



Modulation of cytochrome P450 and induction of DNA damage in *Cyprinus carpio* exposed *in situ* to surface water treated with chlorine or alternative disinfectants in different seasons

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ABSTRACT

Epidemiological studies have shown an association between consumption of disinfected drinking water and adverse health outcomes. The chemicals used to disinfect water react with occurring organic matter and anthropogenic contaminants in the source water, resulting in the formation of disinfection by-products (DBPs). The observations that some DBPs are carcinogenic in animal models have raised public concern over the possible adverse health effects for humans. Here, the modulation of liver cytochrome P450-linked monooxygenases (MFO) and the genotoxic effects in erythrocytes of *Cyprinus carpio* fish exposed *in situ* to surface drinking water in the presence of disinfectants, such as sodium hypochlorite (NaClO), chlorine dioxide (ClO₂) and peracetic acid (PAA), were investigated in winter and summer. A complex induction/suppression pattern of CYP-associated MFOs in winter was observed for all disinfectants. For example, a 3.4- to 15-fold increase was recorded of the CYP2B1/2-linked dealkylation of pentoxyresorufin with NaClO (10 days) and PAA (20 days). In contrast, ClO₂ generated the most notable inactivation, the CYP2E1-supported hydroxylation of p-nitrophenol being decreased up to 71% after 10 days' treatment. In summer, the degree of modulation was modest, with the exception of CYP3A1/2 and CYP1A1 supported MFOs (62% loss after 20 days PAA).

The micronucleus (MN) induction in fish circulating erythrocytes was also analysed as an endpoint of genotoxic potential in the same fish population. Significant increases of MN induction were detected at the latest sampling time on fish exposed to surface water treated with chlorinate-disinfectants, both in winter (NaClO) and summer (NaClO and ClO₂), while no effect was observed in fish exposed to PAA-treated water.

These results show that water disinfection may be responsible for harmful outcomes in terms of MFO perturbation and DNA damage; if extrapolated to humans, they ultimately offer a possible rationale for the increased urinary cancer risk recorded in regular drinking water consumers.

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Abbreviations: DBPs, disinfection by-products; THMs, trihalomethanes; HAAs, haloacetic acids; CYP, cytochrome P450; NaClO, sodium hypochlorite; ClO₂, chlorine dioxide; PAA, peracetic acid; MN, micronucleus; APND, aminopyrine N-demethylase; PNH, p-nitrophenol hydroxylase; PROD, pentoxyresorufin O-dealkylase; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; TH, testosterone hydroxylase; AOX, adsorbable organic halogens; TOC, total organic carbon; TTHMFP, trihalomethane formation potential; MFOs, mixed function monooxygenases; ROS, reactive oxygen species.

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1. Introduction

Drinking water disinfection is one of the major public health advances in the 20th century, reducing the risk of epidemics from water microbial pathogens. Today, the most common water disinfectants are chlorine, chlorine dioxide, ozone and chloramines. Such chemicals are effective for killing pathogens, but may oxidize the organic and inorganic matter (e.g. humic and fulvic acids, bromide and iodide) naturally occurring in most source waters (lakes, rivers, and groundwaters), generating a variety of highly toxic disinfection by-products (DBPs) which constitute an important public health concern [1]. Source waters are also contaminated by industrial and urban emissions, which may react with disinfectants producing additional carcinogenic DBPs [2]. Water source conditions, such as concentration of natural organic matter (e.g.

organic carbon), bromide or iodide, and other physical factors (pH, temperature), together with the class and amount of disinfectant used, have a significant influence on the formation of the final DBP mixtures [3,4], a phenomenon difficult to predict and assess.

To date, more than 600 DBPs have been identified and reported in the literature; nevertheless, they represent less than half of all possible environmental DBPs. Among these, the halogenated trihalomethanes (THMs) (e.g. chloroform, dibromochloromethane and bromoform) and haloacetic acids (HAAs) (e.g. dichloroacetic acid, trichloroacetic acid and bromate) are the most prevalent classes of DBPs in chlorinated drinking water [5]. Their formation occurs in plant treatments and in distribution systems, generally present at sub- $\mu\text{g/L}$ (ppb) levels.

Some of these products have been shown to be genotoxic in various biological systems [1,6], as well as in humans [7–9], while others have been shown to be non-genotoxic carcinogens in animal models [1]. Several epidemiological studies suggest a link between consumption of chlorinated drinking water and reproductive and developmental outcomes, such as increased spontaneous abortions and intrauterine growth retardation [10–14], and bladder and gastrointestinal tract cancers [15–21]. At present, which by-products in chlorinated drinking water could be responsible for increasing cancer risk in human beings is not well established, because the potential synergistic interactions of chlorinated by-products and their role in the molecular mechanisms of carcinogenesis are still poorly understood.

To reduce the risk of adverse health effects, alternative water disinfectants may be considered as replacements for chlorine-containing chemicals, though DBPs might still form. Over the last years, peracetic acid ($\text{CH}_3\text{COO}_2\text{H}$, PAA), a strong antimicrobial agent with many applications in hospitals, laboratories and factories, has been regarded as a potential alternative disinfectant [22–24]. Disinfection of surface water with PAA was shown to produce, as DBPs, only carboxylic acids, not identified as mutagenic, with a very low level of genotoxicity [25,26]. Furthermore, the number of studies with alternative disinfectants is limited, and there has not been a complete assessment of the involvement of some chemical and physical variables (i.e., source water conditions), which may have a dramatic impact on the generation of various unexpected DBPs and on their production rate, during the water disinfection process [27].

In the absence of definitive epidemiological data, estimations of human health risks posed by DBP contamination may be developed through toxicological evaluations of complex mixtures after *in situ* exposure, rather than the assessment of each individual chemical. To better characterize and mimic real human exposure, the effects of two traditional chlorinated disinfectants (NaClO and ClO_2) and an alternative disinfectant (PAA) were investigated on the modulation of liver CYP-linked monooxygenases of *Cyprinus carpio* fish, exposed *in situ* (up to 20 days) to treated surface waters from Lake Trasimene near Perugia, Italy. As CYP induction is linked to an overproduction of free radical and mutagenic metabolites (co-mutagens), these endpoints are regarded as possible epigenetic mechanisms for DBP carcinogenesis [28–32], which are totally ignored in most works. A parallel estimation of the genotoxic effect of the three disinfectants was also carried out through the micronucleus (MN) test in circulating erythrocytes of the same *C. carpio* fish populations. Due to its highly predictive value for genotoxic effects, the MN test is a widely employed assay, performed in a broad spectrum of eukaryotic organisms, from plants [33,34] to mammalian systems [35]. In the last two decades its application to circulating fish-erythrocytes has been proposed, because it was shown to be simple, reliable and sensitive [2,36].

