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Comet assay and micronucleus test in circulating erythrocytes of *Cyprinus carpio* specimens exposed in situ to lake waters treated with disinfectants for potabilization

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Abstract

The detection of a possible genotoxic effect of surface water treated with disinfectants for potabilization is the aim of the present work. The Comet assay and the micronucleus test were applied in circulating erythrocytes of *Cyprinus carpio*. Young specimens (20–30 g) were exposed in experimental basins, built within the potabilization plant of Castiglione del Lago (Perugia, Italy). In this plant the water of the Trasimeno Lake is treated and disinfected for potabilization before it is distributed to the people in the net of drinkable water. A continuous flow of water at a constant rate was supplied to basins; the water was continuously treated at a constant concentration with one of the three tested disinfectants (sodium hypochlorite, peracetic acid and chloride dioxide), one control basin being supplied with untreated water. Three sampling campaigns were performed: October 2000, February 2001 and June 2001. Repeated blood samplings through intracardiac punctures allowed to follow the same fish populations after different exposure times: before introduction of the disinfectant, and 10 or 20 days afterwards. An additional blood sampling was performed 3 h after addition of the disinfectant in other, simultaneously exposed, fish populations. Genotoxic damage was shown in fish exposed to water disinfected with sodium hypochlorite and chloride dioxide. The Comet assay showed an immediate response, i.e. DNA damage that was induced directly in circulating erythrocytes, whereas micronuclei reached their highest frequencies at later sampling times, when a genotoxic damage in stem cells of the cephalic kidney is expressed in circulating erythrocytes. The quality of the untreated surface water seems to be the most important parameter for the long-term DNA damage in circulating erythrocytes.

Keywords: Water potabilization; Disinfectants; Micronuclei; Comet assay; Circulating erythrocytes; *Cyprinus carpio*

1. Introduction

Chlorine as drinking water disinfectant may produce toxic by-products, such as haloalkenes, haloacetic acids, haloacetonitriles, haloketones and haloaldehydes, in particular if water is obtained from

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surface sources [1]. Water chlorination has been shown to give rise to formation of mutagenic/carcinogenic by-products which derive from reactions of chlorine with organic compounds (humic and fulvic acids) present in natural water [2–6]. Epidemiological studies generally support the correlation between consumption of chlorinated drinking water and an increased risk for cancer of the urinary and gastrointestinal tracts [7–9]; studies on bladder cancer provide further evidence for this correlation [6]; long-term carcinogenicity studies in rats supplied with sodium hypochlorite (NaClO) in drinking water showed an increase in leukaemia in females [10]. Among disinfectants alternative to chlorine, chlorine dioxide (ClO_2) is widely used, while peracetic acid (CH_3COO_2H, PAA) deserves to be studied for its application in drinking water disinfection; PAA is a potent antimicrobial agent with many applications in hospitals, laboratories and factories [11–13]. Disinfection of lake and river water for potabilization with PAA produced only carboxylic acids, not recognized as mutagenic, and resulted in a very low level of genotoxicity [14].

A useful approach to detect mutagenicity in water is the in vivo treatment/in situ exposure of plants, e.g. *Zea mays* [15], *Vicia faba* [16], *Allium cepa* [17] and *Tradescantia* spp. [18,19] or aquatic animals, e.g. newts [20–22], polychaetes [23] or molluscs [24–26]. For example, sodium hypochlorite by-products with humic substances increased the micronucleus frequency in erythrocytes of newt larvae [27]. The micronucleus test in circulating erythrocytes of fish has been widely employed for both in situ exposure to environmental waters [28–32] and laboratory treatments in vivo [33–37]; in particular this test has been employed in *Cyprinus carpio* [38–40]. The micronucleus test is a very sensitive and useful method that can detect both clastogenic and aneugenic activity (for a review, see [41,42]). The test appears to be a promising tool for the monitoring of drinking water.

An additional sensitive indicator of genetic damage is DNA strand breakage, which can be detected by the alkaline single-cell gel electrophoresis assay (Comet assay). This assay has been applied in aquatic environments both on vertebrate and invertebrate organisms [43–46]. In particular, in vivo data on fish were obtained with redbreast sunfish (*Lepomis auritus*), hard head cat fish (*Anus felis*), bullhead (*Ameirurus nebulosus*) and carp (*C. carpio*) [45,47]. Furthermore, the German Federal Environmental Agency proposes the Comet assay as a useful test in a graduated testing battery for a reliable detection of genotoxicity of surface waters [48].

