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Genotoxicity of source, treated and distributed water from four drinking water treatment plants supplied by surface water in Sardinia, Italy

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ABSTRACT

High levels of disinfection by-products (DBPs) are constantly found in drinking water distributed in Sardinia, an Italian island with a tourist vocation and critical issues related to the drinking water supply. To reduce the concentration of trihalomethanes the disinfectant in use was changed – chlorine dioxide was adopted instead of hypochlorite. However, this caused the appearance of other DBPs (*e.g.*, chlorites) in water distributed to the population. Thus, the use of monochloramine as a secondary disinfectant (associated with chlorine dioxide as the primary disinfectant) was evaluated in four drinking water treatment plants supplied by artificial basins located in the central-northern part of Sardinia. Raw, disinfected and distributed waters were studied for genotoxicity using a battery of *in vitro* tests on different cells (bacteria, plant and mammalian cells) to detect different genetic endpoints (*i.e.*, point and chromosome mutations and DNA damage). Moreover, a chemical and microbiological characterisation of water samples was performed. All samples of water distributed to the people showed mutagenic or genotoxic effects in different cells/organisms. In particular, chromosome aberrations in plant cells and DNA damage in human cells were observed. In this study, the use of chloramines associated with other disinfectants did not eliminate the mutagenicity present in the raw water and when the raw water was not mutagenic it introduced mutagenic/genotoxic substances. A careful management of drinking water is needed to reduce health hazards associated with the mutagenicity of drinking water.

1. Introduction

The quality of drinking water depends on several factors, among which the most relevant are the supply source, the disinfection treatment and the distribution system. In particular, drinking water obtained from surface sources and disinfected with chlorine and its compounds could be a potential health risk because of the presence of disinfection by-products (DBPs) whose formation is proportional to the concentration of natural organic matter, naturally abundant in surface water (Li et al., 2017). An adequate concentration of chlorine is essential to achieve the necessary level of microbial risk reduction, but, on the other hand, chlorine reacts with the organic substances producing the DBPs (WHO, 2011).

The use of other disinfectants, such as chlorine dioxide and ozone, has been also associated with the formation of numerous DBPs (WHO, 2011). Moreover, a piped distribution system may encourage the formation of by-products due to organic matter (the thickness of the biofilm) and disinfectant residue. Further factors can influence the quality of water in the distribution system, since it can remain for a long time after disinfection in water networks before reaching users. The quality of water in the distribution system is attributable not only to the quality of the starting water, but also to the hydraulic conditions of the network and the possible introduction of external contaminants, and to phenomena of corrosion and release of substances by the materials used for the pipes. Furthermore, the physicochemical characteristics of water (*e.g.*, temperature, pH, salts, metals, etc.) can influence microbial

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growth and the formation of biofilm, as well as the reaction between organic substances and disinfectants, and, consequently, determine the transformation of inactive compounds into compounds capable of inducing toxic, genotoxic and epigenetic effects (Mercier Shanks et al., 2013).

DBPs can be toxic and mutagenic/genotoxic compounds (Monarca et al., 2005; Guzzella et al., 2004; Richardson et al., 2007; Krasner, 2009; Leme and Marin-Morales, 2009; Cortés and Marcos, 2018). The genotoxicity of individual DBPs and drinking water was widely studied in different cell types (Richardson et al., 2007; Cortés and Marcos, 2018). Toxicological studies have shown that some DBPs cause cancer and adverse reproductive or developmental effects in laboratory animals (Boorman et al., 1999). Moreover, epidemiological studies have evidenced a slightly increased risk for bladder, colon and rectal cancers in humans who were exposed to chlorinated surface waters for many years. Associations between the consumption of chlorinated drinking water and adverse reproductive or developmental health effects, such as spontaneous abortion or congenital anomalies, in particular ventricular septal defects were also reported (Nieuwenhuijsen et al., 2000, 2009; Villanueva et al., 2004; Legay et al., 2010).

Sardinia (Italy), the second-largest island in the Mediterranean Sea, has a marked tourist vocation with a strong increase in the resident population during the summer leading to a consequent change in the demand for drinking water. In this Italian Region, drinking water is mainly obtained by surface waters collected and regulated in water reservoirs (*i.e.*, artificial basins). Water coming from artificial basins is largely exposed to anthropogenic contamination and consequently, because of its low quality, it is necessary to apply complex disinfection treatments, even providing high dosages, with an increased risk linked to the formation of DBPs. Finally, there are many concerns related to the condition of pipelines, such as type and age of materials, and poor maintenance (Azara et al., 2004; Contu et al., 2004; Dettori et al., 2016). It should also be taken into consideration that the future climate change scenarios in Sardinia, in terms of low rainfall and higher temperature, will influence future water availability and, consequently, water management has to take into account this transient condition (Zingaro et al., 2017). For all these reasons, Sardinia has a very singular situation in Italy that requires attention from public health authorities (Dettori et al., 2019).

In particular, as early as the 90s high levels of DBPs were constantly found in drinking water distributed in Sardinia (Romano et al., 1991), and water disinfected by hypochlorite showed trihalomethanes (THMs) concentration higher than the maximum permissible value set by Italian law (Azara et al., 2010). Starting from this evidence, to reduce the THMs concentration the disinfectants in use were changed. Thus, chlorine dioxide was adopted instead of hypochlorite, but this caused the appearance of other DBPs (*e.g.*, chlorites) in water distributed to the population.

Owing to the considerable difficulties in managing the drinking water treatment process, on the one hand, this condition led to the issuing of a ministerial derogation, granted until the December 31, 2006 due to the presence of chlorites in the drinking water supply (Azara et al., 2018). On the other hand, efforts in obtaining good quality drinking water have led to experimentations aimed at optimising the process of disinfection and at reducing chlorite concentration. Thus, the use of monochloramine as a secondary disinfectant (associated with chlorine dioxide as the primary disinfectant) was evaluated. Drinking water guidelines indicate the disinfection efficacy and the greater stability of this compound used in secondary disinfection, as well as the lower production of mutagenic by-products compared to other disinfectants (WHO, 2011; NHMRC NRMMC, 2014).

