Modulating effects of humic acids on genotoxicity induced by water disinfectants in *Cyprinus carpio*

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Abstract

The use of chlorinated disinfectants during drinking-water production has been shown to generate halogenated compounds as a result of interactions of humic acids with chlorine. Such chlorinated by-products have been shown to induce genotoxic effects and consumption of chlorinated drinking-water has been correlated with increased risk for cancer induction in human populations. The aim of this work was to test the potential genotoxic effects on circulating erythrocytes of the fish *Cyprinus carpio* exposed in vivo to well-waters disinfected with sodium hypochlorite (NaClO), chlorine dioxide (ClO$_2$) or peracetic acid (CH$_3$COO$_2$H, PAA), in the absence or presence of standard humic acids (HA). The effects were measured by use of the micronucleus (MN) and the single-cell gel electrophoresis (Comet) assays at different sampling times after a 3-day exposure period. The exposure to chlorine disinfectants without the addition of HA produced a clear toxic effect. Significant cytogenetic damage (i.e. MN induction) was detected in fish populations exposed to both NaClO and ClO$_2$ with humic acids. In the Comet assay, a significant decrease of DNA migration was observed in erythrocytes of specimens after exposure to NaClO-disinfected water without HA. No effects were observed in any other experimental condition.
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1. Introduction

Disinfection of surface waters for drinking-water production is widely conducted with chlorination, which is known to generate toxic by-products as a result of reactions of chlorine with naturally occurring humic acids [1–3]. These organic halogenated compounds induce genotoxic/mutagenic effects [4–8]. Humic acids (HA) constitute the major portion of organic material present in surface waters, deriving from living or decaying vegetation or microbial decomposition processes [5]; they are usually quantified in terms of total organic carbon (TOC).

A correlation between consumption of chlorinated drinking-water and an increased risk for cancer has been

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shown from epidemiological studies in human populations [9–12]. Such a correlation has also been confirmed by long-term carcinogenicity studies in rats [13]. Thus, the concern for effects on human health from drinking-water contaminated with genotoxic compounds is the reason for intensive studies, where the widest spectrum of biological systems and exposure conditions are needed. Indeed, results obtained from different organisms used as bio-indicators have indicated several genotoxic and mutagenic effects of disinfected waters, including drinking-water, after direct exposure of vertebrates [14–18], invertebrates [19,20] and plants [21,22].

Due to the increasing evidence of adverse effects of chlorinated compounds, in some instances – such as in hospitals, laboratories and factories – the use of peracetic acid (CH$_3$COO$_2$H, PAA) has been experimentally introduced as an alternative compound for water disinfection [23–25]. PAA was shown to produce, as disinfection by-products, only carboxylic acids, which exhibit a very low level of genotoxicity [26].

For investigations on the quality of treated waters or polluted aquatic environments with respect to genotoxicity, a useful approach is the in vivo treatment or the in situ exposure of living organisms. The micronucleus (MN) test performed in proliferating cell systems of such exposed organisms is a very common and recommended approach for the detection of genotoxicity of treated waters [14–22]; the use of fish as a suitable bio-indicator has been proposed because of its high sensitivity for the detection of both clastogenic and aneugenic activity (reviewed in [27,28]). The application of the MN assay to circulating erythrocytes of fish has been considered a promising tool for the screening of drinking-water. It has been employed for both in situ exposure to environmental waters [29–33] and laboratory treatments in vivo [33–38]. In particular, this test has been performed in *Cyprinus carpio* [18,39–41].

