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Cytogenetic studies of PCB77 on brown trout (*Salmo trutta fario*) using the micronucleus test and the alkaline comet assay

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Polychlorinated biphenyls (PCBs) are stable pollutants, which can be found in almost every compartment of terrestrial and aquatic ecosystems. They are very lipophilic and therefore have the potency of accumulating in the fat stores of animals. The mechanisms by which PCBs exert their adverse effects are still unclear. It is known that PCBs induce some important biotransformation enzymes, but their mutagenic properties are still controversial. The DNA breakage and clastogenic potency of a planar PCB77 (3,3′,4,4′-tetrachlorobiphenyl) was determined *in vivo* in fish, using the single cell gel electrophoresis or comet assay and the micronucleus test, on erythrocytes of the brown trout exposed for 3, 9 and 14 days to initial PCB concentrations of 780 and 918 pg/ml, dissolved in the water. Blood was taken by a caudal puncture and the erythrocytes were either deposited in an agarose gel (0.6%) for the comet assay or smeared directly on slides for the micronucleus test. Five fish were studied per treatment and 50 and 2000 erythrocytes per concentration and per animal were analysed for the comet assay and the micronucleus test respectively. Ethyl methanesulphonate (EMS) at a concentration of 25 mg/l water was used as a positive control. Although EMS induced a statistically significant increase of single strand breaks in the comet assay, in neither of the two tests used, were mutagenic effects due to PCB exposure observed.

Introduction

Polychlorinated biphenyls (PCBs) are listed by the US Environmental Protection Agency as 'priority pollutants' because they have been shown to be both toxic and carcinogenic (Butterworth *et al.*, 1995). Although PCBs are known to be toxic, especially the coplanar ones with two para-, at least two meta- and no ortho-substituents (De Voogt *et al.*, 1990), the mechanisms by which they exert their toxic effects has yet to be elucidated.

The use of PCBs has been limited since the 1970s, however PCBs can still be found in almost every compartment of the ecosystem. This pollution, almost without exception, originates from the use of technical mixtures of PCBs, or commercial formulations containing such mixtures. PCBs are extremely persistent in the environment and like many other organochlorines of similar nature are widely spread among living organisms all over the world (Falandysz, 1994). Their biochemical and toxicological properties have already been studied extensively in terrestrial and aquatic organisms, however only a few studies have been conducted to study the mutagenicity of these organochlorines.

Planar PCBs occur as only minor constituents in commercial mixtures, but because of their extremely low water solubility (high lipophilicity) they can accumulate in great amounts in aquatic species, such as fish. Several authors have assumed that organic compounds with a high lipophilicity have a tendency to be taken up by fish via their food, as well as directly from the water (Scura and Thieilacher, 1977). In this report attention was focused on one of the most toxic planar PCBs, i.e. PCB77 (3,3′,4,4′-tetrachlorobiphenyl), which has been found in fish tissue (Hong *et al.*, 1992). Sargent *et al.* (1989) mentioned the clastogenic effect of PCB77 (3,3′,4,4′-tetrachlorobiphenyl) *in vitro* in human lymphocytes, using the chromosome aberration test.

This work was aimed at the study of potential clastogenic effects of PCB77 in brown trout (*Salmo trutta fario*), using the classic micronucleus (MN) test and the alkaline single cell gel electrophoresis (SCGE) or comet assay. The MN test was performed essentially to assess which proportion of DNA breakage seen in the comet assay would lead to chromosome breakage and/or loss.

Brown trout were used as test animals because this species is very sensitive for induction of P450-enzymes by chemicals (e.g. PCBs) (Payne *et al.*, 1987).

The micronucleus test has already been used in genotoxicology studies with fish (Hooftman and Vink, 1981; Hooftman and de Raat, 1982; Das and Nanda, 1986; Al-Sabti, 1986a,b, 1992, 1994; Metcalfe, 1988; Hughes and Hebert, 1991; De Flora *et al.*, 1993; Bahari *et al.*, 1994). The alkaline comet assay (pH >13) was first introduced by Singh *et al.* (1988). This easy and sensitive technique is capable of detecting a wide range of DNA damage including: single strand DNA breaks, any lesion capable of being transformed into a single strand DNA break at the alkaline pH used (i.e. alkali-labile sites), DNA crosslinks (Tice, 1995) and incomplete excision repair events (Gedik *et al.*, 1992; Green *et al.*, 1992). This assay has proven to be extremely useful in the area of genetic toxicology and environmental biomonitoring (for review Fairbairn *et al.*, 1994; Tice, 1995).

This study showed that the highly toxic PCB (IUPAC number 77) does not induce either micronuclei (double strand breaks and/or chromosome loss) or tails with the comet assay (single strand breaks) in erythrocytes of the brown trout.