The experiments described in the present paper are part of a multidisciplinary research project with the aim to assess the presence of genotoxic activity in surface drinking water treated with different disinfectants (NaClO, ClO_2, PAA). For this purposes a pilot plant was built within the potabilization plant of Castiglione del Lago (Perugia, Italy), where the water of lake Trasimeno (l.w.) is disinfected for potabilization. In the pilot plant an integrated approach was taken, including chemical analyses, microbiological tests, and in vitro and in vivo genotoxicity tests (see [49]).

In this paper we report data on the micronucleus test and Comet assay in circulating erythrocytes of *C. carpio* exposed in situ to water containing disinfectants. Fish were exposed for 20 days in basins within the pilot plant; blood samples were taken at different times from the same specimens, in order to study the time course of the mutagenic end points in the same fish populations. Blood samples were also taken 3 h after the start of the exposure from different specimens. The experiments took place in October 2000 and February and June 2001.

2. Materials and methods

2.1. Lake water

Lake Trasimeno, a mesotrophic limnic environment, was chosen as a site with surface water with a high concentration of total organic carbon (TOC) and bromide, both possible precursors of mutagenic disinfection by-products.

2.2. Pilot plant—(see [49])

The following functional units were the main components of the pilot plant:

(1) a lake water capturing system;
(2) a sedimentation system (Corby 10, FZ Fantoni, Bedizzole, Italy), with two 1-m^3 reservoirs to clarify the water;
(3) a filtration system (a 50-μm pore size inox stainless steel filter followed by a 25-μm pore size filter

cloth) to remove suspended particles (Fluxa Filtri S.p.A., Milan, Italy);
(4) a pumping system in which sulphuric acid is added to neutralize the water to pH 7;
(5) one main pipeline divided into four secondary ones that supply filtered water (flux: 300 l/h) to four 300-l stainless basins (*contact basins*);
(6) four contact basins, one with untreated water and three in which water was in contact and interacted with the disinfectants used in the experiments;
(7) four 1-m^3 stainless steel basins (*exposure basins*) used for in situ exposure of bioindicators (fish, molluscs and plants), which received the water flowing from the contact basins.

2.3. Disinfectants

(1) Sodium hypochlorite (NaClO) (Solvay S.p.A., Rosignano, LI, Italy): supplied as a 14.5–15.5% solution through a membrane pump.
(2) Chlorine dioxide (ClO_2), produced in loco directly from an 8% $NaClO_2$ solution and 10% HCl through an automatic generator (Tecme S.r.l., Gardolo di Trento, TN, Italy).
(4) Peracetic acid (CH_3COO_2H) (Promox S.r.l. Leggiuno, VA, Italy): supplied as a 15% solution through a membrane pump.

Disinfectant concentrations were chosen on the basis of chlorine demand of the water, after preliminary laboratory experiments. Free disinfectant residues in disinfected water were monitored twice a day during the exposure of 20 days for a total of 40 determinations with a field photometer (DR 2000, Hach); total and free chlorine concentrations were determined with a standard *N*,*N*-diethyl-*p*-phenylene diamine (DPD) method; ClO_2 concentration was determined with phenol red and residual PAA was measured with a total DPD reaction, after treatment with catalase and a potassium iodide reaction. On this basis the concentrations of the disinfectants were adjusted twice a day to maintain the free residue concentration <0.2 mg/l, in order to avoid direct toxicity to fishes and mussels. The mean ($\pm$S.D.) of the disinfectant concentrations added during the exposure were:

- October 2000: 1.24 ± 0.19 mg/l of NaClO, 1.64 ± 0.21 mg/l of ClO_2 and 1.00 ± 0.19 mg/l of PAA;
- February 2001: 0.71 ± 0.06 mg/l of NaClO, 1.63 ± 0.22 mg/l of ClO_2 and $0.61 + 0.04$ mg/l of PAA;
- June 2001: 0.55 ± 0.09 mg/l of NaClO, 1.84 ± 0.05 mg/l of ClO_2 and 0.90 ± 0.05 mg/l of PAA.

