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Genotoxic effects in the Eastern mudminnow (*Umbra pygmaea* L.) after exposure to Rhine water, as assessed by use of the SCE and Comet assays: A comparison between 1978 and 2005

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Abstract

Surface water used for drinking-water preparation requires continuous monitoring for the presence of toxic compounds. For monitoring of genotoxic compounds fish models have been developed, such as the Eastern mudminnow (*Umbra pygmaea* L.) because of its clearly visible 22 meta-centric chromosomes. It was demonstrated in the late seventies that Rhine water was able to induce chromosome aberrations and sister chromatid exchange in this fish species. Although in vitro mutagenicity studies of the RIWA (Rhine Water Works, The Netherlands) have shown that the genotoxicity of the river Rhine steadily decreased during the last decades, there is still concern about the presence of some residual mutagenicity. In addition, in most studies the water samples have been tested only in in vitro test systems such as the *Salmonella*-microsome test.

For this reason, and in order to be able to make a comparison with the water quality 27 years ago, a study was performed with the same experimental design as before in order to measure the effect of Rhine water on the induction of SCE in the Eastern mudminnow. As a new test system the single cell gel electrophoresis assay (Comet assay) was performed.

Fish were exposed to Rhine water or to groundwater for 3 and 11 days in flow-through aquaria. Fish exposed for 11 days to Rhine water had a significantly higher number of SCE and an increased comet tail-length compared with control fish exposed to groundwater. After exposure for three days to Rhine water there was no difference in SCE and a slightly increased comet tail-length compared with the control. It was concluded that genotoxins are still present in the river Rhine, but that the genotoxic potential has markedly decreased compared with 27 years ago. Furthermore, the Comet assay appears to be a sensitive assay to measure the genotoxic potential of surface waters in fish.

Keywords: SCE; Comet assay; Fish; Umbra; Mudminnow; Surface water; Genotoxicity; Biomonitoring

1. Introduction

The river Rhine is an important source for drinking-water production and for this reason continuously monitoring for quality parameters is necessary. Studies

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by the RIWA (Rhine Water Works, The Netherlands) show that the quality of the river water is slowly improving [1–3]. However, there is still concern because of increasing levels of new chemical species such as estrogens and because residual activity of possibly genotoxic compounds is still present. In the last decades, much attention has been paid to the genotoxic potential of water of the river Rhine. Already in 1978, it was shown that fish exposed to Rhine water developed chromosome aberrations in their gill cells [4]. Other studies also showed the genotoxic potential of water of the river Rhine and other Dutch rivers [5–9]. Although different animal species have been used for genotoxic monitoring of surface and waste waters [10,11], fish models are still very useful. As a vertebrate model, fish is the best available to estimate possible human risks, because they can metabolize and accumulate pollutants [12–23]. So far the different studies indicated that there is a steady decrease of genotoxic potential of Rhine water. However these studies used bioassays that measured effects in non-vertebrate cells or tested specific fractions of the surface water [24] (Fig. 1). So there was no certainty about the presence of compounds exerting health risk for vertebrate cells in situ. This question could only be answered by using a fish model appropriate to measure genotoxic effects. Such a fish model is the Eastern mudminnow (*Umbra pygmaea* L.) as this fish species has a karyotype with a small number (22) of large meta-centric chromosomes which makes cytogenetic analysis very feasible. In addition, this fish species is a non-native species for The Netherlands and bears ambient exposure to river water very well [4,6,7,21]. For this reason, and in order to make a comparison possible with the quality of Rhine water 27 years ago, Eastern mudminnows (*U. pygmaea* L., Fig. 3a) were exposed to water of the river Rhine. Gill cells were then studied for genotoxic effects by means of the sister chromatid exchange (SCE) test. For comparison and to study other genotoxic endpoints the single-cell gel electrophoresis assay (Comet assay) was included in the present study as a new and rapid test [25–28].