Experiments were carried out in different seasons in order to assess the influence of chemical variations and physical characteristics of water (e.g. temperature and pH; bromide, iodide and

acid concentration) on the potential toxicity of these disinfectants.

2. Materials and methods

2.1. Lake water

Lake Trasimene near Perugia, Italy, a mesotrophic limnic environment, was chosen as a test site because the surface water has a high concentration of bromide and total organic carbon, which are possible precursors of mutagenic disinfection by-products.

2.2. Monitoring of physical–chemical variables

Determination of physico-chemical variables was performed measuring temperature, oxygen concentration and pH with a Multiline P4 WTW probe, in each exposure basin at each sampling time (t0, t10 and t20). Moreover, lake-water content of nitrite (colorimetry, diazotization method), nitrate (colorimetry, cadmium reduction method), ammonium (colorimetry, indophenol blue), and phosphorus (colorimetry, ascorbic acid method) was determined after water filtration (glass fibre filters Whatman GF/F) [37]. Total organic carbon (TOC), adsorbable organic halogens (AOX), trihalomethane (THM), UV absorbance at 254 nm and total THM formation potential (TTHMFP) were also determined [38], while concentrates of both raw and disinfected water were analysed for disinfection by-products using gas chromatography/mass spectrometry (GC/MS) [39,40].

2.3. Water treatment pilot plant

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 $50\text{-}\mu\text{m}$ pore size inox stainless steel filter followed by a $25\text{-}\mu\text{m}$ 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 7.0.
- (5) One main pipeline divided into 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 mixed for interaction with each disinfectant used in the experiments.
- (7) Four 1 m^3 stainless steel basins (exposure basins) used for *in situ* exposure of the fish. These basins received the water flowing from the contact basins.

2.4. Disinfectants

Briefly, the disinfectants were added to the water from the Trasimene Lake as follows:

- (1) Peracetic acid (PAA, $\text{CH}_3\text{COO}_2\text{H}$ obtained from Promox S.r.l. Leggiuno, VA, Italy), supplied as a 15% solution through a membrane pump.
- (2) Sodium hypochlorite (NaClO , Solvay S.p.A., Rosignano, LI, Italy), supplied as a 14.5–15.5% solution through a membrane pump.
- (3) Chlorine dioxide (ClO_2), produced on site in the pilot plant from an 8% NaClO_2 solution and a 10% HCl solution through an automated generator (Tecme S.r.l., Gardolo di Trento, TN, Italy).

The three disinfectants were used at concentrations that are usually employed for water disinfection. The mean ($\pm\text{SD}$) concentration of each disinfectant added during the exposure was: $1.00 \pm 0.19\text{ mg/L}$ of PAA, $1.64 \pm 0.21\text{ mg/L}$ of ClO_2 , and $1.24 \pm 0.19\text{ mg/L}$ of NaClO .

2.5. Chemicals

Nicotinamide adenine dinucleotide phosphate in its oxidized and reduced forms (NADP⁺ and NADPH), pentoxylresorufin, methoxyresorufin, 7-ethoxyresorufin, p-nitrophenol, aminopyrine, glutathione, 16α -hydroxytestosterone, corticosterone and androste-4-ene-3,17-dione were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and cytochrome c were from Boehringer–Mannheim (Germany). HPLC grade methanol, tetrahydrofuran and dichloromethane were acquired from Labscan Ltd. Co. (Dublin, Ireland). 7α , 6β and 1β -hydroxytestosterone were from Steraloid (Wilton, NH, USA), 6α , 2α and 2β -hydroxytestosterone were a generous gift from Dr. P. Gervasi (CNR, Pisa, Italy). All other chemicals were of the highest purity commercially available.

2.6. Fish acclimatization and exposure; preparation of subcellular fractions

Adult individuals of *C. carpio* were supplied by the 'Centro Ittiogenico di Sant'Arcangelo' (Perugia, Italy).

Male samples, weighing between 340 g and 420 g, were divided into four groups. After a 20 days acclimatization, each group was placed in a different exposure basin containing surface water from the lake treated with one of three different disinfectants: PAA (as an alternative drinking water disinfectant), NaClO, or ClO₂. The fourth group, in untreated lake water, served as an unexposed control. After an exposure period of up to 10 or 20 days, the liver was rapidly removed from each fish, homogenized and then centrifuged at 9000 × g. The post-mitochondrial supernatant was then centrifuged for 60 min at 105,000 × g, after which the pellet was resuspended in 0.1 M K₂P₂O₇, 1 mmol/L EDTA (pH 7.4) and centrifuged again for 60 min at 105,000 × g to give the final microsomal fraction. Washed microsomes were then suspended with a hand-driven potter Elvehjem homogenizer in a 10 mmol/L Tris–HCl buffer (pH 7.4) containing 1 mmol/L EDTA and 20% (v/v) glycerol. The fractions were immediately frozen in liquid nitrogen, stored at –80 °C and used within a week for enzymatic analyses [41].

2.7. Aminopyrine N-demethylase (APND) as a probe of CYP3A1/2

Preferential activity of CYP3A1/2 was determined using aminopyrine N-demethylase (APND) as a probe by quantification of formaldehyde release, according to Mazel [42]. The total incubation volume was 3 mL, composed of 0.5 mL of a buffer solution of 50 mM aminopyrine and 25 mM MgCl₂, 1.48 mL of 0.60 mM NADP⁺, 3.33 mM glucose 6-phosphate in 50 mM Tris–HCl buffer (pH 7.4), 0.02 mL glucose 6-phosphate dehydrogenase (0.93 U/ml), and 0.125 mL of sample (0.5 mg of protein); under these conditions, the method was linear up to ~50 nmol mg⁻¹ min⁻¹. After 5 min of incubation at 37 °C, the yellow color, caused by the reaction with the formaldehyde released with the Nash reagent, was read at 412 nm and the molar absorption of 8000 was used for calculation [43].

