

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

Contaminants of emerging concern in drinking water: Quality assessment by combining chemical and biological analysis

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Contaminants of emerging concern in drinking water: Quality assessment by combining chemical and biological analysis / Valbonesi P.; Profita M.; Vasumini I.; Fabbri E.. - In: SCIENCE OF THE TOTAL ENVIRONMENT. - ISSN 0048-9697. - STAMPA. - 758:(2021), pp. 143624.1-143624.10. [10.1016/j.scitotenv.2020.143624]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/787211> since: 2021-01-07

*Published:*

DOI: <http://doi.org/10.1016/j.scitotenv.2020.143624>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Valbonesi P.; Profita M.; Vasumini I.; Fabbri E.: *Contaminants of emerging concern in drinking water: Quality assessment by combining chemical and biological analysis*

SCIENCE OF THE TOTAL ENVIRONMENT. Vol. 758 ISSN 0048-9697

DOI: 10.1016/j.scitotenv.2020.143624

The final published version is available online at:

<https://dx.doi.org/10.1016/j.scitotenv.2020.143624>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

**When citing, please refer to the published version.**

# Contaminants of emerging concern in drinking water: quality assessment by combining chemical and biological analysis

Paola Valbonesi <sup>1</sup>, Marilin Profita <sup>1</sup>, Ivo Vasumini <sup>2</sup>, Elena Fabbri <sup>1,3,§</sup>

<sup>1</sup>*Department of Biological, Geological and Environmental Sciences (BIGEA) University of Bologna, Italy*

<sup>2</sup>*Romagna Acque Società delle Fonti SpA, Forlì (Italy)*

<sup>3</sup>*Interdepartment Centre for Environmental Science Research, University of Bologna, Campus of Ravenna, Italy*

<sup>§</sup> Corresponding Author: Via S. Alberto 163, 48123 Ravenna, Italy. Tel.: +39 0544937311. E-mail: elena.fabbri@unibo.it (E. Fabbri)

**Keywords:** Contaminants of emerging concern; Drinking water; LC-MS/MS; E-screen assay; Micronuclei test

## 24 ABSTRACT

25 Drinking water quality is a priority issue of the environmental policy agenda, however regulation on  
26 Contaminants of Emerging Concern (CECs) is limited. A proposal to revise the Drinking Water  
27 Directive has recently been approved (EU Council 2020), which updates the quality standards and  
28 introduces the watch list mechanism, including for the first time endocrine disruptors and  
29 pharmaceuticals. The purpose of this study was to evaluate the occurrence of selected CECs in surface  
30 water at the entrance of drinking water treatment plants (DWTPs) and in treated water, ready for  
31 distribution in the network. Samples were collected at three different DWTPs (Italy) and CECs  
32 assessed by LC-MS/MS were the following: bisphenol A (BPA), nonylphenol (NP), octylphenol,  
33 perfluorooctanesulfonic and perfluorooctanoic acids (PFOS and PFOA), atenolol, caffeine (CFF),  
34 carbamazepine (CBZ), estrone, 17- $\beta$ -estradiol, 17- $\alpha$ -ethinyl estradiol, diclofenac, and ibuprofen. In  
35 addition, biological analyses were performed to ascertain cumulative estrogenic and/or genotoxic  
36 potential of the samples. CFF, NP, PFOA, BPA, and CBZ were the most frequently detected  
37 contaminants, found in treated water in the following ranges: CFF 12.47-66.33 ng/L, NP 7.90-53.62  
38 ng/L, PFOA <LOQ-12.66, ng/L, BPA <LOQ-6.27 ng/L, and CBZ <LOQ-1.20 ng/L. While  
39 treatments were generally efficacious in reducing BPA, CFF and CBZ, they were sometimes  
40 ineffective for NP and PFOA. According to the low concentrations and/or regulation limit for each  
41 single contaminant, the water analyzed met the criteria of good quality. No estrogenic or genotoxic  
42 activities were induced by the water assessed, with the exception of one sample. Although drinking  
43 water may not represent a significant source of human exposure to CECs, their incomplete removal  
44 and potential cumulative effects in the mixture deserve implementation of strategies for detection and  
45 removal.

46

47

## 1. Introduction

Contaminants of emerging concern (CECs) comprise a vast array of contaminants, that have only recently been discovered in water supply, or that are of recent concern because they have been detected at concentrations significantly higher than expected. The risk they pose to human health and the environment is not yet fully understood. Examples include pharmaceuticals and personal care products, industrial and household chemicals, pesticides, manufactured nanomaterials, and their transformation products (Glassmeyer et al., 2017), (Krzeminski et al., 2019). CECs are ubiquitous in the aquatic environment, and because water eligible for human consumption is drawn from surface water, the removal of known or suspected CECs during the purification process is needed. Conventional drinking water treatments may be not sufficient to completely eliminate CECs from source waters, because they are not specifically designed to this purpose (Padhye et al., 2014). Among CECs, caffeine and ibuprofen are removed effectively in water treatment plants, whereas other pharmaceuticals such as carbamazepine and diclofenac (DCF) are removed at a much lower efficiency, and are detected even in tap water (Kwon et al., 2017).

Many CECs have been reported to act as endocrine disruptors, including as expected natural and synthetic hormones, but also a variety of other compounds widely used (Kiyama and Wada-Kiyama, 2015).

In 2000, the European Union launched the Directive 2000/60/EC to establish a framework for Community action in the field of water policy (EU, 2000). The subsequent Directive 2008/105/EC established a list of Priority Substances and Environmental Quality Standards with the aim of reaching a good ecological and chemical status for EU surface water (EU, 2008). A further Directive proposed a revised list of priority substances (45 compounds) and launched a Watch List of potential water pollutants to be carefully monitored by the EU Member States to support future prioritization exercises (EU, 2013), which was published in the Decision 2015/495/EU. This panel, which is updated every two years, comprised about 15 substances among which for the first time, some

73 hormones and pharmaceuticals (17- $\beta$ -estradiol, E2; 17- $\alpha$ -ethinylestradiol, EE2; and DCF) were  
74 included (EU Commission, 2015).

75 Although water quality is one of the priority issues of the environmental policy agenda due to the  
76 increasing demand for safe and clean water, regulation of CECs in drinking water is limited. Only  
77 recently, the EU Council approved a proposal to revise the Drinking Water Directive, which updates  
78 quality standards and introduces the watch list mechanism, including for the first time endocrine  
79 disruptors and pharmaceuticals (EU Council, 2020). In view of their endocrine disrupting properties  
80 E2 and nonylphenol (NP) are included in the watch list under definition, while bisphenol A (BPA)  
81 has been directly added to the Directive (EU Council, 2020).

82 The updates of regulatory limits cover only part of the issue. In fact, chemical analysis based often  
83 on liquid chromatography tandem mass spectrometry (LC-MS/MS) able to detect concentrations as  
84 low as parts per trillion (Ibáñez et al., 2012), do not account for synergetic effects of contaminant  
85 mixtures on ecosystems and human health, which may take place even at low concentrations, from  
86 ng/L to low  $\mu$ g/L (Arnold et al., 2014). For a more comprehensive assessment of water quality,  
87 chemical analysis may be complemented by cell-based bioassays that target health-relevant biological  
88 endpoints. In a real environmental scenario, a multiplicity of interactions and synergies among  
89 different compounds take place, which chemical investigations are unable to account for. Escher and  
90 coworkers recommended to use a purpose-tailored panel of bioassays for routine monitoring of water  
91 quality and to assess efficacy of water treatment processes, suggesting as the most health relevant  
92 endpoints xenobiotic metabolism, hormone-mediated modes of action, genotoxicity, and adaptive  
93 stress response pathway (Escher et al., 2014).