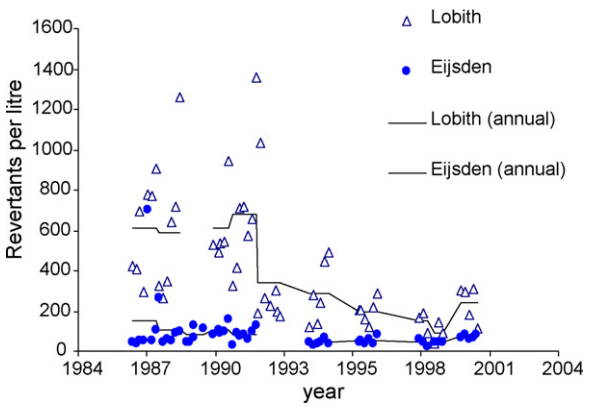

Fig. 1. Mutagenicity of Rhine water near Lobith and of river Meuse water near Eijsden, two different locations in The Netherlands, measured in the period 1981–2001 in the *Salmonella*-microsome test (data from [24]).

2. Materials and methods

2.1. Chemicals

All chemicals were of pro-analysis quality. Ethyl methanesulphonate (EMS) was obtained from Fluka (Buchs, Switserland). Collagenase, bovine serum albumin (BSA), lauroyl sarcosine, bromodeoxyuridine (BrdU), colchicine, Hoechst 33258, phosphate-buffered saline (PBS), Giemsa, normal melting point (NMP) agar and low melting point (LMP) agarose were obtained from Sigma–Aldrich (Zwijndrecht, Netherlands). KCl, NaCl, acetic acid, phosphate and citrate salts for buffers, Triton X-100 and DMSO were from Merck (Amsterdam, Netherlands) and EDTA was purchased from Baker (Deventer, Netherlands).

2.2. Fish husbandry and exposure to Rhine water

Fifty Eastern mudminnows (*U. pygmaea* L.) were collected from small ponds in the National Park "De Groote Peel", a nature preserve in the south of The Netherlands, in collaboration with the Dutch Forest Service, and after permission of the Animal Welfare Committee of the Wageningen University. The fish were transported to the 'Waterlaboratorium' at Nieuwegein, located at the river Rhine, so that the fish could be exposed directly to Rhine water. As the peat water of the ponds had a pH of 3, fish were adapted slowly to the higher pH and the conditions of Rhine water and control water (defined below) in order to prevent stress. Prior to the experiment, the fish were placed for 14 days in mixtures of peat and control water of which the pH was gradually increased from about 3.0 to 7.0. Fish were fed standard feed consisting of frozen mosquito larvae (*Chironomid*) alternated with live feed (*Tubifex*) before and during the experiment.

Fish were exposed to Rhine or control water in the same way and at approximately the same location as in a previous experiment in 1978 [6]. The control water was natural groundwater of drinking-water quality. It has been retained in deep aquifers for over 100 years. This water, which is not treated with chlorine or any other disinfectant, is aerated and rapidly filtered through sand before distribution. The lack of influence from infiltrating river water makes this groundwater ideal for control experiments. NaCl was added until the conductivity was the same as that of Rhine water (about 700 μS/cm). So only the pH and the conductivity were adjusted and there was no other relation between the control water and the Rhine water. The control can, thus, be considered as a laboratory control.

In March 2005, two groups of 12 fish, 8 fish for the SCE-test and 4 fish for the Comet assay, were exposed to Rhine water for