Despite the recommended and wide use of this test system, little information is available on fish erythrocyte cell kinetics. Several studies carried out mainly on zebra fish show that the kidney marrow is the organ of definitive hematopoiesis in adult fish, analogous to the mammalian bone marrow, where hematopoietic stem cells are resident: all blood-cell lineages and their precursors are found as a heterogeneous population between the renal tubules and the blood vessels. The kidney marrow displays a cellular complexity comparable to that of the mammalian bone marrow [42]. In some fish, such as trout (*Salmo trutta*) and perch (*Perca fluviatilis*), the spleen is an additional or the only site of adult hematopoiesis, respectively [43]. For these cells, a direct estimation on the length of the cell-cycle is not reported in the literature. Direct and indirect estimations on cell-cycle dynamics of different cell types, both from early studies on in vivo proliferating systems [44] and from explanted cell cultures [45,46] indicate an average cell-cycle length of about 3 days. Based on radioisotope labelling experiments, an average lifespan of about 150 days has been estimated for red blood cells in *Tinca tinca* [47]. According to data from different authors, the estimated time for detection of MN in erythrocytes in circulating blood ranges from the 2nd and 3rd week after a clastogenic treatment [33,36,41], which is compatible with the requirement of at least 100 days for a complete turnover of circulating erythrocytes.

The appearance of MN in circulating fish erythrocytes is the ultimate step in their development: they are generated by segregational errors (due to clastogenic and/or aneugenic events) taking place in the dividing erythropoietic stem cells in the cephalic kidney. The time interval occurring between their initial induction and their detection in the circulating erythrocyte cell population is rather long, depending on the cell-cycle duration, the number of the occurring mitotic events and the time of their detention in the kidney marrow needed for cell differentiation and maturation. Once micronucleated erythrocytes are produced, they persist in the red blood cell population until their removal.

The DNA damage measured by DNA strand breakage represents another sensitive indicator of genotoxicity. In particular, the alkaline single-cell gel electrophoresis assay (Comet assay) has been applied to the aquatic environment, both on vertebrate [48,49,18] and invertebrate organisms (for a review, see [50]). This assay has also been proposed by the German Federal Environmental Agency as a useful test in a graduated testing battery for a reliable detection of genotoxicity of surface waters [51].

In our previous study [18], the genotoxic effects of disinfected lake waters were investigated in different seasons on carps exposed in situ to lake waters treated with three different compounds: two chlorinated chemicals, i.e. sodium hypochlorite and chlorine dioxide, and the newly introduced non-chlorinated compound peracetic acid. The micronucleus test and the Comet assay performed in fish erythrocytes indicated the induction of genotoxic effects by the chlorinated disinfectants. Supported by literature data on the mutagenic effects induced as a result of humic acid chlorination, our positive results were discussed taking into account the formation of genotoxic intermediate compounds, such as adsorbable organic halogens (AOX), which were also found in the treated lake waters [22]. However, some confounding factors had been identified, such as temperature and/or other seasonal/environmental variables.

Since humic acids are the main organic component of surface fresh waters and because their interaction with chlorinated disinfectants generates genotoxic by-products [4–8], our attention in the present work is focused on the role of these disinfectants in the induction of genotoxic or mutagenic effects in specific physiological conditions of animals and in a controlled experimental environment.

Genotoxicity assays were performed on circulating erythrocytes of in vivo exposed *C. carpio* for the analysis of the genotoxic/mutagenic effects of three disinfectants, sodium hypochlorite (NaClO), chlorine dioxide (ClO_2) and peracetic acid (CH_3COO_2H, PAA), by use of the MN and Comet assays. Fish were exposed to the disinfectants dissolved in water, with or without humic acids. In view of the possible relevance of the results in relation to drinking-water consumption, we tested the disinfectants at low concentrations: the highest concentration employed (0.2 ppm) corresponds to the maximum free disinfectant residue adopted in surface water disinfection experiments [18,22]. The humic acids concentration (0.1 ppm) was chosen to obtain a 1:0.32 molar-ratio C:Cl for the highest NaClO concentration and a 1:0.08 molar-ratio for the lowest concentration (0.05 ppm), so as to achieve almost complete reaction of chlorine with organic carbon, according to carcinogenicity/mutagenicity studies [4,52,53].