94 In response to the increasing concern on drinking water quality, the aim of this study was to evaluate  
95 the occurrence of selected CECs in surface water at the entrance of drinking water treatment plants  
96 (DWTPs) and in treated water, ready for distribution in the network, and assess the efficacy of  
97 treatments. In addition, biological analyses were performed to ascertain treated water cumulative  
98 estrogenic and/or genotoxic potential. Water samples were collected at three different DWTPs serving

99 the Romagna region (Italy). Chemical analyses were carried out by LC-MS/MS, addressed to a panel  
100 of CECs, most of which showing endocrine disruptor properties. Assessment of estrogenic and  
101 genotoxic activity were carried out by E-screen assay and Micronuclei test, respectively.

102

## 103 **2. Materials and methods**

### 104 2.1. Chemicals and reagents

105 Table 1 shows the panel of CECs evaluated in this study. All non-labelled standards were purchased  
106 from Merck Life Science (Milan, Italy). Isotope-labeled compounds used as internal standards were  
107 purchased by Cambridge Isotopes Laboratories Inc. (Lab Service Analytica Srl, Anzola dell'Emilia,  
108 Bologna, Italy) ( $^{13}\text{C}_3$ -Caffeine), CDN Isotopes (Quebec, Canada) (E2-d<sub>2</sub> and BPA-d<sub>6</sub>), Wellington  
109 Laboratories Inc. (Guelph, ON, Canada) ( $^{13}\text{C}_4$ -PFOA), and Merck Life Science (Ibuprofen-d<sub>3</sub>).  
110 Solvent reagents from Merck Life Science were of LC-MS analytical grade.

### 111 2.2. Sampling sites and sample storage

112 Two sampling campaigns per year were carried out during 2018 and 2019, in July and  
113 September/October, corresponding to the dry season with the purpose of analyzing the worst scenario  
114 regarding CECs in the study area, when rivers are drier and the expected concentration of pollutants  
115 is greater. Pre- and post- treatment water samples were collected from the three main Romagna's  
116 waterworks operated by the company Romagna Acque-Società delle Fonti (Figure 1). Capaccio  
117 (Forli-Cesena) is fed by the large reservoir of Ridracoli, in the National Park of the Casentinesi  
118 Forests (high Tuscan-Romagna Apennines). Differently, NIP and Standiana receive water from areas  
119 with many anthropic activities, NIP (Bassette, Ravenna), receiving water mainly from the Lamone  
120 river (integrated, in particularly dry periods, from the Reno River) and from the CER (the Emilia-  
121 Romagna channel that branches off the Po river and brings its water in the Romagna area); Standiana  
122 (Standiana, Ravenna), active since 2015, using more advanced water treatment techniques, such as  
123 ultrafiltration through 0.04  $\mu\text{m}$  membranes, to obtain high quality water starting from the CER.

Differently from Capaccio, both NIP and Standiana plants are equipped with activated carbon filters for the elimination of organic and inorganic micro-pollutants. In particular, NIP is equipped with granular activated carbon (GAC), and Standiana with the biological activated carbon (BAC). GAC is used as a filter through which the water is pumped, regularly backwashed, and does not need to be replaced until it is exhausted, which may take several years. It is mainly used in drinking water treatment to remove dissolved organic contaminants. Microbial activity occurs naturally on GAC during the treatment of waters containing biodegradable materials. Adsorption of biodegradable organics to GAC provides extended contact times for degradation of certain dissolved organic contaminants by microorganism, thereby extending the service life of GAC beds as well as treatment efficiency. GAC converts to BAC due to natural biological growth on GAC media.

For each sampling point, 3 L of water were collected in 1L-PE bottles and stored at 4 °C until analysis.

### 2.3 Sample processing

All samples were processed essentially as previously reported (Pignotti et al., 2017). Briefly, for chemical analysis 1 L of water was spiked with a mixture of labeled internal standards (E2-d<sub>2</sub>, BPA-d<sub>6</sub>, <sup>13</sup>C<sub>4</sub>-PFOA, Ibuprofen-d<sub>3</sub> at a concentration of 30 ng/L, and <sup>13</sup>C<sub>3</sub>-Caffeine at 15 ng/L), filtered with glass microfiber filters (1.60 µm) and then with cellulose acetate filters (0.45 µm). Solid-phase extraction was subsequently performed through Oasis HLB cartridges (6 cm<sup>3</sup>, 200 mg; Waters S.p.A., Sesto San Giovanni, Milan, Italy). Cartridges were eluted with 6 mL of methanol, evaporated under a N<sub>2</sub> gentle stream up to a volume of 250 µL, and split in two vials of 125 µL each. The first set of vials were additioned with 125 µL of water (finally 50:50 water/methanol) for the first set of LC-MS-MS analysis (group 1, Table 2). The remaining vials were further evaporated to 25 µl and reconstituted in 250 µl of a mixture of water/methanol (90:10) for further two sets of LC-MS-MS analysis (group 2 and group 3, Table 2). Samples were then centrifuged (17,000 × g, 5 min), filtered and transferred into glass vials. For the biological analysis the same protocol was applied to water samples, except for spiking with the labeled internal standards. Eluted samples reached 50 µL and



149 were additioned first with 200  $\mu$ L of pure water, then with steroid-free experimental medium to obtain  
150 a final concentration factor of 20, containing 0.1% methanol, and finally sterilized with 0.20  $\mu$ m  
151 syringe cellulose acetate filters. These experimental conditions did not cause any toxicity on cell  
152 culture, as assessed by a viability test (data not shown).

#### 153 2.4. Chromatographic conditions and mass spectrometry detection

154 Chemical analysis were carried out with an HPLC system (Agilent 1.200 series, Agilent Technologies  
155 Italia S.p.A, Cernusco sul Naviglio, Milan, Italy) coupled with a MS/MS spectrometer, equipped with  
156 an electrospray ionization source (Quattro Premier XE Micromass, Waters S.p.A.). Separation of  
157 compounds was achieved through an XBridge C<sub>18</sub> 3.5 $\mu$ m 2.1  $\times$  150 mm column (Waters S.p.A.) and  
158 the volume injection was 20  $\mu$ L. Mass analyses were performed in multiple reaction monitoring  
159 (MRM) mode. Table 2 summarizes the mass transitions selected for each compound and further MS  
160 parameter details. For group 1 compounds, analyses were carried out in negative ion mode using  
161 0.1% ammonium hydroxide in Water (A) and 0.1% ammonium hydroxide in Acetonitrile (B) as  
162 mobile phases, with a flow rate of 0.2 mL/min. The elution gradient started at 5% B and rapidly  
163 increased to 80% B (2 min), kept at isocratic conditions for 6 min, then to 99% B in 1 min and kept  
164 at isocratic conditions for 6 min, followed by 2 min linear gradient back to initial conditions, and then  
165 kept for 12 min to equilibrate the column before a new injection. The optimized mass spectrometry  
166 parameters were as follows: capillary 2.90 V; desolvation temperature 400  $^{\circ}$ C; desolvation gas flow  
167 800 L/h; cone gas 80 L/h. For group 2 compounds, analyses were conducted in negative ion mode  
168 using 10 mM ammonium acetate in Water (A) and Acetonitrile (B) as mobile phases, with a flow rate  
169 of 0.2 ml/min. Elution gradient started with 5% B and gradually increased to 99% in 7 min and to  
170 99% in 5 min, followed by 5 min isocratic elution and a 2 min linear gradient back to initial  
171 conditions, and then kept for 7 min to equilibrate the column before a new injection. The optimized  
172 mass spectrometry parameters were as follows: capillary 2.70 V; desolvation temperature 350  $^{\circ}$ C;  
173 desolvation gas flow 850 L/h; cone gas 85 L/h. For group 3 compounds, analyses were done in