The results were very encouraging both for the reduced concentrations of DBPs and for the tenor of the other parameters indicating the good functioning of the treatment plants. Nowadays, the majority of Sardinian drinking water treatment plants use the combination of chloride dioxide and monochloramine to disinfect water distributed for the human consumption.

The aim of this study was to evaluate the formation and/or presence of genotoxic substances in water sources, disinfected and distributed drinking water in northern Sardinia, District of Sassari. The final objective is to provide useful information for managers of drinking water treatment plants to improve drinking water quality. It is, in fact, mandatory to maintain a high microbiological quality and reduce the risks to human health related to DBPs.

2. Material and methods

2.1. Drinking water treatment plants

In the present study, we evaluated the genotoxic effects of chlorinated drinking water processed from raw water collected from four reservoirs/artificial basins in the northern part of Sardinia (Fig. 1). These four water networks serve about 1/3 of the whole population of Sardinia.

In Italy, classification of water sources for drinking water production is based on the Italian Legislative Decree 152/2006. Classification of the four artificial basins included in this study is reported in Table 1S.

The four drinking water treatment plants considered in this study use different processes for pre-oxidation/disinfection:

(A) Pattada/*Monte Lerno* - chlorine dioxide (1.41 g/m^3) in pre-oxidation and chloramine (ammonia 0.16 g/m^3 + sodium hypochlorite 2.46 g/m^3) in post-disinfection;
(B) *Bidighinzu* - potassium permanganate (1.5 g/m^3) in pre-oxidation and chlorine dioxide (0.2 g/m^3) + chloramine (ammonia 1.9 g/m^3 + sodium hypochlorite 33 g/m^3) in post-disinfection;
(C) Cuga/*Monte Agnese*) - potassium permanganate (2.3 g/m^3) in pre-oxidation and chlorine dioxide (0.79 g/m^3) in post-disinfection;
(D) Casteldoria/*Pedra Majore* - chlorine dioxide (2.3 g/m^3) in pre-oxidation and chloramine (ammonia 1.7 g/m^3 + sodium hypochlorite 16 g/m^3) in post-disinfection.

2.2. Sample collection, physicochemical and microbiological analyses

Raw (from artificial basins), treated (at the water treatment plants) and distributed (along the pipelines) drinking water samples were collected from four water networks.

Three sampling points were considered for each water network: (i) artificial basins, raw water; (ii) treatment plant (disinfection stage), disinfected water; and (iii) distribution system (along the pipeline, at least 5 km far from the treatment plant), distributed water.

A total of 100 L water (five 20 L samples) were collected for each sampling point in spring (Cuga/*Monte Agnese*), summer (Pattada/*Monte Lerno*, and Bidighinzu/*Bidighinzu*), or autumn (Casteldoria/*Pedra Majore*).

Water samples were studied for genotoxicity using a battery of *in vitro* tests on different cells (bacteria, plant and mammalian cells). Moreover, chemical and microbiological analyses were carried out on water samples.

2.3. Physical and chemical parameters determination

Analysis of water samples aimed at evaluating physical and chemical parameters followed standards set by the national Environmental Protection Agency (APAT-IRSA-CNR) (APAT, 2003) and the Legislative Decree 31/2001 (Italian implementation of Council Directive 98/83/EC on the quality of water intended for human consumption).

The pH value, as well as air and water temperature and water

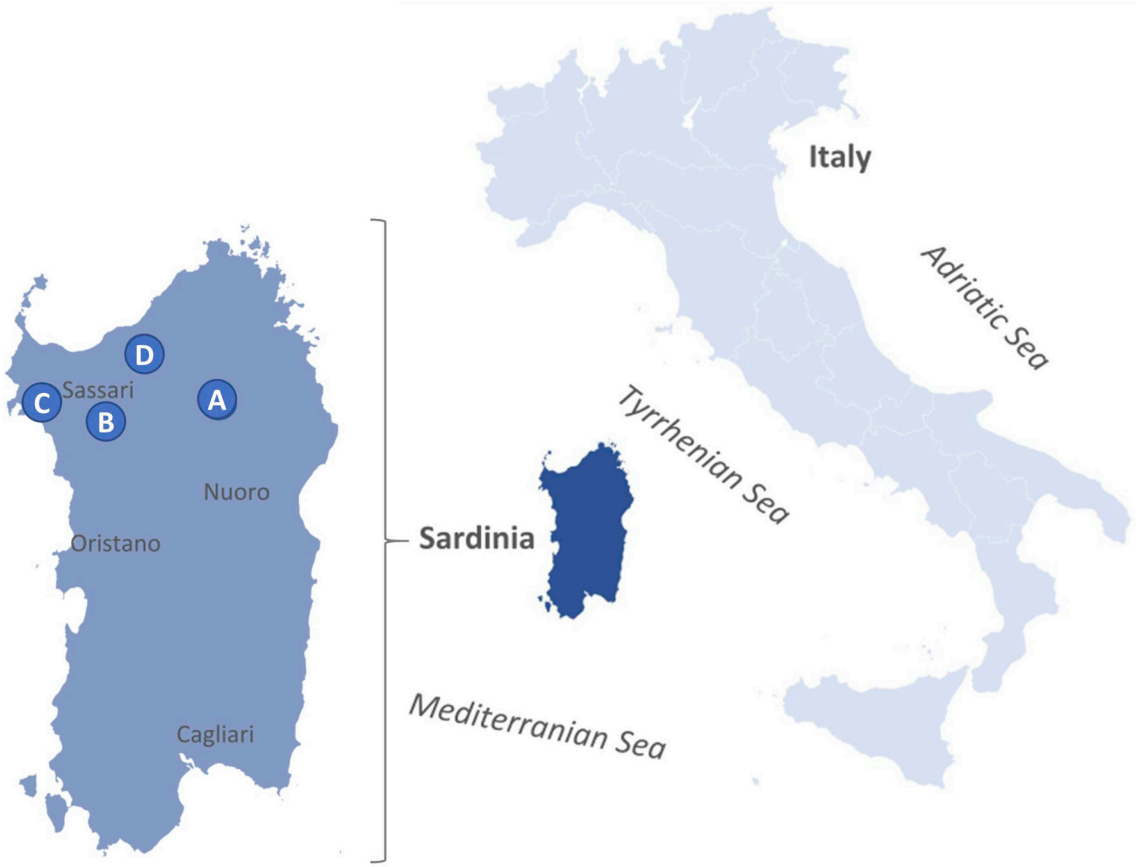

Fig. 1. Geographical location of Sardinia, Italy and of the four artificial basins and water treatment plants in northern Sardinia, District of Sassari: (A) Pattada - Monte Lerno; (B) Bidighinzu; (C) Cuga, Coghinas - Monte Agnese; (D) Casteldoria - Pedra Majore.