2.4. Fish exposure and blood sampling

The study consisted of 20-day long experiments and was carried out in different seasons (October 2000, February 2001 and June 2001) in order to assess the effects of disinfection in different physical and chemical conditions of the lake water. Young specimens of *C. carpio* (age: <1 year; weight: 20–30 g), were supplied by the 'Centro Ittiogenico di Sant'Arcangelo' (Perugia, Italy). Specimens were introduced in each basin 20 days before the start of the exposure, to allow acclimatization. Blood samples were taken through intracardiac puncture with heparinized syringes from each fish (eight specimens for Comet assay [50] and 10 for MN, eight of which were also employed for the Comet assay), anaesthetized with 0.1 g/l of MS-222 Finquel (SCUBLA S.n.c.). After a 5-min recovery period in filtered lake water, fishes were replaced in their own basin. The first sampling was performed immediately before the disinfectants were supplied to the water in the contact basins, for both micronucleus test and Comet assay. Different sampling times were chosen after the start of exposure: 3 h for the Comet assay, 10 days for the micronucleus test and 20 days for both micronucleus test and Comet assay. Samples were repeatedly taken from the same fish specimens of each basin, except for the 3 h sampling, due to the fact that successive cardiac puncture must be made with an interval of at least 24 h (preliminary experiments, unpublished data). Some specimens died before the end of the exposure period. Therefore, blood samples were taken from nine specimens only at 10 and 20 days in February 2001 in the basins containing ClO_2-disinfected lake water, at 20 days in February 2001 in the basins containing NaClO-disinfected lake water, and at 20 days in June 2001 in the basins containing untreated lake water.

2.5. Monitoring of physical–chemical variables

The physicochemical variables were examined in the exposure basins at the start of the experiment and at the 10- and 20-day sampling times. Temperature,

oxygen concentration and pH were measured in situ using a Multiline P4 WTW probe. The following chemical analyses were performed on water, after filtration (glass fibre filters Whatman GF/F): nitrite (colorimetry, diazotization method), nitrate (colorimetry, cadmium reduction method), ammonium (colorimetry, indophenol blue), and phosphorus (colorimetry, ascorbic acid method) [50].

2.6. Micronucleus test

Blood smears were done immediately after sampling, fixed with absolute ethanol for 20 min, stained with Feulgen and mounted with dissolved polystyrene in xylene (DPX; Fluka). For each specimen, 25 000 erythrocytes were scored at 1000× magnification. After blind scoring, statistical analysis was carried out for each experiment with non-parametric tests (Mann–Whitney test after a Kruskal–Wallis non-parametric ANOVA). Comparisons were made between fish populations before the start of the exposure to disinfected water and within the same population, exposed to the same disinfected water, before exposure and at the different exposure times: 10 and 20 days.

2.7. Comet assay

The single-cell gel electrophoresis assay was basically performed according to [51,52]. Freshly withdrawn cells (20 μl) were added to 80 μl of 0.65% low melting agarose (LMA) in PBS and then transferred onto degreased microscope slides previously dipped in 1% normal melting agarose (NMA) for the first layer. The agarose was allowed to set for 5 min at 4 °C before addition of a final layer of LMA. After agarose solidification, the slides were placed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4 °C overnight in the dark.

Alkaline DNA-unwinding was carried out in a gel electrophoresis chamber containing a freshly prepared buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for 10 min and electrophoresis was performed in the same buffer for 20 min at 0.78 V cm^{-1} and 300 mA. DNA unwinding and electrophoresis were performed in an ice-water bath. Once the electrophoresis had been carried out, the slides were washed in a neutralisation buffer (0.4 M Tris–HCl, pH 7.5). All the steps described above were performed under yellow light to minimise additional UV-induced DNA damage.

After staining with 100 μl ethidium bromide (10 μl/ml), observations were made under a fluorescence microscope (Leitz Dialux 20) equipped with an excitation filter BP 515–560 nm and a barrier filter LP 580 nm, using an image-analysis system (Cometa Release®2.1 Sarin, Florence, Italy).