174 positive ion mode using 0.1% formic acid in Water (A) and 0.1% formic acid in Acetonitrile (B) as  
175 mobile phases, with a flow rate of 0.3 mL/min. Elution gradient started with 10% B and rapidly  
176 increased to 48% (0.5 min), kept at isocratic conditions for 6 min, then to 85% B in 0.5 min and to  
177 100% in 4 min. After 2 min at isocratic conditions and 0.5 min linear gradient back to initial  
178 conditions, flow was kept for 11.5 min to equilibrate the column before a new injection. The  
179 optimized mass spectrometry parameters were as follows: capillary 2.80 V; desolvation temperature  
180 350 °C; desolvation gas flow 750 L/h; cone gas 70 L/h.

## 181 2.5. Quantification and method validation

182 Data related to quantification and method validation are reported in Table 3. Each water sample was  
183 analyzed in triplicate. Recovery and repeatability were tested in DWTP waters by mixing 3 L of  
184 entering and 3 L of exiting water (1 L of each DWTP in 2018 July campaign). From this amount, 3 L  
185 were spiked before the extraction procedure with 30 ng/L of the targeted analytes, with the exception  
186 of CBZ (5 ng/L). The remaining 3 L of unspiked samples were analyzed in the same batch to correct  
187 the final concentrations for the amount of analytes already present in DWTP waters. Recoveries and  
188 accuracy were calculated subtracting the concentration of each analyte in unspiked water to the  
189 measured concentration after spiking. Procedural blanks were prepared in parallel to samples in order  
190 to exclude any contamination during sample treatments. Three standard mixtures, containing all the  
191 CECs to be analysed, were prepared before each analytical run by diluting stock solutions to obtain  
192 six-point calibration curves (0–100/300 ng/mL), prepared in a mixture of water/methanol at the same  
193 initial conditions of samples. An instrumental blank containing only the labeled internal standards  
194 was used as control for analytical interference. To rule out any system contamination and check  
195 sensibility drifts, one point of the calibration curve (10 ng/mL) was run every six sample injections.  
196 Detection limits (LODs) of the methods were calculated as the amount of native standard (pg) loaded  
197 that yielded a signal to noise ratio of 3 and quantification limits (LOQs) of the methods corresponded  
198 to the concentration that yielded a signal to noise ratio of 10, using real water samples, to take into

199 account the matrix effect. LOQ values were used as cut- off values for quantification of the analytes.  
200 Intra- and inter-day precision were calculated by injection of one point of the calibration curve (10  
201 ng/mL) and calculating the relative standard deviation (RSD, %) (n = 3). Concentrations below the  
202 LOQ were considered as half the LOQ.

## 203 2.6 Cell culture conditions

204 Human breast cancer cells MCF-7 were kindly provided by Prof. M. Marino (University Roma Tre,  
205 Rome, Italy). Cells were grown in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37 °C, in Dulbecco's  
206 modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 2  
207 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mg/mL  
208 gentamicin and 1% of non- essential amino acids. Phenol red-free DMEM supplemented with 5%  
209 charcoal-dextran treated fetal calf serum was used as experimental medium, containing DWTP water  
210 extracts or mineral water extracts as laboratory blank samples. Cell culture reagents were from Merck  
211 Life Science.

## 212 2.7 E-screen assay

213 Estrogenic activity assessment was performed by E-screen assay, as described by Korner (Korner,  
214 1999), with some modifications. Cells were plated into 24-well plates at initial concentration of  
215 10,000 cells/well. After 24 h, the seeding medium was replaced by the experimental medium  
216 containing DWTP extracts or different concentrations of E2. After a 5-day exposure, cell proliferation  
217 was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as  
218 described by Mosmann (Mosmann, 1983). Results are expressed as Proliferative Effect (PE), i.e. the  
219 ratio of the cell number achieved in the treated wells, and the cell number of the negative controls.  
220 E2 dose-dependent cell proliferation curve fitting was performed in order to express estrogenic  
221 activity in terms of equivalent estradiol (EEQ, ng/L). The negative control with pure water extract  
222 and the internal positive control with 10<sup>-10</sup> M E2 were added to each assay.

## 223 2.8 Micronuclei test

224 Genotoxic activity assessment was performed by the micronuclei test, as described by Fenech  
225 (Fenech, 2000), with some modifications (Espinoza et al., 2019). Cells were plated into 12-well plates  
226 at initial concentration of 80,000 cells/well. After 24 h, the medium was replaced by the experimental  
227 medium containing DWTP extracts or 0.1  $\mu$ M BPA as a positive control. After 48 h of exposure, the  
228 medium was replaced with experimental medium containing 2  $\mu$ g/mL cytochalasin B. Following a  
229 further 24-h incubation, the medium was removed, and cells were trypsinized, spread on slides and  
230 fixed in Carnoy solution (methanol/acetic acid 3:1). Slides were air dried and stained with DAPI (4',6-  
231 diamidino-2-phenylindole), at a concentration of 100 ng/mL. About thousand binucleated cells were  
232 scored for each slide with a microscope (Eclipse 80i, Nikon Instruments Europe B.V. Amsterdam,  
233 Netherlands) equipped for fluorescence microscopy at 1000  $\times$  magnification. Data are expressed as  
234 the number of micronuclei/1000 binucleated cells scored in each slide.

## 235 2.9 Statistical analysis

236 For biological assays the experimental data were obtained from the replication of at least four  
237 independent experiments (N = 4). In fact, for each sampling point, 2 L of water were processed  
238 independently and each eluate was tested at least twice in independent experiments. For the E-Screen  
239 assay, results are expressed in terms of PE as the mean  $\pm$  standard error (SE). Results of MN test are  
240 reported as the mean  $\pm$  standard deviation (SD), obtained from 4 independent experiments (for each  
241 replicate 1000 binucleated cells were scored). E2 dose-dependent cell proliferation curve fitting was  
242 performed using a commercial graphical package (SigmaPlot software, ver 13, Systat Software Inc.).  
243 Data groups were compared using one-way ANOVA and followed by Dunnett post-hoc test; a  
244 statistical difference was accepted when  $p < 0.05$  (Sigma Stat, SPSS Science, Chicago IL, USA).

245

## 246 3. Results and Discussion

### 247 3.1. Chemical analysis

248 Tables 4 and 5 summarize the concentrations of compounds for industrial use recognized as endocrine  
249 disruptors and pharmaceuticals, respectively, in the water entering and exiting the DWTPs.