conductivity were measured in-field. To analyze the pH value, a glass electrode combined with a suitable reference electrode was used, following its calibration with two standard buffer solutions brought to the same temperature of the sample. Conductivity was evaluated using a conductivity-meter with temperature compensation.

In order to assess calcium, magnesium, sodium, potassium, iron and manganese measurements, a determination by atomic absorption spectrometry with flame atomisation was performed.

Conversely, EPA methods (EPA, 1997; EPA, 1983) were followed to evaluate the values of chlorites and chlorates (ion chromatography), as well as residual free chlorine, and total active residual chlorine (DPD-Colorimetric method).

Bromine and iodine measures were obtained by performing a potentiometric determination with a gaseous diffusion membrane electrode.

Moreover, organic carbon was determined by performing a high temperature catalytic oxidation using a catalyst which consists of platinum supported on an inorganic matrix (*e.g.*, alumina, quartz).

Finally, the UV absorbance at 254 nm was directly read using a spectrophotometer with molecular absorption.

2.4. Microbiological parameters determination

The microbiological parameters determination in water samples followed the Ministry of Health Decree indications (Ministerial Decree of June 14, 2017, implementation of Commission Directive EU, 2015/1787 amending Annexes II and III to Council Directive 98/83/EC on the quality of water intended for human consumption), which refers to standards set by the International Organisation for Standardisation (ISO), adopted by UNI (Ente Nazionale Italiano di Unificazione).

Accordingly, the microbial colony count at 22 °C and 36 °C followed the UNI EN ISO 6222:2001 method (2001), whereas UNI EN ISO 9308–1:2017 (2017) and 9308–2:2014 (2014) were followed to determine the presence of *Escherichia coli* and coliform bacteria in the water samples.

2.5. Preparation of organic extracts for genotoxicity testing

Water samples (100 L per sample) were adsorbed on trifunctional silica tC18 cartridges (Sep-Pak Plus tC18 Environmental Cartridges, Waters Chromatography) according to the USEPA 525.2 method, with minor modifications (EPA, 1995; Monarca et al., 2004; Guzzella et al., 2006) to study *in vitro* genotoxicity.

Briefly, water samples were passed on filter paper to eliminate the suspended solids, acidified with H_2SO_4 at pH 3.5, and passed on trifunctional silica tC18 cartridges, previously washed with 40 ml of ethyl acetate, 40 ml of acetone, 40 ml of methanol and 40 ml of distilled water. Twenty litres of water samples were adsorbed on each cartridge (flow 10–15 ml/min). At the end of adsorption the cartridges were maintained in the refrigerator (2–6 °C) until elution. The cartridges (5 per sample) were eluted with 40 ml of ethyl acetate, 40 ml of acetone and 40 ml of methanol. For each sampling point, the eluates were reduced to a small volume by means of a rotating vacuum evaporator, pooled, and dried under nitrogen flow. The dry residue was dissolved in dimethylsulfoxide (DMSO) to have 3 L equivalent of water (L_{eq}) in 50 µl of DMSO, and stored in the dark at −20 °C until the mutagenicity assays were performed (for each sample, 50 L_{eq} were allocate for the Ames test, and 25 L_{eq} for the comet assay and the cytokinesis-block micronucleus test, respectively).

Distilled water (60 L) was adsorbed on tC18 cartridges (blank cartridge) to exclude effects related to the concentration method (E sample).

At the same time unconcentrated water (3 L per sample) was collected before and after disinfection treatment and at the distribution system to assay using a plant mutagenicity test to reveal DNA damage, namely chromosomal mutations and micronuclei, in root cells of *Allium cepa*. Furthermore, water samples were analysed for microbiological and chemical-physical characterisation.

2.6. Salmonella/microsome (Ames) test

Water samples were tested in duplicate at increasing doses corresponding to 1, 2 and 3 L equivalent, using *Salmonella typhimurium*, TA98 and TA100 strains, with and without microsomal activation, to highlight the presence of promutagens and direct mutagenic substances, respectively. TA98 strain detects frame-shift mutagens and TA100 strain responds to base-pair substitution mutations (APHA, 2012; Maron and Ames, 1983). Based on the results some samples were also tested at lower doses (0.1 and 0.5 L_{eq}/plate). 2-nitrofluorene for TA98 (10 μg/plate) and sodium azide for TA100 (10 μg/plate) were used as positive control in the test without S9; 2-aminofluorene (20 μg/plate) for both strains in the test with S9. DMSO was a negative control. Blank cartridge was assayed too.

The results, obtained from the average of duplicate plates, were expressed as mutagenicity ratio (MR), dividing the revertants/plate by the spontaneous mutation rate. Results 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 (APHA, 2012). Only in the event of a positive response, the net revertants per litre of water (net revertants/L), were calculated by linear regression analysis of the dose-response curve.

2.7. Allium cepa test

Allium cepa test to detect chromosome aberrations (CA), namely bridges, laggard or lost chromosomes, fragments, c-mitosis and multipolar anaphases, and micronuclei (MN) was carried out on unconcentrated water samples (Cabaravdic, 2010; Ma et al., 1995). Along with CA and MN were also considered binucleated cells, buds, lobulated nuclei, polyploidizated and polynuclear cells as a sign of nuclear abnormalities.

