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Tools for monitoring toxicological and genotoxicological changes in a drinking water treatment plant in Northeast Italy

Carlotta Alias[1,2] | Donatella Feretti[1,2] | Laura Benassi[1] | Ilaria Zerbini[1,2] | Claudia Zani[2] | Sabrina Sorlini[1,3]

[1]B+LabNet Environmental Sustainability Laboratory, University of Brescia, Brescia, Italy

[2]Department of Medical and Surgical Specialties, Radiological Sciences, and Public Health, University of Brescia, Brescia, Italy

[3]Department of Civil, Environmental, Architectural Engineering and Mathematics, University of Brescia, Brescia, Italy

Correspondence
Donatella Feretti, Department of Medical and Surgical Specialties, Radiological Sciences, and Public Health, University of Brescia, viale Europa 11, 25123 Brescia, Italy.
Email: donatella.feretti@unibs.it

Funding information
University of Brescia

Abstract

Drinking water quality can be compromised at different stages, from raw water to treated one. This research aimed to evaluate the toxicity and genotoxicity of groundwater contaminated by fluorinated compounds treated in a drinking water treatment plant, through several bioassays. Water samples underwent chemical analyses and were assayed on *Daphnia magna*, *Pseudokirchneriella subcapitata*, *Allium cepa*, human leukocytes and *Salmonella typhimurium*. Physical–chemical parameters were always within the Italian legislation limits. Water after filtration and disinfection caused slight toxicity in *D. magna*; the sample after filtration inhibited the proliferation of *P. subcapitata*. None of the water samples exerted toxicity in *A. cepa*. All the analysed samples had genotoxic effects on *A. cepa* and human leucocytes, while only disinfected water caused mutations in *S. typhimurium*. A battery composed of tests on *D. magna*, *P. subcapitata*, *S. typhimurium* and *A. cepa* could represent a useful tool to verify the toxicity/genotoxicity through the water treatment stages and to improve drinking water quality management.

KEYWORDS
Allium cepa tests, Ames test, comet assay, *Daphnia magna*, *Pseudokirchneriella subcapitata*

1 | INTRODUCTION

The availability of safe and clean drinking water is crucial for human life (United Nations, 1977). However, drinking water quality may be impaired by chemical and microbiological pollution (WHO, 2017). Among chemical pollutants, the occurrence of organic micropollutants in drinking water has become a growing concern (Brunner et al., 2020; Ren et al., 2020; Tröger et al., 2018; Zhang et al., 2020). Pesticides, pharmaceuticals, personal care products and perfluorinated compounds, are frequently detected both in surface water and groundwater destinated to human consumption (Galindo-Miranda et al., 2019; Kurwadkar et al., 2020; Wilkinson et al., 2017). Perfluoroalkylated and polyfluoroalkylated substances (PFAS), a group of man-made chemicals, are contaminants of concern due to their high stability in the environment (EPA and US EPA, 2020), their activity as endocrine disruptors and the potential carcinogenic activity exerted by several of them (Temkin et al., 2020). Above all, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are the most extensively produced and studied (US EPA, 2020). Moreover, PFOA was classified as 'possibly carcinogenic to humans' (group 2B) by the International Agency for Research on Cancer (IARC, 2017). Perfluorinated compounds are employed as additives in everyday life, such as in textiles and paper, in food packaging products, in fire foams, in music instruments and ammunition, in insecticides, cosmetics and many other applications (Glüge et al., 2020). Due to their innumerable uses, their diffusion in the environment is very wide. Their high solubility,

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coupled with their persistence, makes them very mobile in aquatic environments, resulting in long-distance, diffusive and large-scale contamination (Naidu et al., 2020). PFAS emission has been identified as an emerging chemical risk in the European Union and immediate actions have been proposed to rapidly address this pressing issue (European Environment Agency, 2019). The population may be exposed to PFAS through contaminated food (Domingo & Nadal, 2017; Knutsen et al., 2018) and drinking water (Domingo & Nadal, 2017; Mastrantonio et al., 2018). To address this last route of exposure, the European Union has revised its Directive on the quality of water intended for human consumption including the PFOA and PFOS among new parameters of water quality, because of their persistency, bioaccumulative capacity and toxicity (Council of the European Union, 2020). This has led to the revised Drinking Water Directive (EU) 2020/2184 of the European Parliament and of the Council, which was formally adopted on 16 December 2020, and entered into force on 12 January 2021. Member States will have 2 years to transpose it into national legislation, with the introduction of the following limits: 0.5 μg/L for total PFAS (the totality of perfluoroalkyl and polyfluoroalkyl substances) and 0.1 μg/L for the sum of PFAS (sum of perfluoroalkyl and polyfluoroalkyl substances considered a concern as regards water intended for human consumption).

In 2013, significant PFAS pollution of groundwater and surface freshwater was discovered in three provinces (Vicenza, Padova and Verona) of the Veneto Region (north-eastern Italy) (Polesello et al., 2013). Thereafter, the authorities of the Veneto Region, supported by the Italian Ministry of the Environment and the National Institute of Health, fixed threshold concentrations for PFAS in drinking water (Regione Veneto, 2014, 2017a, 2017b). Starting from the PFAS discovery, the Lonigo drinking water treatment plant (DWTP), serving the Province of Vicenza, implemented a granular activated carbon (GAC) filtration system to remove the PFAS contamination (Bertanza et al., 2020). Among the currently available systems for the effective removal of such molecules (EPA, 2020; Kucharzyk et al., 2017; Sorlini et al., 2019), GAC is indeed the most commonly used technology applied by many full-scale DWTPs (Kim et al., 2020; Liu & Zhang, 2019; Tröger et al., 2020).

Water intended for human consumption must also be microbiologically safe. To achieve this public health target, disinfection procedures of treated water are performed, commonly through the addition of chlorine (WHO, 2017). Notwithstanding, as a result of the interaction between chlorine and naturally occurring organic matter precursors (e.g., humic substances), various by-products could be generated, such as trihalomethanes (THMs), haloacetic acids (HAAs), haloketones, haloacetonitriles and nitrosamines (Mazhar et al., 2020). Such disinfection by-products (DBPs) exert toxic and genotoxic/mutagenic actions towards living organisms, including humans (Ceretti et al., 2016; Chaves et al., 2019; Cortés & Marcos, 2018; Feretti et al., 2020) and are potentially associated with reproductive disorders (Kali et al., 2021) and human bladder cancer (Diana et al., 2019).

