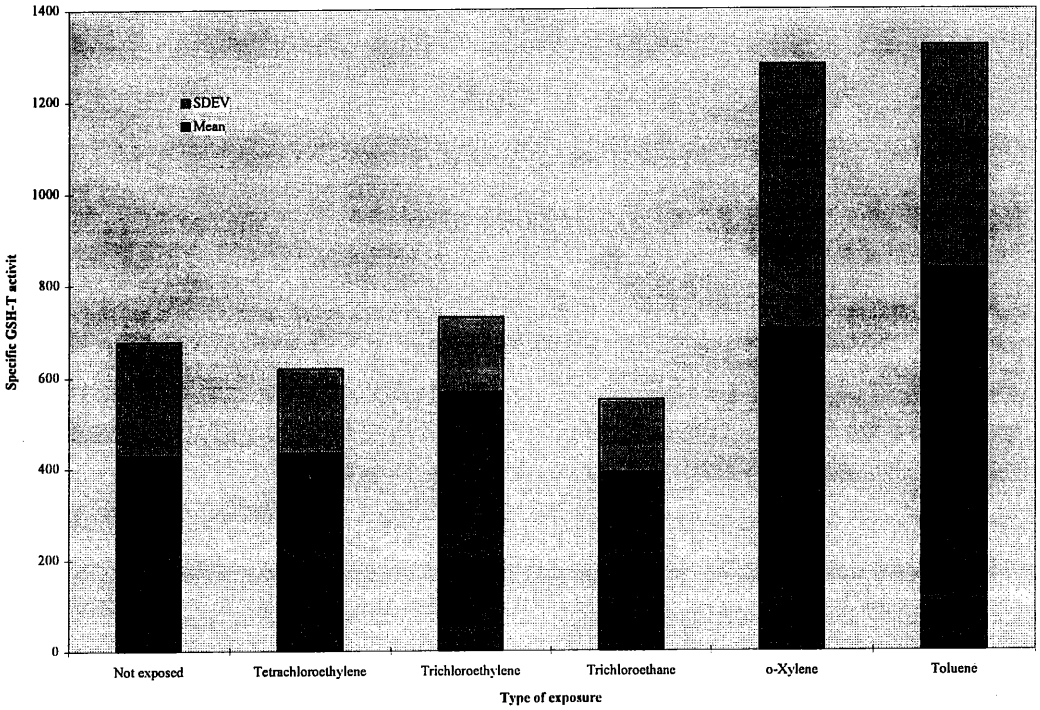
the possible effects of substituted benzenes on phase II biotransformation enzymes especially because benzene readily induces conjugating enzymes (Kalf *et al.*, 1987).

Table V. 3.2.1.2-1 : Hepatic glutathione S-transferase activities in sole (*Solea solea*) exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene compared to non-exposed animals.

Type of exposure	dA/min.[a]	mg prot.[b]	Specific activity [c]
Not exposed	0.021	0.02	102.83
	0.113	0.03	430.96
	0.085	0.02	423.91
	0.114	0.03	381.00
	0.062	0.01	803.75
Tetrachloroethylene exposed	0.115	0.02	546.62
	0.093	0.01	669.90
	0.056	0.01	451.53
	0.046	0.02	238.20
	0.026	0.01	265.00
Trichloroethylene exposed	0.054	0.02	369.28
	0.088	0.01	639.57
	0.298	0.04	848.92
	0.048	0.01	489.23
	0.042	0.01	511.74
	0.182	0.03	543.29
1,1,1-Trichloroethane exposed	0.032	0.01	230.50
	0.092	0.02	406.32
	0.141	0.02	651.11
	0.041	0.01	324.14
	0.086	0.03	340.82
o-xylene exposed	0.081	0.03	286.04
	0.099	0.05	202.37
	0.196	0.03	740.54
	0.047	0.04	118.12
	0.09	0.01	648.29
	0.198	0.01	1645.61
Toluene exposed	0.207	0.04	606.75
	0.198	0.01	1680.06
	0.176	0.03	671.24
	0.07	0.02	444.92
	0.148	0.02	761.53

a increase in extinction per minute
b protein content of the incubation mixture
c specific glutathione S-transferase expressed as nmoles S-2,4 dinitrophenylglutathion formed per min. and per mg protein

Figure V. 3.2.1.2-1 : Specific hepatic glutathion S-transferase activities in sole (*Solea solea*) exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene compared to non-exposed animals.



