Cyto- and genotoxic profile of groundwater used as drinking water supply before and after disinfection

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ABSTRACT

The assessment of the toxicological properties of raw groundwater may be useful to predict the type and quality of tap water. Contaminants in groundwater are known to be able to affect the disinfection process, resulting in the formation of substances that are cytotoxic and/or genotoxic. Though the European directive (98/83/EC, which establishes maximum levels for contaminants in raw water (RW)) provides threshold levels for acute exposure to toxic compounds, the law does not take into account chronic exposure at low doses of pollutants present in complex mixture. The purpose of this study was to evaluate the cyto- and genotoxic load in the groundwater of two water treatment plants in Northern Italy. Water samples induced cytotoxic effects, mainly observed when human cells were treated with RW. Moreover, results indicated that the disinfection process reduced cell toxicity, independent of the biocidal used. The induction of genotoxic effects was found, in particular, when the micronucleus assay was carried out on raw groundwater. These results suggest that it is important to include bio-toxicological assays as additional parameters in water quality monitoring programs, as their use would allow the evaluation of the potential risk of groundwater for humans.

Key words | cytotoxicity, disinfection by-products, genotoxicity, groundwater

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INTRODUCTION

A directive of the European Union (98/83/EC) regulates the quality of drinking water, and defines the microbiological and physical–chemical parameters to allow its use for humans. The promulgation of the directive became very useful, as both groundwater and surface water have been polluted in recent decades, mainly because of human activities.

The quality of surface water has been analyzed in several studies (Alves *et al.* 2014; Mendoza *et al.* 2014; Wilbers *et al.* 2014), while groundwater has received less attention as it has been considered pollution-free. However, several recent studies have reported that contaminants, including pharmacologically active compounds (e.g. pharmaceutical drugs, hormonal substances, and drugs of abuse), industrial compounds, various products used for personal hygiene, and pesticides, have been detected in groundwater (Petrovic *et al.* 2003; Radjenovic *et al.* 2007; Dieter 2010; Martínez Bueno *et al.* 2012). These contaminants and their degradation products can be very persistent, thereby becoming a risk for human health (Postigo *et al.* 2008; Soares *et al.* 2008; Reungoat *et al.* 2010). In particular, pollutants with genotoxic properties may lead to serious adverse effects, including cancer, even when present at low concentrations (Jurado *et al.* 2012; Lapworth *et al.* 2012; Pal *et al.* 2014). The contemporary use of several complementary *in vitro* tests able to identify different types of genotoxic damage is a widespread monitoring approach to assess the presence of genotoxins in water environments (Lah *et al.* 2005; Marabini *et al.* 2007). Among the available tests, the most used are the Ames test, which allows the identification of mutagens with a different mechanism of action (Maron & Ames 1983), the Alkaline version of the Comet assay that detects primary DNA damage (Olive &

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Banath 2006) and the micronucleus assay, which mainly measures clastogenic and aneugenic effects (Fenech *et al.* 1999). The assessment of raw water (RW) is interesting to predict the type and quality of tap water. In fact, several studies have shown that the formation of disinfection by-products (DBPs) is connected to the chemical and physical properties of RW (Fulladosa *et al.* 2004; Roy & Bickerton 2011; Huang *et al.* 2014). In particular, Teksoy *et al.* (2008) have shown a relationship between the formation of DBPs and the pH, the temperature and the concentration of organic matter, and of bromine dissolved in the water supply. Several studies have shown how contaminants in groundwater (e.g. pentavalent arsenic and bromine) can affect the disinfection process, resulting in the formation of substances that are cytotoxic, genotoxic and teratogenic (Hunter *et al.* 1996; Plewa *et al.* 2002; Richardson *et al.* 2008; Richardson 2009; Huang *et al.* 2014). Though the levels of these compounds in water are low, and the exposure of humans through water consumption is rather limited, the severity of the health risk may be high because such exposure is maintained throughout life (Carraro *et al.* 2000; Hrudey 2009). Several studies have indeed reported a link between the consumption of chlorinated drinking water and cancer of the urinary, gastrointestinal, and nervous systems, as well as reproductive disorders (Ijsselmuiden *et al.* 1992; Koivusalo *et al.* 1994, 1995; Nieuwenhuijsen *et al.* 2000; Woo *et al.* 2002; Villanueva *et al.* 2004). For this reason, the Environmental Protection Agency of the United States (US EPA) recommends a level of zero in drinking water for compounds that are known or probable carcinogens.

The goal of this study was to identify a methodological approach to better evaluate the cyto- and genotoxic effects induced by groundwater. In addition to the compounds found through chemical and physical analysis, many unknown compounds are present in groundwater. Known and unknown toxic substances become the components of complex environmental mixtures that may induce side effects on human health. In fact, though the European directive (98/83/EC) establishes maximum levels for contaminants in RW (introducing threshold levels for acute exposure to toxic compounds), this does not take into account chronic low dose exposure to pollutants, generally present in complex mixtures. Consequently, water pollution may be a serious problem for public health and the aquatic ecosystem.