### 250 *3.1.1. Surfactants and Plasticizers*

251 Alkylphenols, widely employed as surfactants in chemical industry, are frequently detected in the  
252 environment at concentrations in the order of  $\mu\text{g/L}$ ; further they are toxic, persistent and able to  
253 bioaccumulate (Sousa et al., 2018). Among this group, nonylphenol (NP) and octylphenol (OP)  
254 belong to category 1 of the Endocrine Disruptor Priority List (EU Commission, 2007). Both  
255 compounds are among the 33 priority substances in the European water framework directive (EU,  
256 2013) and are classified as priority hazardous substances. The document identifies the environmental  
257 quality standards (EQS) in the water column, corresponding to average values of 0.1 mg/L and 0.3  
258 mg/L, for OP and NP, respectively. The same 0.3 mg/L concentration was initially proposed by the  
259 WHO as the upper limit for NP in drinking water (EU Commission, 2018); then, according with a  
260 following decision, the approved document includes NP in the Watch list (EU Council, 2020).

261 In the present study, OP and NP showed concentrations below the above mentioned limits. In fact,  
262 OP has never been detected above the quantification limit (0.66 ng/L), neither in water entering or in  
263 water exiting the DWTPs. NP has been found in all plants and sampling campaigns, and ranged from  
264 7.90 ng/L to 53.62 ng/L in the post-treatment water.

265 NP measured in the water leaving the 6 Italian plants was reported at concentrations up to 100 ng/L,  
266 and similar values have been published by Maggioni and coworkers in drinking water from public  
267 fountains in 35 Italian cities, with NP highest concentrations of 84 ng/L (Maggioni et al., 2013). In  
268 European countries maximum value of 505 ng/L was reported in France (Colin et al., 2014), 16 ng/L  
269 in Germany (Kuch and Ballschmiter, 2001) and 126 ng/L in Spain (Valcárcel et al., 2018).

270 Interestingly, a higher amount of NP after DWTP treatment has been occasionally found. Similar data  
271 were reported by the Italian Institute of Health concerning 6 Italian waterworks monitored between  
272 2008 and 2009. The higher occurrence of NP in post treatment water was possibly related to the use

273 of plastic materials for the pipelines, which could release substances such as alkylphenols, bisphenol  
274 A (BPA), phthalates and PAH into drinking water (Achene et al., 2011).  
275 BPA, one of the highest-volume chemicals produced worldwide, is used as a plastic monomer and  
276 plasticizer in the production of polycarbonate and epoxy resins. In turn, these materials are currently  
277 used as components of many consumer products, including reusable plastic bottles, household  
278 kitchenware, canned food items, and medical equipment (Prins et al., 2019). BPA exposure has been  
279 associated with serious endocrine-disrupting effects in humans and wildlife, thus it belongs to  
280 category 1 of the Endocrine Disruptor Priority List (EU Commission, 2007). The recent revision of  
281 the Drinking Water Directive (EU Council, 2020) represents the first regulation concerning BPA  
282 occurrence in water for human consumption, with the definition of the upper limit of 2.5 µg/L.

283 In the present study, the post-treatment water contained a range of BPA concentration from <LOQ to  
284 0.006 µg/L, well below the limit of EU regulation (EU Council, 2020). BPA was detected in almost  
285 all water samples entering the plants (Table 4), the highest concentration being 0.018 µg/L in  
286 Standiana in October 2018. It is noteworthy that all DWTPs were able to completely or at least  
287 partially remove BPA.

288 BPA concentrations in Italian drinking water ranged from < LOQ to 0.003 µg/L, except for a sample  
289 where value was higher (0.102 µg/L) (Maggioni et al., 2013). The maximum BPA concentration  
290 reported in drinking water varies among European Countries: 0.05 µg/L in Spain and in France (Colin  
291 et al., 2014), (Valcárcel et al., 2018), and 0.002 µg/L in Germany (Kuch and Ballschmiter, 2001).

### 292 3.1.2. *Perfluorinated substances*

293 Per- and polyfluorinated alkyl substances (PFAS) are used in a wide range of industrial applications  
294 and commercial products (e.g. paper coatings, insecticides, paints). Effects on human health  
295 associated to PFAS exposure are related to dysfunction in lipid metabolism, thyroid metabolism,  
296 developmental effects in fetuses during pregnancy or in breastfed infants, and cancer in  
297 occupationally exposed individuals (Ingelido et al., 2018). European legislation regarding PFAS in

298 surface water has been updated at the end of 2015. The European Commission included  
299 perfluorooctanesulfonic acid (PFOS) in the list of priority hazardous substances, to be monitored in  
300 the EU water bodies, setting an EQS of 0.65 ng/L (EU Commission, 2015). Moreover, a list of not  
301 yet priority substances was included in the European Directive 2013/39/EC, for which EQSs are  
302 suggested to be monitored in order to achieve of a good ecological status by December 2027. Among  
303 these, perfluorooctanoic acid (PFOA) is included, with average EQS value of 0.1 µg/L for inland  
304 surface waters. The recently approved revision of the Drinking Water Directive included PFAS in the  
305 list of chemicals to be monitored (EU Council, 2020): member States shall take the measures  
306 necessary to ensure that water intended for human consumption complies with the parametric values  
307 set to 0.1 µg/L for individual PFAS and 0.5 µg/L for PFAS in total.

308 In the present study, both PFOS and PFOA have occasionally been detected in the water leaving the  
309 DWTPs, at maximum concentrations of 0.81 ng/L and 12.66 ng/L respectively, well below the limits  
310 suggested by the revision of the Drinking Water Directive. Both maximum values were found in the  
311 sampling campaign of July 2018 in Standiana. Comparing PFOS occurrence in the three DWTP, we  
312 observed that it has never been detected in Capaccio. In NIP, PFOS has only been found in entering  
313 water, while in Standiana traces of PFOS have always been detected also in the water leaving the  
314 plant. Conversely, PFOA has been detected in all water samples analysed with the only exception of  
315 Capaccio in July 2018.

316 As a comparison with other Italian data, occurrence of PFOA and PFOS in drinking water in the  
317 Veneto region dropped to maximum concentrations of 386 ng/L and 36 ng/L, respectively, after the  
318 abatement of an important water contamination detected in 2014, due to the draining of PFAS from  
319 a manufacturing company (WHO, 2016). PFOA and PFOS mean concentrations in tap water near the  
320 Maggiore lake were 2.4 ng/L and 8.1 ng/L, respectively (Loos et al., 2007), while PFOA in drinking  
321 water from an industrialized area in North of Milan reached 47 ng/L (Castiglioni et al., 2015). In  
322 France the highest concentrations reported for PFOA and PFOS were 12 and 22 ng/L, respectively

323 (Boiteux et al., 2012); in Germany, drinking water showed a maximum concentration of PFOA and  
324 PFOS of 519 and 22 ng/L, respectively (Skutlarek et al., 2006); in Spain the highest concentrations  
325 in drinking water corresponded to 2.40 and 1.81 ng/L, for PFOA and PFOS, respectively (Domingo  
326 et al., 2012).