In a preliminary toxicity assay, 12 equal-sized young bulbs of onion were exposed for 96 h in the dark to undiluted and diluted water (undiluted water, 1:2, 1:10, and 1:100 dilution), changing the sample solution every day. Root length was used to calculate the EC_{50} value of each sample and to identify the concentration to undergo the *Allium cepa* genotoxicity assay, being the highest correspondent to the EC_{50} value identified (the concentration that gives a 50% reduction in root growth). Other macroscopic parameters (turgescence, consistency, change in colour, root tip shape) were used as toxicity indexes (Fiskesjö, 1995).

Afterwards the genotoxicity test was performed using six equal-sized young bulbs per sample and after 72-h pre-germination in saline solution to have 2–2.5 cm long roots, the bulbs were exposed to the water samples for 24 h. After exposure, the roots were cut and fixed in acetic acid and ethanol (1:3) for 24 h and lastly stored in 70% ethanol. Only for the MN test the bulbs, after exposure, were replaced in saline solution for 44 h of recovery time, to cover two rounds of mitosis (in order for damage induced in chromosomes during mitosis to be visible as micronuclei in interphase cells), consequently, we proceeded as above. Distilled water (24-h exposure) and maleic hydrazide (10^{-2} M, 6-h exposure) were used as negative and positive control, respectively.

The root tips underwent Feulgen staining. Five roots of each sample were considered for microscopic analysis (1000 ×): 1000 cells/slide (5000 cells/sample) were scored for mitotic index (MI) – as a measure of cell division and hence of sample toxicity – 200 in mitosis cells/slide (1000 cells/sample) for CA and 2000 interphase cells/slide (10,000 cells/sample) were scored for MN frequency analysis. Chi square test was performed for mitotic index and CA data analyses; the analysis of variance and Dunnett's *t*-test were performed for MN. A value of $p < 0.05$ was considered statistically significant.

2.8. Single-cell microgel-electrophoresis (comet) assay

HepG2 human hepatocellular carcinoma cells (ATCC HB-8065) were routinely grown as monolayer cultures in Minimal Essential Medium (MEM) supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, sodium pyruvate (1%), non-essential amino acids (1%) at 37 °C in a humidified atmosphere containing 5% CO_2 (Villarini et al., 2014). The cells were subcultured in 12-well tissue culture plates at an initial concentration of 2.5×10^5 cells/well. After 24 h culture, the cells were incubated for 4 h in MEM complete medium containing water extracts with concentrations equivalent to 1, 2, and 3 L_{eq}/ml. Immediately after the *in vitro* treatment, the cells were washed twice with PBS, harvested by trypsinization (150 μl of 0.05% trypsin-EDTA, 5 min) and centrifuged for 5 min at 720×*g*. Cell viability was evaluated by the Trypan blue dye exclusion assay using a Countess™ automated cell counter (Villarini et al., 2014).

For genotoxicity testing, HepG2 cells were processed in the comet assay under alkaline conditions (alkaline unwinding/alkaline electrophoresis, pH > 13), basically following the original procedure (Singh et al., 1988; Tice et al., 1990, 2000), with minor modifications (Dominici et al., 2013; Lombardi et al., 2015). Cell pellets (~5×10^5 cells) were resuspended in 0.7% low melting-point agarose (LMPA; w/v in PBS); 30 μl of cell suspension in LMPA were pipetted onto 1% normal melting-point agarose pre-coated conventional microscope slides to obtain microgels containing approximately 1×10^5 cells. Agarose microgels were allowed to spread using a cover slip and maintained on an ice-cold flat tray for 10 min for solidification. After removal of the cover slip, a 75 μl top layer of 0.7% LMPA was added to protect agarose microgels. Two slides were prepared for each experimental point. Lysis of nuclear and cellular membranes of cells included in the agarose microgels was performed overnight at 4 °C by immersing the slides in ice-cold freshly prepared high-salt solution with detergents (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, and 1% Triton X-100 added just before use; pH 10). The slides were then drained and placed in a horizontal electrophoresis box filled with the ice-cold electrophoresis solution (10 mM Na_4EDTA, 300 mM NaOH; pH > 13). After 20 min of alkaline pre-electrophoresis, the electrophoresis was carried out in the same buffer for 20 min by applying an electric field of 25 V (1 V/cm) and adjusting the current to 300 mA. Afterwards, the slides were neutralised with tris buffer (0.4 M Tris-HCl; pH 7.5) and the agarose microgels dehydrated by placing the slides in absolute ethanol for 5 min. Immediately before scoring, air-dried slides were stained with 50 μl of ethidium bromide (20 μg/ml). Analysis of blind samples was carried out by using an epi-fluorescence microscope (excitation filter 515–560 nm, emission filter 590 nm) at a 200 × magnification equipped with a high-sensitivity black and white CCD camera and connected with a computerised system for the analysis of images ("Comet Assay III", Perspective Instruments Ltd., Suffolk, UK). One hundred cells were analysed for each experimental point (50 cells/slide); the median of the scored comets for each slide was used to calculate the group means.

2.9. Cytokinesis-block micronucleus (CBMN) test

After 24 h culture, the cells were incubated for 24 h in complete MEM medium containing water extracts with concentrations equivalent to 1, 2, and 3 L_{eq}/ml. The CBMN assay was performed according to the original method (Fenech, 2007) with marginal modifications for adaptation to HepG2 cells (Vannini et al., 2018). Immediately after the *in vitro* treatment, the medium was removed and replaced by fresh medium containing cytochalasin B (final concentration 4.5 μg/ml) to

inhibit cell division after mitosis. The cells were then incubated further for 24 h. After that the cells were harvested by trypsinization, re-suspended in hypotonic solution (3 ml of 0.56% KCl) at 37 °C and fixed with 3 ml of ice-cold Carnoy's reagent (methanol:glacial acetic acid - 5:1 v/v). Cell suspensions were centrifuged again for 5 min at 720 × *g* and resuspended in 6 ml of fixative. Next, the tubes were centrifuged for 5 min, the supernatants removed, and the cell suspensions dropped onto glass slides (two slides per tested concentration). After drying, the slides were stained with 4% Giemsa solution in Sörensen buffer (0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4, pH 6.8) for 8 min, washed with distilled water, air-dried and finally mounted with Eukitt.