2.8. p-Nitrophenol hydroxylase (PNH) as a probe of CYP2E1

The incubation medium consisted of 2 mM p-nitrophenol in 50 mM Tris–HCl buffer (pH 7.4), 5 mM MgCl₂ and a NADPH-generating system consisting of 0.4 mM NADP⁺, 30 mM isocitrate, 0.2 U of isocitrate dehydrogenase and 1.5 mg of proteins in a final volume of 2 mL, as previously described [44]. In brief, after 10 min at 37 °C, the reaction was stopped by adding 0.5 mL of 0.6 N perchloric acid. Under these conditions, the reaction was linear up to ~5.5 nmol mg⁻¹ min⁻¹. Precipitated proteins were removed by centrifugation, and 1 mL of the supernatant was mixed with 1 mL 10 N NaOH. Absorbance at 546 nm was immediately measured and the concentration of 4-nitrocatechol determined ($\epsilon = 10.28 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.9. Pentoxyresorufin O-dealkylase (PROD) as a probe of CYP2B1/2, ethoxyresorufin O-deethylase (EROD) as a probe of CYP1A1, methoxyresorufin O-demethylase (MROD) as a probe of CYP1A2

For measurement of PROD activity, the reaction mixture consisted of 0.025 mM MgCl₂, 200 μM pentoxyresorufin, 0.32 mg of proteins and 130 mM NADPH in 2.0 mL 0.05 M Tris–HCl buffer (pH 7.4). Resorufin formation at 37 °C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin (excitation 562 nm, emission 586 nm) [45]. EROD and MROD activities were measured in the same way, except that the substrates were 1.7 μM ethoxyresorufin and 5 μM methoxyresorufin, respectively [46].

2.10. Testosterone hydroxylase (TH)

An incubation mixture was prepared containing liver microsomes (equivalent to 1–2 mg protein), 0.6 mM NADP⁺, 8 mM glucose 6-phosphate, 1.4 U glucose 6-phosphate dehydrogenase and 1 mM MgCl₂, in a final volume of 2 mL 0.1 M phosphate Na⁺/K⁺ buffer (pH 7.4). The mixture was pre-incubated for 5 min at 37 °C. The reaction was performed at 37 °C by shaking and started by the addition of 80 mM testosterone (dissolved in methanol). After 10 min, the reaction was stopped with 5 mL ice-cold dichloromethane and 9 nmol corticosterone (internal standard) in methanol. After 1 min of mixing, phases were separated by centrifugation at 2000 × g for 10 min and the aqueous phase was extracted once more with 2 mL dichloromethane. The organic phase was extracted with 2 mL 0.02 N NaOH to remove lipid constituents, dried over anhydrous sodium sulphate and transferred to a small tube. Dichloromethane was evaporated at 37 °C under nitrogen and the dried samples stored at –20 °C. The samples were dissolved in 100 μL methanol and analysed by HPLC [47].

Chromatographic separations were performed using a system consisting of a high-pressure pump (Waters Model 600E, Multisolute Delivery System), a sample injection valve (Rheodyne Model 7121, CA, USA) with a 20 μL sample loop and an ultraviolet (UV) detector (254 nm, Waters Model 486, Tunable Absorbance Detector) connected to an integrator (Millennium 2010, Chromatography Manager). For reversed-phase separation of metabolites, a NOVA-PAK C18 analytical column (60 Å, 4 mm, 3.9 mm × 150 mm, Waters) was used for the stationary phase. The mobile phase consisted of a mixture of solvent A [7.5% (v/v) tetrahydrofuran in water] and

solvent B [7.5% (v/v) tetrahydrofuran and 60% (v/v) methanol in water] at 1 mL/min flow rate. Metabolite separation was performed by a gradient from 30 to 100% (v/v) of solvent B over 30 min. The eluent was monitored at 254 nm and the area under the absorption band was integrated. The concentration of metabolites was determined by the ratio between respective metabolite peak areas and corticosterone (the internal standard), and the calibration curves were obtained with synthetic testosterone derivatives [47].

2.11. Protein concentration

Protein concentration was determined according to the method described by Lowry [48] as revised by Bailey [49], using bovine serum albumin as a standard. Samples were diluted at least 200 times to provide a suitable protein concentration.

2.12. Micronucleus test

The MN test was performed on circulating erythrocytes as described previously [50] from a subset of the same *C. carpio* fish. Erythrocytes were obtained at 0, 10 and 20 days after exposure to the different types of disinfected water.

Blood samples from seven fish were studied per experimental point. Heparinized (sodium heparin, CLARISCO®, Schwarz Pharma S.p.A., Milano, Italy) whole blood smears were done immediately after sacrifice, fixed with absolute ethanol for 20 min, then stained with Feulgen (1 h in Schiff reagent (Merck, Germany)) after 15 min of acid hydrolysis at 60 °C in 1 N HCl and mounted with dissolved polystyrene in xylene (DPX, Fluka, Milano, Italy).

For each specimen, 20,000 erythrocytes were analysed under a light microscope (1000× magnification). Only nucleated erythrocytes with an intact cellular and nuclear envelope were scored. Rounded particles not larger than 1/5 of the main nucleus and without any connection to the nucleus were accepted as MN [51]. The scoring criteria were given in detail previously [50–52].

2.13. Statistics and computer analysis

Statistical analysis for the monooxygenase activities was performed using the Wilcoxon's rank method, as reported by Box and Hunter [53]. Non-parametric tests (Kruskal–Wallis ANOVA and two-tailed Mann–Whitney) were performed on MN induction, for control data evaluation among the different sampling times, as well as for pairwise comparison of MN frequencies observed at each experimental point of the exposed groups with the corresponding (exposure time) control.