Drinking water is a complex mixture, containing many chemicals not easily detectable by chemical standard analysis and others not known at all. Moreover, many of them act synergistically with potential long-term effects on human health. The monitoring of such substances by short-term bioassays could allow a better evaluation of the global human exposure to drinking water toxics.

Here, a novel procedure was applied to investigate toxic, mutagenic and genotoxic traits of water intended for human consumption. The implemented battery of biotests was composed of toxicity tests on *Daphnia magna*, *Pseudokirchneriella subcapitata* and *Allium cepa*, mutagenicity assay on *Salmonella typhimurium*, and genotoxicity assays on *A. cepa* and human leucocytes. To our knowledge, this is the first study in which several short-term bioassays were applied on PFAS-contaminated groundwater intended for human consumption to assess the usefulness of such toxicological and genotoxicological tools.

2 | MATERIAL AND METHODS

2.1 | Water treatment plant and water samples collection

This study was performed considering the Lonigo water treatment plant located near Vicenza (Italy), and managed by Acque Veronesi S. c.a.r.l, because of the known PFAS groundwater contamination.

The DWTP schematic representation and the sampling points are summarized in Figure 1.

The groundwater is drawn from six artesian wells (over 100 m deep—maximum flow rate of 550 L/s) and is collected in an initial storage tank of 1000 m^3. The water treatment encompasses the following:

- Five rapid sand filtration units (SF—capacity of 100 L/s each), which allow the physical separation of the suspended solid particles by gravity filtration.
- Two banks in series with 10 filters per bank of GAC adsorption units (capacity of 50 L/s each). Each GAC filter (downflow configuration) consists of a 4 m diameter steel cylinder. Approximately 13 000 kg of carbon per filter is used. The GAC is obtained from coconut shells and physically activated using steam. The empty bed contact time is approximately 13 min.
- Final disinfection (NaClO).

Four different water samples were collected: capture site (point A), water after sand filtration and the first GAC treatment (point B), water after the second GAC treatment and the disinfection phase (point C) and water along the drinking water distribution system (point D).

Thirty litres from each point was sampled in polypropylene (PP) containers once a week for three consecutive weeks. A total amount of 90 L per point was collected and properly pooled to obtain a representative sample of each sampling site over time.

2.2 | Analytical characterization of water samples

The characterization of water samples was performed according to standards set by the National Environmental Protection Agency

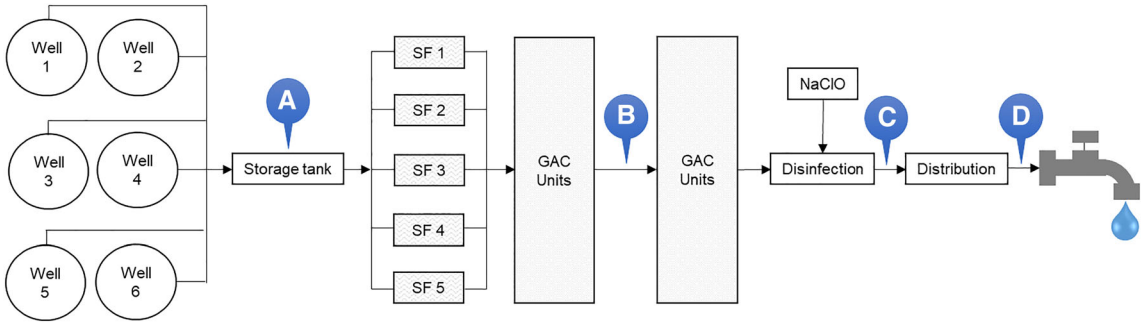


FIGURE 1 DWTP stages and schematic overview of the sampling sites: A) groundwater from wells, B) water after sand filtration (SF) and first granular activated carbon (GAC) filtration, C) water after second GAC filtration and disinfection with sodium hypochlorite (NaClO) and D) along the distribution system

TABLE 1 PFAS limit concentration values set by Italian National Health Institute guidelines and Regional Decree of Veneto n. 1590/2017

	Limit concentration values (ng/L)	
	Italian National Institute of Health	Regional Decree of Veneto n. 1590/2017
PFOA-sum	500	-
PFOA-PFOS-sum	-	90
PFOS-sum	30	30
others PFAS	500	300

(EPA, 1995) and the Italian legislation on the quality of water intended for human consumption (Legislative Decree n. 31 02.02.2001, 2001). Water samples were also characterized for the presence of several PFAS following the guidelines set by the Environmental Regional Agency of the Region of Veneto (ARPAV, 2019), the Italian National Institute of Health (Istituto Superiore di Sanità) (ISS, 2014) and the Regional Decree of Veneto n. 1590/2017 (Regione Veneto, 2017a). Limit concentration values are established only for some of such molecules, as summarized in Table 1.

2.3 | Unconcentrated water samples

After each weekly sampling, 1 L from each sampling point was filtered on fibreglass filters with particle retention of 1.2 μm and stored at −20°C until the last specimen was collected. Then, samples from the same sampling point were melted, mixed and used for toxicity and genotoxicity tests.

2.3.1 | *D. magna* acute immobilization test

The *D. magna* acute immobilization assay was conducted following the ISO 6341 (UNI EN ISO 6341, 2013) by using Daphtoxkits F (Ecotox LDS). Twenty daphnids (<24 h old) were incubated with 10 ml of unconcentrated water for each sample. The mobility of neonates was examined after 24 and 48 h of treatment. Standard freshwater was used as a negative control. Experiments were performed in duplicate. The assay is considered valid if the immobilization rate in the negative control is below 10%. The data were expressed as the percentage of immobilized animals.

2.3.2 | *P. subcapitata* growth inhibition test

The *P. subcapitata* growth inhibition assay was conducted following the ISO 8692 (UNI EN ISO 8692, 2012) by using Algaltoxkit F (Ecotox LDS). A mini-scale test method was applied. The initial algal density was 10^4 cells/ml in 2.5 ml of undiluted sample, adjusted to culturing conditions with concentrated nutrient solutions. The samples were incubated for 72 h with orbital agitation (200 rpm), at 23°C and at 10 000 lx. Algal growth medium was used as a negative control. The test was performed in triplicate. The algal growth rates were calculated based on the daily manual counting of cell density. The assay is considered valid if the average growth rate in the control is at least 1.4/day and the variation coefficient of the growth rate in the control replicates does not exceed 5%. Toxicity was expressed as the percentage of growth inhibition (I%) in comparison with the control. The statistical analysis was performed using the Student's *t* test.