Micronuclei (MN) were scored in 2000 binucleated cells for each sample (two replicates, in each 1000 cells per slide were scored). Cytostatic effects were determined by calculating the nuclear division index (NDI) using the following formula:

$$NDI = \frac{[(1 \times M_1) + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]}{N}$$

where $M_1 - M_4$ represent the number of cells with 1–4 nuclei in the same cytoplasm after staining with Giemsa dye, and N is the total number of viable cells scored (*i.e.*, 500 cells from each experimental point) (Eastmond and Tucker, 1989).

2.10. Statistical analysis on data from comet assay and CBMN test

Each result is expressed as the mean ± standard deviation (SD) of at least two independent experiments. Data obtained were submitted to statistical evaluation using ANOVA univariate test with post hoc Bonferroni correction, the significance was calculated in comparison to the negative (untreated) and positive control, respectively. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chemical analyses

The physical and chemical parameters of the water from four basins are summarized in Table 1. The highest TOC values were observed in the raw waters of basins B and D. The concentration of chlorite was high in the water in distribution of all basins, exceeding the concentration limit of 0.2 mg/L established in Italy's Legislative Decree no. 31/2001, and in particular in basins A and B, exceeding the provisional health-based guideline value of 0.7 mg/L for chlorite and chlorate ions in drinking water established in the 2011 WHO Guidelines for Drinking Water Quality.

With regard to metals, in the raw water of basin A manganese and iron were much higher than the required parameter values. Manganese was also above the limit in basin D. After treatment both metals were within the concentration limits.

3.2. Microbiological analyses

All disinfectant treatment caused a reduction in the total bacteria count and completely removed coliforms *Escherichia coli* and enterococci (these results are reported in Supplementary material, Table 2S). Only disinfected water of B basin (sample B2) maintained a high bacteria count both at 22 °C and 36 °C even after disinfection (> 300 colony-forming unit, CFU), but not in distributed water.

3.3. Ames test

The results of Ames test, expressed as MR, are presented in Table 2. According to the twofold rule (MR > 2) for positive results, some samples displayed mutagenic activity in Salmonella. In particular samples B3, C3, D2 and D3 induced point mutations in TA98 strain, which detects substances capable of inducing frameshift mutations. With the TA100 strain a borderline effect was observed only for the D2 sample at the highest dose tested.

Some samples showed a toxic effect on bacteria which inhibited the growth of revertant colonies (C1, C2, C3, D1, D2, D3). These samples and those showing mutagenic activity already present at the lowest dose were tested again at lower doses (0.1 and 0.5 L_{eq}/plate) to allow the calculation of net revertants using regression linear analysis. Table 3 shows the net revertants per L of water for the samples with mutagenicity and with a clear dose-response trend. For the samples that despite having mutagenicity ratios greater than 2 failed to show a dose-response relationship the net revertants/L calculation has not been possible. The samples with the most evident mutagenic effect and for

Table 1
Physical and chemical parameters of water samples.

	A) *Monte Lerno*			B) *Bidighinzu*			C) *Monte Agnese*			D) *Pedra Majore*		
	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
pH	7.50	6.57	7.45	8.33	7.01	7.85	7.84	6.89	6.84	8.98	7.40	7.95
T (°C)	19	20.5	22	24	22	22	16	19	20	15	16	17
Conducibility (μS cm^{-1})	259	267	283	351	399	427	305	392	382	465	530	507
TOC (mg/L)	8.4	7.2	7.5	11.6	5.6	5.6	9.2	5.0	5.5	12.3	9.7	9.4
Ca (mg/L)	11.9	10.2	12.8	32.6	30.4	28.0	21.4	17.9	18.6	21.4	22.4	22.2
Mg (mg/L)	6.8	6.7	6.7	10.0	9.2	8.8	5.9	8.4	8.6	9.8	9.8	10.0
Na (mg/L)	34.0	28.5	33.5	42.0	46.0	53.0	41.0	51.0	50.0	54.0	61.0	64.0
K (mg/L)	1.92	1.90	1.97	4.05	4.25	4.30	3.13	3.92	3.79	4.20	3.75	4.10
Fe (μg/L)	570	15	116	125	2	1	212	9	67	201	9	6
Mn (μg/L)	93	20	13	29	20	22	24	3	3	83	18	19
Br (mg/L)	0.87	0.71	0.75	0.94	0.81	0.83	1.06	0.74	0.72	1.03	0.85	0.78
I (mg/L)	0.083	0.072	0.064	0.097	0.078	0.073	0.089	0.065	0.061	0.077	0.053	0.051
Chlorite (μg/L)	–	1000	1200	–	–	2100	–	–	700	–	500	700
Chlorate (μg/L)	–	990	1250	–	–	570	–	–	50	–	370	390
$UV_{254\ nm}$ (absorbance)	0.21	0.15	0.14	0.18	0.04	0.04	0.39	0.07	0.06	0.16	0.05	0.05
RFC (mg/L)	–	0.08	0.09	–	–	0.07	–	–	0.05	–	0.05	0.23
TARC (mg/L)	–	0.09	0.65	–	–	1.60	–	–	0.10	–	0.12	0.37

Raw (A1, B1, C1, D1), disinfected (A2, B2, C2, D2) and distributed (A3, B3, C3, D3) water samples.
TOC, total organic carbon; RFC, residual free chlorine; TARC, total active residual chlorine.

Table 2
Results of Ames test expressed as mutagenicity ratio (RM).