MATERIALS AND METHODS

Chemicals

Reagents for the biological assays and general laboratory chemicals were from Sigma-Aldrich (St Louis, MO, USA). C18 cartridges (Sep-Pak Plus tC18 Environmental Cartridges) were from Waters Chromatography (Milford, MA, USA).

Water sampling and concentration

Sampling of groundwater was performed at two water treatment plants from the same aquifer system of the same river basin in Northern Italy, which together serve 127,000 inhabitants. At these plants, the only treatment performed on waters is disinfection. Chlorine dioxide is used as a disinfectant for the first plant (Plant 1), while sodium hypochlorite is used in the second one (Plant 2). Fifty liters of water was sampled in spring and autumn of the same year in each plant before (RW) and after disinfection (disinfected water (DW)). According to the US EPA 525.2 method (US EPA 1994), the water samples were immediately taken to the laboratory, acidified with hydrochloric acid (pH 2–2.5), and filtered through tri-functional C18 cartridges. Cartridge activation was performed with 40 mL of the following solvents: ethyl acetate, dichloromethane and methanol (Guzzella *et al.* 2006). The water was sucked through the cartridge (20 L/cartridge, at a constant flow of about 20 mL/min) with a pump in a multi-sample concentration system (VAC ELUT SPS 24, Varian, Leini, Italy). The elution of each cartridge was made using ethyl acetate, dichloromethane and methanol (40 mL/each). The eluates were then reduced to a small volume by means of a rotating vacuum evaporator, dried under nitrogen flow, and re-dissolved in dimethyl sulfoxide (DMSO) at an extract concentration equivalent to 25 L of water per 1 mL of DMSO (Pellacani *et al.* 2006). RW and DW (~1 L) were also sampled for chemical analysis.

Physical and chemical parameters

Physical and chemical analyses were performed at the treatment plants, using the analytical methods for the determination of physical/chemical parameters in water intended for human consumption according to the Italian Legislative Decree 31/2001 (transposition of European Directive 98/83/EC) and its integrations (Ottaviani & Bonadonna 2007).

Salmonella typhimurium reversion test

The mutagenic effect of water samples was studied using *S. typhimurium* (strains TA 98 and TA 100) with or without microsomal activation (S9 liver extract of rats treated with Aroclor 1260) to detect direct and indirect mutagenic compounds (Maron & Ames 1983). Positive controls were 2-aminofluorene (5 μg/plate) and hycanthone (25 μg/plate), while DMSO (80 μL/plate) was tested as a negative control. The results of the Salmonella assay are reported as the mean of three replicates with their relative standard deviation. The results of the assay were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the solvent control and at least two of these consecutive doses showed a dose-response relationship. For each sample tested, three concentrations of extract corresponding to 0.5, 1.0 and 2.0 L of water and for each concentration three independent replicates were performed (three repeated measurements of the sample that represent independent measures of the error associated with equipment). The revertants were counted after 48 hours of incubation at 37 °C. Cytotoxicity was detected by a clearing or diminution of the background lawn.

Micronucleus assay

The micronucleus assay was performed using blood samples from healthy donors who provided written, informed consent for use of their blood sample in the study. Whole blood cultures were set up adding 0.5 mL (about 1×0^6 lymphocytes/5 mL) of blood to 4.5 mL of culture medium containing RPMI 1640, 15% heat-inactivated fetal calf serum, 1% phytohemoagglutinin, 1 mM L-glutamine, 100 IU penicillin, and 100 mg/mL streptomycin. The cultures were incubated at 37 °C, 5% CO_2 for 72 h. Treatments were carried out at 24 h of incubation by adding water extracts dissolved in DMSO (0.15, 0.30, 0.60 and 1.2 Leq/5 mL; Leq = liters equivalent). Mitomycin C (0.3 μM) and vinblastine (40 ng/mL) were used as positive controls, and DMSO (12 μ/mL) as negative control. Each treatment was performed in duplicate. Cytochalasin-B (6 μg/mL) was added to all culture tubes at 44 h. The cultures were harvested at 72 h and treated with a hypotonic solution (KCl 0.075 M) for 2–3 minutes at room temperature; the cells were then fixed twice in ice-cold acetic acid: methanol (10:1) solution. Pre-coded slides were cytocentrifuged, air dried and stained with May Grunwald-Giemsa. Micronuclei were scored using a light microscope at 400× magnification (Surrallés *et al.* 1995). In each slide, 1,000 binucleated lymphocytes were examined to discover the presence of micronuclei, and the micronuclei frequency was then calculated for each treatment. Cell-cycle parameters were evaluated by classifying the 500 cells according to the number of nuclei. The cytokinesis block proliferation index (CBPI) was calculated using the formula: $CBPI = [M_1 + 2M_2 + 3(M_3 + M_4)]/N$, where M_1 through M_4 indicate the number of cells with 1–4 nuclei, and N indicates the total number of cells scored (Eastmond & Tucker 1989; OECD 2014). The cytotoxicity of compounds is assessed as a decrease in CBPI as a consequence of suppressed cell proliferation (Surrallés *et al.* 1995). Necrotic and apoptotic cells (discriminated and recognized by morphological criteria) were scored according to the guidelines of Fenech *et al.* (1999).