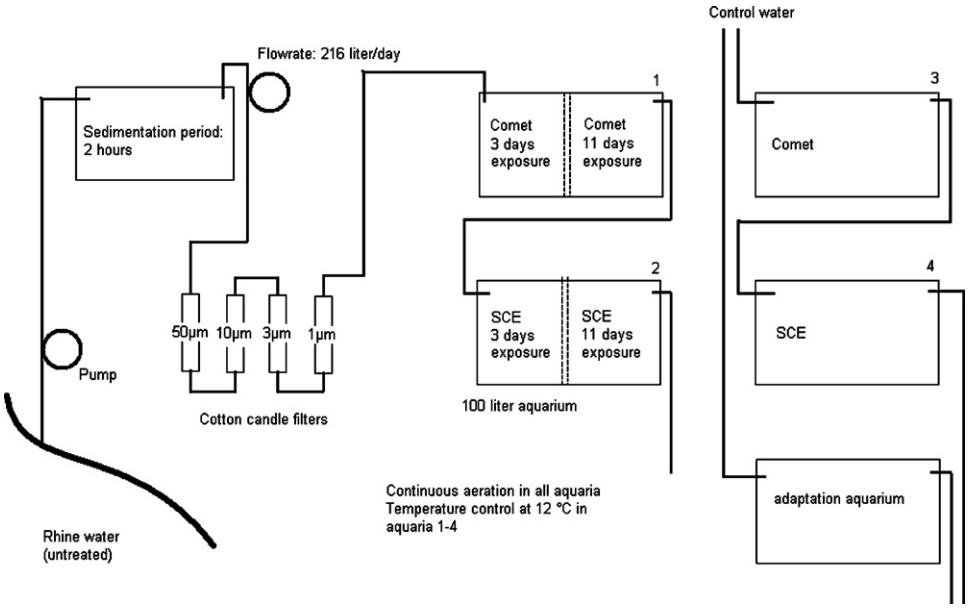

Fig. 2. The experimental design for exposure of fish to Rhine and control water.

3 and 11 days, respectively. Two groups of 12 fish were kept in control water for the same periods. Fish for the SCE-test and Comet assay were kept in separate 100 L all-glass flow-through aquaria with a flow rate of 216 L/day and continuous aeration. A temperature of 12 °C was maintained by heating the incoming water. Silt was removed from the Rhine water by a 120-min sedimentation period and by a serial filtration with cotton candle filters (50, 10, 3 and 1 μm)(Fig. 2). By removing the large particles, only dissolved substances and substances adsorbed to particles <1 μm are considered in this experiment. As a positive control, 5 fish were exposed in a 5-L aquarium to ethyl methanesulfonate (120 mg/L) for 3 days.

2.3. Sister chromatid exchange test

The sister chromatid differentiation technique in vivo, as described by Kligerman and Bloom [14] was used with slight modifications. Fish were injected once intraperitoneally (i.p.) with 0.5 mg BrdU/g fish and were exposed to this base analogue for 10 days (2 cell cycles). Fish to be exposed for 3 days to Rhine water were first kept in control water for 7 days after injection with BrdU. At the end of the exposure period the fish were injected i.p. with 0.25 mg colchicine/g fish and killed 5–19 h later. After decapitation of the fish, the gills were removed and placed in a 0.4% hypotonic solution of KCl for 30 min. The tissues were then fixed in methanol-acetic acid (3:1). Cell preparations were made by the solid-tissue technique [15]. The cells were dried for at least 24 h, then stained according to a modified fluorescence-plus-Giemsa method [29]. Preparations were first treated with Hoechst 33258 (50 μg/mL) in Sorensen's buffer (pH 7.0) for 10 min in the dark, rinsed in distilled water and then exposed to UV radiation (HPW 125W-T, Philips, Belgium) for 4 h in a phosphate–citrate buffer (pH 7.0). Subsequently, preparations were heated in 2 × SSC at 60 °C for 40 min and stained in 5% Giemsa in Sorensen's buffer (pH 6.8) for 10 min.

The preparations were dried for at least 48 h and the SCEs were scored double blind by two different persons, in metaphases of at least eight chromosomes. Mean values and standard errors were determined. For differences between means, Student's *t*-test was used with significance levels at $P < 0.05$.

2.4. Comet assay

The Comet assay, a technique that allows to detect and quantify chromosome damage in single cells, was a modification of the standard method for zebra mussels, *Dreissena polymorpha* [30], adapted according to a procedure described for Zebrafish [26]. In short, the procedure was as follows.

After preparation of the gills a cell suspension was obtained by treatment with a collagenase solution for 20 min. After filtration, which was needed to get rid of undigested tissue, and centrifugation the pellet was re-suspended in PBS with 0.1% BSA. The cell suspension was then mixed with LMP agarose and transferred to a slide pre-coated with NMP agarose. Subsequently, the slide was coated with 1:1 LMP agar in PBS with 0.1% BSA. Per fish 4 slides were prepared.

Subsequently, the cells – not the nuclei – were lysed in a lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% sodium lauroyl sarcosine, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C for at least 1 h. Single-strand DNA was prepared by unwinding the DNA in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 30 min. Then DNA fragments were separated during micro-electrophoresis (Hoeffer supersub, Pharmacia biotech) for 20 min at 25 V and 400 mA.