V.3.2.1.3 ACETYLCHOLINESTERASE

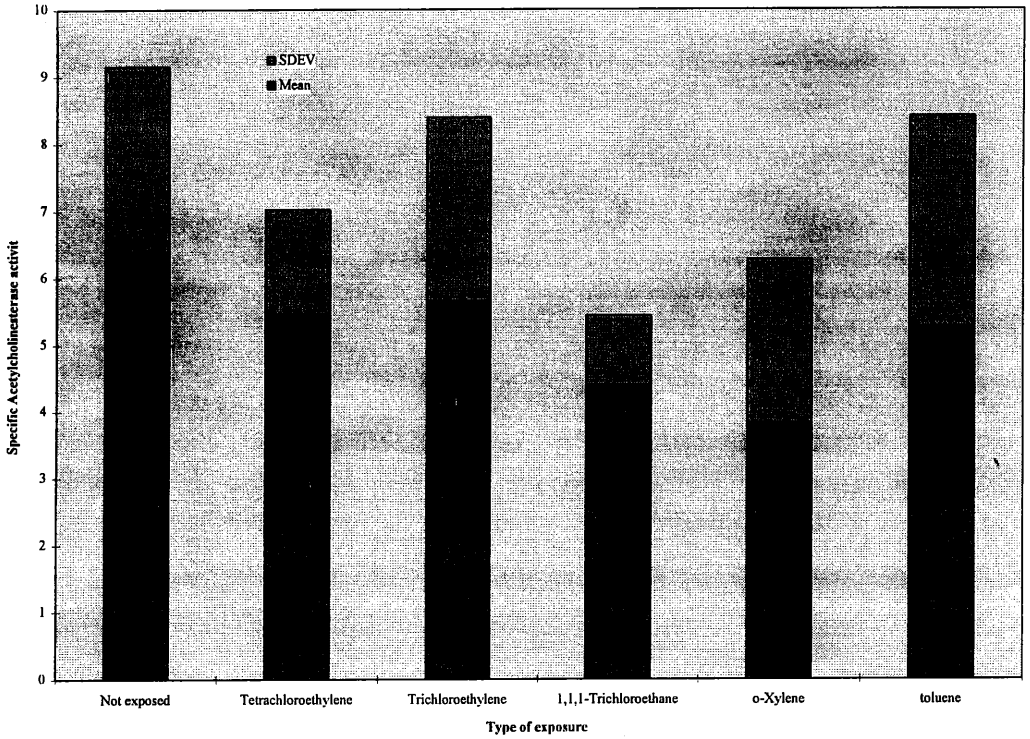
Table V. 3.2.1.3-1 shows the specific hepatic acetylcholinesterase activities in sole exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene during 7 days, compared to non-exposed animals.

Table V. 3.2.1.3-1 : Specific hepatic acetylcholinesterase activities in sole (*Solea solea*) exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene compared to non-exposed animals.

Type of exposure	dA/30min.[a]	mg prot.[b]	Specific activity [c]
Not exposed	0.205	0.04	4.82
	0.263	0.05	4.81
	0.25	0.04	5.98
	0.314	0.06	5.04
	0.181	0.02	11.26
Tetrachloroethylene exposed	0.143	0.04	3.26
	0.172	0.03	5.95
	0.151	0.03	5.84
	0.192	0.04	4.77
	0.153	0.02	7.49
Trichloroethylene exposed	0.156	0.03	5.12
	0.252	0.03	8.79
	0.199	0.07	2.72
	0.359	0.02	17.56
	0.14	0.02	8.19
	0.24	0.07	3.44
1,1,1-Trichloroethane exposed	0.114	0.03	3.94
	0.168	0.05	3.56
	0.183	0.05	4.06
	0.163	0.03	6.19
	0.223	0.05	4.24
o-xylene exposed	0.129	0.06	2.19
	0.223	0.10	2.19
	0.205	0.06	3.72
	0.124	0.08	1.50
	0.16	0.03	5.53
	0.197	0.03	7.86
Toluene exposed	0.307	0.07	4.32
	0.276	0.02	11.24
	0.234	0.05	4.28
	0.134	0.06	2.15
	0.13	0.03	3.97
	0.229	0.04	5.66

a increase in extinction per 30 minutes
b protein content of the incubation mixture
c specific acetylcholinesterase activities expressed increase in extinction per 30

Figure V. 3.2.1.3-1 : Specific hepatic acetylcholinesterase activity in sole (*Solea solea)* exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene compared to non-exposed animals.