2.3.3 | *A. cepa* toxicity test

The *A. cepa* toxicity test was performed using equal-sized commercial onion bulbs of *A. cepa* cleaned and washed without destroying the primordial roots. Twelve bulbs germinated for 72 h in the dark were soaked in each solution of water samples (undiluted, 1:2, 1:10 and 1:100). Distilled water was used to dilute the samples and as a negative control. The roots' mean length was used to calculate the EC50 value (Fiskesjö, 1985, 1995). Turgidity, consistency, colour change and root tip shape were also used as toxicity indexes.

2.3.4 | *A. cepa* chromosome aberration (CA) test

A genotoxicity test was carried out on water samples by using *A. cepa* to detect CAs in mitotic cells (Aswathi & Thoppil, 2019; Cabaravdic, 2010; Prajitha & Thoppil, 2017). The test was performed on six equal-sized young onion bulbs for each sample. At the end of a 72 h pregermination period in a saline solution (Rank's solution)

(Rank, 2003), the bulbs were exposed for 24 h to undiluted samples due to the results obtained in the toxicity test. The roots of all bulbs were cut, fixed in 1:3 acetic acid–ethanol for 24 h and stored in 70% ethanol at 4°C until the microscopic observation. Root tips were stained with 2% acetic orcein for the microscopic analysis (1000× magnification). Five roots from each sample were considered: 5000 cells (1000 cells/slide) were scored for the mitotic index (MI) indicating the number of dividing cells and 1000 cells in mitosis (200 mitotic cells/slide) for CA frequency. All experiments were performed in duplicate. Rank's solution was used as the negative control, while a positive control was performed using maleic hydrazide (10 mM, 6 h exposure). MI and CA were analysed by using the chi-square test.

2.3.5 | *A. cepa* comet test

The test was conducted on three equal-sized young onion bulbs for each sample. At the end of the 48-h pregermination period in Rank's solution, the bulbs were exposed for 24 h to undiluted samples. Negative and positive controls were carried out using Rank's solution and ethyl methanesulfonate (EMS, 8 mM), respectively. Fifty meristematic root tips (5 mm long) were cut and collected in an ice-cold dish. The tips were finely chopped with a scalpel and 1 ml of ice-cold nuclei isolation buffer (200 mM Tris, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% Triton-X) added. The suspension was ground with a pestle and let to sediment on ice for a few minutes. One hundred eighty microliters of supernatant was diluted 1:1 in low melting agarose (LMA, 0.7%). Eighty microliters of suspension was distributed on an agarose-coated glass slide, and a coverslip was immediately placed on the top of the cell–gel mixture. The slides were then placed for 20 min at 4°C, to allow the solidification of the agarose. After that, the coverslips were gently removed, and the samples were subjected to 1-h unwinding (pH > 13) and 20-min electrophoresis (pH > 13, 0.8 V/cm and 26 V at the limit). The slides, stained with GelRed Nucleic Acid Gel Stain (Biotinum), were examined under a fluorescence microscope (Olympus CX 41RF) equipped with a BP 515–560 nm excitation filter and an LP 580 nm barrier filter. Levels of DNA damage were evaluated by the comet parameter 'tail intensity' (TI) (percentage of DNA migrated in the tail) detected by an automatic image analysis software (Komet 5, Kinetic Imaging Ltd, UK). Each experiment was conducted in duplicate. The statistical analysis was performed using ANOVA univariate and Dunnett's multiple comparison test.

2.4 | Concentrated water samples

2.4.1 | Solid phase extraction

After each sampling, waters were filtered on fibreglass filters with particle retention of 1.2 μm and acidified with 37% HCl (1.0–1.3 ml/L) to reach pH 4–4.5. Samples were immediately adsorbed on trifunctional silica C18 cartridges with an optimal pH working range of 3–7 (10 g Sep-Pak Plus tC18 Environmental Cartridges, Waters Chromatography), following the EPA 525.2 method (EPA, 1995), with some modification (Monarca et al., 2004). Before use, cartridges were activated with ethyl acetate, acetone and methanol and rinsed with distilled water (40 ml each). Water samples were pumped through the cartridges (10 L/cartridge) in a multisample extraction manifold (Waters Corporation) at a maximum flow rate of 15 ml/min. After this, nine cartridges per sample were refrigerated (2°C to 8°C) until elution. The elution of the adsorbed substances was performed with ethyl acetate, acetone and methanol (40 ml/cartridge, respectively). The eluates were reduced in a rotating vacuum evaporator and dried under a light flow of nitrogen gas. The residue was dissolved in dimethyl sulfoxide (DMSO) to produce an approximately 50 000-fold concentration, which was then stored in the dark vials at −20°C. Distilled water (90 L; 30 L/cartridge) was treated as the drinking water samples (blank cartridge).

2.4.2 | *A. cepa* comet test

The test was conducted on three equal-sized young onion bulbs for each sample. At the end of the 48-h pregermination period in Rank's solution, the bulbs were exposed for 24 h to extracts of each sample equivalent to 1, 2 and 3 L of water. The detailed procedure has already been described in Section 2.3.5.

2.4.3 | Human leukocytes comet test

Human leukocytes were collected from a young, male, healthy non-smoker donor (Dhawan & Anderson, 2009). The leukocytes were treated with extracts of each sample equivalent to 1, 2 and 3 L of water diluted in phosphate buffer saline (pH 7.4) at 37°C for 1 h. Negative and positive controls were carried out using DMSO and ethyl methanesulfonate (EMS, 2 mM), respectively. After treatment, the cellular survival was evaluated using the Trypan Blue exclusion technique, and the test was carried out only on samples with viability >70%, according to the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). At the end of slides preparation and cell lysis (pH 10, 4°C, overnight), samples were subjected to 20-min unwinding (pH > 13) and 20-min electrophoresis (pH > 13, 0.8 V/cm and 300 mA). The slides were stained and examined as described above for the *A. cepa* comet test. Each experiment was conducted in duplicate. The statistical analysis was performed using ANOVA univariate and Dunnett's multiple comparison test.