3. Results

3.1. Physical–chemical parameters (Table 1)

Water temperatures ranged between 5 and 10 °C in the cold season (mean value: 8.1 °C, February) and between 22 and 24 °C in the hot season (mean value: 23.6 ± 1.2 °C SD, late June), showing seasonal fluctuations that are typical in Central Italy, while pH values (7.3–7.8) did not show significant variations. Oxygen concentrations showed an inverse pattern, partially due to the different solubility of oxygen in water at different temperatures. Chemical analyses revealed high concentrations of TOC (5.8–9.5 mg/L) in raw and disinfected lake water, whereas TTHMFP in raw water was 158 mg/L and 475 mg/L in February and June, respectively. At the doses employed in the pilot plant, NaClO was the only disinfectant that produced low levels of THM, which also formed unknown organo-chlorinated compounds through its reaction with the high level of organic substances. AOX concentration increased only in NaClO-disinfected water, in both seasons. Finally, nutrient concentrations were always compatible with the rearing demand.

3.2. Mixed function monooxygenases

The exposure of *C. carpio* for 10 or 20 consecutive days to the disinfected waters in the different seasons resulted in variable modulation of several liver CYP-metabolizing enzymes, as reported in Tables 2–5.

3.2.1. Specific biomarkers

In the winter experiments (Table 2), treatment with the ClO₂ disinfectant decreased the following MFOs: APND (CYP3A1/2-preferential, 36.4% loss, $p < 0.01$), PNH (CYP2E1, 70.5% loss, $p < 0.01$), EROD (CYP1A1, 44.4% loss, $p < 0.01$) and MROD (CYP1A2, 58.8% loss,

Table 1

Mean values (\pm SD) of some of the physico-chemical parameters measured during fish exposure in both seasons, in each experimental basin. Water temperatures were $8.1 \pm 2.2^\circ\text{C}$ (winter) and $23.6 \pm 1.2^\circ\text{C}$ (summer).

Season	None		PAA		NaClO		ClO ₂	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
pH	7.8 \pm 0.1	7.4 \pm 0.1	7.7 \pm 0.1	7.3 \pm 0.1	7.7 \pm 0.1	7.3 \pm 0.1	7.6 \pm 0.1	7.3 \pm 0.1
O ₂ (% sat.)	90.1 \pm 2.5	67.3 \pm 10	94.0 \pm 1.1	74.2 \pm 7.5	93.4 \pm 1.6	74.9 \pm 7.5	93.7 \pm 3.2	79.6 \pm 7.0
N-NO ₂	3.3 \pm 0.8	6.0 \pm 2.6	3.7 \pm 1.5	5.7 \pm 3.8	5.3 \pm 1.2	4.7 \pm 1.2	4.7 \pm 0.6	4.3 \pm 1.2
N-NO ₃	1.1 \pm 0.1	0.7 \pm 0.1	1.2 \pm 0.0	0.6 \pm 0.2	1.4 \pm 0.1	1.0 \pm 0.4	1.2 \pm 0.1	0.6 \pm 0.1
NH ₄ ⁺	66.7 \pm 5.8	66.7 \pm 20.8	50.0 \pm 10.0	60.0 \pm 26.5	76.7 \pm 40.4	66.7 \pm 20.8	43.3 \pm 5.8	63.3 \pm 11.6
PO ₄ ³⁻	10 \pm 0.0	167 \pm 66.6	17 \pm 1.2	137 \pm 55.1	17 \pm 1.2	223 \pm 60.3	20 \pm 10.0	163 \pm 90.7
TOC (mg/L)	9.4	5.8	9.5	5.9	9.5	5.9	8.7	6.0
AOX (mg/L)	12	8	13	10	43	39	15	12
TMH (mg/L)	<0.10	<0.10	<0.10	<0.10	9.3	1.34	<0.10	<0.10
TTHMPF (mg/L)	158	475	-	-	-	-	-	-

TTHMPF, potential formation of Total THM (raw water).

Table 2

CYP-linked enzymatic activities in hepatic microsomes of *Cyprinus carpio* exposed to PAA, NaClO or ClO₂ (male – winter).

Linked monooxygenases	t = 10				t = 20			
	None	PAA	NaClO	ClO ₂	None	PAA	NaClO	ClO ₂
Aminopyrine N-demethylase (CYP3A1/2) (nmol mg ⁻¹ min ⁻¹)	1.51 \pm 0.13	1.01 \pm 0.07**	1.17 \pm 0.11**	0.96 \pm 0.05**	1.42 \pm 0.10	1.72 \pm 0.12**	1.42 \pm 0.09	1.31 \pm 0.12
p-Nitrophenol hydroxylase (CYP2E1) (pmol mg ⁻¹ min ⁻¹)	75.22 \pm 5.85	32.64 \pm 3.21**	33.47 \pm 2.51**	22.23 \pm 2.75**	62.71 \pm 3.12	24.54 \pm 2.32**	81.67 \pm 7.94**	56.44 \pm 4.78
Pentoxoresorufin O-dealkylase (CYP2B1) (pmol mg ⁻¹ min ⁻¹)	0.59 \pm 0.05	0.98 \pm 0.04**	0.85 \pm 0.07**	0.56 \pm 0.02	0.56 \pm 0.05	0.78 \pm 0.05**	0.59 \pm 0.02	0.48 \pm 0.01*
Ethoxyresorufin O-deethylase (CYP1A1) (pmol mg ⁻¹ min ⁻¹)	0.36 \pm 0.02	0.40 \pm 0.02*	0.54 \pm 0.03**	0.20 \pm 0.01**	0.34 \pm 0.03	0.27 \pm 0.02*	0.45 \pm 0.03**	0.46 \pm 0.05**
Metoxyresorufin O-demethylase (CYP1A2) (pmol mg ⁻¹ min ⁻¹)	3.91 \pm 0.21	2.29 \pm 0.15**	2.89 \pm 0.18*	1.61 \pm 0.12**	4.17 \pm 0.43	5.88 \pm 0.61**	4.96 \pm 0.18*	3.82 \pm 0.34

Each value represents the mean \pm SD of six experiments on six different carps for each studied group, at different time intervals (t = days).

* P < 0.05, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

** P < 0.01, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

Table 3
CYP-linked enzymatic activities in hepatic microsomes of *Cyprinus carpio* exposed to PAA, NaClO or ClO₂ (male – summer).