Processing of the data sets by Kruskal-Wallis nonparametric ANOVA and convenient multiple comparison tests did not reveal any differences. However sublethal levels of toluene have been shown to inhibit acetylcholinesterase in exposed tilapia (*Sarotherodon mossambicus*) and indicated impairment of the nervous function (Dange and Masurekar, 1981).

V.3.2.2 BEHAVIOURAL ANALYSIS OF :

V.3.2.2.1 SWIMMING BEHAVIOUR

The fish remained embedded in the sediment most of the time. No notable avoidance and no abnormal swimming behaviour were observed.

V.3.2.2.2 OPERCULAR BEATS

Table V. 3.2.2.2-1 contains the results of the respirometrical analysis during the exposure experiments.

Table V. 3.2.2.2-1: Opercular beats per minute of sole (*Solea solea*) exposed to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, o-xylene and toluene compared to non-exposed animals.

Type of exposure	1 day	3 days	5 days
Not exposed	20	26	22
	28	26	28
	25	23	22
	19	30	26
	26	22	25
Tetrachloroethylene exposed	23	21	30
	24	22	29
	28	21	24
	20	26	23
	23	26	25
Trichloroethylene exposed	24	21	22
	24	24	28
	22	23	27
	24	24	22
	25	27	23
1,1,1-Trichloroethane exposed	28	26	29
	26	26	27
	24	22	24
	24	28	22
		20	
o-xylene exposed	21	25	23
	27	23	25
	26	27	26
	23		25
			27
Toluene exposed	20	28	19
	27	26	27
	24	23	27
	23	24	25
	25	23	26

Kruskal-Wallis nonparametric ANOVA and convenient multiple comparison tests did not reveal any differences between the data sets. These results indicate that the homeostatic costs in all tanks were similar, even in the toluene-exposed tank where the fish were severely affected (see section 3.2.3).

V.3.2.3 OCCURRENCE OF EXTERNAL VISIBLE DISEASES AND LIVER LESIONS

Exposed fish and controls were examined for the presence of externally visible diseases. Special attention was paid to the eventual occurrence of body surface lesions (ulcers, hyperplasia pigment anomalies, lipid metabolic disorder and fin rot), (X-cell) gill lesions and liver nodules.
No body surface lesions were found in control fish nor after exposing fish to tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane and o-xylene during 7 days. The gills were normal without swellings or discolorations. No abnormalities were observed on the liver surface.
Fish exposed to toluene during 7 days exhibited serious skin disruptions as shown in fig V.3.2.3-1. The lesions were situated anywhere on the upper body surface and did not occur on the bottom surface. The lesions were in some cases spread over large areas and were recognised as slightly raised, opaque, creamy white areas with sometimes haemorrhagic centres. In advanced lesions scale loss was observed.
The disease differs significantly from known macroscopic body surface lesions such as epidermal hyperplasias/papillomas, skin ulcers or other skin disruptions and it is therefore assumed that toluene caused the effect and not other primary causes such as viral or bacterial infections. On the other hand, a causal relationship with toluene is difficult to establish because of two reasons : (1) the water and sediment concentrations of the compound remained undetectable (< 0.2 ng/ml) while the water concentrations of toluene in tanks exposed to other compounds were significantly higher (see Table V. 3.1.1-2) and, (2) other compounds are also present in detectable amounts. However the toluene concentrations measured in muscle tissue were significantly ($p = 0.0021$) higher in the toluene-exposed fish compared to the other. It may therefore be concluded that at least a part of the total administered amount of toluene (155 mg) was available to the fish. The low concentrations in fish and water phases can then be explained by enhanced biotransformation of the compound although that this was not reflected in enhanced EROD (3.2.1.1) and/or changed glutathione S-transferase (3.2.1.2) metabolism. An important drawback in the study on effects of contaminants on the phase I metabolism capacities in fish is the lack of knowledge on cytochromes P450 types other than the P450IA1 in fish. The absence of EROD induction is therefore no closely-reasoned evidence that no compound breakdown was initialised. Indicative of phase I biotransformation of toluene and o-xylene are the seemingly raised glutathion S-transferase activities measured in toluene and o-xylene-exposed fish (figure V.3.2.1.2-1). However, inductive effects of toluene and o-xylene were not confirmed by statistical analysis.
No distortions of the bottom surfaces of the fish were observed. The sand bed seems to have protected that part of the skin from injury since the fish were layered on and in the sand bed most of the time. This protective phenomenon can be explained by the absence of toluene in the sand phase in detectable amounts.
In view of the serious injuries and the occurrence of large affected areas of the skin surface it may be concluded that the injury might proliferate by secondary viral and/or bacterial infections and finally result in irreversible harm to the organism. The role of toluene in sublethal pollution should therefore be further examined at concentrations that approach those occurring in the (marine) environment despite the lowest