2. Material and methods

2.1. Well-water analysis

Measurements of physical–chemical parameters in the aquarium water were carried out before the start of the experiments after filtration (glass fibre filters, Whatman GF/F) determining nitrite (colorimetry, diazotization method), nitrate (colorimetry, cadmium-reduction method), ammonium (colorimetry, indophenol blue) and phosphorus (colorimetry, ascorbic acid method) [54]. Temperature, oxygen concentration and pH were determined by a Multiline P4 WTW probe. Total organic carbon was also measured (see below).

2.2. Treatment solutions

2.2.1. Disinfectants

Sodium hypochlorite (NaClO; CAS #10022-70-5; 15%, w/v) was from Solvay S.p.A. (Rosignano, LI, Italy). Chlorine dioxide (ClO_2; CAS #10049-04-4) from Caffaro (Brescia, Italy) was produced by mixing a hydrochloric acid solution (10% weight) and a sodium chlorite solution (8% weight) in a weight ratio of 1:1. Peracetic acid (CH_3COO_2H; CAS #79-21-0) was from Promox S.r.l. (Leggiuno, VA, Italy): the peracetic acid solution contains peracetic acid (15%, w/w), hydrogen peroxide (23%, w/w) and acetic acid (20%, w/w). NaClO and

ClO_2 were always titrated [55] before use, because of their instability: NaClO with an iodometric titration and ClO_2 with a DPD method (Katalase from Merck, KgaA, Darmstadt, Germany; DPD Total Chlorine Reagent Powder Pillow, HACH Company, CO, USA).

2.2.2. Solutions

The disinfectants were used at 0.05 and 0.20 ppm (final concentration), both with and without humic acids.

Before use, all treatment solutions containing the disinfectants alone were freshly prepared (1000× concentrated) in 100 ml distilled water. HA solutions (1000× concentrated) were prepared by adding up to about 20 mg of humic acids (Fluka, Milano, Italy, CAS #1415-93-6, ignition residue about 20%) in 100 ml distilled water under magnetic stirring, until a TOC value of 100 ppm was reached, as measured by TOC-V CPH (total organic analyzer, SHIMADZU). After disinfectant addition (at 200 and 50 ppm), the solutions were stored at 4 °C for at least 3 days before use, in order to let interactions take place. The C:Cl molar-ratios of the mixture containing HA + NaClO at the highest and lowest chlorine concentrations were about 1:0.32 and 1:0.08, respectively. Each concentrated solution was added to 100-l aquariums filled with well-water.

Finally, due to a dramatic effect of lethality observed within the first 24-h of exposure to 0.2 ppm of both NaClO and ClO_2 (pure disinfectants) these concentrations were halved: thus, for these two chlorinated compounds, the highest concentration of pure disinfectant employed for fish exposure was 0.1 ppm.

2.3. Fish rearing and experimental conditions

Young specimens of *C. carpio*, weight range 30–40 g, obtained by artificial reproduction in the Laboratory of Experimental Ecology and Aquaculture of Tor Vergata, were used in the experiments. Before and after the exposure time (72 h), fishes were maintained in facilities consisting of 100-l aquariums fed with filtered well-water (open system) at a temperature of 16–17 °C.

Fish were exposed to the three disinfectants NaClO, ClO_2 and PAA at different final concentrations both with and without humic acids. Two control populations exposed to well-water with or without humic acids were also assessed. Exposure of fish specimens (10 per experimental point) was carried out for 72 h followed by a recovery time of 30 days. During the treatment time, fish were not fed and aquariums were hermetically closed; a complete renewing of the treatment solutions was carried out every 24 h to minimize volatilization of the compounds. At the end of the treatment, the usual rearing conditions were given to the fishes until the end of the experiments.

Positive controls were not built into the experimental design, because of the strict regulations for discharging high volumes (100 l) of contaminated water. On the other hand, our previous study [18] on carps exposed to lake water with or without NaClO, ClO_2 and PAA disinfection showed the sensitivity of the MN and Comet assays in monitoring environmental stress and pollutants. Other authors also reported the ability of

the Comet assay to detect DNA damage induced by environmental contaminants [56,57].