Materials and methods

Chemicals

Ethidium bromide, Giemsa, EDTA, Tris(hydroxymethyl)-aminomethane, sodium hydroxide, sodium chloride and ethyl methanesulphonate (EMS), were obtained from Merck (Darmstadt, Germany). Low melting point agarose and normal melting point agarose were purchased from Gibco BRL. *N*-lauroylsarcosine, DMSO and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pure PCB77 powder was purchased from Schmidt (Amsterdam, The Netherlands).

Fish

Immature brown trout (*Salmo trutta fario*) aged 10–15 months old, weighing on average 20.7 ± 2.3 g with an average length of 13.0 ± 1.59 cm, were obtained from a fish hatchery, near Waver, Belgium. The fish were kept in 100 l aquarium and fed daily with artificial fish food (Gold Medal). The water temperature was controlled at 11 ± 1°C.

Treatment of the fish

For the standardization of the techniques a well known clastogen (EMS) was used. EMS is a direct acting mutagen. It induces comets in human lymphocytes *in vitro* (Belpaeme *et al.*, 1996) and micronuclei in fish erythrocytes (Hoofman and De Raat, 1982; Al-Sabti, 1994). It was used as a positive control to define the experimental conditions for the comet assay in fish erythrocytes; therefore different concentrations (25, 50, 100 and 200 mg EMS/l water) and treatment times (3, 9 and 14 days) were compared. The water with the test substance was renewed every 24 h. For later experiments 25 mg EMS/l was used as a positive control.

As far as PCBs are concerned, we attempted to achieve the highest concentrations soluble in water: 5.4 mg PCB77 was dissolved in 3 ml hexane and added to a small ball of cotton. The impregnated cotton was then put in a glass tube through which water was driven by a pump-system. Water samples (100 ml) were taken to analyse the PCB77 concentration in the water, until a stable concentration was reached (after ~7 days). After the concentration had stabilized the fish were put in the aquarium for treatment. After treatment three to five fish and 250 ml of water were sampled for analysis at each time interval. The fish were not fed during the exposure.

PCB77 measurements

In fish: the fish livers were dried with a lyophilizator for 24 h at 20°C. The dry samples were weighed and extracted for lipids and lipophylic substances with hexane/acetone (9/1) for 10 h and purified with Florisil columns eluted with hexane/ether (1/1).

In water: 250 ml of water was taken at each sampling time, and extracted twice with 10 ml hexane. The extract was then evaporated to 1 ml for analysis.

The final samples of tissue and water were analysed by an electron capture gas chromatograph (on column injection, dual splitting into two capillary columns with different polarities (DB 17-01 and DB 5-02) connected to two electron capture detectors, automatic samplers and automatic integrater systems; Carlo Erba Instruments, GC 8000 series).

Alkaline comet assay

The alkaline comet assay was performed under yellow light as described by Tice *et al.* (1992) and by Belpaeme *et al.* (1996) with some modifications. Briefly, 10 µl of diluted (100×) fresh fish blood was put in the second gel layer (100 µl 0.5% LMP agarose). This layer was sandwiched between a bottom layer (300 µl 0.6% NPM agarose) and a top layer (100 µl 0.5% LMP agarose). After preparation the slides were put in the electrophoresis buffer for 40 min, to allow unwinding of DNA. Electrophoresis (300 mA, 0.7 V/cm) was performed in the same buffer for 20 min at 18°C.

Before analysis the cells were stained with 100 µl ethidium bromide (20 µg/ml water) for 10 min, rinsed with distilled water and kept in a moist chamber at 4°C. For analysis, images from a Zeiss fluorescence microscope (magnification ×300) were captured with an air-cooled camera (Photonic Science, type coolview) on a DT 2855 frame grabber type. Fifty non-overlapping images were selected randomly on the slides. The DNA content of the image was calculated from the sum of light intensities of all pixels in that area. For each separate image the background intensity was subtracted from the image intensity. The comet length was measured by defining manually on the screen the centre of the nucleus, the leading edge of the nucleus and the end of the tail. All the data were transferred to, and analysed with a Macintosh Quadra 650. The features extracted from the measurements were essentially the 'DNA content' defined as the total fluorescence associated with an image, 'tail length' measured from the centre of the nucleus to the end of the tail and 'tail moment' defined as the fraction of DNA in the tail (DNA content in tail/DNA content of the image) multiplied by the tail length.

Fish micronucleus test

Blood was collected with a small syringe, wetted with heparin, by cardiac puncture. One drop of blood was used to make smears. After fixation in pure methanol for 20 min, the prepared slides were left to dry at room temperature. Finally the smears were stained with 20% Giemsa in Sorenson buffer for 15 min. A light microscope was used to scan the blood smears under oil immersion at ×100. The number of micronucleated erythrocytes (MN) per 1000–2000 erythrocytes for each fish was determined. The number of MN was expressed as number per thousand (‰) MN. The criteria for scoring the micronuclei were: no connection with the main nucleus, and area ~1/10–1/30 smaller than the main nucleus.

Statistics

For the comet assay differences between treatments were determined using the Friedman analysis of variance (ANOVA) test for repeated measurements, which is based on the median and the distribution of the measurements. For the MN test, statistical differences were determined with the χ^2 test.