327 As from Table 4, while PFOS concentrations were always reduced by the treatment, PFOA levels  
328 were occasionally higher in post- with respect to pre- treatment waters. Rahman and coworkers  
329 reviewed PFAS fate in drinking water and noted the same PFOA behaviour, providing some  
330 explanations, such as the possible breakdown of certain precursor compounds to PFOS and PFOA  
331 during treatments, or the leaching from Teflon-coated components and desorption from GAC filters  
332 that had been in service for long periods of time without reactivation (Rahman et al., 2014).

### 333 3.1.3. Pharmaceuticals

334 This class of contaminants are synthetic or natural chemicals found in prescription medicines, over-  
335 the-counter therapeutics and veterinary drugs. Because drinking water limits for pharmaceuticals  
336 have not been established yet, and little has been published on safe long-term exposure levels, the  
337 evaluation of drinking water quality is challenging. The need to collect monitoring data relative to  
338 pharmaceutical occurrence in water for human consumption was confirmed in 2015, when diclofenac  
339 (DCF), 17- $\beta$ -estradiol (E2), and 17- $\alpha$ -ethinyl estradiol (EE2) were included in the Watch List of  
340 Decision for the compounds posing a significant risk to the aquatic environment, with insufficient  
341 monitoring data at European Union level (EU Commission, 2015). The first monitoring results,  
342 reported by Higher Institute for Environmental Protection and Research (ISPRA, 2017), showed that  
343 DCF was one of the most frequently detected pharmaceutical, found in 22 of the 35 Italian stations,  
344 at concentrations ranging from 5 to 683 ng/L. Due to its documented occurrence in the environment,  
345 bioaccumulation and adverse effects on the health of aquatic fauna, the EU Joint Research Centre  
346 removed DCF from the Watch list in the most recent update (Loos et al., 2018), and the definition of  
347 specific legislation is expected shortly.



On the basis of the precautionary principle, E2 is included in the first Watch list of the Drinking Water Directive revision (EU Council, 2020).

Present results (Table 5) indicate that water samples did not contain the natural hormone E1 or the synthetic hormone (EE2) over their LOQ values. Differently, E2 was detected in July 2018 in two samples of pre-treatment water, and subsequently removed. Atenolol, ibuprofen and DCF have been occasionally found only in water entering the plants, demonstrating the removal efficacy of the DWTPs. Conversely, caffeine (CFF) and carbamazepine (CBZ) have been the two most frequently detected pharmaceuticals. CFF is ubiquitous in the environment, and it has been detected in surface water almost all over the world (Glassmeyer et al., 2017). CFF occurrence is linked to the high consumption of drugs as well as of drinks that contain it, thus it is considered an indicator of anthropogenic impacts.

CFF has been found in each sample analyzed (Table 5). The highest concentration of CFF was detected in water entering NIP in both 2018 campaigns, when a value as high as 2.58 µg/L was reached. Nevertheless, after treatment the concentration of CFF was reduced in the range of 12.89 to 66.33 ng/L, showing a good DWTP effectiveness in retaining the contaminant.

The range of CFF concentration in water samples leaving the DWTPs is similar to those previously assessed in drinking water in Italy, between 10 and 53 ng/L (Loos et al., 2007), in France, from 5 to 82 ng/L (Mompelat et al., 2011), and Spain, from 15 to 75 ng/L (Valcárcel et al., 2011).

CBZ occurred in all the pre-treatment samples from NIP and Standiana, and often also in the water leaving the plants, although reduced by at least 10 times. The drug has never been found in Capaccio. The maximum concentration of CBZ found in post-treatment water was 1.20 ng/L.

Previous studies reported CBZ water levels of 10.3 ng/L in Italy (Riva et al., 2018), 59 ng/L in Spain (Leusch et al., 2018), 14 ng/L in Portugal (de Jesus Gaffney et al., 2015), 6.0 ng/L in Poland (Kot-Wasik et al., 2016), and in France CBZ was detected in tap water at a concentration of 43.2 ng/L (Togola and Budzinski, 2008). The wide occurrence of CBZ is related to its high resistance to environmental degradation independent of seasonality (Kot-Wasik et al., 2016). In agreement, a

374 monitoring study of 31 pharmaceuticals along Lisbon's drinking water documented that CBZ,  
375 together with CFF, was the most ubiquitous compounds with a detection frequency of 96% in  
376 drinking water (de Jesus Gaffney et al., 2015).

377 Overall, the comparison of contaminants occurrence in the different DWTPs indicates that water  
378 entering Capaccio contained the lowest levels of pharmaceuticals, showing only the anthropic tracer  
379 CFF. Conversely, all pharmaceuticals have been detected in water entering NIP; nevertheless, their  
380 concentration in post-treatment waters was always significantly reduced.

### 381 3.2. Biological analysis

#### 382 3.2.1. Evaluation of estrogenic activity by E-screen assay

383 As previously mentioned, all the environmental contaminants evaluated in this study are reported to  
384 affect human health. Thus, chemical assessments have been integrated with biological analysis  
385 aiming to evaluate the potential effects of water as a mixture containing non-measured compounds  
386 and/or transformation products (Lv et al., 2016), (Leusch et al., 2018). Estrogen-like compounds are  
387 known as the major contributors to endocrine disrupting activity of water samples, acting at  
388 concentrations ranging from pg to ng/L (Farré et al., 2007), (Vulliet et al., 2007), (Chen and Chou,  
389 2016). The E-screen assay has been employed as a complementary tool to ascertain the overall  
390 estrogenic activity of the water, due to a mix of known and unknown chemicals potentially leading  
391 to additive or synergistic effects (Cocci et al., 2015).

392 MCF-7 cells were exposed for 5 days to increasing amounts of E2, ranging from  $10^{-15}$  to  $10^{-8}$  M, then  
393 the proliferative effect (PE) was evaluated (Figure 2A). E2 induced a dose-dependent cell  
394 proliferation, with a maximum PE at  $10^{-10}$  M, which was inhibited by the presence of the estrogen  
395 receptor blocker tamoxifen (TAM), confirming the involvement of estrogen receptors in this  
396 response. The minimum E2 concentration showing a significant response was  $10^{-13}$  M, corresponding  
397 to about 0.03 ng/L. The dose-response curve of E2, analyzed by non-linear regression ( $r^2 = 0.987$ ,  
398 dotted curve), allows to quantify the PE in terms of equivalent estradiol (EEQ) concentration. Figure

2B indicates that the water samples analyzed did not show a PE different from control cells, with the exception of the post-treatment water sampled in Capaccio in July 2019. This result was corroborated by further analysis, which found the estrogenic activity also in pre-treatment water of the same sampling campaign, and confirmed by TAM exposure test, which abolished the E-screen positive response (data not shown). The estrogenic activity, quantified by the dose-response curve and corrected for the concentration factor, corresponded to 24.6 and 9.06 pg/L EEQ in pre- and post-treatment water, respectively. Similar results were found in drinking waters in 16 out of 35 Italian cities, with a maximum of 13.6 pg/L EEQ, judged by the Authors as a low estrogenic activity (Maggioni et al., 2013). Estrogenic activity was also observed in bottled water commercialized in Europe, ranging from 1.9 to 12.2 pg/L EEQ (Wagner and Oehlmann, 2011).

The weak but significant estrogenic response was recorded in the Capaccio samples, although estrogens (E1, E2, or EE2) and simil-estrogens (BPA or NP) were at concentrations similar to other samples analyzed. Thus, a biological effect caused by either synergistic effects, or unidentified chemicals present in the mixture was hypothesized.