cytotoxicity of toluene, measured by reduced lysosomal uptake of neutral red, compared to other substituted monoaromatic compounds. The rank order of cytotoxicity is methyl mercury > pentachlorophenol > 2,3,5,6-tetrachlorophenol > 2,3,5-trichlorophenol > 2,3-dinitrotoluene > 2,4,6-trichlorophenol > 2,4-dichlorophenol > 2,4-dichlorotoluene > 6-chloro-3-hydroxytoluene > o-chlorotoluene > 4-chlorophenol > 2,-chlorophenol , 2,4-dimethylphenol > 2,4-dinitrophenol > 4-nitrophenol > 3-methylphenol > phenol > toluene according to Babich and Borenfreund (1987).

Figure V. 3.2.3-1: Upper skin surface of sole exposed to toluene. Note the milky white areas on the skin.

V.4. SUMMARY AND CONCLUSIONS

The bioavailability of the chlorinated hydrocarbons tetrachloroethylene, trichloroethylene and 1,1,1-trichloroethane and the monoaromatic compounds toluene and o-xylene were studied in sole (*Solea solea*). The compounds were introduced in the water tanks via the air input so that exchanges between air, water, sediment and fish could be studied. After 7 days exposure, water, sediment and fish samples were chemically analysed and sublethal effects studied. Special attention was paid to the bioaccumulation from the water since accumulation via feeding was reported to be unimportant.

The chemical results revealed that bioconcentration did not to moderately occur. These values differ thoroughly from the bioconcentration factors calculated from the field data. The latter values may be expected more realistic as the octanol-water partition coefficients of the compounds allow to suggest a certain bioconcentration to occur. A state of non-equilibrium may be the explanation for the low bioconcentration during the *in vitro* experiment resulting in too high water and too low muscle tissue concentrations.

Sublethal effects were studied at the biochemical level by the measurement of ethoxyresorufin O-deethylase, glutathione S-transferase and acetylcholinesterase, swimming behaviour, homeostatic cost (measurement of opercular beats) and the occurrence of external visible diseases and liver lesions. No differences in the biochemical results, swimming behaviour, opercular beats and livers were observed between exposed fish and controls. There are indications of raised glutathion S-transferase activities in toluene and o-xylene-exposed fish. However, inductive effects of toluene and o-xylene were not confirmed by statistical analysis.

External visible lesions were solely present after exposure to toluene. All test animals were affected but the lesions were only present on the upper site (site towards the water phase) and not on the lower site. It seems that the sediment layer protected the lower site of the fish, a phenomenon that can only be explained by the absence or unavailability of the administered compounds in the sediment phase. The sediment concentrations of the compounds were indeed totally undetectable. The results also imply that the lesions were caused by direct interactions between water components and the body surface. The lesions occurred on large areas of the upper body surface and were recognised as raised, milky white opaque areas with sometimes haemorrhagic centres. A more evolved stadium was the loss of scales. The lesions did not correspond to know external visible diseases such as epidermal hyperplasias/papillomas or skin ulcers. It may thus be excluded that the main origin is viral or bacterial. The seriousness of the lesions allows to suggest that proliferation may be lethal to the animal. This observation should lead to more long-term studies of the sublethal effects of toluene especially because the compound concentrations in the phases were realistic.

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