Comet assay

For this assay, a whole blood sample from healthy volunteers (who provided written, informed consent for its use in the study) was centrifuged twice in an erylyse solution (155 mM NH_4Cl, 5 mM $KHCO_3$, 0.005 mM Na_2EDTA, pH 7.4) for leukocyte isolation, and was then washed and resuspended at 10^6 cell/mL in phosphate buffered saline. The cell suspension was added to a microcentrifuge tube together with water extract for 1 h at 37 °C, to produce the following concentrations: 0.25, 0.50, 0.75, 1.00, and 1.25 Leq/mL (Pellacani *et al.* 2006). DMSO (50 μL/mL) was used as a negative control, while ethyl

methanesulfonate (2 mM) served as a positive control. The Comet assay was performed with cell viability >70% (Tice *et al.* 2000), and cell viability was determined using the fluorescein diacetate/ethidium bromide assay (Merk & Speit 1999). Following this passage, the Comet assay was performed as described by Singh *et al.* (1988), with minor modifications as described by Buschini *et al.* (2001). Alkaline DNA unwinding (pH > 13) was carried out for 20 minutes, and electrophoresis was conducted for 20 minutes at 0.78 V cm^{-1} and 300 mA. After staining with 100 μL ethidium bromide (10 μL/mL), the slides were examined with a fluorescence microscope (Leica DMLS, 400×) 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). Fifty randomly-selected cells per slide (two slides per sample) were analyzed. The samples were coded and evaluated blind. The Comet parameter total migration length (TL) was used to measure DNA damage, and cells with complete disintegration of the head region (ghost cells (GC)/hedgehog) were also recorded to evaluate the necrosis/apoptosis phenomena.

Statistical analysis

An SPSS 11 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The median values of DNA migration (TL) of each experiment (Comet assay) were used for analysis of variance (ANOVA) (Dunnett's *post hoc* test). The statistical analysis of micronuclei frequency was performed using the χ^2-test. CBPI data were analyzed by ANOVA (Tukey *post hoc* test).

RESULTS

Physical and chemical parameters

All chemical and physical parameters of RW from both plants had results within the limits required by the Italian legislative decree 31/2001 (D.Lgs 31/2001 and subsequent amendments and additions) transposing the European Directive 98/83/EC (Table 1). Nevertheless, some molecules of toxicological interest were present above the detection limit but under the maximum allowable concentration (MAC) described by the Italian law. In particular, the following contaminants were identified: chloroform, bromoform, carbon tetrachloride, 1,1,1-trichloroethane, and tetrachlorethylene (Table 1). In most cases, levels were similar in the two plants at both sampling periods. The concentration of total trihalomethanes (THMs) in the RW collected in June and October presents similar values in both plants. THMs were also measured in water in both plants after disinfection (Table 1). As shown in Table 2, disinfection of water with chlorine dioxide (Plant 1) did not modify the levels of THMs; in contrast, in Plant 2, disinfection of water with sodium hypochlorite caused a four- to five-fold increase in THM levels (Table 2).

Salmonella typhimurium reversion test

Table 3 shows the results of the mutagenicity studies. In general, samples of raw and DW, in both plant and at both sampling points, were negative in both Salmonella strains.

Although no significant result has been achieved, it should be noted that both aqueous extracts (RW and DW) taken in Plant 1 in June are able to induce a doubling of the spontaneous frequency of revertants in strain TA98 (without metabolic activation) (Table 3). The same samples, in the presence of metabolic activation (S9), showed no mutagenic activity, highlighting detoxifying activity of S9.

Micronucleus test in human lymphocytes

Figure 1 shows the results of the micronucleus assay; CBPI and the number of micronuclei induced are reported. In general, a significant decrease ($P < 0.05$) in cell proliferation was observed, as attested by a decreased CBPI in relationship to the dose. This finding suggests that water samples contain substances that are able to interfere with the cell cycle.