2.4. Blood sampling

The blood samplings were carried out by intra-cardiac puncture on anaesthetized fishes (0.1 g/l of MS-222 Finquel, SCUBLA AQUACULTURE, Udine, Italy) with heparinized syringes (sodium heparin, CLARISCO®, Schwarz Pharma S.p.A., Milano, Italy). After a 5-min recovery period in well-water, fish were replaced in their own aquarium.

In order to study the same fish population during the experiment with the two different tests, we adopted the following protocol, which combines with several constraints, such as the ethical limitation in specimen number, the need to prevent animal stress or killing due to successive cardiac punctures, and the need of different sampling times in relation to the peculiar characteristics of each test (the Comet assay detects DNA damage that is induced very early after exposure to genotoxic compounds; MN are the result of chromosome breaks and/or chromosome malsegregation that require necessarily a passage through mitosis to be revealed).

Blood samples were repeatedly taken from the same fish specimens in each aquarium, for both MN and Comet assays. The first sampling was conducted immediately before the treatment solutions were added to the water ($t = 0$), for both assays. Then, blood samples for the Comet assay were collected at 3 days [56,57] starting from the introduction of disinfectants into the aquarium, while the samples for the analysis of MN were taken at 15 and 30 days.

2.5. Comet assay

The Comet assay was basically performed according to [58]. Cells directly withdrawn from the fish (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 for the first layer. The agarose with cells was allowed to solidify 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$_2$EDTA, 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$_2$EDTA, 300 mM NaOH, pH 13) for 20 min and electrophoresis was performed in the same buffer for 10 min at 0.78 Vcm^{-1} and 300 mA. DNA unwinding and electrophoresis were performed in an ice-water bath. After the electrophoresis, the slides were washed in neutralization buffer (0.4 M Tris–HCl, pH 7.5). All the steps described above were performed under yellow light to minimize 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 (50 cells/slide) were analyzed; the number of clouds of DNA fragments – or ghost cells, which represent cellular toxic events such as apoptosis and/or necrosis [59] – was recorded, but 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 present the data on genotoxic effects.

Data were analyzed using the statistical and graphical functions of SPSS 11 (SPSS Inc., Chicago, IL, USA). The median tail-moment values were used in a one-way analysis of variance test (GLM, general linear model). If a significant F-value of $P < 0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted.

2.6. Micronucleus test

Whole-blood smears were fixed in absolute ethanol for 20 min immediately after sampling; slides were then Feulgen stained (1 h Shiff's reagent after acid hydrolysis for 15 min at 60 °C in 1N HCl) and mounted with dissolved polystyrene in xylene (DPX, Fluka, Milano, Italy). Twenty thousands erythrocytes per fish (10 specimens per experimental point) were analysed blind for the presence of micronuclei, at $1000\times$ magnification under a light microscope. Only nucleated erythrocytes with intact cellular and nuclear membranes were scored and rounded particles without any connection with main nucleus were accepted as micronuclei (for scoring criteria see also [33,41,60]. Statistical evaluation was carried out by non-parametric tests (Mann–Whitney test after a Kruskal–Wallis non-parametric ANOVA). Comparisons were made among fish populations at the first sampling time ($t = 0$). Pairwise comparisons were made for each treatment between samples taken at $t = 0$ and samples taken at $t = 15$ or 30 days. Finally, regression analyses were carried out on the data obtained for each disinfectant with or without HA, at each sampling time.

3. Results

3.1. Well-water analysis

Physical–chemical values (mean ± S.D.) of the water parameters measured in the aquariums before the start of the experiments were as follows: temperature, 16.4 ± 0.5 °C; pH 7.4 ± 0.3; O$_2$ concentration, 7.6 ± 0.2 mg/l; O$_2$% saturation, 80.3 ± 2.5; N-NO$_2 < 0.02$ mg/l; N-NO$_3 = 0.91 \pm 0.03$ mg/l; N-NH$_4 < 0.2$ mg/l; P-PO$_4 < 0.02$ mg/l. The mean TOC value was 9.6 ± 3.1 µg/l. In addition, measurements of heavy metal content (cadmium, copper, mercury,

zinc), performed for water-quality assessment in the aquaculture plant, showed values (data not reported) far below the limits recommended to protect the health of cold- and warm-water fish in intensive culture [61]. The data altogether indicate a good water quality, which is adequate to minimize the sources of confounding factors.