2.4.4 | Salmonella/microsome mutagenicity test

The *Salmonella*/microsome assay (Maron & Ames, 1983) was performed using *S. typhimurium* TA98 and TA100 strains (Trinova Biochem GmbH) with and without *in vitro* metabolic activation (±S9, Trinova Biochem GmbH). The extracts dissolved in DMSO were tested at increasing doses equivalent to 1, 2 and 3 L of water/plate. Positive controls were 2-nitrofluorene (10 μg/plate) and sodium azide

(10 μg/plate) for the TA98 and the TA100 without S9, respectively, and 2-aminofluorene (20 μg/plate) for both strains with S9. Negative control was carried out using DMSO. A blank cartridge was assayed too. The results were expressed as mutagenicity ratio (MR), dividing the revertants/plate by the spontaneous mutation rate. According to the Standard Methods for the Examination of Water and Wastewater (APHA, 2017; Mortelmans & Zeiger, 2000), the results of the *Salmonella*/microsome test 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. The net revertants per litre of water (net revertants/L) were calculated by linear regression analysis of the dose–response curve.

3 | RESULTS

3.1 | Chemical analyses

Water samples were characterized through chemical analyses according to the Italian legislation on drinking water quality (Legislative Decree n. 31 02.02.2001, 2001). All the analysed parameters of each water sample were within the legislative limit values (complete datasets are shown in Tables A1–A5). Also, according to the national and regional guidelines (ARPAV, 2019; ISS, 2014; Regione Veneto, 2017a), the presence of several PFAS was investigated (Table 2). Many PFAS were detected in water from the captation site (sample A): PFBA, perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, PFOA-isomers, PFOA-sum, PFOS, PFOS-isomers, PFOS-sum, PFBS, perfluorohexane sulfonic acid (PFHxS), PFOA-PFOS-sum and other PFAS. Two of these parameters were above the defined limit values: PFOS-sum (36 ng/L, limit value [ISS, 2014]: 30 ng/L) and PFOA-PFOS-sum (242 ng/L, limit value [Regione Veneto, 2017a]: 90 ng/L). A weak concentration (6 ng/L) of the only PFOA was detected after SF and the first GAC treatment (sample B), whereas a complete removal of all PFAS was obtained after the second GAC treatment and disinfection (sample C) and maintained along the distribution system (sample D).

TABLE 2 Perfluoroalkyl substances analyses of water samples according to the Environmental Regional Agency list of PFAS, the Italian National Health Institute guidelines and to the Regional Decree of Veneto 1590/2017

	Samples			
Fluorinated compounds (ng/L)	A	B	C	D
PFBA	61	<Q.L.	<Q.L.	<Q.L.
PFPeA	38	<Q.L.	<Q.L.	<Q.L.
PFHxA	38	<Q.L.	<Q.L.	<Q.L.
PFHpA	10	<Q.L.	<Q.L.	<Q.L.
PFOA	138	6.0	<Q.L.	<Q.L.
PFOA-isomers	67	<Q.L.	<Q.L.	<Q.L.
PFOA-sum	206	6.0	<Q.L.	<Q.L.
PFNA	<Q.L.	<Q.L.	<Q.L.	<Q.L.
PFDeA	<Q.L.	<Q.L.	<Q.L.	<Q.L.
PFDoDA	<Q.L.	<Q.L.	<Q.L.	<Q.L.
PFUnDA	<Q.L.	<Q.L.	<Q.L.	<Q.L.
PFOS	22	<Q.L.	<Q.L.	<Q.L.
PFOS-isomers	14	<Q.L.	<Q.L.	<Q.L.
PFOS-sum	**36**	<Q.L.	<Q.L.	<Q.L.
PFBS	80	<Q.L.	<Q.L.	<Q.L.
PFHxS	13	<Q.L.	<Q.L.	<Q.L.
PFOA-PFOS-sum	**242**	6.0	<Q.L.	<Q.L.
HFPO-DA	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Others PFAS	240	<Q.L.	<Q.L.	<Q.L.

Abbreviation: Q.L., quantification limit. Parameters above the limit values are indicated in bold.

3.2 | Toxicity

The toxicity of unconcentrated water samples was evaluated through *D. magna*, *P. subcapitata* and *A. cepa* tests.

Results obtained from the *D. magna* acute immobilization test are summarized in Table 3. Only two samples had an effect on *D. magna*. Sample B caused 20% of daphnids immobilization after 48 h of treatment, while sample C affected the mobility of crustaceans during the time, immobilizing 10% of organisms after 24 h and 20% after 48 h. Samples A and D did not produce any toxic effect on *D. magna*.

The *P. subcapitata* assay outcomes are described in Table 4. Sample B caused a significant reduction in the algae proliferation, in

TABLE 3 Results of *Daphnia magna* acute immobilization test expressed as immobilization rate of daphnids after 24 and 48 h of treatment with unconcentrated water samples

	Immobilization (%)	
Samples	24 h	48 h
A	0	0
B	0	20
C	10	20
D	0	0

TABLE 4 Results of *Pseudokirchneriella subcapitata* growth inhibition test expressed as percentage of inhibition after 72 h of treatment with unconcentrated water samples

Samples	Inhibition (%)
A	−16
B	78**
C	−30*
D	−7

*$p < 0.05$, statistically significant versus negative control. **$p < 0.001$, statistically significant versus negative control.

comparison with the negative control ($p < 0.001$). On the other hand, sample C strongly promoted the growth of microalgae ($p < 0.05$), while samples A and D demonstrated a slight stimulation of the algal proliferation.

The *A. cepa* toxicity test revealed the absence of toxicity (dataset in Table A6). Neither undiluted nor diluted samples negatively affected the length of the roots, although slight signs of toxicity were observed macroscopically for B and C samples, such as the presence of some hooks and apical tumours. Due to the absence of clear toxic effects in roots, undiluted samples were assayed in the *A. cepa* genotoxicity tests.

3.3 | Genotoxicity and mutagenicity

Genotoxicity tests were performed through *A. cepa* and human leukocytes, while the mutagenicity test was carried out using *S. typhimurium* TA98 and TA100 strains.

The cytotoxic/genotoxic effects of unconcentrated water samples in *A. cepa* were evaluated through the MI and the frequency of induced CAs. None of the samples affected the proliferation rate of root cells, as indicated by the similar MIs. However, all four samples induced a statistically significant increase in CA frequency compared with the negative control (Table 5).

Water samples were also tested for genotoxicity by using the comet assay in both *A. cepa* and human cells.