In Plant 1, all water samples induced a decrease of CBPI, with the exception of the DW sample collected in June, which had no significant effects on the CBPI. None of the water samples caused any induction of micronuclei, it is conceivable that the reduced proliferation could have masked possible genotoxic effects. No effects on

Table 1 | Physical and chemical characteristics of the RW of the two different plants (Plant 1 and Plant 2) during the summer (June) and autumn (October) campaigns

		Plant 1		Plant 2	
Physical and chemical characteristics	**MAC**	**June**	**October**	**June**	**October**
Temperature (°C)		15	15	14	14.5
Conductivity (μS/cm)		640	646	723	723
Hardness (°F)		38.25	39.93	41.93	44.74
pH	6.5–9.5	7.3	7.2	7.2	7.3
Bicarbonate (mg/L)		386	365	394	413
Calcium (mg/L)		126.0	133.7	128.5	137.8
Chloride (mg/L)	200.0	24.3	24.0	20.8	21.5
Iron (mg/L)	0.20	0.01	nd	nd	0.01
Fluoride (μg/L)	1,500	94	86	154	144
Phosphate (mg/L)		nd	nd	nd	nd
Magnesium (mg/L)	50.0	16.5	15.9	23.9	25.1
Nitrate (mg/L)	50.0	25.7	28.0	25.1	27.7
Ammonia nitrogen (mg/L)	0.5	nd	nd	nd	nd
Nitrite (mg/L)	0.1	nd	nd	nd	nd
Potassium (mg/L)		2.5	2.6	3.2	2.9
Sodium (mg/L)	200.0	23.8	19.4	25.5	27.4
Sulfates (mg/L)	250.0	50.4	48.5	45.7	46.8
TOC (μg/L C)		353	491	497	522
Ox Kubel (mg/L O_2)	5.00	0.17	0.23	0.23	0.22
THM tot. (μg/L)	30.00	0.25	0.48	0.21	0.40
Chloroform (μg/L)		0.11	0.07	0.06	0.04
Bromoform (μg/L)		0.12	0.39	0.13	0.34
Chlorobromomethane (μg/L)		nd	nd	nd	nd
Bromodichloromethane (μg/L)		nd	nd	nd	nd
Carbon tetrachloride (μg/L)		0.01	nd	0.01	0.01
1,2-Dichloroethane (μg/L)	3	nd	nd	nd	nd
1,1,1-Trichlorethane (μg/L)		0.20	nd	0.06	0.03
1,1,2-Trichlorethane (μg/L)		nd	nd	nd	nd
Trichloroethylene (μg/L)	10.00	nd	nd	nd	nd
Tetrachloroethylene (μg/L)		0.01	nd	0.03	0.03

MAC: maximum allowable concentration (D.Lgs 31/2001 and subsequent amendments and additions). nd: not detectable. Ox Kubel: Kubel method to reveal the presence of organic compounds in the water. THM: trihalomethanes. TOC: total organic carbon.

proliferation was induced by the RWs from Plant 2, while waters sampled immediately after disinfection with sodium hypochlorite induced anti-proliferative effects at the highest concentrations tested (Figure 1). However, the RW sampled in June (at the concentration equivalent to 1.2 L) induced breakage and /or loss of chromosomes, detected from micronuclei counts (Figure 1).

Comet assay on human leukocytes

Figure 2 shows data on the cytotoxic and genotoxic effects induced by the water samples on human leukocytes, as determined in the Comet assay. In most cases, a strong cytotoxic effect was observed, as determined by a reduction in viable cells (evaluated through 'fluorescein diacetate/

Table 2 | Level of THMs measured in RW and after disinfection with chlorine dioxide (Plant 1) or sodium hypochlorite (Plant 2) during the two sampling periods

	THMs (μg/L)	
	June	October
Plant 1		
RW	0.25	0.48
DW	0.27	0.57
Plant 2		
RW	0.21	0.40
DW	1.14	1.72

ethidium bromide 'double straining' assay), and by an increase of GC. Table 4 reports some parameters that could describe the toxicity load of the water samples: LC50 (Lethal Concentration, 50%) and Lowest Effective Dose (LED). RW samples showed comparable LC_{50} and LED at the two plants in the two sampling periods: in June, Plant 1 $LC_{50} = 0.25$ L_{eq}/mL and Plant 2 $LC_{50} =$ 0.22 Leq/mL; in October, Plant 1 $LC_{50} = 0.45$ Leq/mL and Plant 2 $LC_{50} = 0.44$ Leq/mL. In general, the DW samples at both plants, in both samplings, showed substantially less toxicity compared with the RW samples. A stronger detoxifying effect was detected after sodium hypochlorite disinfection in Plant 2: in June, before disinfection LC_{50} was 0.22 Leq/mL and after disinfection 1.20 Leq/mL; in October, LC_{50} was 0.40 Leq/mL and after disinfection 1.30 Leq/mL. The DWs, independent of the biocide used, showed a decrease in the induction of GC. All water samples induced a dose-dependent DNA migration. The presence of a high cytotoxicity, with a cell survival less than 70%, does not allow the DNA fragmentation detected as genotoxicity to be identified.