After staining with ethidium bromide (20 μg/mL) for 10 min, the tail-length was measured by means of an Olympus BH-2 fluorescence microscope (excitation wavelength, 515–560 nm) equipped with image-analysis software (Perceptive Instruments, Haverhill, UK). Per slide the tail-length of 50 comets was measured. Scoring was done in a double blind fashion.

2.5. Statistics

Differences between groups were studied using the Student's *t*-test with significance levels at $P < 0.05$. Each fish was considered as a test unit as described by others [31,32].

3. Results

3.1. Sister chromatid exchange test

In Fig. 3b a typical example is given of a metaphase of a gill cell of the Eastern mudminnow showing sister chromatid differentiation and exchange. Not all the fish showed sister chromatid differentiation. For the control group six fish and for the experimental groups five fish were used for calculating the data. After 11 days of exposure to Rhine water there was a significant increase, almost a doubling, in the number of SCEs per chromosome compared with the control ($P = 0.013$) and also compared with the 3-day exposure group ($P = 0.023$). No induction in SCEs was seen after 3 days of exposure (Table 1 Fig. 4).

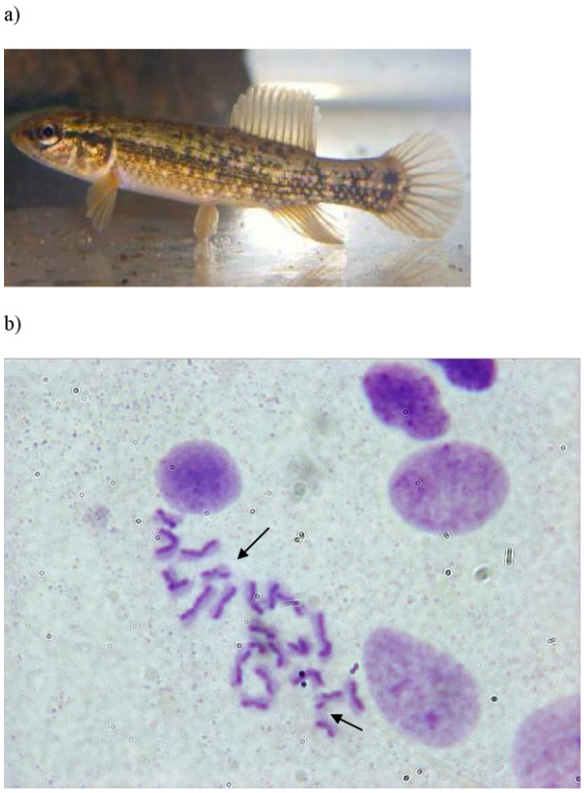

Fig. 3. (a) Eastern mudminnow (*Umbra pygmaea* L.) and (b) metaphase image of a gill cell of the Eastern mudminnow showing two sister chromatid exchange events (see arrows).

3.2. Comet assay

In order to differentiate between DNA damage due to cytotoxicity or genotoxicity, cell viability was assessed using the trypan blue assay. The viability of the cell suspensions of all fish was between 88 and 96%. Validation studies have not been conducted to identify acceptable cytotoxicity levels for in vivo comet assays [32]. However, cell viability below 70–80% of that in the control animals may be considered excessive [32]. The viability of cell suspensions used in this study was above these values and therefore considered acceptable. Thus, it is unlikely that cytotoxicity of the Rhine water or of the positive control had an effect on the outcome of the experiments.

In Fig. 5, comets are shown of gill cell DNA derived from fish exposed to Rhine water for 11 days. As shown in Fig. 6 and Table 2 there was a significant difference between the comet tail-length of fish exposed for 11 days to Rhine water compared with the groundwater control ($P < 0.05$). After 3 days of exposure to Rhine water the comet tail-length was slightly longer than in the control, but the difference was not significant. Exposure to EMS significantly ($P < 0.05$) increased the comet tail-length.