Hu and coworkers demonstrated that when BPA reacted with high concentrations of chlorine, derivatives were still present after 60 min and are more difficult to biodegrade than BPA; furthermore, by-products were detected at the exit of the DWTPs showing an estrogenic activity greater than the parent compounds at lower concentrations (Hu et al., 2002). The effects of by-products from chlorination cannot be ruled out, because not analysed in our samples. However, due to the higher estrogenicity found in the corresponding pre-treatment water, we suggest that an occasional peak of contaminants in the water feeding the plant determined the estrogenic effects observed in the specific samples.

### 3.2.2. *Evaluation of genotoxic activity by Micronuclei test*

A further issue relates to the occurrence of genotoxic chemicals, due not only to direct or indirect discharges after industrial, domestic, and agricultural usages but also to disinfection treatments,

424 particularly when water is obtained from surface sources and then chlorinated. Thus, short-term  
425 genotoxicity tests predictive of carcinogenic activity have been suggested to assess the potential  
426 genotoxic activity of such complex mixtures in drinking water (Buschini et al., 2004), (WHO, 2011),  
427 (Ceretti et al., 2016). Many estrogen-like chemicals induce multiple effects *in vivo* that cannot be  
428 related only to estrogenic activity. For example, BPA is also a genotoxic compound, that leads to  
429 DNA damage, detectable by an increase of micronuclei (MN) number in exposed cells (Ramos et al.,  
430 2019). For carcinogenic compounds, the United States Environmental Protection Agency  
431 recommends zero level in drinking water (US EPA, 2017). Despite the risks associated with the  
432 presence of mutagenic/carcinogenic substances in water intended for human consumption, the current  
433 legislation does not provide for the application of mutagenesis tests.

434 We presently used MN test for its sensitivity and reliability. MN test has already been applied for the  
435 assessment of the quality of drinking water (Maffei et al., 2009), (Zeng et al., 2015) (Buchner et al.,  
436 2019). Table 6 shows the frequency of MN evaluated in MCF-7 cells after 48 h treatment with the  
437 different sampled water extracts. None of the water extracts induced any statistically significant  
438 increase in the MN frequency compared to negative controls. The positive control BPA 0.1  $\mu$ M  
439 showed a significant variation ( $p < 0.05$ ), thus indicating the sensitivity of the test.

440

#### 441 4. Conclusions

442 The quality of drinking water and the efficacy of treatments in relation to CECs are a matter of  
443 concern, because the risk they pose to human health and the environment is not yet fully understood.  
444 A chemical and biological integrated approach is here proposed to evaluate the occurrence of selected  
445 CECs and the overall estrogenic and genotoxic potential of waters eligible for human consumption.  
446 The water analysed in the present investigation met the criteria of good quality, according to the low  
447 concentration and/or regulation limit for each single contaminant. Chemical analysis indicated that  
448 NP, PFOA, BPA, CFF and CBZ were the most frequent contaminants in water samples, thus  
449 confirming that these substances are ubiquitous contaminants in the water cycle. While the

450 waterworks treatment was generally effective in reducing BPA, CFF and CBZ, it was sometimes  
451 ineffective for NP and PFOA. For some of the studied CECs, occurrence in the incoming water was  
452 different among waterworks, which are fed by water coming from areas with lower (Capaccio) and  
453 higher (NIP and Standiana) anthropogenic impact. Water feeding Capaccio in fact was neither  
454 contaminated by pharmaceuticals nor by PFOS, while PFOA concentration was at least 5 times lower  
455 than in other plants. All CECs were instead detected in water entering NIP and Standiana.  
456 Interestingly, BPA and NP occurred in all plants at very similar concentrations, regardless the area of  
457 origin of the incoming water. Some of the chemicals investigated are included in the Watch list of  
458 substances for which EU-wide monitoring data need to be gathered to support future prioritization.  
459 Present data therefore provide information on the fulfilling of the purposes of EU Water Framework  
460 Directive (EU, 2013) and of the recently revised Drinking Water Directive (EU Council, 2020).  
461 Biological analyses were performed to ascertain the absence of cumulative estrogenic and genotoxic  
462 activities in the waters from the DWTPs. Although previous reports are available on this possibility  
463 (Maggioni et al., 2013), no estrogenic or genotoxic activities were shown by the waters analyzed,  
464 with the exception of one sample. The recorded estrogenic activity remained an isolated phenomenon,  
465 of low entity and in line with estrogen concentrations previously reported in drinking waters.  
466 However, this may not always be the case, and high frequency monitoring are suggested for a  
467 comprehensive assessment of the risks associated with exposure to CEC mixtures.  
468 It is a recurrent suggestion that drinking waters do not represent a relevant source for human exposure  
469 to CEC as asserted for NP (Soares et al., 2008), (Colin et al., 2014), BPA (Arnold et al., 2013), PFAS  
470 (Domingo and Nadal, 2019) and pharmaceuticals (WHO, 2017). The above considerations, however,  
471 cannot bridge the knowledge gaps in terms of assessing the risks associated with long-term, low-level  
472 exposures, and possible combined effects of chemicals in the mixture. Overall, the present study  
473 points out the usefulness of an integrated chemical and biological approach as a screening tool for  
474 drinking water quality.

475 In conclusion, health effects related to the consumption of drinking water containing a cocktail of  
476 CECs are still unknown and difficult to predict. Thus, more information and proactive measures to  
477 treat and remove these compounds are advisable, despite the costs and uncertain benefits.

478

## 479 **5. Acknowledgements**

480 This paper is dedicated to the memory of Dr. Andrea Gambi, General Director of Romagna Acque,  
481 Società delle Fonti (Italy), recently passed away for COVID-19. With immense foresight and  
482 visionary practicality, he always supported the scientific training as the basis of industrial progress.  
483 The Authors are grateful to Romagna Acque, Società delle Fonti (Italy) for allowing the water  
484 samplings. This work was funded by MIUR Italy (RFO 2018 and 2019 to EF).

485 **Declaration of interests:** The Authors declare that there are no conflict of interests regarding the  
486 publication of this work.

487

## 488 **6. References**

- 489 Achene, L., Bogialli, S., Lucentini, L., 2011. Interferenti endocrini nelle acque da destinare al  
490 consumo umano in Italia: strumenti metodologici per un'indagine conoscitiva estesa a diversi  
491 sistemi idrici. Rome: Italian Institute of Health (ISTISAN Reports 11/18)  
492 <https://www.iss.it/rapporti-istisan>.
- 493 Arnold, K.E., Brown, A.R., Ankley, G.T., Sumpter, J.P., Arnold, K.E., 2014. Medicating the  
494 environment : assessing risks of pharmaceuticals to wildlife and ecosystems. *Philos Trans R*  
495 *Soc L. B Biol Sci* 369, 20130569. <https://doi.org/https://doi.org/10.1098/rstb.2013.0569>
- 496 Arnold, S.M., Clark, K.E., Staples, C.A., Klecka, G.M., Dimond, S.S., Caspers, N., Hentges, S.G.,  
497 2013. Relevance of drinking water as a source of human exposure to bisphenol A. *J. Expo. Sci.*  
498 *Environ. Epidemiol.* 23, 137–144. <https://doi.org/10.1038/jes.2012.66>
- 499 Boiteux, V., Dauchy, X., Rosin, C., Boiteux, J.F.V., 2012. National screening study on 10