DISCUSSION

In Italy, the main source (ISTAT 2012) of drinking tap water is groundwater. Generally, this water is not subjected to purification treatments, due to its good quality resulting from filtration by permeable layers and by protection from overlying impermeable layers (De Giglio *et al.* 2015). However, in the last decades the quality of groundwater has decreased, principally because of the considerable exploitation of water resources that induces a progressive deterioration of their quality (Widory *et al.* 2004; Burkart & Stoner 2007; Huang *et al.* 2012). The quality of groundwater also depends on the features of the soil and its filtration ability, which may reduce the degree of water contamination (Goeppert & Goldscheider 2011; Atherholt *et al.* 2013). Moreover, seasonal variation and climatic factors may change the natural replenishment of aquifers, and may worsen the quantity of groundwater (Makwe & Chup 2013; Rea *et al.* 2014; Sun *et al.* 2014; Tornevi *et al.* 2014).

The safety of groundwater resources represents a primary issue, and complex activities are needed to preserve their integrity. Biological monitoring allows detection of toxic substances undetectable through chemical and physical analysis, and allows identification of antagonistic/synergistic effects caused by toxic substances present in complex mixtures (Pellacani *et al.* 2005, 2006). Environmental risks are often evaluated analyzing the single pollutants, and do not take into consideration the effect of chemical mixtures, thus underestimating the risk to human health (Backhaus & Faust 2012). A more complex approach, involving a battery of bio-toxicological tests, is an important tool to properly assess the quality of groundwater and drinking water. To evaluate the drinking water's mutagenic and/or genotoxic risks and to get information in the short term, several biological assays, such as the Ames test, the micronucleus test and Comet assay, may be used, as in the present study.

Though the RW of both plants at both sampling periods complied with D.Lgs 31/2001 (and subsequent amendments and additions), a number of contaminants were detected (carbon tetrachloride, 1,1,1-trichloroethane, tetrachlorethylene, bromoform and chloroform). Though the exposure level to any single pollutant is low, chronic consumption of the complex mixture may represent a risk factor for human health due its ability to generate synergic effects between toxic compounds.

Carbon tetrachloride is considered a possible carcinogen to humans (IARC 1999). This molecule results in toxicity to the liver and kidney (WHO 2004a). It does not appear mutagenic to *Salmonella typhimurium*, however it induces DNA damage and mutations in single studies with *Escherichia coli* (IPCS 1999). *In vitro*, carbon tetrachloride produces a low induction of DNA damage, unscheduled

Table 3 | Mutagenicity of RW and DW in *Salmonella* assay

June

Plant 1	RW												DW											
	TA98			TA98 +			TA100			TA100 +			TA98			TA98 +			TA100			TA100 +		
Leq	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*
0	12	2	1.0	13	4	1.0	81	5	1.0	76	7	1.0	12	2	1.0	13	4	1.0	81	5	1.0	76	7	1.0
0.5	14	1	1.2	14	0	1.1	88	9	1.1	70	2	0.9	13	0	1.1	13	1	1.0	72	4	0.9	84	2	1.1
1.0	16	1	1.4	16	4	1.2	79	3	1.0	69	2	0.9	26[a]	4	2.2	20	2	1.5	99	9	1.2	78	4	1.0
2.0	27[a]	2	2.3	17	1	1.3	97	10	1.2	83	6	1.1	21	1	1.8	23	4	1.7	80	4	1.0	72	10	1.0
Plant 2	**P1**												**P2**											
	TA98			TA98 +			TA100			TA100 +			TA98			TA98 +			TA100			TA100 +		
Leq	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*
0	12	2	1.0	16	3	1.0	75	14	1.0	81	9	1.0	12	2	1.0	16	3	1.0	75	14	1.0	81	9	1.0
0.5	15	1	1.2	16	3	1.0	58	8	0.8	73	9	0.9	11	1	0.9	14	2	0.9	68	8	0.9	74	1	0.9
1.0	15	2	1.2	15	2	1.0	54	2	0.7	64	4	0.8	14	3	1.2	14	1	0.9	66	4	0.9	71	10	0.9
2.0	16	3	1.3	16	2	1.0	54	5	0.7	65	3	0.8	21	2	1.7	18	1	1.1	77	5	1.0	99	3	1.2