Results

Preliminary experiments

Before starting the experiments with PCB77, the fish exposure conditions were optimized, for the micronucleus test and the comet assay, using a well-known mouse clastogen, EMS. Fish

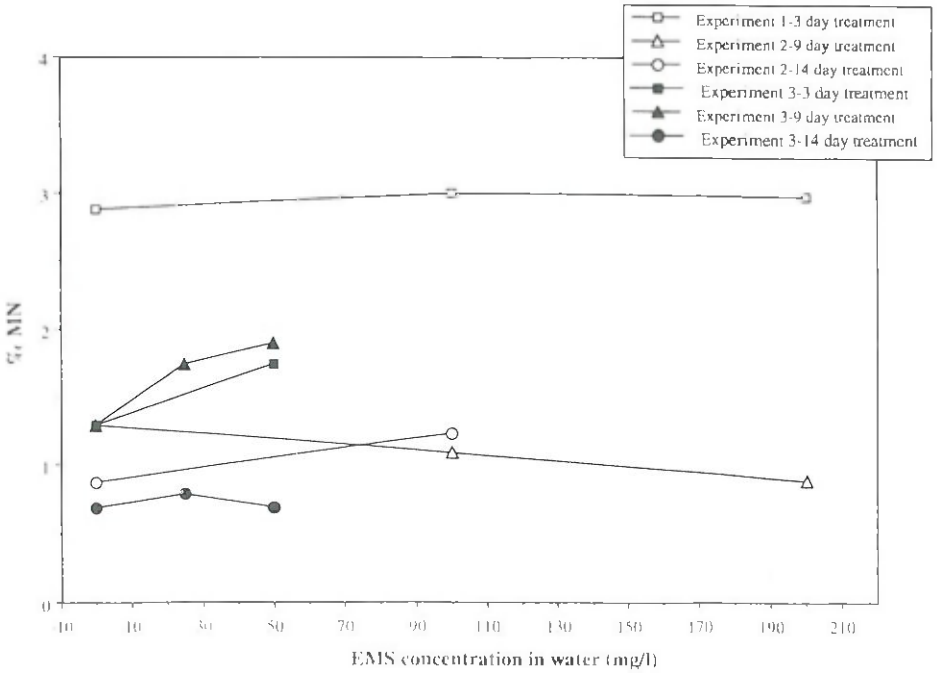

Fig. 1. Mean number per thousand (‰) micronuclei (MN) in fish erythrocytes exposed to EMS dissolved in water. In experiment 1, at least 1000 erythrocytes were counted, and in experiments 2 and 3, at least 2000 erythrocytes per fish. Each point in the graph represents the mean of four or five fish.

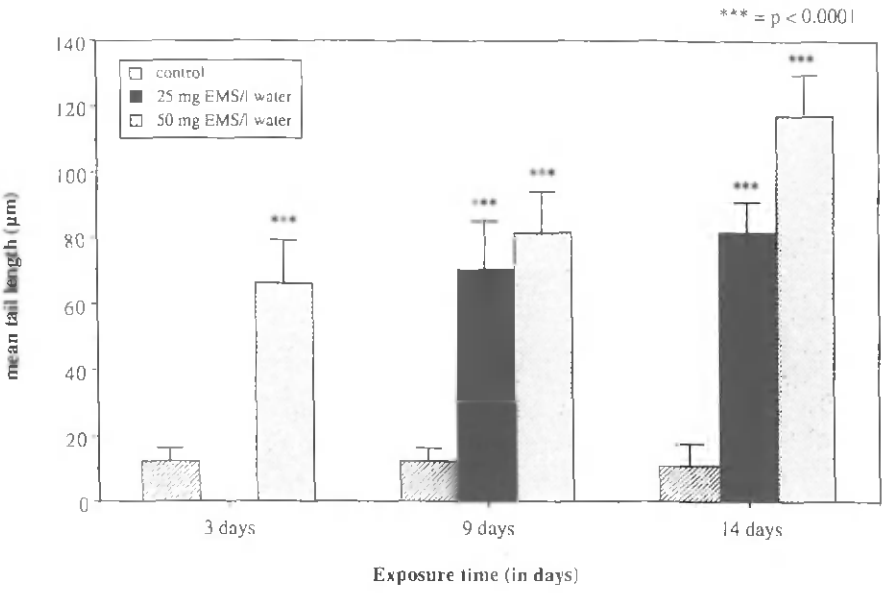

Fig. 2. Mean tail length measured in fish erythrocytes after exposure to EMS dissolved in water. Fifty cells were measured per animal. The bar represents the mean of three fish (i.e. 150 cells) with the SD. The tail length was measured from the centre of the nucleus to the end of the tail.

were exposed for 3, 9 and 14 days to 25, 50, 100 and 200 mg EMS/l water. No statistically significant increase in micronucleus frequencies was seen after EMS treatment of the animals (Figure 1). However, EMS was clearly positive in the comet assay, where a time-dependent and dose-dependent increase in tail length was observed (Figure 2). In these preliminary experiments only tail length was taken into account. For EMS concentrations >100 mg/l the erythrocyte DNA was completely destroyed, making the measurements for the comet assay difficult, if not impossible.