Linked monooxygenases	t = 10				t = 20			
	None	PAA	NaClO	ClO ₂	None	PAA	NaClO	ClO ₂
Aminopyrine N-demethylase (CYP3A1/2) (nmol mg ⁻¹ min ⁻¹)	1.97 ± 0.18	1.85 ± 0.20	0.93 ± 0.10**	1.06 ± 0.10**	1.83 ± 0.15	1.92 ± 0.17	1.57 ± 0.13*	1.42 ± 0.12**
p-Nitrophenol hydroxylase (CYP2E1) (nmol mg ⁻¹ min ⁻¹)	0.23 ± 0.02	0.21 ± 0.01	0.12 ± 0.01**	0.20 ± 0.01	0.24 ± 0.02	0.22 ± 0.02	0.20 ± 0.01	0.19 ± 0.02*
Pentoxoresorufin O-dealkylase (CYP2B1) (pmol mg ⁻¹ min ⁻¹)	0.52 ± 0.05	0.65 ± 0.06	0.80 ± 0.05**	0.59 ± 0.05	0.46 ± 0.05	0.60 ± 0.06**	0.41 ± 0.04	0.64 ± 0.05**
Ethoxyresorufin O-deethylase (CYP1A1) (pmol mg ⁻¹ min ⁻¹)	0.57 ± 0.04	0.62 ± 0.05	0.29 ± 0.02**	0.54 ± 0.02	0.64 ± 0.02	0.43 ± 0.04**	0.63 ± 0.06	0.66 ± 0.06
Metoxyresorufin O-demethylase (CYP1A2) (pmol mg ⁻¹ min ⁻¹)	0.56 ± 0.05	0.65 ± 0.05*	0.72 ± 0.06**	0.70 ± 0.05**	0.52 ± 0.04	0.68 ± 0.03**	0.28 ± 0.01**	0.74 ± 0.04**

Each value represents the mean ± SD of six experiments on six different carps for each studied group, at different time intervals (t = days).

* P < 0.05, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

** P < 0.01, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

Table 4
Testosterone hydroxylase in hepatic microsomes of *Cyprinus carpio* exposed to PAA, NaClO or ClO₂ (male – winter).

Linked monooxygenases	t = 10				t = 20			
	None	PAA	NaClO	ClO ₂	None	PAA	NaClO	ClO ₂
6α-Hydroxytestosterone (CYP2A1, CYP1A1) (pmol mg ⁻¹ min ⁻¹)	3.83 ± 0.01	2.40 ± 0.19**	10.75 ± 0.35**	1.79 ± 0.06**	3.33 ± 0.13	4.22 ± 0.18**	2.42 ± 0.14**	2.12 ± 0.05**
7α-Hydroxytestosterone (CYP2A1/2, CYP1A1/2) (pmol mg ⁻¹ min ⁻¹)	3.83 ± 0.31	2.69 ± 0.27**	4.40 ± 0.01	3.71 ± 0.06	3.05 ± 0.20	3.61 ± 0.28**	2.37 ± 0.21**	2.29 ± 0.12**
6β-Hydroxytestosterone (CYP3A1, CYP1A1/2) (nmol mg ⁻¹ min ⁻¹)	7.81 ± 0.69	5.58 ± 0.61**	33.85 ± 3.46**	5.76 ± 0.13**	7.66 ± 0.61	8.68 ± 0.46*	5.09 ± 0.35**	4.17 ± 0.28**
16α-Hydroxytestosterone (CYP2B1, CYP2C11) (pmol mg ⁻¹ min ⁻¹)	1.15 ± 0.05	1.18 ± 0.08	9.17 ± 0.64**	1.05 ± 0.06	1.19 ± 0.07	2.57 ± 0.01**	0.60 ± 0.04**	0.69 ± 0.01**
16β-Hydroxytestosterone (CYP2B1) (pmol mg ⁻¹ min ⁻¹)	5.55 ± 0.14	7.44 ± 0.08**	83.65 ± 5.44**	3.34 ± 0.06**	6.42 ± 0.60	21.90 ± 0.42**	7.35 ± 0.10**	5.52 ± 0.16**
2α-Hydroxytestosterone (CYP2C11) (nmol mg ⁻¹ min ⁻¹)	1.79 ± 0.09	4.42 ± 0.35**	5.20 ± 0.39**	1.10 ± 0.01**	1.67 ± 0.12	3.68 ± 0.10**	2.42 ± 0.04**	1.85 ± 0.01*
2β-Hydroxytestosterone (CYP3A1/2, CYP1A1) (pmol mg ⁻¹ min ⁻¹)	0.46 ± 0.01	1.18 ± 0.11**	0.82 ± 0.01**	0.46 ± 0.01	0.43 ± 0.02	0.37 ± 0.01**	1.01 ± 0.04**	0.43 ± 0.03
4-Androsten-3,17-dione (CYP3A1) (nmol mg ⁻¹ min ⁻¹)	0.55 ± 0.05	0.47 ± 0.02*	2.26 ± 0.07**	0.40 ± 0.01**	0.43 ± 0.03	0.79 ± 0.02**	0.42 ± 0.02	0.37 ± 0.01*

Each value represents the mean ± SD of six experiments on six different carps for each studied group, at different time intervals (t = days).

* P < 0.05, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

** P < 0.01, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

Table 5
Testosterone hydroxylase in hepatic microsomes of *Cyprinus carpio* exposed to PAA, NaClO or ClO₂ (male – summer).