Unconcentrated waters were tested in *A. cepa* root cells. As shown in Figure 2, the greatest DNA damage, expressed as TI, was induced by sample A (17.8 ± 3.1, $p < 0.001$), followed by samples C and D (13.2 ± 4.4 and 11.7 ± 2.3, respectively, $p < 0.01$). Sample B caused slight DNA damage, significantly smaller than that induced by raw water (sample A). Complete datasets are reported in Table A7.

Concentrated water samples were also tested in *A. cepa* root cells by using the comet test. As shown in Figure 3, a dose–response trend of DNA damage in plant cells, expressed as TI, was demonstrated by all samples. A statistically significant increase in DNA damage was induced by sample A at the doses 2 and 3 L_{eq} (2 L_{eq}, TI = 11.7 ± 1.4; 3 L_{eq}, 14.6 ± 6.2) and by samples C and D at the highest dose (3 L_{eq}, TI = 12.3 ± 5.2 and 11.5 ± 2.3, respectively), in comparison with the negative control.

Similarly, a dose–response trend of DNA damage was demonstrated by concentrated samples in human leukocytes (Figure 4). A statistically significant increase in DNA damage was induced by samples A and B only at the highest dose (3 L_{eq}, TI = 4.2 ± 2.2 and 6.5 ± 1.7, respectively). Samples C and D caused significant damage both at 2 (TI = 7.5 ± 0.5 and 3.7 ± 2.0, respectively) and 3 L_{eq} (TI = 10.0 ± 1.1 and 8.4 ± 1.7, respectively), when compared with the negative control. Moreover, sample C (doses 2 and 3 L_{eq}) and sample D (dose 3 L_{eq}) demonstrated significant genotoxic effect when compared with sample A. Complete datasets are reported in Table A8.

The results of *Salmonella*/microsome mutagenicity test showed that water samples did not have mutagenic effects in the TA100

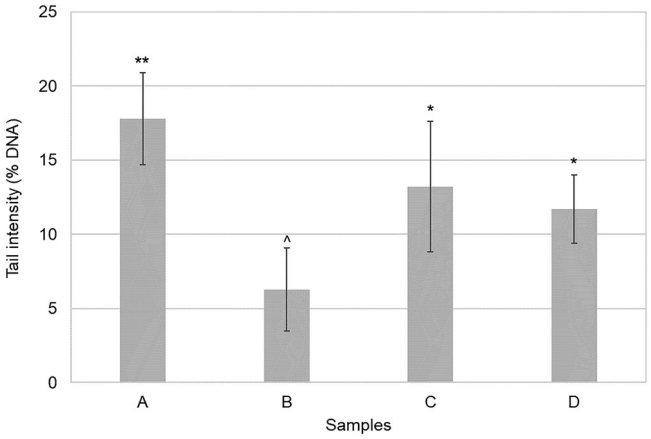


FIGURE 2 Results of comet test in *Allium cepa* root cells on unconcentrated water samples. Data are expressed as mean percentage of tail intensity ± SD. Statistically significant versus negative control: $*p < 0.01$, $**p < 0.001$; statistically significant versus sample A (raw water): $\hat{}p < 0.05$

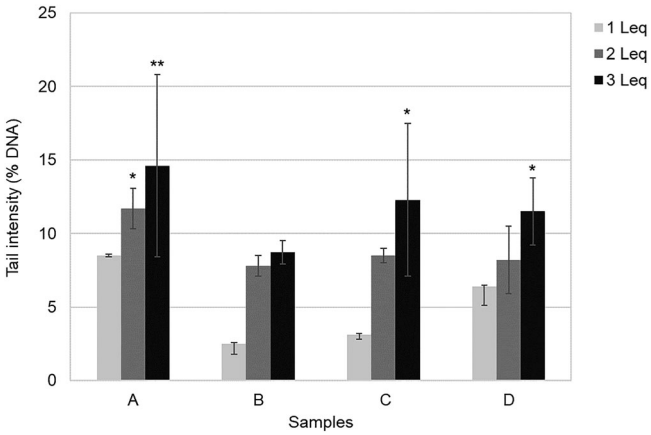


FIGURE 3 Results of comet test in *Allium cepa* cells on concentrated water samples. Data are expressed as mean percentage of tail intensity ± SD. Statistically significant versus negative control: $*p < 0.05$; $**p < 0.01$.

TABLE 5 Results of *Allium cepa* genotoxicity test on unconcentrated water samples, expressed as mitotic index (MI) and frequency of total chromosome aberrations (CAs)

Samples	MI (%)	Total CA (%)
A	10.5	6.8***
B	10.7	5.9**
C	10.5	5.2*
D	10.5	4.8*
Negative control	11.3	3.3

$*p < 0.05$, statistically significant versus negative control. $**p < 0.01$, statistically significant versus negative control. $***p < 0.001$, statistically significant versus negative control.

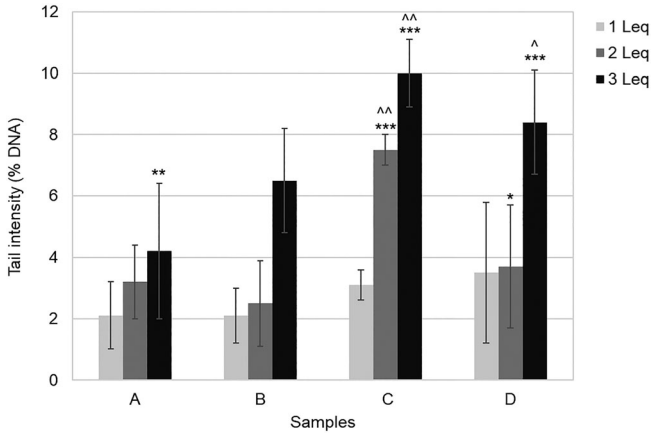


FIGURE 4 Results of comet test in human leucocytes on concentrated water samples. Data are expressed as mean percentage of tail intensity ± SD. Statistically significant versus negative control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; statistically significant versus sample A (raw water): $^{\wedge}p < 0.01$; $^{\wedge\wedge}p < 0.001$