Cytogenetic effects of PCB77 on the brown trout

Fish were exposed for 3, 9 or 14 days to initial PCB77 concentrations of 780 and 918 pg PCB77/ml water, in experiments 1 and 2 respectively. As a reference mutagen, 25 mg EMS/l water was used. The PCB77 concentrations measured in the water and the fish livers are given in Table I.

The PCB77 concentration in the water decreased after the fish were placed in the aquarium; this was probably due to absorption of the PCBs to the fish body. In experiment 1 an unexplained increase was observed at day 9 of the experiment. In both experiments very high PCB77 concentrations were found in the fish liver. In experiment 1 the measured concentration increased with treatment time. In experiment 2 the concentrations were comparable after 9 and 14 days of treatment.

Figure 3a and b shows that no increase in micronucleus frequencies was observed after exposure to PCB77 or EMS. The actual micronucleus frequencies of each fish in all the experiment are shown in Table II. In Figure 4 a picture of a fish erythrocyte with and without a micronucleus is shown.

Data for the comet assay are given in Table III and Table IV for experiments 1 and 2 respectively. PCB77 treatment did not lead to a significant increase in either tail length or tail moment, whereas EMS clearly induced an increase. When viewing the stained erythrocytes under the microscope the nuclei were identical to those of the control cells. This shows that the PCB concentrations were not cytotoxic.

Table I. PCB77 concentrations for two parallel experiments, measured in the water (pg PCB77/ml water) and in the fish livers (ng PCB77/g liver fresh weight)

Exposure time (days)	Experiment 1		Experiment 2	
	Water (pg PCB77/ml)	Liver (ng PCB77/g)	Water (pg PCB77/ml)	Liver (ng PCB77/g)
0	780	–	918	–
3	396	296.0	–	–
9	1027	1054.9	429	987.2
14	–	–	343	626.5

The values given for the livers are obtained from the mean of four or five pooled fish livers.

Discussion

Since the 1930s, researchers have tried to find a causal link between chemical contamination and acute toxicity in fish populations (Macek, 1980). The first genetic toxicology studies were conducted at the beginning of the 1970s (Ames *et al.*, 1973; Miller and Miller, 1975; McCann *et al.*, 1975; Kligerman *et al.*, 1975; Hooftman and Vink, 1981; Prein *et al.*, 1987), and many waterborne pollutants have cytotoxic properties which have been shown to cause enhanced frequencies of chromosomal aberrations in fish (Kligerman, 1982).

Cytogenetic methods are probably the most sensitive and efficient ways to detect the effects of genotoxins. However, fish are not normally very popular for certain cytogenetic techniques (e.g. chromosome aberration test, sister chromatid exchanges), because of their large number of small chromosomes. On the other hand, fish provide an excellent source of material for the study of the mutagenic and/or carcinogenic potential of water samples since they are aquatic vertebrate organisms that can metabolize, concentrate and store waterborne pollutants (Al-Sabti, 1991). Several other genetic techniques do not need karyotyping, and hence the number and size of the chromosomes does not matter, e.g. the micro-