October

Plant 1	RW												DW											
	TA98			TA98 +			TA100			TA100 +			TA98			TA98 +			TA100			TA100 +		
Leq	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*
0	17	5	1	14	3	1	98	2	1	93	8	1	17	5	1	14	3	1	98	2	1	93	8	1
0.5	15	3	0.9	14	4	1	96	2	1	91	12	1	17	1	1	15	2	1.1	97	1	1	94	3	1
1.0	15	2	0.9	15	3	1.1	89	1	0.9	112	15	1.2	22	5	1.3	14	1	1	96	9	1	112	5	1.2
2.0	14	1	0.8	19	1	1.4	98	9	1	119	4	1.3	15	2	0.9	18	1	1.3	97	2	1	121	9	1.3
Plant 2	**P1**												**P2**											
	TA98			TA98 +			TA100			TA100 +			TA98			TA98 +			TA100			TA100 +		
Leq	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*	*mean*	*sd*	*T/C*
0	14	2	1	16	4	1	94	7	1	94	15	1	14	2	1	16	4	1	94	7	1	94	15	1
0.5	14	2	1	16	2	1	93	1	1	108	6	1.1	14	3	1	17	4	1.1	106	8	1.1	96	8	1
1.0	15	2	1.1	16	1	1	100	4	1.1	93	6	1	19	5	1.4	17	3	1	104	2	1.1	92	9	1
2.0	14	3	1	17	2	1.1	101	3	1.1	93	11	1	26	2	1.9	17	2	1.1	101	10	1.1	112	13	1.2

Revertants per liter of water induced in strain TA98 and in strain TA100 by samples collected in Plant 1 (chlorine dioxide) and in Plant 2 (sodium hypochlorite) in both seasons (June and October). Mean, standard deviation (SD) and induced revertants treated/control ratio (T/C) of three independent experiments are reported. += with microsomal activation (S9 liver homogenate of rats treated with Aroclor 1260). Leq: liters equivalent.
[a]Doubling of revertants.

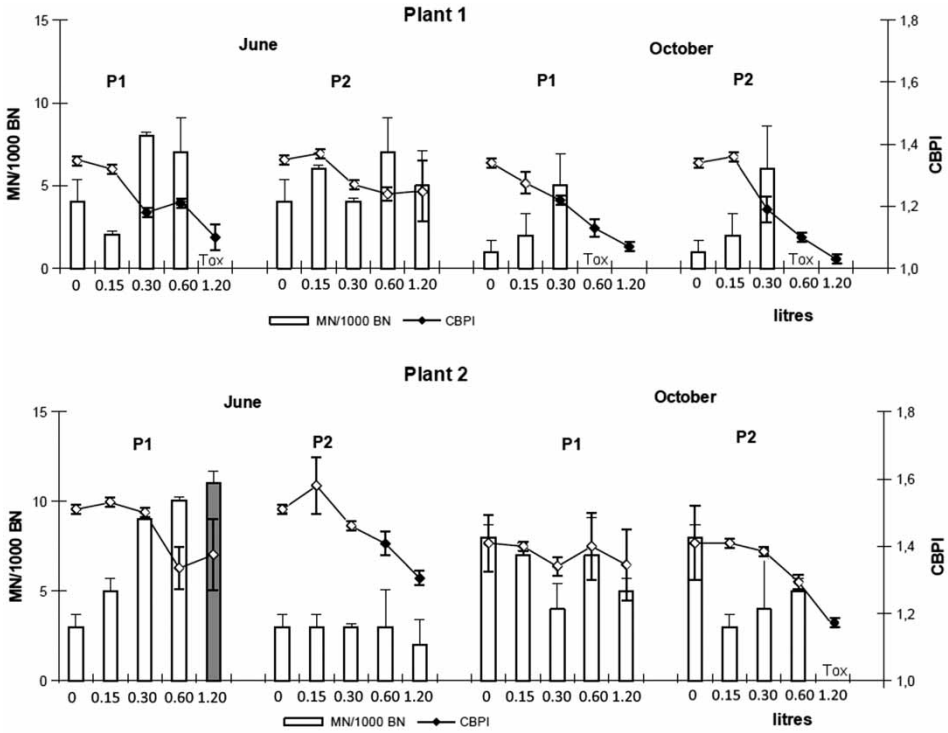

Figure 1 | CBPI (solid line) and mean frequency of micronuclei (MN-bars) in human lymphocytes treated with water sampled in two seasons (June and October) in two treatment plants (1 and 2). Results represent the mean ± SD of three independent experiments. Raw groundwater (water before disinfection); disinfected groundwater (water after disinfection); CBPI: the filled symbols show a statistically significant difference compared to the control value ($P < 0.05$); MN: filled bars indicate significant differences with respect to control ($P < 0.05$); tox: toxic.

DNA synthesis, sister chromatid exchange or chromosomal aberrations (IPCS 1999). This chemical is also able to induce damage in mammalian cells during cytokinesis (McGregor & Lang 1996).

1,1,1-trichloroethane is a substance that can cause liver effects after repeated or long-term exposure (IARC 1999). Several studies evaluated genotoxic effects of 1,1,1-trichloroethane (US EPA 2005). The results in the Ames Salmonella assay are contradictory: in fact some test results were negative, while others, performed to minimize evaporation of the compound, were positive (ATSDR 2006). 1,1,1-trichloroethane induces chromosomal aberrations in Chinese hamster ovary cells *in vitro* and is positive in most mammalian cell transformation assays (ATSDR 2006).