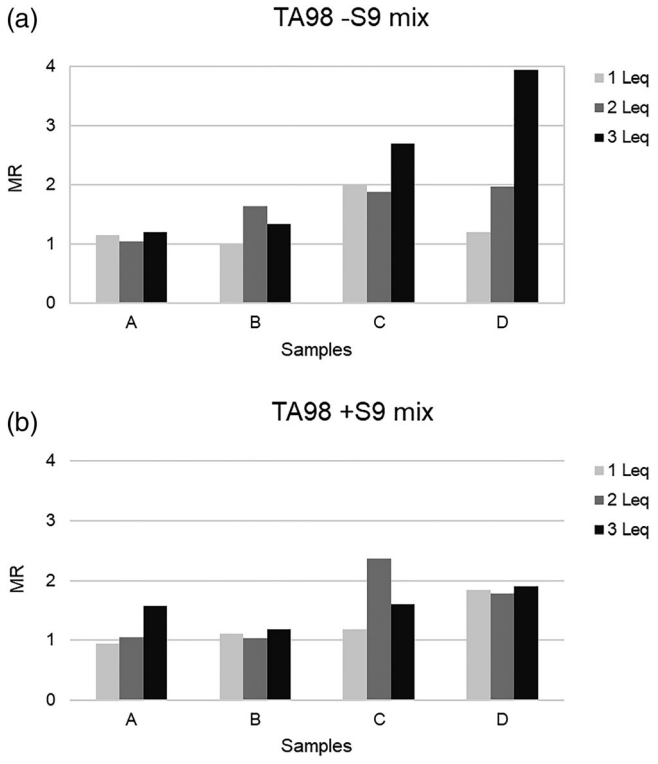


FIGURE 5 Results of Ames test expressed as mutagenicity ratio (MR) in *Salmonella typhimurium* TA98 strain without (−S9 mix, panel a) and with metabolic activation (+S9 mix, panel b)

strain, whereas exert slight activity towards the TA98 strain (Figure 5). According to the twofold rule (MR > 2) for positive results, samples A and B did not cause mutagenicity, while sample C increased its mutagenicity at the dose 3 L_{eq} without metabolic activation (MR = 2.7) and at the dose 2 L_{eq} with S9 mix (MR = 2.4). Sample D induced mutagenic effects at the highest dose (3 L_{eq}) without S9 (MR = 3.9). The net revertants/L were calculated only for samples C and D without metabolic activation, obtaining the values of 12.5 and 19.5, respectively. Data of revertants/plate in both *S. typhimurium* TA98 and TA100 strains are outlined in Table A9.

4 | DISCUSSION

The present study evaluated the toxicity and genotoxicity of PFAS-contaminated drinking water along an entire supply chain. Several endpoints of toxicity (immobilization, algal and onion roots growth inhibition) and genotoxicity (point mutations, CAs and DNA damage) in organisms from different trophic levels (crustaceans, algae, plant, bacteria and human cells) were analysed.

The treatments applied by the Lonigo DWTP were effective in the removal of PFAS pollution derived from the source water. The chemical characterization of the distributed water was fully in compliance with the legislative limit values, according to the national and regional guidelines (Legislative Decree n. 31 02.02.2001, 2001; Regione Veneto, 2017a). Indeed, the effective removal of PFAS compounds is achievable through GAC filtration, as demonstrated by several studies on large-scale treatment plants worldwide (Dauchy, 2019; Reade et al., 2019). However, the disinfection process may introduce potentially mutagenic/carcinogens by-products derived from the reaction between NaClO and the organic substance present in the water (Ceretti et al., 2016; Richardson, 2003; Richardson et al., 2007; Richardson & Postigo, 2012).

The potential effects on living organisms were investigated through different bioassays that covered a broad range of toxicity mechanisms.

The *D. magna* acute immobilization test, one of the most internationally used bioassays for screening and monitoring the activity of chemicals, effluents and contaminated waters (Martins et al., 2007), showed low toxicity of water samples after sand and GAC filtration (sample B) and after disinfection (sample C).

Similarly, water after sand filtration and GAC treatment (sample B) exerted toxicity in the *P. subcapitata* growth inhibition assay.

The *A. cepa* test did not reveal any cytotoxic effect on the root cells, indicating good tolerability to the tested samples, even those with a presence of perfluoroalkyl compounds. On the other hand, all the samples exerted genotoxic effects, in terms of CA and DNA damage, the latest determined through the comet test. These findings might be attributable to the PFAS concentration, in the order of ng/L, and to the DBPs in the tested samples. Cytotoxic/genotoxic effects of the PFOS on *A. cepa* were recently observed at concentrations well above 10 mg/L (Sivaram et al., 2021), as well as an increase in total CA and DNA damage which was attributed to bromoform and chloroform, two of the major DBPs, at a concentration ranging between 50 and 200 μg/ml (Khallef et al., 2015).

Regarding genotoxicity/mutagenicity, responses in different tests certainly indicate the presence of multiple substances that can interact in various ways with DNA. The Ames test showed mutagenic

activity only in disinfected samples on the TA98 strain, sensitive to agents that cause frame-shift mutations, especially the mutagens present in water (Claxton et al., 2008). In particular, the mutagenic activity was highlighted without metabolic activation, thus indicating the presence of direct mutagens in the water, as expected (Claxton et al., 2008; Feretti et al., 2020). As is well known, numerous mutagenic DBPs were identified in organic extracts of drinking water samples by GC/MS analysis (Ceretti et al., 2016; Kali et al., 2021; Neale et al., 2012). On the other hand, raw water (sample A) did not show any mutagenic activity in *Salmonella*, which could be attributable to the inability of the Ames test to detect the action of perfluoroalkyl substances. Indeed, PFAS, in particular PFOA, are not able to induce gene mutations in several *S. typhimurium* strains (TA98, TA100, TA102 and TA104) in the range of 100–500 μM (Fernández Freire et al., 2008).

In the comet assay performed on human leucocytes, all samples caused a significant increase in the DNA damage, when tested at the dose correspondent to three litres of water. Furthermore, the disinfected and distributed water (samples C and D) significantly increased the DNA damage compared with raw water at the same dosage. The comet assay on organic extracts was more sensitive than the Ames test, and similarly to *A. cepa* test, it was able to reveal the effects of both substances present in the untreated water and the DBPs in the disinfected water. Several studies have employed the alkaline comet assay to provide evidence that PFAS and DBPs are related to oxidative stress (Feretti et al., 2020; Guo et al., 2016; Wielsøe et al., 2015; Yao & Zhong, 2005).