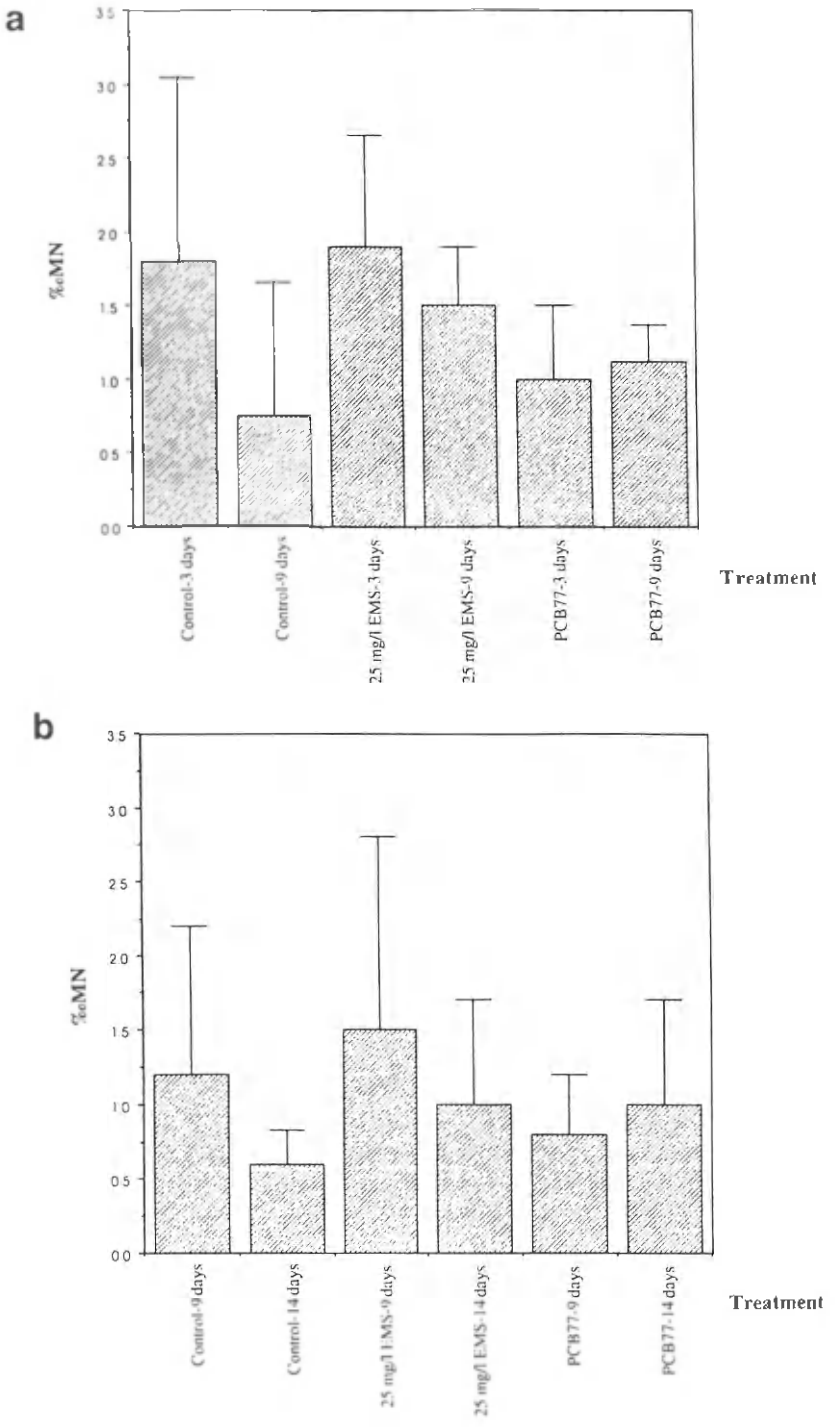

Fig. 3. Mean number per thousand (‰) MN in fish erythrocytes exposed to 25 mg/l EMS or 780 or 918 pg PCB77/ml water in (**a**) experiment 1 and (**b**) experiment 2 respectively. In both experiments at least 2000 erythrocytes were counted per fish. Each bar represents the mean of four or five fish.

nucleus test and the comet assay. In the comet assay many fish tissues can be used, e.g. brain, liver, gills, but as the use of non-destructive methods, i.e. without killing the animal, is highly preferred (Fossi, 1994) we tried to develop the comet assay on blood.

Our aim was to study the possible clastogenic potency of a planar PCB congener (PCB77), using the micronucleus test and the comet assay.

To optimize the treatment conditions a known clastogen was used (EMS) since it had already been shown to induce micronuclei in eastern mudminnow, *Umbra pygmaea* (Hooftman and de Raat, 1982; Metcalfe, 1988), in brown bullheads, *Ictalarus nebulosus* (Metcalfe, 1988) and in *Xenopus* larvae (Van Hummelen *et al.*, 1989). However, no data were available concerning the comet assay in fish.

It is difficult to control the concentration of PCB77 in the water, because this biphenyl compound is extremely hydrophobic and absorbs very quickly to the glass walls and other substances present in the aquarium (faeces, fish, etc.). By first dissolving the PCBs in hexane and putting this solution

Table II. Actual MN frequencies (number per thousand) of each fish and the mean MN frequencies in all the experiments. The initial PCB77 concentrations were 780 and 918 ng/ml in experiments 1 and 2, respectively