500 perfluorinated compounds in raw and treated tap water in france. Arch. Environ. Contam.  
 501 Toxicol. 63, 1–12. <https://doi.org/10.1007/s00244-012-9754-7>  
 502 Buchner, E., Happel, O., Schmidt, C.K., Scheurer, M., Schmutz, B., Kramer, M., et al., 2019.  
 503 Approach for analytical characterization and toxicological assessment of ozonation products in  
 504 drinking water on the example of acesulfame. Water Res. 153, 357–368.  
 505 <https://doi.org/10.1016/j.watres.2019.01.018>  
 506 Castiglioni, S., Valsecchi, S., Polesello, S., Rusconi, M., Melis, M., Palmiotto, M., et al., 2015.  
 507 Sources and fate of perfluorinated compounds in the aqueous environment and in drinking  
 508 water of a highly urbanized and industrialized industrialized area in Italy. J. Hazard. Mater.  
 509 282, 51–60. <https://doi.org/doi:10.1016/j.jhazmat.2014.06.007>  
 510 Chen, K., Chou, P., 2016. Detection of endocrine active substances in the aquatic environment in  
 511 southern Taiwan using bioassays and LC e MS / MS. Chemosphere 152, 214–220.  
 512 <https://doi.org/10.1016/j.chemosphere.2016.02.115>  
 513 Cocci, P., Palermo, F.A., Quassinti, L., Bramucci, M., Miano, A., Mosconi, G., 2015. ScienceDirect  
 514 Determination of estrogenic activity in the river Chienti ( Marche Region , Italy ) by using in  
 515 vivo and in vitro bioassays. JES 43, 48–53. <https://doi.org/10.1016/j.jes.2015.07.018>  
 516 Colin, A., Bach, C., Rosin, C., Munoz, J.F., Dauchy, X., 2014. Is drinking water a major route of  
 517 human exposure to alkylphenol and bisphenol contaminants in France? Arch. Environ.  
 518 Contam. Toxicol. 66, 86–99. <https://doi.org/10.1007/s00244-013-9942-0>  
 519 de Jesus Gaffney, V., Almeida, C.M.M., Rodrigues, A., Ferreira, E., Benoliel, M.J., Cardoso, V.V.,  
 520 2015. Occurrence of pharmaceuticals in a water supply system and related human health risk  
 521 assessment. Water Res. 72, 199–208. <https://doi.org/10.1016/j.watres.2014.10.027>  
 522 Domingo, J.L., Ericson-Jogsten, I., Perelló, G., Nadal, M., Van Bavel, B., Kärrman, A., 2012.  
 523 Human exposure to perfluorinated compounds in Catalonia, Spain: Contribution of drinking  
 524 water and fish and shellfish. J. Agric. Food Chem. 60, 4408–4415.  
 525 <https://doi.org/10.1021/jf300355c>

526 Domingo, J.L., Nadal, M., 2019. Human exposure to per- and polyfluoroalkyl substances (PFAS)  
527 through drinking water: A review of the recent scientific literature. *Environ. Res.* 177, 108648.  
528 <https://doi.org/10.1016/j.envres.2019.108648>

529 Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., et al., 2014.  
530 Benchmarking Organic Micropollutants in Wastewater, Recycled Water and Drinking Water  
531 with In Vitro Bioassays. *Environ. Sci. Technol.* 48, 1940–1956.  
532 <https://doi.org/10.1021/es403899t>

533 Espinoza, F., Cecchini, L., Morote, J., Marcos, R., Pastor, S., 2019. Micronuclei frequency in  
534 urothelial cells of bladder cancer patients, as a biomarker of prognosis. *Environ. Mol.*  
535 *Mutagen.* 60, 168–173. <https://doi.org/10.1002/em.22252>

536 EU, 2013. Directive 2013/39/EU of the European parliament and of the Council of 12 August 2013  
537 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field  
538 of water policy. *Off. J. Eur. Union.* [https://eur-lex.europa.eu/legal-](https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX32013L0039&from=EN)  
539 [content/EN/TXT/PDF/?uri=CELEX32013L0039&from=EN](https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX32013L0039&from=EN) 1–17.

540 EU, 2008. Water environmental quality standards. *Off. J. Eur. Union* 348, 84-97. [https://eur-](https://eur-lex.europa.eu/eli/dir/2008/105/)  
541 [lex.europa.eu/eli/dir/2008/105/](https://eur-lex.europa.eu/eli/dir/2008/105/).

542 EU, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000  
543 establishing a framework for Community action in the field of water policy. *Off. J. Eur.*  
544 *Communities.* <https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX32000L0060> 327,  
545 1–73.

546 EU Commission, 2018. Proposal for a Directive of the European Parliament and of the Council on  
547 the quality of water intended for human consumption (recast). *Off. J. Eur. Union.* [https://eur-](https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX52017PC0753)  
548 [lex.europa.eu/legal-content/en/TXT/?uri=CELEX52017PC0753](https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX52017PC0753).

549 EU Commission, 2015. Commission implementing decision (EU) 2015/495 of 20 March 2015  
550 establishing a watch list of substances for Union-wide monitoring in the field of water policy  
551 pursuant to Directive 2008/105/EC of the European Parliament and of the Council. *Off. J. Eur.*



Union. [https://eur-lex.europa.eu/legal-](https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2015.078.01.0040.01.ENG)  
[content/EN/TXT/?uri=uriserv%3AOJ.L\\_.2015.078.01.0040.01.ENG](https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2015.078.01.0040.01.ENG) L260, 6–17.

EU Commission, 2007. Commission Staff Working Document on the implementation for the  
 “Community Strategy for Endocrine Disrupters”- a range of substances suspected of  
 interfering with the hormone systems of humans and wildlife. SEC (2007) 1635,  
[https://ec.europa.eu/environment/chemicals/endocrine/pdf/sec\\_2007\\_1635.pdf](https://ec.europa.eu/environment/chemicals/endocrine/pdf/sec_2007_1635.pdf).

EU Council, 2020. Safe and clean drinking water: Council approves provisional deal which updates  
 quality standards. Press Off. - Gen. Secr. Counc.  
[https://www.consilium.europa.eu/en/press/press-releases/2020/02/05/safe-and-clean-drinking-](https://www.consilium.europa.eu/en/press/press-releases/2020/02/05/safe-and-clean-drinking-water-council-approves-provisional-deal-which-updates-quality-standards/)  
[water-council-approves-provisional-deal-which-updates-quality-standards/](https://www.consilium.europa.eu/en/press/press-releases/2020/02/05/safe-and-clean-drinking-water-council-approves-provisional-deal-which-updates-quality-standards/).