Table 1
Number of sister chromatid exchange events per chromosome in gill cells of *Umbra pygmaea* after exposure to Rhine water (R) or groundwater (C) for 3 or 11 days, compared with data from 1978 [6]; mean ± S.D., *n* is number of fish or chromosomes

	1978			2005		
	SCEs/chromosome	n_{chr}	n_{fish}	SCEs/chromosome	n_{chr}	n_{fish}
C	0.045 ± 0.012	1149	5	0.044 ± 0.012	1260	6
R3	0.128 ± 0.023	2416	6	0.043 ± 0.017	913	5
R11	0.155 ± 0.021	2317	5	0.072 ± 0.016	917	5

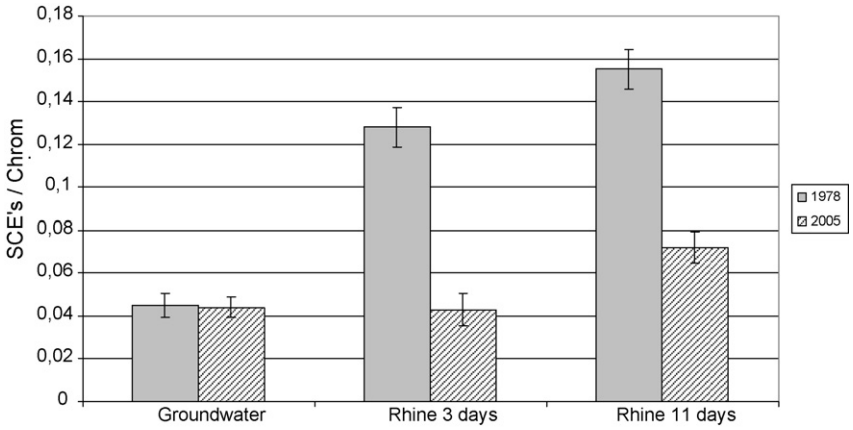

Fig. 4. Effect of Rhine water on sister chromatid exchange in gill cells of the Eastern mudminnow after exposure for 3 and 11 days in 1978 and 2005; mean ± S.E.M., $n = 5–6$.

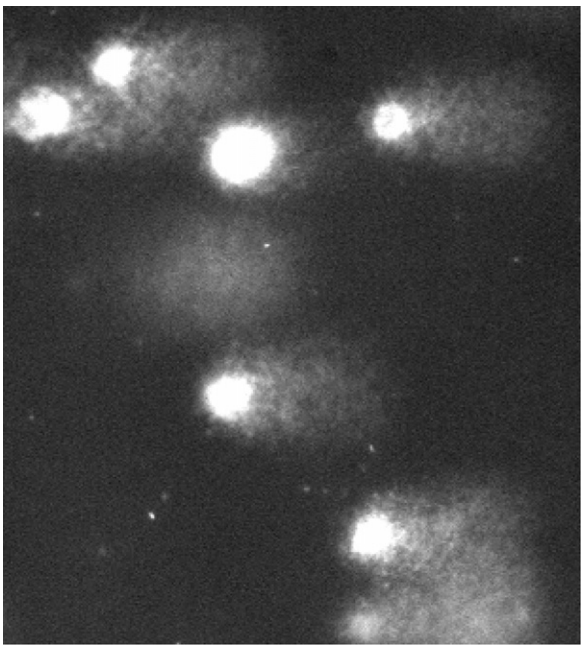

Fig. 5. Comets after single-cell gel electrophoresis of gill cell DNA from Eastern mudminnows exposed to Rhine water.

Table 2
Significant differences between EMS-exposed and Rhine water-exposed fish compared with their controls in the Comet assay; one-tailed *P*-values in Student's *t*-test

Differences between		*P* one-tailed	Significance
Blank EMS	EMS	0.000394	++
Blank R	R 11	0.013356	+
Blank R	R 3	0.482744	−

−: not significant, +: $P < 0.05$, ++: $P < 0.001$.

4. Discussion

The main conclusion of the present study is that Rhine water still contains genotoxins that are able to induce sister chromatid exchange and single-strand DNA breaks, measured with the Comet assay, in gill cells of fish exposed for 11 days to Rhine water.

After 3 days of exposure no effect was seen on SCE frequency and a slight, but not significant increase of DNA damage in the Comet assay. This suggests that there is a dose- and time-dependent effect.

Twenty-seven years ago, our group observed an increase in SCEs in the same fish species, the Eastern mudminnow, already after a 3-day exposure to Rhine water, and a much higher SCE frequency after an 11-day exposure than in the present study. The SCE frequency in the fish exposed to groundwater was exactly the same as before [6]. Based on the SCE assay, it can be concluded that the quality of the water of the river Rhine with respect to the presence of genotoxic compounds has improved during the last decades.