Tetrachloroethylene has been found in drinking water and in groundwater worldwide (WHO 2003). It persists in water, where volatilization does not occur, but does not cause bioaccumulation in food chains (ATDSR 1993). It is able to generate developmental toxic effects (WHO 2003) and it is considered a probable human carcinogen (IARC 1995). A short-term assay demonstrated that tetrachloroethylene causes single-strand DNA breaks, but it could not induce chromosomal aberrations. Moreover, this compound showed negative effects in *Salmonella typhimurium, Escherichia coli*, and *Saccharomyces cerevisiae*, both with and without microsomal activation (WHO 1984, 2003).

Bromoform is a substance that can cause liver toxicity (IARC 1999). It is considered a mutagen (IARC 1991; GlobalTox 2002). Bromoform was positive in the Ames test with or without metabolic activation (ATSDR 2005). The National Toxicology Program (ATSDR 2005) indicates that bromoform is able to generate micronuclei formation and it is positive to SCE assay. However, bromoform presents negative results for chromosomal aberrations in mouse bone marrow (ATSDR 2005). Potter *et al.* (1996) found that bromoform is not able to generate DNA strand breaks in male F344 rats.

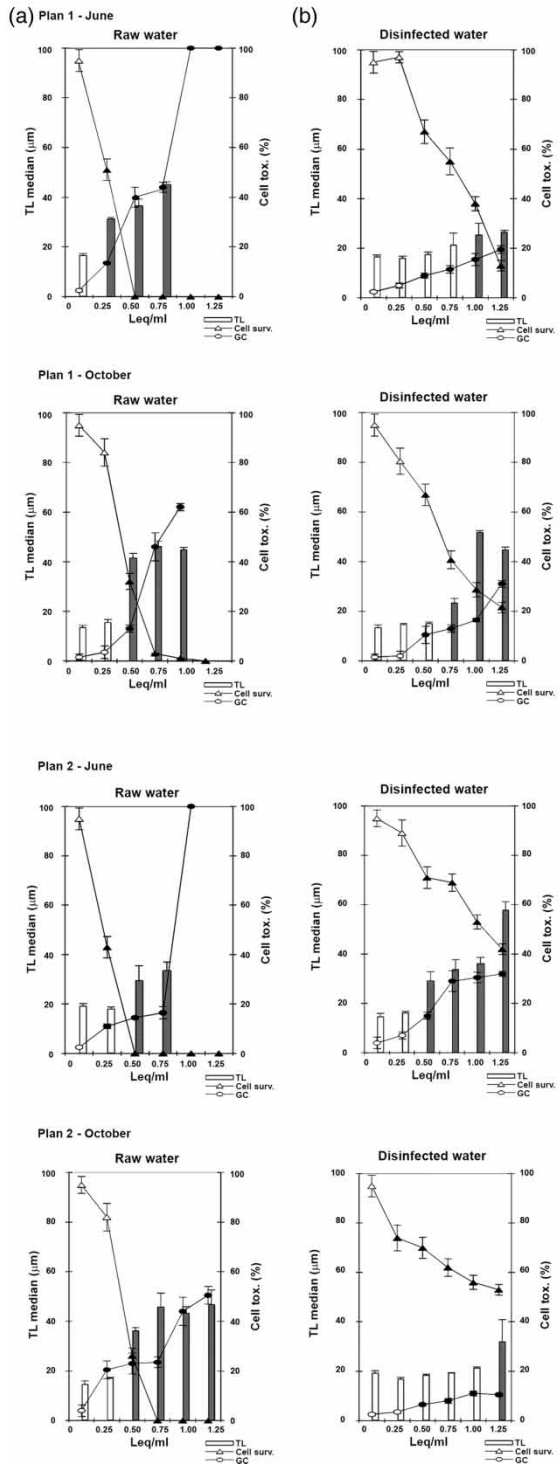

Figure 2 | Comet assay on human leukocytes treated with extracts of raw groundwater (a) and disinfected groundwater (b) in two treatment plants (Plant 1; Plant 2). Migration of DNA (bars), as length of the tail (TL), cell survival (Δ) and percentage of ghost cells (O) are reported (mean ± S.D. of three independent experiments). Cell survival and ghost cells (Y axis, labelled Cell tox.%): filled symbols, $P < 0.05$; TL:* $P < 0.05$, with respect to control (Dunnett's C).