3.2. Biological assays

A strong toxic effect was evident for pure chlorinated disinfectants (Tables 1A and 2A); this effect was completely erased by the concomitant use of humic acids (Tables 1B and 2B).

3.2.1. Comet assay

The data obtained with the Comet assay in erythrocytes of *C. carpio* exposed in situ to different concentrations of NaClO, ClO$_2$ and PAA with or without HA are reported as median TM values in Table 1. The data concerning the percentage of ghost cells detected, which was never >10% and did not show a clear dose–response relationship, are not reported.

The comparison of the DNA migration detected in the fish population at the beginning ($t = 0$) of exposure and that in the two control populations (0 ppm) at the end of the exposure time ($t = 3$ days) in well-water without (Table 1A) or with (Table 1B) humic acids did not show any significant differences.

After 3 days of exposure ($t = 3$ days), an overall reduction in migration compared with the control population (0 ppm) was found with the two pure chlorine-containing disinfectants without humic acids (Table 1A). The effects were significant ($P < 0.01$) for 0.1 ppm NaClO. Significant variations in DNA migration were not observed in the presence of humic acids (Table 1B) at this time point.

3.2.2. Micronucleus test

The observed frequencies from MN analysis are presented in Table 2. Micronucleus frequencies of all fish populations sampled at the start ($t = 0$) were very similar, indicating a good homogeneity in the starting conditions.

No significant increase in MN frequency was found in blood samples taken at $t = 15$ and 30 days compared with samples taken at $t = 0$, for fish populations exposed to disinfectants alone. Significant dose-dependent increases in MN frequency (linear regression analysis) were found for some experimental points only (Table 3).

On the other hand, significant increases in MN frequency were found for blood samples taken at successive time points compared with samples taken at $t = 0$, in fish populations exposed to NaClO + HA, at

Table 1
Median tail-moment (mean ± S.D.) observed in erythrocytes of *Cyprinus carpio* (100 cells per specimen) after exposure of 3 days ($t = 3$ days) to different concentrations of NaClO, ClO$_2$ and CH$_3$COO$_2$H without (A) or with (B) humic acids (10 specimens per experimental point if not indicated otherwise: severe decrease in number was due to lethal effects)

0 ppm	NaClO			ClO$_2$			CH$_3$COO$_2$H	
	0.05 ppm	0.1 ppm	0.2 ppm	0.05 ppm	0.1 ppm	0.2 ppm	0.05 ppm	0.2 ppm
A: Without humic acid								
17.7 ± 6.1 (n=20)	14.7 ± 1.6 (n=2)	7.2 ± 1.8** (n=4)	TOX	14.2 ± 2.6 (n=6)	12.6 ± 1.7 (n=3)	TOX	18.8 ± 3.6 (n=9)	21.7 ± 4.8
B: With humic acid								
21.6 ± 6.5 (n=20)	22.1 ± 11.1	nd	21.8 ± 3.8	24.3 ± 11.6 (n=9)	nd	19.4 ± 1.3	25.0 ± 10.8	24.1 ± 7.3
Starting population ($t=0$): 20.2 ± 6.1 (n=160)								

The median TM (mean ± S.D.) of the starting population is also shown.
** $P < 0.01$, Dunnet's C test; TOX = 100% lethality.