Furthermore, the present study shows that the Comet assay can be applied successfully in fish, for genotoxic monitoring of surface waters. As far as we know this is the first time that the Comet assay is used for genotoxic monitoring in the Eastern mudminnow.

The Eastern mudminnow, *U. pygmaea* L., a non-native species, is abundantly present in pools in the south of The Netherlands. In previous studies this fish appeared to be an appropriate model for cytogenetic studies, because of the restricted number of well-visible metacentric chromosomes, and because this fish species can be kept easily under laboratory conditions [4,6,12–15]. As the sister chromatid differentiation (SCD) technique is a laborious method, especially in vivo, because it

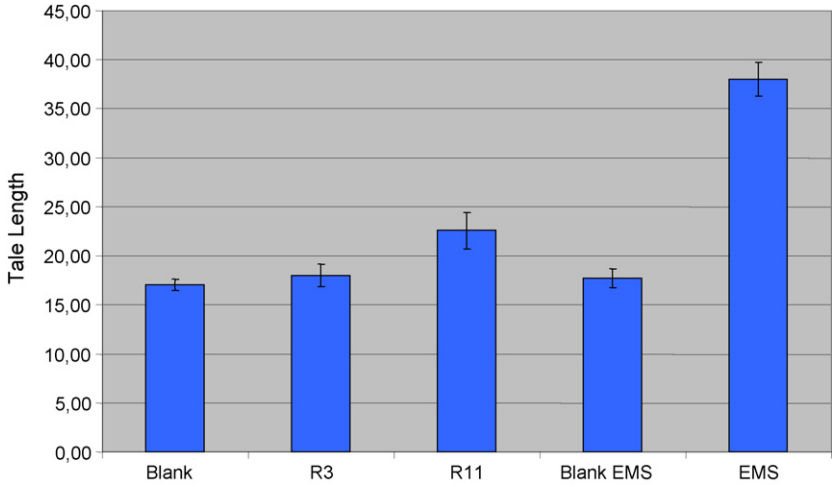

Fig. 6. Effect of 3 and 11 days of exposure to Rhine water on chromosome breaks in gill cells of the Eastern mudminnow, measured as comet tail-length after single-cell gel electrophoresis; positive control: 120 mg/L EMS; mean ± S.E.M., $n = 4$.

needs dividing cells, the Comet assay was applied in order to compare the results with the SCE test. The Comet assay has many advantages, such as being independent of chromosome number, not requiring animal pre-treatment with BrdU and colchicine, and being less time-consuming due to automatic scoring of the Comets by use of image-analysis software [26,32,33]. For most compounds both assays exhibit a similar sensitivity, although there are some differences in types of DNA damage detected [34]. SCEs reflect a DNA-repair process and they may occur spontaneously in normal cycling cells, suggesting a link between SCE and DNA replication. Although their molecular basis still remains obscure, homologous recombination may be one of the principal mechanisms responsible for SCEs in vertebrate cells [35]. The Comet assay detects primary DNA lesions such as DNA strand breaks, but may also detect genomic instability, repair of double-strand breaks, DNA-adduct formation and DNA cross-links [34]. The widespread applicability of the Comet assay is reflected in the wealth of data that appeared in the last few years.

In this study, the data of the SCE test and the Comet assay are in agreement. A comparison of the recent results with data obtained in the previous study 27 years ago [6] shows a marked decrease in SCE frequency, suggesting the presence of less genotoxins. This corresponds with recent studies of the RIWA and the 'Waterlaboratorium' showing a decrease in mutagenicity of water extracts measured in the *Salmonella*-microsome test [24]. Other studies show also an improvement of the water quality of the river Rhine [2]. The observed correspondence in sensitivity between the Comet and SCE assays in this study is also in agreement with the literature, where for most chemicals tested a similar sensitivity was found for the two assays [34]. As the Comet assay measures primary DNA lesions and the SCE test reflects DNA-repair processes, our data suggest that compounds present in Rhine water still display a broad spectrum of genotoxic effects.