Treatment	Frequencies of MN (‰) per fish. Four or five fish per treatment in each experiment									
	EMS						PCB			
	experiment 1 3 days	experiment 2 9 days	14 days	experiment 3 3 days	9 days	14 days	experiment 1 3 days	9 days	experiment 2 9 days	14 days
Control per fish (‰)	3.4,5.2,1	0.5,1.5,1.2,5.1	1.0,5.1,5.0,5	3.5,1.0,5.0,0.5,1	3.5,1.0,5.0,5.1	0.1,0.5,0.5,1.5	1.5, 1, 1.5, 4, 1	0, 2, 1, 0	0.5, 0, 2.4, 2, 1	0.5,0.5,1.0,5.0,5
mean (‰) SD	2.87 ± 1.7	1.30 ± 0.8	0.87 ± 0.5	1.30 ± 1.2	1.30 ± 1.2	0.70 ± 0.6	1.80 ± 1.2	0.75 ± 1	1.18 ± 1.0	0.60 ± 0.2
25 mg EMS/l per fish (‰)	–	–	–	–	2.3,5.0,1.5	1.1,1.0,1	2.1,2.5,1.5,2.5	1.1,5.2,1.5	0.5,3,1	0.5,1.5
mean (‰) SD				–	1.75 ± 1.4	0.80 ± 0.4	1.90 ± 0.6	1.50 ± 0.4	1.50 ± 1.3	1.0 ± 0.7
50 mg EMS/l per fish (‰)	–	–	–	1,1.5,4,0.5	1.5,2,3,1.5,1.5	0.0.5,0.5,1,1.5	–	–	–	–
mean (‰) SD				1.75 ± 1.6	1.90 ± 0.6	0.70 ± 0.6				
100 mg EMS/l per fish (‰)	4,2.2.4,3	2.5,1.5,1.0,5.0	1.5,1	–	–	–	–	–	–	–
mean (‰) SD	3.00 ± 1.0	1.10 ± 1.0	1.25 ± 0.4							
200 mg EMS/l per fish (‰)	4,2.9,0.9,2.4	0.5,2.0,5,1.0,5	–	–	–	–	–	–	–	–
mean (‰) SD	2.96 ± 1.3	0.90 ± 0.6								
PCB 77 per fish (‰)	–	–	–	–	–	–	0.5,1.5,1.1.5,0.5	1.5,1.1.1	0.5,1.5,0.5,0.5,1	1.5,1.5,1.5,0.5,0
mean (‰) SD							1.0 ± 0.5	1.12 ± 0.2	0.80 ± 0.4	1.0 ± 0.7

on a cotton ball in a glass tube, a high concentration of PCBs was reached by a flow of water through the tube. The stabilized initial concentrations in the aquarium were 780 and 918 pg PCB77/ml water in experiments 1 and 2 respectively. These concentrations were much higher than those found in natural aquatic systems, for example the highest concentration ever found was 178 pg/l in a German bay (De Voogt *et al.*, 1990).

To check whether the fish had actually had taken up the PCBs, the PCB77 concentrations were measured in the fish livers, a target organ for PCB accumulation. Because of the small size, the livers were pooled before measurement. The measured PCB77 concentrations in the fish livers (Table I), were higher than the concentrations found in the environment, e.g. 37.1 ng PCB77/g in fish (Hong *et al.*, 1992).

In our experiments, the micronucleus frequencies of EMS and PCB77 treated fish were not significantly higher when compared with the controls. This result is rather unexpected because previous reports mention the induction of micronuclei by EMS, in amphibia *in vivo* (Van Hummelen *et al.*, 1989), and in fish (Hooftman and de Raat, 1982; Metcalfe, 1988). This contradiction may be explained in several ways. First the most important condition to be met is that the studied cells should divide. It is known that the mitotic rate of fish erythrocytes is very low (Al-Sabti, 1994), which might also explain the low frequencies of micronuclei in previous reports. Secondly, another possibly explanation for the lack of effect is that micronucleated erythrocytes are destroyed by the immune system, and hence not observed. A third possibility is that the EMS concentrations applied might be cytotoxic for the fish erythrocytes. However, indications for apoptosis in the comet assay were only observed at concentrations >100 mg/l, which does not explain the absence of micronuclei at lower concentrations of EMS, which are capable of inducing comets. The results suggest that the erythrocytes of the brown trout are sensitive to EMS but that the MN test is not a good indicator for DNA breaks in this species under the described experimental conditions. The positive responses observed by Metcalfe (1988) and Williams and Metcalfe (1992) in the MN test in mudminnows, brown bullheads and rainbow trout respectively, were obtained after i.p. injection with EMS, which is not directely comparable to exposure in the water.

Despite these difficulties, the micronucleus test has been recommended as an accurate indicator in environmental studies under laboratory conditions and in the field (Hooftman and Vink, 1981; Hooftman and de Raat, 1982; Landolt and Kocan, 1983; Longwell *et al.*, 1983; Al-Sabti, 1986a,b; Das and Nanda, 1986; Hose *et al.*, 1987; Metcalfe, 1988; Hughes and

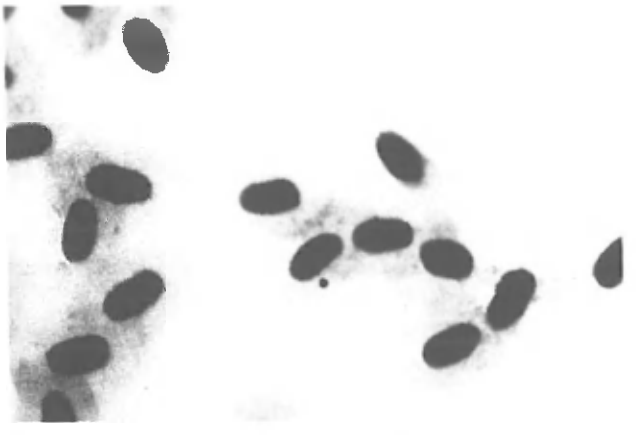

Fig. 4. Trout erythrocytes with and without a micronucleus. The blood smear was stained with Giemsa.