Table 2
Mean frequency values (±S.E.) of micronuclei (MN/20,000 cells) observed in erythrocytes of *Cyprinus carpio* (20,000 cells per specimen) after 72 h exposure to different concentrations (0.05–0.2 ppm) of NaClO, ClO_2 and CH_3COO_2H without (A) or with (B) humic acids (0.1 ppm TOC)

Control	NaClO			ClO_2			CH_3COO_2H	
	0.05 ppm	0.1 ppm	0.2 ppm	0.05 ppm	0.1 ppm	0.2 ppm	0.05 ppm	0.2 ppm
A: Without humic acid								
$t = 0$								
0.30 ± 0.13 ($n = 20$)	0.20 ± 0.13	0.40 ± 0.22	nd	0.50 ± 0.22	0.40 ± 0.16	nd	0.30 ± 0.21	0.0 ± 0.15
$t = 15$ days								
0.35 ± 0.13 ($n = 20$)	1.00 ± 0.00 ($n = 2$)	0.75 ± 0.48 ($n = 4$)	TOX	0.50 ± 0.34 ($n = 6$)	1.67 ± 0.33 ($n = 3$)	TOX	0.33 ± 0.16 ($n = 9$)	0.80 ± 0.25
$t = 30$ days								
0.35 ± 0.13 ($n = 20$)	1.50 ± 0.50 ($n = 2$)	1.25 ± 0.25 ($n = 4$)	TOX	1.00 ± 0.36 ($n = 6$)	1.00 ± 0.58 ($n = 3$)	TOX	0.44 ± 0.17 ($n = 9$)	1.20 ± 0.33
B: With humic acid								
$t = 0$								
0.40 ± 0.13 ($n = 20$)	0.30 ± 0.15	nd	0.50 ± 0.22	0.33 ± 0.24 ($n = 9$)	nd	0.20 ± 0.13	0.40 ± 0.16	0.40 ± 0.22
$t = 15$ days								
0.40 ± 0.13 ($n = 20$)	1.00 ± 0.26	nd	2.20 ± 0.29[**]	0.70 ± 0.26	nd	1.40 ± 0.42[*]	0.50 ± 0.22	1.10 ± 0.23
$t = 30$ days								
0.60 ± 0.15 ($n = 20$)	1.30 ± 0.30[*]	nd	2.90 ± 0.53[***]	0.90 ± 0.23	nd	1.70 ± 0.39[**]	0.70 ± 0.30	1.10 ± 0.31

Statistical comparisons were made for each treated group between blood samples at $t = 0$ and at $t = 15$ or 30. Three sampling times are studied: immediately before the beginning of exposure ($t_0 = 0$) and 15 or 30 days ($t_1 = 15$ days, $t_2 = 30$ days) after the start of exposure. Ten specimens per experimental point (20 for controls) were analysed, when not indicated otherwise (severe decreases in numbers were due to lethal effects).

[*] $P < 0.05$ (Mann–Whitney test).
[**] $P < 0.01$ (Mann–Whitney test).
[***] $P < 0.001$ (Mann–Whitney test).

Table 3
Results of linear regression analysis on micronucleus (MN) frequencies in fish erythrocytes (see Table 2), obtained from a 72-h exposure to different concentrations (range: 0.05–0.2 ppm) of NaClO, ClO$_2$ and CH$_3$COO$_2$H with (+) or without (−) humic acids (HA: 0.1 ppm TOC): two sampling times (15 and 30 days) were examined after the treatment

Disinfectant	Humic acids	Slope	Correlation coefficient (r)
NaClO (15 days)	−	0.0213	0.240
NaClO (30 days)	−	0.0471[*]	0.491
ClO$_2$ (15 days)	−	0.0639[**]	0.536
ClO$_2$ (30 days)	−	0.0377	0.316
CH$_3$COO$_2$H (15 days)	−	0.0230	0.302
CH$_3$COO$_2$H (30 days)	−	0.0430[**]	0.460
NaClO (15 days)	+	0.0893[***]	0.714
NaClO (30 days)	+	0.1144[***]	0.675
ClO$_2$ (15 days)	+	0.0498[**]	0.430
ClO$_2$ (30 days)	+	0.0550[**]	0.475
CH$_3$COO$_2$H (15 days)	+	0.0363[**]	0.444
CH$_3$COO$_2$H (30 days)	+	0.0251	0.248