Samples	Dose L eq/plate	Mutagenicity ratio			
		TA98 (-S9)	TA98 (+S9)	TA100 (-S9)	TA100 (+S9)
A1	1	1.3	1.1	0.9	1.1
	2	1.7	1.7	1.0	1.2
	3	1.5	1.5	1.1	1.5
A2	1	1.3	1.1	1.0	1.2
	2	1.7	1.4	1.4	1.3
	3	1.6	1.5	1.4	1.3
A3	1	1.1	1.3	1.2	1.2
	2	1.5	1.4	1.2	1.1
	3	1.7	1.7	1.5	1.4
B1	1	1.4	1.4	1.1	1.2
	2	1.4	1.8	1.1	1.3
	3	1.5	1.5	1.2	1.2
B2	1	1.3	1.4	1.1	1.1
	2	1.6	1.9	1.2	1.0
	3	1.0	1.9	1.3	1.4
B3	1	**2.0**	**2.4**	1.0	1.2
	2	**2.1**	**3.4**	1.3	1.5
	3	**2.3**	**3.6**	1.2	1.5
C1	1	1.1	1.3	0.7	1.1
	2	1.4	1.6	0.9	1.2
	3	Tox	1.7	0.9	1.3
C2	1	1.2	1.1	0.8	1.1
	2	1.3	1.2	0.8	1.2
	3	Tox	1.4	0.8	1.3
C3	1	1.9	1.8	0.9	1.2
	2	**2.6**	**2.2**	0.8	1.3
	3	Tox	**3.2**	0.7	1.4
D1	1	1.2	1.4	1.1	1.2
	2	Tox	1.3	1.4	1.3
	3	Tox	1.5	1.2	1.7
D2	1	1.7	1.7	1.3	1.4
	2	Tox	**2.9**	1.2	1.6
	3	Tox	**2.8**	1.2	2.1
D3	1	1.4	1.7	1.2	1.3
	2	1.9	**2.4**	1.5	1.5
	3	Tox	**2.7**	0.9	1.6

Significant results (explanation in the text) are reported in bold type.
Negative control: DMSO, 50 μl/plate. Negative control results: TA98 (-S9) = 22.8 ± 0.5 revertants/plate; TA98 (+S9) = 24.0 ± 4.2 revertants/plate; TA100 (-S9) = 117.0 ± 15.3 revertants/plate; TA100 (+S9) = 124.8 ± 4.9 revertants/plate.
Positive control (-S9): TA98 10 μg/plate 2-nitrofluorene; TA100 10 μg/plate sodium azide; positive control (+S9): TA98, TA100 20 μg/plate 2-aminofluorene. Positive control results: TA98 (±S9) > 1000 revertants/plate; TA100 (±S9) > 1000 revertants/plate.

Table 3
Ames test results expressed as net revertants/L.

Samples	Net revertants/L	
	TA98 (-S9)	TA98 (+S9)
A1	-	-
A2	-	-
A3	-	-
B1	-	-
B2	-	-
B3	13	20
C1	-	-
C2	-	-
C3	-	21
D1	-	-
D2	-	17
D3	-	15

which the net revertants/L calculation was possible are distributed water (B3, C3 and D3). Mutagenicity was detected only with the TA98 strain and was present without and with exogenous metabolic activation, revealing the presence of both direct-acting mutagens and pro-mutagens, respectively, while the predominant effect is with S9.

The blank cartridge did not induce mutagenicity.

Table 4
Results of *Allium cepa* tests: mitotic index (MI), micronuclei (MN) frequency and chromosome aberrations (CA) in root cells.

Samples[a]		MN test		AC test	
		MI (%)	% MN (mean ± SD)	MI (%)	TAC (%)
A) *Monte Lerno*	A1	11.1	0.10 ± 0.08**	12.9	3.2
	A2	11.6	0.08 ± 0.07*	12.2	3.4
	A3	11.4	0.04 ± 0.04	10.5	4.9**
	Negative control	10.2	0.03 ± 0.04	11.3	3.2
B) *Bidighinzu*	B1	9.6	0.04 ± 0.03	11.3	2.3*
	B2	10.2	0.04 ± 0.04	10.1	2.5*
	B3	9.7	0.04 ± 0.07	11.3	1.2
	Negative control	10.6	0.01 ± 0.02	10.0	1.6
C) *Monte Agnese*	C1	8.3	0.03 ± 0.04	11.0	3.3
	C2	8.7	0.02 ± 0.02	10.3	3.1
	C3	9.3	0.05 ± 0.08	10.8	2.1
	Negative control	10.9	0.02 ± 0.03	10.4	2.8
D) *Pedra Majore*	D1	9.7	0.09 ± 0.10	9.9	4.8**
	D2	10.1	0.08 ± 0.06	11.3	4.2*
	D3	10.7	0.14 ± 0.11	10.3	4.9*
	Negative control	12.6	0.07 ± 0.03	11.1	3.0

Mitotic index, MI, micronuclei, MN, total aberrant cells, TAC.
Negative control: distilled water.
Positive control: 10^{-2} M maleic hydrazide (MN test: MI 7.5%, MN 16.4 ± 5.9%; AC test: IM 9.1%, AC 10.6%).
*$p < 0.01$, **$p < 0.001$: statistically significant according to Dunnett's test.
[a] Raw (A1, B1, C1, D1), disinfected (A2, B2, C2, D2) and distributed (A3, B3, C3, D3) water samples.

3.4. Allium cepa test

The toxicity test in *Allium cepa* showed that the samples, both undiluted and diluted, do not have any influence on the reduction of the radical elongation. In addition, no sign of toxicity was present in macroscopic parameters, therefore, the undiluted samples were assayed in the following genotoxicity tests.

The results of CA and MN tests are reported in Table 4. Some samples (A3, B1, B2, D1, D2 and D3) induced a statistically significant increase in aberrant cells compared to the negative control and an increase in micronuclei frequency was observed only in samples A1 and A2.

The types of aberrations in dividing cells are listed in Table 3S. The most frequent aberrations are spindle defects such as laggard, polar slip, anaphase and telophase multipolar, and c-mitosis.