Table 4 | Cytotoxicity of RW and DW on human leukocytes

		Cell toxicity				Comet assay GC	
		LC_{50} (Leq/mL)		LED (Leq/mL)		LED (Leq/mL)	
Plant	Sampling	RW	DW	RW	DW	RW	DW
1	June	0.25	0.80	0.25	0.50	0.25	0.50
	October	0.45	0.70	0.50	0.50	0.50	0.50
2	June	0.22	1.20	0.25	0.50	0.25	0.50
	October	0.40	1.30	0.50	0.25	0.25	0.50

LC_{50}: lethal concentration, 50%. LED: lowest effective dose. GC: ghost cells.

Chloroform is a probable human carcinogen (IARC 1999). Its principal removal process is volatilization (US EPA 1984). However, in groundwater chloroform is persistent, due to restricted volatilization and slow biodegradation (Environment Canada & Health Canada 2001). Chloroform presents contradictory mutagenic activity in *Salmonella typhimurium* and *Escherichia coli* bacteria assays (WHO 1984, 2003). Several studies did not indicate genotoxic potential (chromosome aberrations, sister chromatid exchange and micronuclei formation) *in vitro* and *in vivo* (IPCS 2004). However, other studies reported that chloroform is able to induce micronuclei formation and chromosome damage in rats (Robbiano *et al.* 1998; Fujie *et al.* 1990). Moreover, Morimoto & Koizumi (1983) indicate a positive SCE result in mouse bone marrow. In conclusion, chloroform is slightly mutagenic and it does not appear to be genotoxic (WHO 2004b).

Taking into account the pollutants detected in the RWs analyzed, our data seem to confirm literature evidence that stated that the Ames test and Comet assay, in general, are not able to detect the genotoxic effects of these molecules (Hartmann & Speit 1995; IPCS 1999; WHO 2003; ATSDR 2006). As an example, tetrachloroethylene induces a clear cytotoxic effect on human blood cells treated *in vitro* (Perocco *et al.* 1983; Hartmann & Speit 1995), but does not alter DNA migration in the Comet assay when tested in the absence or presence of metabolic activation (Hartmann & Speit 1995).

The main indication obtained from the three assays carried out in this study is that the prevalent effect induced by water samples was cell toxicity, as indicated by the reduction of viability, the increase of GC, and by the

reduction of CBPI. Furthermore, the results show that cytotoxicity is mainly observed with RW samples rather than samples after disinfection. As reported in the literature, the presence of toxic chemicals in RW may influence the toxic properties of the same water after disinfection (Nikolaou *et al.* 2004; Teksoy *et al.* 2008; Feretti *et al.* 2012; Huang *et al.* 2014). The results indeed show that the disinfection process (in both seasons) alters the characteristics of RW, reducing its cytotoxicity and improving its quality, independent of the biocidal chemical utilized (chlorine dioxide or sodium hypochlorite). Peculiar results were detected through MN assay when the RW of Plant 2, sampled in June, was analyzed. In the absence of anti-proliferative effects, the RW induced a significant increase in micronucleated cells. After disinfection, DW induced anti-proliferative effects and no genotoxic effect. These data support the hypothesis that genotoxic data obtained may be underestimated/masked by the strong cytotoxic effect. Nevertheless, the water toxicity decrease after disinfection, detected by the reduction of leukocyte viability and GC increase when a Comet assay was performed, could be related to a possible detoxication of compounds present in the RW. It is unclear how the chlorination processes can break down the toxic load of raw groundwater, as detected in our study. Chlorine is able to transform several water micropollutants; oxidation, addition and electrophilic substitution reactions with organic compounds are possible pathways (Deborde & von Gunten 2008). Chlorine is known to interact with dissolved natural organic matter (DNOM). DBPs, such as THMs, can result from the reaction of chlorine and chlorine dioxide with natural organic molecules. In our samples, we found a mild induction of total THMs after disinfection far below the MAC, evidencing a low concentration of DNOM.

The toxicological activity detected in our study could also be related to the occurrence in Italian water resources of emerging water pollutants such as endocrine disruptors, some pesticides, some pharmaceuticals and some azo-dyes and/or their metabolites (Meffe & de Bustamante 2014). We can hypothesize that these pollutants could be detoxified by the disinfection processes. Some of these molecules, such as endocrine disrupting chemicals and pharmaceuticals and personal care products, seem to be degraded through advanced oxidation processes in water effluents (Esplugas *et al.* 2007).

CONCLUSION

The methodological approach used, which involves a combination of chemical and physical analysis and a battery of bio-toxicological tests (with different molecular targets), has allowed the identification of a strong cytotoxic activity of concentrates of raw groundwater that result within the limits required by law. The disinfection processes, in particular the use of hypochlorite, induced an unexpected reduction of toxicity. More extensive studies are needed to identify groundwater micropollutants, single or in mixture, which can produce the toxicological effect detected in this study and that could represent a risk for human health.

Declaration of conflicting interests

The author(s) declare no potential conflicts of interest in the research, authorship, and/or publication of this article.

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