Linked monooxygenases	t = 10				t = 20			
	None	PAA	NaClO	ClO ₂	None	PAA	NaClO	ClO ₂
	6α-Hydroxytestosterone (CYP2A1, CYP1A1) (pmol mg ⁻¹ min ⁻¹)	12.06 ± 1.08	10.74 ± 0.34*	16.53 ± 1.06**	7.98 ± 0.16**	12.53 ± 0.75	10.09 ± 0.18**	14.02 ± 0.20**
7α-Hydroxytestosterone (CYP2A1/2, CYP1A1/2) (pmol mg ⁻¹ min ⁻¹)	7.18 ± 0.62	7.23 ± 0.34	8.61 ± 0.01*	5.39 ± 0.19**	6.69 ± 0.44	3.10 ± 0.06**	4.93 ± 0.26**	4.80 ± 0.01**
6β-Hydroxytestosterone (CYP3A1, CYP1A1/2) (nmol mg ⁻¹ min ⁻¹)	39.04 ± 1.98	46.84 ± 2.39**	47.65 ± 3.32**	31.46 ± 0.70**	51.87 ± 1.72	23.16 ± 0.73**	31.85 ± 0.07**	35.29 ± 0.19**
16α-Hydroxytestosterone (CYP2B1, CYP2C11) (pmol mg ⁻¹ min ⁻¹)	8.71 ± 0.13	9.31 ± 0.65	9.22 ± 0.13*	7.47 ± 0.18**	10.24 ± 1.02	7.45 ± 0.42**	10.32 ± 0.76	11.79 ± 0.01**
16β-Hydroxytestosterone (CYP2B1) (pmol mg ⁻¹ min ⁻¹)	3.35 ± 0.06	4.22 ± 0.19**	2.90 ± 0.01	3.69 ± 0.06	6.98 ± 0.23	3.31 ± 0.24**	8.47 ± 0.23**	11.52 ± 0.10**
2α-Hydroxytestosterone (CYP2C11) (nmol mg ⁻¹ min ⁻¹)	3.92 ± 0.01	3.62 ± 0.17	4.74 ± 0.43**	3.66 ± 0.01	4.21 ± 0.38	2.69 ± 0.12**	3.67 ± 0.07**	3.66 ± 0.01**
2β-Hydroxytestosterone (CYP3A1/2, CYP1A1) (pmol mg ⁻¹ min ⁻¹)	4.31 ± 0.13	3.53 ± 0.29**	1.70 ± 0.09**	4.41 ± 0.15	5.74 ± 0.01	2.15 ± 0.24**	4.84 ± 0.07**	5.00 ± 0.01**
4-Androsten-3,17-dione (CYP3A1) (nmol mg ⁻¹ min ⁻¹)	1.23 ± 0.07	1.44 ± 0.07	1.61 ± 0.04*	1.39 ± 0.02	1.27 ± 0.05	0.88 ± 0.09**	1.31 ± 0.06	1.56 ± 0.06**

Each value represents the mean ± SD of six experiments on six different carps for each studied group, at different time intervals (t = days).

* $p < 0.05$, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

** $p < 0.01$, significant differences between exposed groups and their respective controls, using the Wilcoxon's rank method.

$p < 0.01$) after 10 days; after 20 days' exposure, significant effects were seen for CYP2B1- and CYP1A1-linked monooxygenases (14.3% loss, $p < 0.05$ and 35.3% increase, $p < 0.01$) only.

The exposure of fish to water containing the NaClO disinfectant for 10 consecutive days up-regulated PROD (+44.1%, $p < 0.01$), EROD (+50%, $p < 0.01$) and inactivated APND (22.5% loss, $p < 0.01$), PNH (55.5% loss, $p < 0.01$) and MROD (26.1% loss, $p < 0.01$) linked oxidases; after 20 days' exposure, inductions for PNH (+30.2%, $p < 0.01$), EROD (+32.4%, $p < 0.01$) and MROD (+18.9%, $p < 0.05$) oxidases were seen.

The PAA disinfectant caused a complex modulation of CYP-supported monooxygenases: after 10 days' exposure, significant increases for PROD (+66.1%, $p < 0.01$), EROD (+11.1%, $p < 0.05$) and inactivations for APND (33.1% loss, $p < 0.01$), PNH (56.6% loss, $p < 0.01$) and MROD (41.4% loss, $p < 0.01$) were shown; after 20 days' treatment, PNH (60.9% loss, $p < 0.01$) and EROD (20.6% loss, $p < 0.05$) oxidases were down-regulated by PAA, but some inductions were also recorded, in particular for CYP1A2-supported monooxygenase (MROD, +41%, $p < 0.01$).

In the summer experiment (Table 3), exposure of fish to ClO₂ disinfectant culminated with a significant decrease of APND (46.2% loss, $p < 0.01$) and an increase of MROD (+25%, $p < 0.01$) activities after 10 days; after 20 days, negative (for example for APND, 22.4% loss, $p < 0.01$) and positive (in particular for MROD, +42.3%, $p < 0.01$) modulations were observed.

The NaClO disinfectant provoked an extensive down-regulation of the tested monooxygenases: APND (52.8% loss, $p < 0.01$), PNH (47.8% loss, $p < 0.01$) and EROD (49.1% loss, $p < 0.01$) activities were decreased after 10 days, while MROD decreased (46.2% loss, $p < 0.01$) after 20 days' treatment. Only PROD oxidase was slightly induced (1.6-fold, $p < 0.01$, 10 days' treatment).

Exposure to PAA resulted in a significant inactivation of EROD (32.8% loss, $p < 0.01$) and increases of PROD and MROD activities (both up to 30.4%, $p < 0.01$) after 20 days' treatment.

3.2.2. Testosterone hydroxylases

Using testosterone as a multiprobe of CYP modulation, in the winter experiments (Table 4), the exposure of fish to ClO₂ disinfectant univocally reduced testosterone hydroxylations, in particular in positions 6α (53.3% loss, reflecting CYP2A1 and CYP1A1, $p < 0.01$), 16β (39.8% loss, CYP2B1, $p < 0.01$) and 2α (37.4% loss, CYP2C11, $p < 0.01$) after 10 days' treatment; inactivations in positions 6α (36.3% loss, $p < 0.01$), 6β (45.6% loss, CYP3A1 and CYP1A1/2, $p < 0.01$) and 16α (42% loss, CYP2B1 and CYP2C11, $p < 0.01$) after 20 days' exposure were seen.

In contrast, the NaClO disinfectant caused notable increases of testosterone hydroxylations in all the different positions studied, from 2.8-fold (6α-TH, $p < 0.01$) up to 15-fold (16β-TH, $p < 0.01$), after 10 days' treatment. After 20 days, the expression of testosterone hydroxylase showed a dual behaviour. Considerable decreases of TH (up to 49.6% loss for 16α-TH, $p < 0.01$) and marked inductions (up to 134.9% for 2β-TH reflecting CYP3A1/2 and CYP1A1, $p < 0.01$) were simultaneously recorded.