For each sample, coded and evaluated blind, 100 cells were analysed; clouds of DNA fragments were registered as number and were not considered for the analysis. The relationship between tail moment (TM) and Comet length was examined by simple linear regression ($R = 0.967$; $P < 0.001$). The Comet parameter TM was chosen to represent the data on genotoxic effects.

Statistical analysis was performed by SPSS 10 for each experiment on the mean of medians of TM values, which is normally distributed, with Student's *t*-test after ANOVA. Comparisons were made, for long-time exposure (20 days), within the same population, exposed to the same disinfected water, at different exposure times (before start of exposure and 20 days after); comparisons were made for short-time exposures (3 h), between each population sampled after 3 h from the start of disinfection and the whole population (32 specimens) sampled at time 0, considered as a control (C_0).

3. Results

3.1. Physical–chemical parameters

The data are presented in Table 1. The pH of the water did not show significant variation (7.3–7.8). The temperature showed the typical seasonal fluctuation of Central Italy (October 2000: 16–17 °C; February 2001: 5–10 °C; June 2001: 22–24 °C). An inverse pattern was seen for the oxygen concentration, which is due partially to a different solubility of oxygen in water at different temperatures. Nitrates reached their highest concentration values during February 2001, while phosphates reached their highest concentration values in June 2001 and the lowest in February 2001. Nutrient concentrations were always compatible with the rearing demand; critical, sub-toxic values were shown

Table 1
Mean values and standard deviations of physico-chemical parameters in the basins during the study period

Sampling	Exposure	pH	O_2		N-NO_2 (ppb)	N-NO_3 (ppm)	NH_4^+ (ppb)	$PO4_2^{3-}$ (ppb)
			ppm	(% sat.)				
October 2000 (16.8 ± 0.5 °C)	Untreated lake water	7.5 ± 0.2	7.6 ± 0.3[a] (1)	80.35 ± 3.7[a] (2)	4.3 ± 0.6	1.03 ± 0.4	80.0 ± 0.0[a] (3)	200 ± 120.0
	l.w. + CH_3COO_2H	7.5 ± 0.0	7.4 ± 0.3	77.9 ± 4.6	4 ± 1.0	0.83 ± 0.2	53.0 ± 5.8	137 ± 5.7
	l.w. + NaClO	7.5 ± 0.0	7.2 ± 0.4	75.8 ± 5.6	3 ± 1.7	0.93 ± 0.1	76.7 ± 32.1	107 ± 15.3
	l.w. + ClO_2	7.4 ± 0.1	7.1 ± 0.1[a] (4)	74.5 ± 0.8[a] (5)	3.7 ± 2.3	0.83 ± 0.2	70.0 ± 28.3[a](6)	137 ± 47.3
February 2001 (8.1 ± 2.2 °C)	Untreated lake water	7.8 ± 0.1	10.5 ± 0.8	90.1 ± 2.5	3.3 ± 0.8	1.07 ± 0.1	66.7 ± 5.8	10 ± 0.0
	l.w. + CH_3COO_2H	7.7 ± 0.1	10.9 ± 0.7	94.0 ± 1.1	3.7 ± 1.5	1.2 ± 0.0	50.0 ±0.0	17 ± 1.2
	l.w. + NaClO	7.7 ± 0.1	10.8 ± 0.7	93.4 ± 1.6	5.3 ± 1.2	1.4 ± 0.1	76.7 ± 40.4	17 ± 1.2
	l.w. + ClO_2	7.6 ± 0.1	10.6 ± 0.1	93.7 ± 3.2	4.7 ± 0.6	1.2 ± 0.1	43.3 ± 5.8	20 ± 10.0
June 2001 (23.6 ± 1.2 °C)	Untreated lake water	7.4 ± 0.1	5.6 ± 1.0	67.3 ± 10.0	6 ± 2.6	0.67 ± 0.1	66.7 ± 20.8	167 ± 66.6
	l.w. + CH_3COO_2H	7.3 ± 0.1	6.0 ± 1.0	74.2 ± 7.5	5.7 ± 3.8	0.57 ± 0.2	60.0 ± 26.5	137 ± 55.1
	l.w. + NaClO	7.3 ± 0.1	6.2 ± 0.6	74.9 ± 7.5	4.7 ± 1.2	0.97 ± 0.4	66.7 ± 20.8	223 ± 60.3
	l.w. + ClO_2	7.3 ± 0.1	6.6 ± 0.6	79.6 ± 7.0	4.3 ± 1.2	0.6 ± 0.1	63.3 ± 11.6	163 ± 90.7

Measurements were performed at the blood sampling times, during a continuous exposure to the water of lake Trasimeno lake treated with different disinfectants (CH_3COO_2H, NaClO and ClO_2). Concentrations of chemicals are expressed as ppm, unless otherwise specified.