Hebert, 1991; Williams and Metcalfe, 1992; De Flora *et al.*, 1993; Al-Sabti and Metcalfe, 1995). In most of these reports the observed micronucleus frequency was low and many cells had to be counted to find a significant response. The biological significance of these micronucleus results should thus be questioned. Higher significant micronucleus frequencies were found in studies by Al-Sabti (1994a,b) after exposure of fish to selenium, organic and inorganic mercury (1–37‰ MN), chromium (5–41‰ MN) and caesium (maximum 27.5‰ MN), and in a study by Bahari *et al.* (1994) after exposure of fish to γ-radiation and mitomycin C. Al-Sabti mentioned that the mitotic activity in peripheral erythrocytes of fish is low, but that there is relatively little known about the rate of haematopoiesis and erythrocyte replacement in fish species (Al-Sabti and Metcalfe, 1995). This research area should be given special attention in future studies. The duration of the cell cycle in organisms will be dependent on the time needed for DNA replication. Therefore the results of the micronucleus test will be dependent on the species used. Carrasco *et al.* (1990) and Burgeot (1995) considered that the use of the fish micronucleus test was highly questionable as a laboratory or field indicator of biological effects in wild fish exposed to chemical contaminants. No definitive or consistent correlation was found between any observed variation of nuclear morphology and any measured level of chemical contamination. The authors suspect that the extremely low frequencies of the observed erythrocyte nuclear lesions may not have originated from a genotoxic event as a result of exposure to xenobiotic contaminants.

Table III. Results of the comet assay (in μm) in experiment 1 after treatment of fish with PCB77 and 25 mg/l EMS. The initial PCB77 concentration was 918 pg/l. Tail length was measured from the centre of the nucleus to the end of the tail. Tail moment = tail length × relative DNA content of the tail

Exposure time (days)	Treatment median	*n* mean ± SD	Tail length median	Tail length mean ± SD	Tail moment	Tail moment
3	Control	46	9.40	9.38 ± 1.82	2.94	3.13 ± 1.01
		50	8.92	9.02 ± 1.32	2.75	2.69 ± 0.70
		50	9.08	9.08 ± 1.21	3.46	3.58 ± 1.03
	EMS	50	43.08***	43.54 ± 14.80***	29.31***	30.13 ± 11.71***
	(25 mg/l)	48	39.94***	39.27 ± 13.75***	25.85***	26.61 ± 10.84***
		50	51.42***	54.11 ± 16.29***	33.67***	36.82 ± 15.90***
	PCB77	50	9.40	10.51 ± 4.26	3.52	4.07 ± 2.53
		49	9.73	10.42 ± 4.28	3.65	3.99 ± 2.19
		50	9.73	10.15 ± 2.18	3.88	4.08 ± 1.37
9	Control	50	8.43	8.88 ± 1.33	2.97	2.98 ± 0.73
		50	7.63	8.58 ± 4.61	2.62	2.92 ± 2.19
		50	9.18	9.32 ± 3.41	2.84	3.06 ± 1.37
	EMS	49	84.15***	83.94 ± 10.49***	79.30***	78.65 ± 11.41***
	(25 mg/l)	50	81.73***	80.69 ± 7.12***	78.88***	77.42 ± 8.08***
		48	121.28***	119.26 ± 13.51***	116.76***	118.30 ± 22.43***
	PCB77	50	9.74	9.77 ± 1.96	3.00	3.11 ± 0.94
		50	10.06	12.29 ± 1.53	3.81	5.51 ± 7.94
		49	9.08	9.32 ± 1.3	3.94	3.95 ± 1.06

n = number of cells measured per fish.
***$P < 0.0001$ in comparison with control.

Table IV. Results of the comet assay (in μm) in experiment 2 after treatment of fish with PCB77 and 25 mg/l EMS. The initial PCB77 concentration was 918 pg/l. Tail length was measured from the centre of the nucleus to the end of the tail. Tail moment = tail length × relative DNA content of the tail

Exposure time (days)	Treatment	*n*	Tail length median	Tail length mean ± SD	Tail moment median	Tail moment mean ± SD
9	Control	46	10.05	10.14 ± 2.67	3.81	4.13 ± 1.41
		49	9.73	9.44 ± 1.49	4.59	4.62 ± 1.01
		49	10.05	10.64 ± 2.86	4.30	4.891 ± 0.85
	EMS	50	103.35***	105.22 ± 12.19***	99.92***	101.12 ± 12.94***
	(25 mg/l)	50	112.01***	108.11 ± 19.44***	108.84***	102.90 ± 22.85***
		49	80.53***	79.03 ± 8.35***	76.52***	74.66 ± 8.75***
	PCB77	49	10.05	10.19 ± 2.17	4.30	4.44 ± 1.05
		50	10.70	10.26 ± 1.57	4.88	4.81 ± 1.10
		49	10.05	9.75 ± 1.43	3.94	4.16 ± 0.99
14	Control	50	9.89	10.61 ± 3.72	3.49	3.91 ± 1.50
		50	9.44	9.51 ± 1.21	3.42	3.51 ± 0.81
		50	9.73	10.22 ± 3.23	3.46	3.66 ± 1.81
	EMS	48	98.31***	97.97 ± 11.80***	97.08***	97.05 ± 12.13***
	(25 mg/l)	48	108.97***	110.12 ± 12.05***	107.45***	108.33 ± 12.40***
	PCB77	50	9.08	9.94 ± 3.29	3.84	4.20 ± 1.45
		50	10.02	9.95 ± 1.49	4.07	4.01 ± 0.85

n = number of cells measured per fish.
***$P < 0.0001$ in comparison with control.