The disinfectant PAA was able either to significantly induce testosterone hydroxylation in positions 2α and 2β (both up to 2.5-fold, $p < 0.01$) or reduce 6α-TH (37.4% loss, $p < 0.01$) after 10 days' treatment; in contrast, after 20 days, PAA produced remarkable monooxygenase up-regulations, in particular in positions 16β (3.4-fold, $p < 0.01$) and 2α (2.2-fold, $p < 0.01$).

In the summer experiment (Table 5), treatment with the ClO₂ disinfectant decreased testosterone hydroxylation in positions 6α (33.8% loss, $p < 0.01$) and 7α (CYP2A1/2 and CYP1A1/2-linked, 25% loss, $p < 0.01$) after 10 days of fish exposure and in positions 7α (28.3% loss, $p < 0.01$) and 6β (32% loss, $p < 0.01$) after 20 days. A marked up-regulation was recorded for 16β TH (+65%, $p < 0.01$, 20 days' treatment).

The NaClO disinfectant was able to significantly decrease testosterone hydroxylation in position 2 β (60.6% loss, $p < 0.01$) after 10 days, and in positions 7 α (26.3% loss, $p < 0.01$) and 6 β (38.6% loss, $p < 0.01$) after 20 days' treatment. The hydroxylation in position 17 of testosterone, namely 4-androsten-3,17-dione-supported monooxygenase (17-TH), was slightly affected (up to 30.9% increase, $p < 0.01$, 10 days' treatment). Moderate inductions were also recorded for 6 α -TH (+37%, $p < 0.01$, 10 days) and 16 β testosterone hydroxylation (+21.3%, $p < 0.01$, 20 days).

Exposure of fish to the PAA disinfectant resulted in a significant decrease of testosterone hydroxylation in the different positions (from 19.5% loss for 6 α -TH, up to 62.5% loss in position 2 β , $p < 0.01$) after 20 days; only moderate modulations were obtained after 10 days: increase for 16 β TH (up to 26%, $p < 0.01$) and decrease for 2 β TH (18% loss, $p < 0.01$).

3.3. Micronucleus test

Data on MN frequencies are shown in Table 6. An increasing trend of MN induction over time was present in all treated fish samples, in both seasonal conditions, while unexposed samples did not show variations of MN frequency in fish erythrocytes. This indicates that the experimental conditions, namely rearing conditions and acclimation time, were adequate and that seasonal variations of physico-chemical parameters of the water in the different seasons did not determine any effect.

On the other hand, the highest and statistically significant MN increases ($p < 0.01$) were observed in summer at the latest sampling time ($t = 20$) of fish samples exposed to both chlorinated disinfectants. In winter the same trends were detectable, with a lower MN increase detected after exposure to all disinfected waters, of which significant increases were only found after exposure to NaClO-disinfected water. No significant MN increase was detected after fish exposure to PAA-treated natural lake water.

4. Discussion

Disinfected drinking water generates a complex mixture of DBPs, the composition of which varies mainly on the basis of the water quality and the disinfectant employed. Here we investigated the *in vivo* effects on phase-I mixed function monooxygenases and genotoxicity in *C. carpio* fish exposed to surface water treated with two ordinary disinfectants, NaClO and ClO₂, or with a relatively new one, PAA. The disinfectants were employed at the actual concentrations used for surface water treatment. The drinking water plant allowed the *in situ* simultaneous exposure of fish to the same water treated with the aforementioned chemicals. The experiments were conducted in different seasons (winter and summer) in order to evaluate the influence of different physical/chemical water conditions on the studied endpoints.

Testosterone (as multibioprobe) and specific substrates for different CYPs were used to study oxidative reactions. In the *C. carpio* population, we recorded a complex pattern of CYP modulations in terms of both inductions and suppressions for all the tested disinfectants. This suggests that the three chemicals studied may affect the CYP profile in a rather complex manner. As such, they may exercise either antagonistic or agonistic effects or, in some cases, even a synergistic one. The CYP450 MFOs were mainly modulated in the winter for all disinfectants and, in particular, after 10 days' exposure. The most significant inductions were observed for CYP2B1-linked penthoxyresorufin O-dealkylation, with a 15-fold increase after exposure with NaClO after 10 days and a 3.4-fold increase after 20 days of exposure with PAA. In contrast, ClO₂ was able to generate the most notable inactivations and, in particular, CYP2E1 was decreased up to 71% after 10 days' treatment.

A similar heterogeneous pattern of induction/suppression of phase I MFO modulation was also shown in a previous pilot experiment carried out in autumn (October) [32]. It was reported that the most induced CYP isoforms were CYP1A1 and CYP2B1, in particular with PAA and NaClO disinfectants after 10 days' treatment while the most notable inactivation regarded CYP2E1 activity by ClO₂ in both time periods. This trend seems to fit with the data collected in winter.

In summer, the degree of the modulation was lower with respect to winter, where the modest increase of CYP2B1 MFO after 10 days' exposure in the presence of NaClO and an inhibition of 62.5% for the CYP3A1/2 and CYP1A1 MFOs after 20 days with PAA were the most considerable variations detected.

Altogether, these results indicate that all tested disinfectants are able to markedly affect the liver microsomal metabolism, in terms of both induction and inactivation of cytochrome P450-dependent enzymes, with different magnitudes, plausibly related to changes in water conditions in different periods.