[a] Computations were made excluding critical values due to overcrowding, which were shown before the start of exposure during the experiment in October 2000, when all fish were stored in the basin for untreated lake water and for ClO_2-disinfected lake water; critical values were the following—O_2 (ppm): (1) = 2.68, (4) = 1.03; O_2 (% sat): (2) = 27.9, (5) = 11.5; NH_4^+ (ppb): (3) = 170, (6) = 380.

Table 2
Average micronucleus frequency per 25 000 red blood cells ± S.E.

Month and year	Sampling time (days)	Exposure			
		Untreated lake water	l.w. + CH_3COO_2H	l.w. + NaClO	l.w. + ClO_2
October 2000	0	0.4 ± 0.16	0.7 ± 0.26	0.6 ± 0.22	0.5 ± 0.17
	10	0.5 ± 0.17	0.6 ± 0.22	0.9 ± 0.31	0.7 ± 0.21
	20	0.5 ± 0.22	0.8 ± 0.25	2.5 ± 0.50**	1.7 ± 0.42**
February 2001	0	0.3 ± 0.15	0.2 ± 0.13	0.2 ± 0.13	0.3 ± 0.21
	10	0.4 ± 0.16	0.3 ± 0.15	0.7 ± 0.21	0.7 ± 0.33
	20	0.3 ± 0.1	0.6 ± 0.22	1.3 ± 0.33*	1.4 ± 0.47
June 2001	0	0.5 ± 0.23	0.4 ± 0.16	0.5 ± 0.22	0.3 ± 0.15
	10	0.4 ± 0.16	0.7 ± 0.26	0.8 ± 0.25	0.9 ± 0.23
	20	0.5 ± 0.24	1 ± 0.33	2.5 ± 0.50**	1.8 ± 0.41**

Observations were made on 25 000 circulating erythrocytes per blood sample in specimens of *C. carpio* following a continuous exposure to the water of lake Trasimeno treated with different disinfectants (CH_3COO_2H, NaClO and ClO_2). Blood samples were taken immediately before exposure and at different times (10 and 20 days) after the start of exposure in the same specimens; untreated lake water was used as a control. Experiments were performed in different seasons (October 2000, February 2001 and June 2001). Statistical analysis was performed using Mann–Whitney test after a Kruskal–Wallis non-parametric ANOVA, by which micronucleus frequencies after 10 or 20 exposure days were compared, for each disinfectant treatment, to that found before exposure (day 0), in the same fish population.

* $P < 0.05$.

** $P < 0.01$.

for oxygen and ammonium concentrations before the start of exposures, in basins for untreated lake water and for ClO_2-disinfected lake water, due to occasional fish overcrowding in October 2000.[1]

3.2. Micronucleus test

Data on micronuclei are given in Table 2. The main results are the following:

(1) There is no significant difference in any experiment in micronucleus frequency among fish populations in the four basins at time 0, that is immediately before the beginning of exposure to disinfected water; it testifies a very good homogeneity in the starting conditions.
(2) For all disinfectants there is an increase in micronucleus frequency within the same fish populations with increasing exposure times; nevertheless, this increase reaches statistical significance in some cases only.
(3) There is a significant increase in micronucleus frequency in fish populations exposed to NaClO-disinfected water in all the experiments and to ClO_2-disinfected water in the experiments of October 2000 and June 2001; a significant increase compared with the micronucleus frequency at $t = 0$ (i.e. before exposure) is reached at $t = 20$, while the difference is not yet significant at $t = 10$.