[*] $P < 0.05$: referred to the slope value (significantly different from 0).
[**] $P < 0.01$: referred to the slope value (significantly different from 0).
[***] $P < 0.001$: referred to the slope value (significantly different from 0).

both 0.2 ppm ($t = 15$ and 30 days) and 0.05 ppm ($t = 30$ days), as well as ClO$_2$ + HA at 0.2 ppm ($t = 15$ and 30 days). A significant dose-dependent increase in MN frequency (linear regression analyses) was shown for the chlorinated disinfectants with HA at both sampling times, and for PAA at $t = 15$ days. The slopes, correlation coefficient (r) and P-values are shown in Table 3.

A clear clastogenic and/or aneugenic effect is induced by chlorinated disinfectants, mainly NaClO, only in the presence of HA, as shown by the MN test.

4. Discussion

The ubiquitous occurrence of trihalomethanes in chlorinated drinking-water and their potential carcinogenic impact has attracted considerable attention. Furthermore, a variety of both chlorinated and non-chlorinated organic products has been shown to be formed as a result of disinfection of water that contains humic material [1–3,62].

In this context, the present study was focused on the assessment of in vivo genotoxic effects on *C. carpio* exposed to different disinfectants in the presence or absence of standard HA. The exposure of fish populations shows evidence for lethal effects induced by the pure disinfectants NaClO and ClO$_2$, dissolved in the

well-water, i.e. in the absence of HA. In particular, the 0.2-ppm concentration of the two chlorinated disinfectants (NaClO and ClO$_2$) appears to be highly toxic, so as to induce 100% lethality within the first 24 h of exposure. High toxicity is still evident even at diminishing disinfectant concentrations. A 'protective effect' of HA against these toxic effects could be suggested by the lack of mortality in fishes exposed to HA-pretreated disinfectant solutions. These findings are in agreement with our previous studies [18] where no lethal effects were detected in *C. carpio* exposed to lake waters with high HA content that were disinfected with comparable chlorine concentrations. Aquatic humic acids have also been found to decrease acute toxicity of a pyrethroid pesticide in grass carp [63].

In general, pure chlorinated disinfectants induced a slight – although not significant – increase in MN frequency with time (up to 30 days) in exposed fish. Beside high toxicity, a weak genotoxic effect was detected by the Comet assay, where a significant reduction of DNA migration was found in surviving specimens after exposure to 0.1 ppm NaClO and, in a non-significant way, ClO$_2$. In the Comet assay, a reduction of DNA migration can be related to intrastrand and interstrand DNA cross-links [64,65]. A reduction in DNA migration, explained as DNA cross-linking, was reported in mice exposed in vivo to dichloroacetic acid, a major disinfection by-product of water chlorination [66]. Pommery et al. [53] proposed that cross-linking by-products could be derived from interactions of chlorine disinfectants with organic molecules present at very low concentrations (high Cl:C ratio) in well-water.

Pre-incubation of chlorinated disinfectants with HA induces clastogenic and/or aneugenic effects, as is shown by the MN test, in which a trend of increasing MN frequency with time (up to 30 days) was detected. This is in agreement with other results previously obtained on carp erythrocytes [33,36,41,18] and explained by the slow release of MN-containing erythrocytes from their stem tissue, the cephalic kidney. These results are confirmed by the dose-dependent increase in MN frequency, suggesting that genotoxic by-products are generated by the interaction of these chlorinated disinfectants with HA. The observed genotoxic effects of NaClO + HA and of ClO$_2$ + HA are in accordance with results obtained from previous studies [18] on MN-carp erythrocytes after in situ exposures to similar concentrations of the disinfectants. The present results are also in agreement with those obtained from in situ plant genotoxicity tests, where clear clastogenic/aneugenic effects of NaClO- and ClO$_2$-disinfected surface waters were reported in different monitoring seasons [22]. Mutagenic activity induced

by chlorinated humic acid compounds was also shown in bacteria [52,1,2]. On the other hand, the Comet assay did not detect any significant increase or decrease in DNA migration.