3.5. Comet assay

The results obtained in the comet assay are reported in Fig. 2; 14 out of 36 extracts caused a significant increase of DNA strand breakage in HepG2 cells, 7 extracts showed a marked toxicity which affected genotoxicity testing. As regards raw water, significantly increased genotoxicity, compared to controls, was found in water sample from reservoir A (sample A1); whereas, samples C1 and D1 were affected by toxicity. The extent of DNA damage was found to be significantly high at the disinfection stage (treatment plant). Water samples from reservoirs A and B (samples A2 and B2) showed a statistically significant increased genotoxicity, compared to controls, at 2 and 3 L equivalent of water (L_{eq}). Samples C2 and D2 also showed genotoxic activity, even though the tests were largely affected by toxicity. Significantly high extents of DNA damage were also observed at the distribution system (pipeline) level, with residual genotoxic activity in water from A, B and C (samples A3, B3, and C3, respectively). The results of sample D3 were

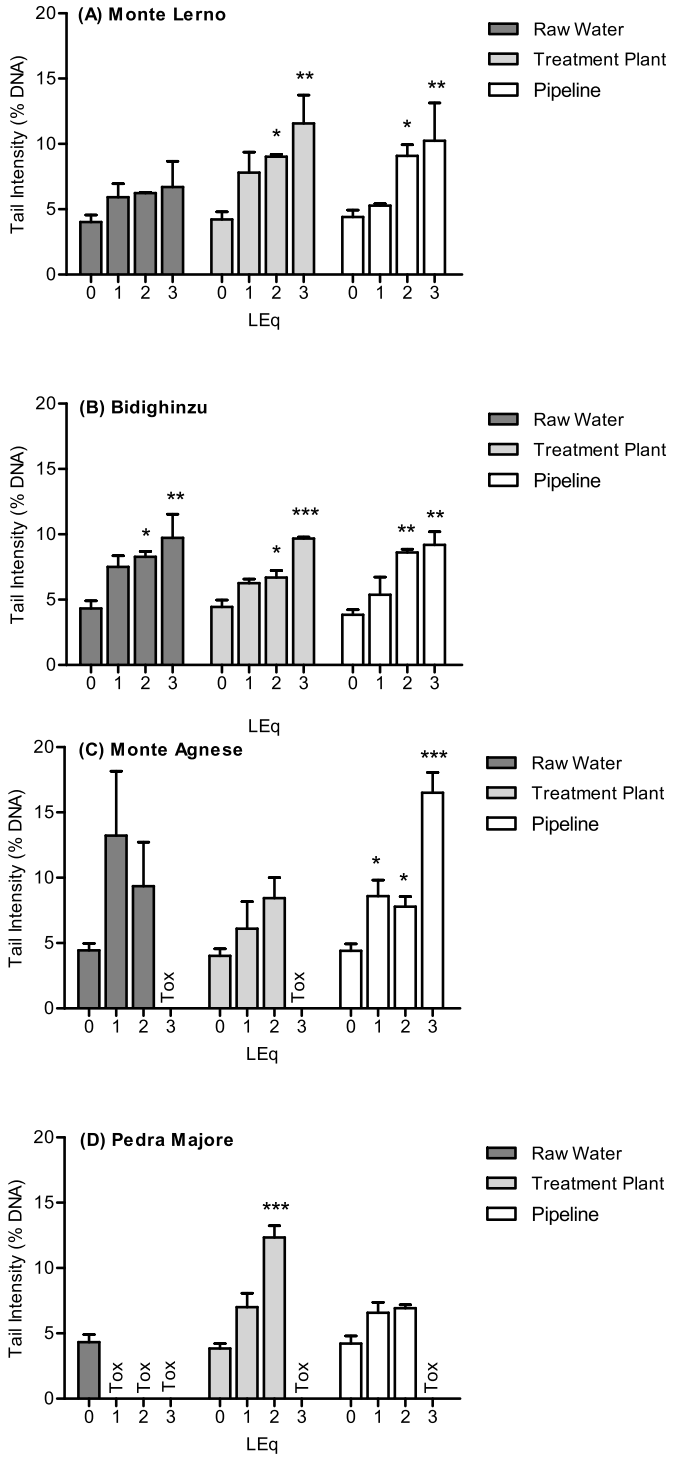

Fig. 2. Comet assay on HepG2 cells treated with increasing doses (Leq) of extracts of raw, disinfected, and distributed water. 0 L*eq* = negative control (1% DMSO). Positive control: 2 μM 4-nitroquinoline *N*-oxide, tail intensity (mean ± SD) = 20.7 ± 2.2.Statistical significance (ANOVA, followed by Bonferroni *post-hoc* analysis): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

affected by toxicity again. Toxic effects in the comet assay were mainly represented by the preponderant presence of so-called 'hedgehogs', corresponding to nucleoids with small or non-existent heads and large, diffuse tails that are assumed to represent apoptotic/necrotic cells.

3.6. CBMN test

The number of MN per 2000 binucleated cells was assessed as a measure of chromosomal abnormalities in HepG2 cells exposed to 1, 2, and 3 L equivalent of water (L_{eq}) in DMSO. None of the water extracts induced any statistically significant increase in the MN frequency compared to negative controls (data not shown). The positive control EMS showed the expected significant variation ($p < 0.01$), thus indicating the sensitivity of the test (data not shown). Additionally, NDI – measured to verify any possible cytostatic effects of water extracts – of samples C2, D2, and D3 –decreased significantly, thus indicating toxicity, when compared to control cells (data not shown).

4. Discussion

The battery of mutagenicity/genotoxicity tests adopted in the present study has clearly shown that genotoxic xenobiotics are present in raw, processed/disinfected, and distributed drinking water from four reservoirs/distribution systems in northern Sardinia.