During the exposure period of the fish in this study, Rhine water was also collected and tested in the *Salmonella*-microsome test (tester strain TA 98, with S9) as described earlier [24]. A doubling of the number of revertants per litre of Rhine water was seen, indicating a weak mutagenic response in the bacterial mutagenicity assay (data not shown). Therefore, it appears that the residual mutagenicity observed in bacterial assays also induces cytogenetic changes in fish. So far the compounds causing these effects are unknown. A list of organic contaminants that have recently been measured in Rhine water is presented in Table 3.

Although in this study testis cells were not included, it may be supposed, based on previous data [6], that the male reproductive system of the fish is still at risk. As this study and others show that exposure during a period of 11 days increases genotoxic effects, it cannot be excluded that long-term exposure to low doses of genotoxins in the surface water leads to marked genotoxic effects in somatic and reproductive cells of fish and other aquatic organisms. Whether bioaccumulation contributes to this effect is unknown and should be a subject of future research. Although at present no mutagenicity can be detected in drinking-water samples derived from Rhine water, it cannot be excluded that small, undetectable amounts of genotoxins are still present in drinking-water. As long as the identity and the actual presence

Table 3
Organic contaminants present in the river Rhine during the experimental period

Substance	CAS-number	Use	Mutagenic[a]	Average concentration (μg/L)
Diethyleneglycol dimethylether (diglyme)	111-96-6	Solvent	No	0.12
Azo-bis-isobutyronitrile (AIBN)	78-67-1	Plasticizer	No	0.04
2-(Trifluoromethyl)aniline	88-17-5	Pesticide	No[b]	0.08
Dipropyleneglycol methylether	13429-07-7	Additive in paint	No	0.03
Triethylphosphate (TEP)	78-40-0	Flame retardant; plasticizer	No	0.06
3,5,5-Trimethylcyclohex-2-enone (isophorone)	78-59-1	Solvent	No	0.03
Triethyleneglycoldimethylether (triglyme)	70992-85-7	Solvent	No	0.37
Diethyl-methyl-carbamodithioaat	8018-01-7	Herbicide	Possible	0.11
Surfynol 104	104-76-7	Surfactant	Yes	0.48
1,1,3,5-Tetramethyl-cyclohexane	4306-65-4	Personal care product	No	0.03
Tetra-ethyleneglycol dimethylether (tetraglyme)	143-24-8	Solvent	No	0.11
Tri-isobutylfosfaat	126-71-6	Pesticide	No[c]	0.05
Tetra-acetylethylenediamine (TAED)	10543-57-4	Detergent	No	0.09
Benzophenone	119-61-9	Personal care product	No	004
N-Ethyl-*p*-toluenesulfonamide	80-39-7	Herbicide	No	0.05
N-Butylbenzenesulphonamide	1907-65-9	Herbicide	–	0.05
Tri (2-chloro-isopropyl) phosphate (Fyrol PCF)	13674-84-5	Flame retardant	No	0.11
Galaxolide (HHCB)	1222-05-5	Personal care product	No	0.08
Dimethylpropylphenol	80-46-6	Pesticide	Possible	0.11
Hexakis(methoxymethyl)melamine (HMMM)	3089-11-0	Adhesive	No	0.30
Xylene	104-76-7	Pesticide	No	0.13
tert-Butylmethylether (MTBE)		Fuel additive	No	0.16
Ethylbenzene	100-41-4	Fuel additive, basic chemical	No	0.11
Carbamazepine	298-46-4	Pharmaceutical	No	0.15

The frequency of sampling was daily. Only compounds determined frequently (>50%) are given.

[a] From: "Chemical Safety Information from Intergovernmental Organizations" (http://www.inchem.org) or the "European Chemical Substances Information System" (http://ecb.jrc.it/esis/), unless otherwise stated.

[b] See Ref. [36].

[c] Toxicological evaluation of triisobutylphosphate; BG Chemie, 2000.

of these genotoxins in drinking-water is unknown, and as long as there is no information on whether these compounds can accumulate in vertebrate organisms, further studies are needed to investigate (a) the presence of genotoxic micropollutants in drinking-water and (b) the long-term genotoxic effects in vertebrates, in order to conclude whether or not chronic exposure of humans to drinking-water derived from surface water requires extra purification steps.

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