Micronuclei are known to originate from DNA-breakage and mal-segregation early events resulting from clastogenic effects, such as those induced by free radicals and other highly reactive compounds, as well as from mitoclastic/aneugenic agents. Their appearance in a cell population, such as that of circulating fish erythrocytes, is the final manifestation of such mutagenic events. A rough estimation of the time interval that occurs between the two 'extremes', i.e., stem cells exposure to mutagens/DNA-damage induction (earliest event) and MN appearance in circulating fish erythrocytes (final event) is about 15–20 days, in relation to the maturation stage of red blood cells [51]. In this view, the significant MN increase detected at 20 days after exposure reflects the expression of clastogenic (and eventually mitoclastic) effects to which fish were submitted at the beginning of the experiment. On the contrary, the observation that the most consistent MFO variations occurs after 10 day exposure can be explained in term of different mechanism involving the cytosolic xenosensor apparatus. According to the biochemical data here presented a genotoxic effect is to be expected after exposure to ClO₂ and NaClO disinfected water, which indeed was confirmed by the significant increase of MN frequencies detected at the latest time ($t = 20$). As far as PAA, only a slight increase of MN frequencies is induced at the same time of analysis ($t = 20$) in both seasons, their values being comparable to those observed for the other two disinfectants at the earlier time ($t = 10$). Yet, if these MN data are compared to the ones obtained for CYP2B1-linked penthoxyresorufin O-dealkylation it can be speculated that a delayed effect might be exerted by this compound on both MFO modulation and DNA damage induction, its initial manifestation starting from 10 days of exposure. Such interpretation might be supported by previous results on genotoxic effects (early-induced DNA lesions, or pre-mutagenic damage) detected by Comet-assay in *C. carpio* exposed to the same disinfectants, which showed significant variation of DNA migration only at late times after exposure to PAA disinfected-water [54], while the two chlorinated disinfectants induced significant increases of DNA damage at the earliest time of analysis (3 h) after the beginning of exposure.

The positive mutagenic effect here detected by the MN induction in fish erythrocytes confirms those previously presented by Sapone et al. [32]. They are also in agreement with results obtained from the longitudinal study on the genotoxic effect (Comet assay and MN test) induced by disinfectants in fish erythrocytes of *C. carpio* [54] which showed a clear genotoxic effect mainly induced by NaClO and to a lesser extent by ClO₂, as opposed to the negative results obtained from PAA.

Similar conclusions on genotoxic effects (MN test and anaphase aberration) of these disinfectants in plant systems (*A. cepa*, *V. faba* and *Tradescantia* spp.) were previously reported in the same

Table 6
Micronucleus frequencies in erythrocytes of *Cyprinus carpio* exposed to PAA, NaClO or ClO₂ (male – winter/summer).

		Control	PAA	NaClO	ClO ₂
Winter	t = 0	0.625 ± 0.26 (n = 8)	–	–	–
	t = 10	0.71 ± 0.36	0.57 ± 0.30	1.60 ± 0.51 (n = 5)	1.14 ± 0.26
	t = 20	0.71 ± 0.29	1.0 ± 0.44	2.83 ± 0.75* (n = 6)	2.14 ± 0.63
Summer	t = 0	0.75 ± 0.31 (n = 8)	–	–	–
	t = 10	0.83 ± 0.31 (n = 6)	0.57 ± 0.20	1.71 ± 0.42	1.29 ± 0.36
	t = 20	0.86 ± 0.26	1.43 ± 0.48	3.86 ± 0.74 ^{o, **}	2.86 ± 0.63 ^{**}

Each value represents the mean ± SE of micronucleus frequencies in carp erythrocytes, observed on 20,000 erythrocytes per specimen, at different time intervals (t = days). Seven specimens were analysed per experimental point (if not differently indicated).

* p < 0.05, significant differences between exposed groups and their respective controls, using the Mann–Whitney Test.

** p < 0.01, significant differences between exposed groups and their respective controls, using the Mann–Whitney Test.

^o P < 0.05 (Mann–Whitney Test): comparison between t = 10 and t = 20 (per disinfectant).

sampling campaigns (October, February and June), showing that the highest effect was induced in the [55].

Seasonal chemical–physical water variations might account for the different outcomes observed in different periods of the year. Actually, physical and chemical analyses of raw lake water revealed seasonal changes with particular regard to temperature, oxygen, nitrate and phosphate concentrations. Polak et al. [56] reported that the most intensive course of the humification process takes place in spring, probably due to the temperature rise as well as the growth of microbiological activity in reservoir water at this time of the year. Furthermore, free radical concentrations of HA extracted from sediments are always higher in spring than in autumn, when the highest maturity of humic acids is reached. Hence, a possible explanation for the stronger MFO modulations observed in the winter might be the increased concentration of DBPs, reasonably attributed to the increment of terminal humic acids at this time. Noteworthy, in raw water, the AOXs in Lake Trasimene shows higher concentrations in autumn and winter with respect to summer (Table 1; see also Monarca et al. [55]). Conversely, the free radicals resulting from the intense humification activity, together with the higher water temperature could explain the major genotoxicity recorded in summer.

Since some XMEs (e.g. CYP 2E1, Glutathione S-transferase theta-1) are responsible for the primary oxidation and activation of THMs, alkanes, alkenes, aromatic and halogenated hydrocarbons, to mutagenic and carcinogenic compounds, and several chemical species in the DBP mixture are potential CYP substrates, any perturbation of their activity might result in an increase of highly toxic metabolite production [21]. Moreover, an up-regulation of CYP isoforms is involved in reactive oxygen species (ROS) generation, thus providing further harmful factors for human health [57,58].

All three tested disinfectants were capable of perturbing fish microsomal metabolism. In contrast, PAA apparently failed to directly produce DNA damage, as determined by the MN assay, but its ability to up-regulate the CYP-dependent metabolism suggests that this compound may still pose a potential hazard through an alternative (possibly non-genotoxic) mechanism. As such, caution should be exercised before PAA can be proposed as a “safe” substitute for chlorine-based disinfection in waste water treatment.

Extrapolating the results to humans, the long-term exposure of individuals to low doses of any of these disinfectants could thus pose an increased carcinogenic risk due to a generalized bioactivation increase of ubiquitous pro-mutagens/pro-carcinogens (co-mutagenesis), leading to a saturation of the enzymatic apparatus involved in DNA repair, and/or increased generation of ROS (linked to CYP induction) [31].

Overall, since they mimic both genotoxic and non-genotoxic (epigenetic) mechanisms of carcinogenesis these data could provide a possible rationale for the observational studies [19] linking the regular consumption of disinfected drinking water to increased incidences of urinary and gastrointestinal cancer.

Conflict of interest

None.

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