3.3. Comet assay

The main results (Table 3) are the following:

(1) No significant differences (ANOVA) were found within the same experiment among the fish populations in the four basins at time 0.
(2) DNA migration of populations samples at $t = 0$ in the June 2001 sampling was significantly higher than in the October 2000 and February 2001 samplings ($P < 0.001$).
(3) The comparison among the 3-h samples and data collected from all the specimens sampled at time 0 (control, C_0) shows a significant DNA migration increase for the basins with chlorinated disinfectant in October 2000 ($P < 0.001$ NaClO; $P < 0.01$ ClO_2) and February 2001 ($P < 0.01$ NaClO and ClO_2).
(4) The blood re-sampling in the same fish populations after a 20 days exposure shows: (i) October 2000: DNA migration was not affected by type

[1] All fish were stored in these two basins before the start of exposure at October 2000.

Table 3
Average median tail moment (TM) ± S.D. in 100 circulating erythrocytes per specimen of *C. carpio* following continuous exposure to the water of lake Trasimeno treated with different disinfectants (CH_3COO_2H, NaClO and ClO_2) in different seasons (October 2000, February 2001 and June 2001)

Exposure	Sampling time	Untreated lake water	l.w. + CH_3COO_2H	l.w. + NaClO	l.w. + ClO_2
October 2000					
Short	C_0	14.30 ± 3.95	14.30 ± 3.95	14.30 ± 3.95	14.30 ± 3.95
	3 h	16.16 ± 2.05	15.62 ± 4.13	22.76 ± 2.66***	19.55 ± 4.95**
Long	0	12.58 ± 1.90	15.87 ± 4.84	14.79 ± 5.01	13.95 ± 3.24
	20 days	12.59 ± 4.68	16.43 ± 2.67	15.53 ± 3.66	12.18 ± 3.21
February 2001					
Short	C_0	15.09 ± 5.19	15.09 ± 5.19	15.09 ± 5.19	15.09 ± 5.19
	3 h	19.09 ± 5.78	19.18 ± 4.76	22.27 ± 4.72**	23.09 ± 3.38**
Long	0	13.56 ± 3.15	12.99 ± 4.30	14.42 ± 8.09	17.57 ± 3.56
	20 days	24.98 ± 9.30*	30.48 ± 4.43***	28.17 ± 7.92**	21.33 ± 5.81
June 2001					
Short	C_0	34.20 ± 11.97	34.20 ± 11.97	34.20 ± 11.97	34.20 ± 11.97
	3 h	37.24 ± 10.29	36.67 ± 11.02	30.62 ± 6.04	39.53 ± 10.58
Long	0	38.21 ± 8.48	27.12 ± 12.19	37.12 ± 12.29	34.38 ± 13.32
	20 days	21.82 ± 7.45***	9.60 ± 1.81**	13.17 ± 5.23**	22.61 ± 7.94*

Blood samples were taken immediately before exposure and 20 days after the start of exposure, in the same specimens; blood samples were taken 3 h after the start in separate fish populations. Statistical analysis was performed using Student's *t*-test after ANOVA for the Comet assay (SCGE) by which DNA migration in the specimens exposed for 20 days was compared to that found before exposure in the same fish population (unexposed sample); DNA migration of the populations exposed for 3 h was compared to the mean values of DNA migration in the unexposed samples (C_0).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

of disinfectant or exposure time; (ii) February 2001: a significant increase in DNA migration compared with the $t = 0$ sampling was observed in basins with untreated water, PAA, and NaClO ($P < 0.001$); (iii) June 2001: a significant decrease of DNA migration was shown in all situations ($P < 0.05$ for untreated water; $P < 0.01$ for PAA and ClO_2; $P < 0.001$ for NaClO).

4. Discussion

The micronucleus test and the Comet assay are sensitive tools for an effective evaluation of field genotoxicity biomarkers. The Comet assay can detect directly DNA strand breaks, which may be induced very early after exposure to genotoxins. Micronuclei are the result of chromosome breaks (or mitotic anomalies) that require necessarily a passage through mitosis to be recognisable. Furthermore, while the Comet assay detects DNA breaks directly in circulating erythrocytes of the fish used in this study, micronuclei reveal chromosome breaks or mitotic anomalies that occur in stem cells in the cephalic kidney, from which circulating erythrocytes, eventually micronucleated, are released very slowly (a complete turnover requires at least 100 days).