Sand and GAC filtration certainly remove many substances (Li et al., 2018) but probably not others which could cause early DNA damage. These compounds could (i) not be measured by chemical analyses; (ii) cause genotoxic damage, such as CA; and (iii) be the same ones responsible for the toxicity revealed in *D. magna* and *P. subcapitata*. Indeed, cytotoxic and genotoxic effects have different endpoints, mainly reliant on the compounds' concentration and the testing system. Chemicals may induce cytotoxic but not genotoxic effects and vice versa (Novak et al., 2017). Moreover, the applied biotests are not substance specific. This represents the strength of such methodology because it allows the evaluation of the '*in toto*' mixture, thus including interactions between living organisms or cells and undetectable substances (Umbuzeiro et al., 2017).

The aim of this work was to evaluate the applicability of this battery of tests for the study of drinking water, starting from a critical water source. A limit of the study is that a single sampling was carried out. However, the sampling performed over three consecutive weeks was representative of a long period. The seasonal sampling was unnecessary due to the stable physical characteristics of groundwater. The number of different tests/organisms used allows for a selection of the most appropriate ones for an assessment of the applied treatments or of the overall water quality. The *D. magna* and *P. subcapitata* tests seem to be sufficient to give an overview of the toxicity. Regarding the evaluation of the genotoxicity, the Ames test must necessarily be adopted, being a standardized method widely utilized for environmental studies. The organic extracts gave the same results in the comet assay on human leukocytes and *A. cepa* root cells. Notably, the results of the comet test in *A. cepa* on the unconcentrated samples overlapped those obtained from organic extracts. This highlights the sensitivity of this system, which allows the detection of genotoxic damage also in presence of an extremely low concentration of pollutants. The comet test in *A. cepa* assumes primary importance within the test battery due to several strengths, such as the capability to detect different substances with high sensitivity, the low costs and the greater convenience due to the fact that no concentration procedure is needed.

5 | CONCLUSION

The results presented here suggest that both raw and treated water could interact with different living organisms. A battery composed of tests on *D. magna*, *P. subcapitata*, *S. typhimurium* and *A. cepa* could be the first line of investigation of a complex mixture, such as water intended for human consumption, in addition to its conventional chemical characterization.

This points out the importance for an integrated chemical–biological approach to have a more detailed assessment of the risk involved in the daily intake of water for the population. It would be desirable for this chemical–biological approach to be adopted by the managers of the water services in critical situations, in order to provide monitored drinking water and optimize the applied treatments according to the quality characteristics of the raw water. Moreover, this information could be useful for the health authorities to better evaluate and monitor water resources, as well as for the legislators, to adapt the legislation in order to guarantee public health. Further studies on different real critical situations will be useful to confirm the selected assays.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Carlotta Alias https://orcid.org/0000-0003-4808-7363
Donatella Feretti https://orcid.org/0000-0002-8455-6174
Ilaria Zerbini https://orcid.org/0000-0002-9337-2166

Claudia Zani https://orcid.org/0000-0002-6454-597X
Sabrina Sorlini https://orcid.org/0000-0001-7057-5473

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APPENDIX A

TABLE A1 Physical–chemical characterization of the samples according to the parameters of Legislative Decree 31/2001, implementation of Directive 98/83/CE

Physical and chemical parameters	Measure unit	Legislative Decree 31/2001 limit value	Sampling point A	Sampling point B	Sampling point C	Sampling point D
Colour	Hazen	Acceptable to consumers and no abnormal change	21.9	16.6	12.2	9.5
Odour	-	Acceptable to consumers and no abnormal change	Not detectable	Not detectable	Not detectable	Not detectable
Turbidity	NTU	Acceptable to consumers and no abnormal change	8.2	28.3	24.1	38.9
pH	-	≥6.5 ≤ 9.5	8.0	7.9	7.9	7.9
Conductivity (20°C)	μS/cm	2500	415	435	444	436
Total alkalinity	mg ($CaCO_3$)/L	-	247	231	223	231
Fixed residue at 180°C	mg/L	1500	312	326	333	327
Bicarbonates	mg ($CaCO_3$)/L	-	244	229	221	229
Total organic carbon	mg/L	No abnormal change	0.3	0.5	0.5	<Q.L.
Water hardness	°F	15–50	21	23	21	22

Abbreviation: Q.L., quantification limit.

TABLE A2 Metals characterization of the samples according to the parameters of Legislative Decree 31/2001, implementation of Directive 98/83/CE

Metals and metalloids	Measure unit	Legislative Decree 31/2001 limit value	Sampling point A	Sampling point B	Sampling point C	Sampling point D
Aluminium	μg/L	200	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Antimony	μg/L	5.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Arsenic	μg/L	10	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Boron	mg/L	1.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Cadmium	μg/L	5.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Cadmium-total	μg/L	50	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Iron	μg/L	200	<Q.L.	25	44	<Q.L.
Manganese	μg/L	50	12	<Q.L.	10	<Q.L.
Mercury	μg/L	1.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Nickel	μg/L	20	<Q.L.	<Q.L.	<Q.L.	2.4
Lead	μg/L	10	<Q.L.	<Q.L.	1.8	3.0
Copper	mg/L	1.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Selenium	μg/L	10	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Vanadium	μg/L	50	<Q.L.	1.1	<Q.L.	<Q.L.
Sodium	mg/L	200	20.9	21.6	20.8	21.9
Potassium	mg/L	-	<Q.L.	<Q.L.	<Q.L.	<Q.L.

Abbreviation: Q.L., quantification limit.

TABLE A3 Organic compounds characterization of the samples according to the parameters of Legislative Decree 31/2001, implementation of Directive 98/83/CE

Organic compounds	Measure unit	Legislative Decree 31/2001 limit value	Sampling point A	Sampling point B	Sampling point C	Sampling point D
Benzene	μg/L	1.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
1,2-Dichloroethane	μg/L	3.0	<Q.L.	<Q.L.	<Q.L.	<Q.L.
cis-1,2-Dichloroethylene	μg/L	-	<Q.L.	<Q.L.	<Q.L.	<Q.L.
trans-1,2-Dichloroethylene	μg/L	-	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Trichloroethylene (TCE)	μg/L	10	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Tetrachloroethylene (PCE)	μg/L	10	<Q.L.	<Q.L.	<Q.L.	<Q.L.
PCE-TCE	μg/L	10	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Chloroform	μg/L	30	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Tribromomethane	μg/L	30	<Q.L.	<Q.L.	0.29	0.52
Bromodichloromethane	μg/L	30	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Dibromochloromethane	μg/L	30	<Q.L.	<Q.L.	<Q.L.	0.17
Trihalomethanes—total	μg/L	30	<Q.L.	<Q.L.	0.31	0.69

Abbreviation: Q.L., quantification limit.