The absence of direct, Comet-detectable primary DNA damage could be related to the Cl:C ratios used in our experiments (1:0.32 or 1:0.08), which are lower than those detected from in situ exposures [21,18]. Furthermore, the data on erythrocytes sampled after 3 days of exposure could be the result of a balance among DNA damage induction, DNA repair processes, and apoptotic events with elimination of damaged cells. The negative results obtained by the Comet assay on the 3rd day from the beginning of a continuous exposure, compared with the positive outcome obtained at an earlier sampling time (3 h) in our previous study [18], seem to suggest that 3 days may be a sampling time that is too long, presumably inadequate for the test in this cell system. Unlike stem cells that undergo cell-cycle progression and full DNA metabolism, circulating erythrocytes might differ, e.g. in terms of repair-system efficacy, and heavily damaged cells may undergo elimination from the erythrocyte cell population within a short lapse of time. Conversely, the occurrence of repair mechanisms, together with an adaptive response to genotoxic exposure, might explain the absence of damaged cells.

Positive results from the MN test – without concomitant DNA damage detectable by the Comet assay – induced by NaClO and ClO$_2$ with HA treatments could be due to a specific response of circulating erythrocytes towards stress, such as detoxication of by-products [67,68]; on the other hand, in stem cells of the cephalic kidney the primary damage may undergo DNA repair, eventually leading to chromosome breakage, detectable as micronuclei in circulating erythrocytes, several days later. Another possible explanation is that the MN test can detect aneugenic effects also.

Chlorination of HA involves a highly complex sequence of reactions. It is essentially an oxidative, halogenating, degradation process with simultaneous substitution, addition, elimination, rearrangement and hydrolytic reactions taking place, resulting in a large variety of end products, not all fully characterized [5,62]. This chain of reactions is highly dependent upon the level of chlorination, total organic carbon, and other physico-chemical parameters [5,53]. Many of the halogenated compounds are direct-acting mono- or bifunctional alkylating agents and possibly responsible for a significant part of the mutagenic activity of the chlorinated humic acids detected in the *Salmonella* microsome assay [5]. Pommery et al. [53] showed that a low con-

centration of chlorine together with HA was unable to produce by-products with significant genotoxic activity. Hutchinson et al. [19] did not find any significant genetic damage in embryo–larval polychaetes exposed to NaClO-disinfected sewage, i.e. in the presence of high concentrations of organics. Pommery et al. [53] suggested that, at low concentration, chlorine may damage humic materials without complete degradation, whereas at high concentrations it can also react with first-formed reaction intermediates, thereby producing genotoxic by-products.

The dose-dependent increase in MN frequency found for PAA, both in combination with HA and alone, suggests that its genotoxic intermediate(s) can be produced independently of the organic carbon content of treated waters. Its effect was not detectable by the Comet assay. Negative results on genotoxic effects of PAA have also been reported [18] with *C. carpio* erythrocytes. The Comet assay was able to detect the genotoxic activity of pure PAA with an increase in DNA migration, measured 1 h after the beginning of exposure, in human lymphocytes [69]. Positive results have also been reported [22] in plant genotoxicity assays after in situ exposure to surface waters disinfected with NaClO, ClO$_2$ and PAA. The genotoxicity of PAA was found to be lower than that induced by the two other chlorinated disinfectants.

Pure chlorinated disinfectants appeared to be highly toxic and their genotoxic potential could not be pointed out within the present experimental schedule. On the other hand, two main effects of humic acids could be observed: (i) a genotoxic effect after their reaction with chlorinated compounds and (ii) a protective effect against the toxicity of the chlorinated compounds, the reduction of which rendered the clastogenic effects detectable.

The use of the in vivo Comet assay and MN test, together with evaluation of survival, has enabled us to point out the complexity of the reaction pathways of the different disinfectants, especially chlorine biocides and/or their by-products with organic compounds present in the water.

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