The time course of micronucleus induction recorded in the present experiments is in good agreement both with data from laboratory treatments in the same [40] and other species [35] and during in situ exposure [32]. It can be explained on the basis of the time required for the turnover of red blood cells in fish. The lack of variation among the micronucleus frequencies at the start of the exposures and the regularity of their change over time suggest that they depend mainly on the presence of genotoxins rather than other environmental factors. Therefore, even if the frequency of micronuclei is very low in this system and a very large cell sample is required, this test showed to be reliable. In particular the present protocol, which allowed us to follow the same fish population over time, could avoid

the inter-individual variation in the response to genotoxic compounds, which plays a role when different individuals are sampled at different times.

The variation in DNA migration detected by the Comet assay shows a strong seasonal influence both in terms of baseline level and sensitivity towards the treatments, in agreement with our previous studies in aquatic organisms [53]. The primary DNA damage, before the induction of the repair system, is clearly visible after 3 h exposure as an increase in DNA migration in the blood samples collected from the fish populations maintained in water treated with NaClO or ClO_2 in October 2000 and in February 2001. On the other hand, after 20 days of exposure the response of the fish seems to be modulated by the environmental seasonal differences more than by the disinfectants or disinfection by-products. An exposure of 20 days seems to be too long to detect DNA primary damage induced by disinfection processes; in this case, temperature variations and seasonal variations in pollutant content of the lake water could be the major confounding factors. Xenobiotic-metabolising enzymes are known to be strongly modulated, in fish, in response to environmental conditions such as temperature, stress, diet, reproductive activity and presence of pollutants [54,55]. Water temperature could alter the baseline of DNA damage detected by the Comet assay as reported by other authors [56]. Acclimation response that involves the regulation of gene expression [57] is considered to be markedly significant for eurythermal fish such as carp, which can survive under a wide temperature range from near zero to over 30 °C. In carp, while low environmental temperatures induce a lower RNA trasduction activity [58], increasing temperatures directly stimulate the protein synthesis [59]. Repair mechanisms could also be increased with a consequent large number of repair-intermediates, i.e. DNA strand breaks. Hyperthermia was shown to be able to modulate DNA repair processes, with a shortening of the time required for DNA repair, as assessed by the Comet assay [60]. In this context, the increment of DNA baseline migration in June 2001 could reflect a faster metabolism in relation to the higher temperature, which could have masked the effect of the chlorinated disinfectants detected in the other seasons. The acclimation processes could also be modulated by the different amounts of pollutants: genotoxic compounds present in the water could increase the baseline DNA damage as suggested by the data obtained in February 2001 after a long-time exposure to untreated lake water.

A correspondence between short-term DNA damage, as detected by the Comet assay and aneugenic/clastogenic effects, as detected by the MN test in different fish populations was found for chlorinated disinfectants during the October 2000 and February 2001 campaigns. A clear correlation between long-term DNA damage and MN induction in the same specimens was not found, confirming the results of previous works on aquatic organisms [61] and human biomonitoring studies [62]. However, direct effects on DNA, detected in the Comet assay, represent an independent and sensitive end-point to assess a synergistic DNA-damaging potential of mixtures containing compounds producing repairable DNA breakage or alkali-labile sites [63].

Chlorinated disinfectants, i.e. ClO_2 and NaClO, seem to represent the major source of clastogenic/aneugenic by-products following superficial water disinfection for potabilization, as assessed by the MN assay (October 2000 and June 2001, after 20-day exposures) and by the Comet assay, after 3-h exposures (October 2000 and February 2001) even if not in the same fish populations. These results are in agreement with the possible formation of genotoxic compounds, such as adsorbable organic halogens (AOX), after treatment of water with chlorinated disinfectants as shown by data on the production of trihalomethanes and AOX, detected in the same sampling campaigns (October 2000, February 2001 and June 2001) in the pilot plant at the beginning of the exposure [64].

With respect to the results of other mutagenicity tests performed with other systems in the same sampling campaigns (October 2000, February 2001 and June 2001) in the pilot plant, there is a good correspondence with data on mutagenicity in plants (*Tradescantia* spp., *A. cepa* and *V. faba*), which showed the strongest mutagenic effect in the temperate–warm seasons following exposure to NaClO- and ClO_2-disinfected waters [64].

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