Two serious problems with the fish micronucleus test will always remain according to Carrasco *et al.* (1990). First, the low frequency of micronuclei and other nuclear lesions would require the use of impractically large sample sizes to assess marginally polluted areas. Secondly, the micronucleus test in fish may not be a sensitive measure of exposure to specific genotoxic chemicals.

Finally, the remaining advantages of the test are that it is cheaper compared with the comet assay, and measures an established chromosomal change.

The results of the comet assay indicated a clear time and dose-dependent increase of induced tail length after EMS treatment of the fish, but no such increase was seen after treatment with PCB77. After 3 days of treatment with 25mg/l EMS a highly significant effect ($P < 0.0001$) was observed. It is important to mention that with the very high concentration of EMS (>100 mg/l) the DNA was so fragmented that the main nucleus was reduced to only a spot, and almost the total amount of nuclear DNA migrated to the tail. This could possibly reflect apoptosis. For this reason, in the following PCB experiments 25 mg/l EMS was used as a positive control. To see an effect in the comet assay there is no need for cell division. This eliminates the problems met with the micronucleus test, so the comet assay could be much more sensitive in ecogenotoxicological studies. It has already been tested on brain, gill, liver and blood tissue of *Medaka*, gill, siphon and mantle tissue of mussels and blood and liver tissue of bullfrog tadpoles (R.Tice, personal communication). This single cell gel electrophoresis assay is waiting now for standardization before it can be used for biomonitoring studies, but it has already proven its usefulness in many other experimental conditions (for review see Fairbairn *et al.*, 1995).

The most important drawbacks of the comet assay are that: (i) sampling errors can occur; (ii) not all mutation types can be detected; (iii) no quantification of the number of breaks is possible; (iv) not all DNA breaks lead to fixed mutations; (v) there is a species variability in response. The advantages are that: (i) information is given at the single cell level, providing information on the intercellular distribution of DNA damage; (ii) only small samples are needed (5000–50 000 cells); (iii) virtually any eukaryotic cell population can be used; (iv) the test is quick, sensitive, simple and low cost. In the future attention should be focused on the optimization and standardization of the comet assay, the relationship between DNA migration and various types of DNA damage and toxic processes which lead to DNA degradation and also on the possibility of preserving tissue for biomonitoring purposes.

PCB77 did not seem to induce any mutations that would induce micronuclei (double strand breaks or chromosome loss) or tails in the comet assay (single strand breaks or alkali labile sites). These data agree very well with the results mentioned by Safe (1989) and Silberhorn *et al.* (1990): PCBs *per se* are non-mutagenic and because they do not form covalent adducts with cellular DNA and are probably non-genotoxic. Several recent studies however point in the direction of mutagenicity: Sargent *et al.* (1989) (human lymphocytes, chromosome aberration test); Meisner *et al.* (1992) (rat bone marrow, chromosome damage); Butterworth *et al.* (1995) (*Drosophila*, recombinogenesis); Jensen (1995) (Chinese hamster ovary cells, c-mitosis test).

Sargent *et al.* (1989) proposed that the clastogenic effects of PCB77, observed in human lymphocytes, may be due to direct intercalation to the DNA (Wassom *et al.*, 1978), or to a cascade of toxic effects associated with binding to the Ah receptor (Poland and Glover, 1980). Other proposed mechanisms were that PCB77 may act through free radical generation or induction. An other explanation for the toxic action of PCBs was given by Al-Sabti and Metcalfe (1995); they proposed that PCBs could inactivate the spindle apparatus.

The best known effect of planar PCBs so far is the induction of the P-450 enzyme system, which could possibly lead to an indirect activation of promutagens present in the cell. A good review of the possible toxic effects of PCBs is given by Safe (1992).

In conclusion, our data indicate that the planar PCB77 (3,3′,4,4′-tetrachlorobiphenyl) does not induce double- or single-stranded breaks detectable with the micronucleus test and the comet assay, in the erythrocytes of brown trout. The absence of clastogenic effects with PCB77 might be extrapolated to other PCBs, however structually different PCBs might have different mutagenic capacities.

The alkaline comet assay or single cell gel electrophoresis assay appears to be much more sensitive than the micronucleus assay as far as ecogenotoxicological studies are concerned (Pandrangi *et al.*, 1995).

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