TABLE A4 Inorganic compounds characterization of the samples according to the parameters of Legislative Decree 31/2001, implementation of Directive 98/83/CE

Inorganic compounds	Measure unit	Legislative Decree 31/2001 limit value	Sampling point A	Sampling point B	Sampling point C	Sampling point D
Ammonia	mg/L	0.5	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Fluoride	mg/L	1.5	0.071	0.073	0.074	0.073
Chloride	mg/L	250	21	19	20	21
Nitrite NO_2	mg/L	0.5	<Q.L.	<Q.L.	0.068	<Q.L.
Nitrate NO_3	mg/L	50	18	16	17	18
Sulphate	mg/L	250	36	36	35	36
Cyanide	µg/L	50	<Q.L.	<Q.L.	<Q.L.	<Q.L.

Abbreviation: Q.L., quantification limit.

TABLE A5 Pesticides characterization of the samples according to the parameters of Legislative Decree 31/2001, implementation of Directive 98/83/CE

Pesticides	Measure unit	Legislative Decree 31/2001 limit value	Sampling point A	Sampling point B	Sampling point C	Sampling point D
Herbicides—total	µg/L	0.5	0.02	<Q.L.	<Q.L.	<Q.L.
Alachlor	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Ametryn	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Atrazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Cyanazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Desethyl-atrazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Desethyl-terbuthylazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Desisopropylatrazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Desethyldesisopropylatrazine	µg/L	0.1	0.023	<Q.L.	<Q.L.	<Q.L.
Flufenacet	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Isoxaflutole	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Metolachlor	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Molinate	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Oxadiazon	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Pendimethalin	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Prometrina	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Simazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Terbuthylazine	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Terbutryn	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Glyphosate	µg/L	0.1	<Q.L.	<Q.L.	<Q.L.	<Q.L.
Aminomethylphosphonic acid	µg/L	0.1	0.07	<Q.L.	<Q.L.	<Q.L.

Abbreviation: Q.L., quantification limit.

TABLE A6 Results of *Allium cepa* toxicity test, expressed as length of the roots (mean ± SD)

	Root elongation (cm)			
Sample	Undiluted	Dilution 1:2	Dilution 1:10	Dilution 1:100
A	4.1 ± 0.5	4.2 ± 0.7	4.0 ± 0.2	3.3 ± 0.6
B	3.9 ± 0.2	3.3 ± 0.6	3.9 ± 0.3	3.7 ± 0.4
C	3.4 ± 0.5	3.8 ± 0.5	3.7 ± 0.7	3.6 ± 0.7
D	3.8 ± 0.4	4.0 ± 0.6	3.8 ± 0.4	3.1 ± 0.4
Negative control	2.5 ± 0.8			

TABLE A7 Results of comet assay on *Allium cepa* root cells treated with undiluted water samples, expressed as tail intensity (mean ± SD)

Samples	Tail intensity
A	17.8 ± 3.1**
B	6.3 ± 2.8^
C	13.2 ± 4.4*
D	11.7 ± 2.3*
Negative control	4.0 ± 0.8

Note: Positive control: 21.4 ± 1.3.
*$p < 0.01$ versus negative control.
**$p < 0.001$ versus negative control.
^$p < 0.05$ versus raw water (sample A).

TABLE A8 Results of comet assay on leukocytes treated with increasing doses (1, 2 and 3 L_{eq}) of sample extracts, expressed as tail intensity (mean ± SD)

Samples	Dose (L_{eq})	Tail intensity
A	1	2.1 ± 1.1
	2	3.2 ± 1.2
	3	4.2 ± 2.2**
B	1	2.1 ± 0.9
	2	2.5 ± 1.4
	3	6.5 ± 1.7***
C	1	3.1 ± 0.5
	2	7.5 ± 0.5***,^^
	3	10.0 ± 1.1***,^^
D	1	3.5 ± 2.3
	2	3.7 ± 2.0*
	3	8.4 ± 1.7***,^
Negative control	DMSO	0.9 ± 0.4

Note: Positive control: 14.8 ± 3.5.
*$p < 0.05$ versus negative contol.
**$p < 0.01$ versus negative control.
***$p < 0.001$ versus negative control.
^$p < 0.01$ versus raw water (sample A).
^^$p < 0.001$ versus raw water (sample A).

TABLE A9 Results of Ames test using *Salmonella typhimurium* TA98 and TA100 strains treated with increasing doses (1, 2 and 3 L_{eq}) of sample extracts, expressed as revertants/plate (mean ± SD)

Samples	Dose (L_{eq}/plate)	TA98 −S9	TA98 +S9	TA100 −S9	TA100 +S9
A	1	25.5 ± 0.7	33.5 ± 0.5	115.5 ± 7.8	127.0 ± 7.1
	2	23.0 ± 4.2	37.0 ± 12.7	133.0 ± 14.1	131.0 ± 11.3
	3	26.5 ± 2.1	56.0 ± 7.1	130.5 ± 4.9	141.0 ± 14.1
B	1	22.0 ± 4.2	39.5 ± 3.5	127.0 ± 7.1	119.0 ± 1.4
	2	36.0 ± 0.0	36.5 ± 6.4	114.0 ± 35.4	134.5 ± 7.8
	3	29.5 ± 3.5	42.0 ± 5.7	110.5 ± 16.3	127.0 ± 2.8
C	1	44.0 ± 7.1	42.0 ± 17.0	149.5 ± 30.4	151.0 ± 7.1
	2	41.5 ± 4.9	84.0 ± 15.6	168.5 ± 2.1	161.5 ± 29.0
	3	59.5 ± 29.0	57.0 ± 1.4	162.0 ± 7.1	155.0 ± 32.5
D	1	26.5 ± 3.5	65.5 ± 0.7	129.0 ± 4.2	151.0 ± 21.2
	2	43.5 ± 12.0	63.5 ± 14.8	145.5 ± 13.4	145.0 ± 14.1
	3	86.5 ± 4.9	67.5 ± 23.3	172.0 ± 8.5	135.0 ± 15.6
Negative control	DMSO	22.0 ± 5.4	35.5 ± 2.1	117.0 ± 6.1	129.0 ± 7.2

Note: Positive control: >1000 revertants/plate.