Different responses in different tests indicate the presence of various substances that can react in diverse ways on DNA. The highest number of positive results was obtained with the comet assay on human cells. Almost all of the positive samples in the Ames test and/or in the *Allium cepa* test are also positive in the comet assay. Moreover, all of the mutagenicity/genotoxicity tests – including the CBMN test, which did not give positive data – have evidenced toxicity in some samples. In this puzzle of results, except for the basin A, the distributed water always induced mutagenicity in bacteria (B3, C3 and D3), showing the possible influence of the distribution system on water quality, in particular for basins B and C. In contrast, as regards the tests in plant cells, the genotoxic activity is already present in the raw water and remains even after disinfection (A, B and D basins) and after distribution (A and D basins). Only the water from basin C does not contain genotoxic substances for plant cells. These results agree partially with those observed in HepG2 cells in the comet assay. Raw and treated water from basin A induced DNA damage in plant (MN and AC) and human cells (primary DNA damage). Also treated water of basin B induced CA in *Allium cepa* roots and DNA damage in HepG2 cells, showing a possible effect due to disinfection treatment. Distributed water did not induce genotoxicity in plant cells but remained genotoxic in human cells. None of the samples of basin C induced genotoxicity in plant cells while the disinfected and distributed water samples were able to damage the DNA in human cells, also showing toxicity. On the contrary, all of the samples from basin D were genotoxic in plant cells and toxic or genotoxic in human cells. No sample induced MN in HepG2 cells. Among the tests on human cells, the comet assay was found to be more sensitive than the CBMN test. It is worth noting, however, the presence of toxic effects, in particular for the samples from basins C and D that could have masked the genotoxic effects.

Overall, by considering the results obtained in the applied genotoxicity tests, when raw water was genotoxic (basins A, B and D), the different processes adopted for pre-oxidation/disinfection treatment were not able to reduce the genotoxicity. In the case of basin C, where the raw water was not mutagenic, following the pre-oxidation/disinfection treatment we evidenced the presence of genotoxic metabolites.

The chemical characteristics of the raw water were improved by potabilisation treatments, as regards the metals, in particular Fe and Mn, which in the raw water had high values. The TOC abatement was also good, even though it never showed particularly high values. Chlorite and chlorate ions were abundantly present in all disinfected water. Chlorite and chlorate are disinfection by-products resulting from the use of chlorine dioxide as a disinfectant. Chlorine dioxide rapidly

decomposes into chlorite, chlorate and chloride ions in treated water, chlorite being the predominant species. For human health, the primary concern from exposure to chlorite and chlorate is oxidative damage to the red blood cells. The 2011 WHO Guidelines for Drinking Water Quality established a provisional health-based guideline value of 0.7 mg/L for chlorite and chlorate ions in drinking water. In addition, Italy's Legislative Decree no. 31/2001 took the problem of chlorite in drinking water into account and established a concentration limit of 0.2 mg/L for chlorite. The genotoxicity observed in plant cells (*Allium cepa* test), and in HepG2 human cells (comet assay), could be attributable to these ions and their mixtures, as reported in a study in which it was also evidenced that chlorite and chlorate did not show a significant effect on HepG2 cells in the CBMN test (Feretti et al., 2008).

This study considered different genetic end-points (point mutations, chromosome aberrations, micronuclei and DNA damage) in different cell types (bacteria, plant and human cells). A worrying aspect is that all samples of water distributed to the people (taken from all basins) had mutagenic or genotoxic effects in different cells/organisms. The potential health risks of DBPs from drinking water include cancer and adverse reproductive outcomes. These substances are in trace concentration in drinking water but human exposure to them is long-term, thus increasing health risks. It should be remembered that an *in vitro* genotoxicity testing comprising at least two tests (*e.g.*, Ames and MN tests) would be sufficient to detect all relevant carcinogens and genotoxins (Kirkland et al., 2011). Thus, a battery of genotoxicity tests allows for a better evaluation of risks related to drinking water exposure (Ceretti et al., 2016).

The use of chloramines as measure of control for prevention of microbial growth, since they are a more stable secondary disinfecting chemical, is a strategy adopted to maintain the quality of water in the distribution system. The use of chloramines constitutes an advantageous alternative to free chlorine in distribution systems with long residence times and elevated temperatures, despite the potential nitrite formation by organisms in biofilms if chloramination is practised when excess ammonia is present. Monochloramines have been shown to be particularly effective against biofilm and generally produce a lower THMs concentration than chlorine and sodium hypochlorite but could produce other genotoxic DBPs, including cyanogen chloride, haloacetonitriles, organic chloramines, chloramino acids, chloral hydrate, haloketones. However, even inorganic products, such as chlorate and hydrazine, and similarly non halogenated products, such as aldehydes, ketones, *N*-nitrosodimethylamine, may have a role in drinking water mutagenicity (Richardson et al., 2007; WHO, 2011). Moreover, other DBPs could be generated during chemical interactions between disinfectant and substances in raw water. Changes in drinking water disinfection practice could remove organic precursor compounds and some of these compounds could be removed by additional treatments, such as granular activated carbon (GAC) filtration.

This study showed that sometimes the raw water already has genotoxic activity. Only raw water from basin C did not show genotoxic effects. On the other hand, the scarcity of water and the ever-increasing demand make it necessary to use water sources even with low quality characteristics. Nowadays, the majority of Sardinian water treatment plants use the combination of chloride dioxide and monochloramine to disinfect water distributed for human consumption. In this study. the use of disinfectant treatments combining chloramine with chloride dioxide (and sodium hypochlorite) does not seem to improve water quality, regarding genotoxicity. These results may be useful in risk assessment, considering the new approach according to water safety plans (WSP) developed by the WHO which shifted the focus from retrospective control on distributed water to risk prevention and management in the drinking water supply chain, extending from collection to tap. Among others, the risks related to the presence of mutagenic compounds in drinking water could be an important topic for managers of water treatment plants to improve the water quality and reduce the health hazards associated with the mutagenicity of drinking water.

5. Conclusion

In conclusion, the routine chemical analysis indicated an improvement in water quality following the applied treatments, which met the microbiological requirements of drinking water. However, the biological assays have shown in some cases that the treatment can increase the mutagenic activity, which was sometimes already present in the raw water. The use of chloramines associated with other disinfectants did not eliminate the mutagenicity present in the raw water (basins A and D) and when the raw water was not mutagenic it introduced mutagenic/genotoxic substances (basin C). In some cases, however, these observed effects could also be attributable partially to the distribution system.

These results indicate the need to better monitor the water quality of these basins by providing, for example, filtration systems on activated carbon to reduce the presence of potentially mutagenic organic substances in order to reduce their presence in the water distributed to the population and to reduce health hazards associated with the mutagenicity of drinking water.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109385.

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