Farré, M., Kuster, M., Brix, R., Rubio, F., Alda, M.J.L. de, Barceló, D., 2007. Comparative study of  
 an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography-tandem mass  
 spectrometry, and ultra performance liquid chromatography-quadrupole time of flight mass  
 spectrometry for part-per-trillion analysis of estrogens in. *J. Chromatogr. A* 1160, 166–175.  
<https://doi.org/10.1016/j.chroma.2007.05.032>

Fenech, M., 2000. The in vitro micronucleus technique. *Mutat. Res.* 455, 81–95.  
[https://doi.org/10.1016/s0027-5107\(00\)00065-8](https://doi.org/10.1016/s0027-5107(00)00065-8)

Glassmeyer, S.T., Furlong, E.T., Kolpin, D.W., Batt, A.L., Benson, R., Boone, J.S., et al., 2017.  
 Nationwide reconnaissance of contaminants of emerging concern in source and treated  
 drinking waters of the United States. *Sci. Total Environ.* 581–582, 909–922.  
<https://doi.org/10.1016/j.scitotenv.2016.12.004>

Hu, J.Y., Aizawa, T., Ookubo, S., 2002. Products of aqueous chlorination of bisphenol A and their  
 estrogenic activity. *Environ. Sci. Technol.* 36, 1980–1987. <https://doi.org/10.1021/es011177b>

Ibáñez, M., Gracia-Lor, E., Sancho, J. V., Hernández, F., 2012. Importance of MS selectivity and  
 chromatographic separation in LC-MS/MS-based methods when investigating pharmaceutical  
 metabolites in water. Dipyrone as a case of study. *J. Mass Spectrom.* 47, 1040–1046.

578 <https://doi.org/10.1002/jms.3050>

579 Ingelido, A.M., Abballe, A., Gemma, S., Dellatte, E., Iacovella, N., De Angelis, G., et al., 2018.

580 Biomonitoring of perfluorinated compounds in adults exposed to contaminated drinking water

581 in the Veneto Region, Italy. *Environ. Int.* 110, 149–159.

582 <https://doi.org/10.1016/j.envint.2017.10.026>

583 ISPRA, 2017. Primo monitoraggio delle sostanze dell'Elenco di controllo (Watch List). Higher

584 Institute for Environmental Protection and Research.

585 [https://www.isprambiente.gov.it/files2017/pubblicazioni/rapporto/R\\_260\\_17\\_watch\\_list\\_rev.p](https://www.isprambiente.gov.it/files2017/pubblicazioni/rapporto/R_260_17_watch_list_rev.pdf)

586 [df.](https://www.isprambiente.gov.it/files2017/pubblicazioni/rapporto/R_260_17_watch_list_rev.pdf)

587 Kiyama, R., Wada-Kiyama, Y., 2015. Estrogenic endocrine disruptors: Molecular mechanisms of

588 action. *Environ. Int.* 83, 11–40. <https://doi.org/10.1016/j.envint.2015.05.012>

589 Korner, W., 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic

590 activity in municipal sewage plant effluents. *Sci. Total Environ.* 225, 33–48.

591 [https://doi.org/10.1016/s0048-9697\(99\)80015-1](https://doi.org/10.1016/s0048-9697(99)80015-1)

592 Kot-Wasik, A., Jakimska, A., Śliwka-Kaszyńska, M., 2016. Occurrence and seasonal variations of

593 25 pharmaceutical residues in wastewater and drinking water treatment plants. *Environ. Monit.*

594 *Assess.* 188. <https://doi.org/10.1007/s10661-016-5637-0>

595 Krzeminski, P., Tomei, M.C., Karaolia, P., Langenhoff, A., Almeida, C.M.R., Felis, E., Gritten, F.,

596 Andersen, H.R., Fernandes, T., Manaia, C.M., Rizzo, L., Fatta-Kassinos, D., 2019.

597 Performance of secondary wastewater treatment methods for the removal of contaminants of

598 emerging concern implicated in crop uptake and antibiotic resistance spread: A review. *Sci.*

599 *Total Environ.* 648, 1052–1081. <https://doi.org/10.1016/j.scitotenv.2018.08.130>

600 Kuch, H.M., Ballschmiter, K., 2001. Determination of endocrine-disrupting phenolic compounds

601 and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter

602 range. *Environ. Sci. Technol.* 35, 3201–3206. <https://doi.org/10.1021/es010034m>

603 Kwon, D., Tak, S., Lee, J., Kim, M., Lee, Y.H., Han, D.W., et al., 2017. Desorption of

micropollutant from spent carbon filters used for water purifier. *Env. Sci Pollut Res* 17606–17615. <https://doi.org/10.1007/s11356-017-9311-z>

Leusch, F.D.L., Neale, P.A., Arnal, C., Aneck-hahn, N.H., Balaguer, P., Bruchet, A., et al., 2018. Analysis of endocrine activity in drinking water , surface water and treated wastewater from six countries. *Water Res.* 139, 10–18. <https://doi.org/10.1016/j.watres.2018.03.056>

Loos, R., Marinov, D., Sanseverino, I., Napierska, D., Lettieri, T., 2018. Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List. *Publ. Off. Eur. Union.* <https://doi.org/10.2760/614367>

Loos, R., Wollgast, J., Huber, T., Hanke, G., 2007. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy. *Anal. Bioanal. Chem.* 387, 1469–1478. <https://doi.org/10.1007/s00216-006-1036-7>

Ly, X., Xiao, S., Zhang, G., Jiang, P., Tang, F., 2016. Occurrence and removal of phenolic endocrine disrupting chemicals in the water treatment processes. *Sci. Rep.* 6, 1–10. <https://doi.org/10.1038/srep22860>

Maffei, F., Carbone, F., Forti, G.C., Buschini, A., Poli, P., Rossi, C., et al., 2009. Drinking water quality: An in vitro approach for the assessment of cytotoxic and genotoxic load in water sampled along distribution system. *Environ. Int.* 35, 1053–1061. <https://doi.org/10.1016/j.envint.2009.05.007>

Maggioni, S., Balaguer, P., Chiozzotto, C., Benfenati, E., 2013. Screening of endocrine-disrupting phenols , herbicides , steroid estrogens , and estrogenicity in drinking water from the waterworks of 35 Italian cities and from PET-bottled mineral water. *Environ. Sci. Pollut. Res.* 20, 1649–1660. <https://doi.org/10.1007/s11356-012-1075-x>

Mompelat, S., Thomas, O., Le Bot, B., 2011. Contamination levels of human pharmaceutical compounds in French surface and drinking water. *J. Environ. Monit.* 13, 2929–2939. <https://doi.org/10.1039/c1em10335k>

630 Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to  
631 proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.

632 Padhye, L.P., Yao, H., Kung'u, F.T., Huang, C.H., 2014. Year-long evaluation on the occurrence  
633 and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an  
634 urban drinking water treatment plant. *Water Res.* 51, 266–276.  
635 <https://doi.org/10.1016/j.watres.2013.10.070>

636 Pignotti, E., Farré, M., Barceló, D., Dinelli, E., 2017. Occurrence and distribution of six selected  
637 endocrine disrupting compounds in surface- and groundwaters of the Romagna area. *Env. Sci*  
638 *Pollut Res* 24, 21153–21167. <https://doi.org/10.1007/s11356-017-9756-0>

639 Prins, G.S., Patisaul, H.B., Belcher, S.M., Vandenberg, L.N., 2019. CLARITY-BPA academic  
640 laboratory studies identify consistent low-dose Bisphenol A effects on multiple organ systems.  
641 *Basic Clin. Pharmacol. Toxicol.* 125, 14–31. <https://doi.org/10.1111/bcpt.13125>

642 Ramos, C., Ladeira, C., Zeferino, S., Dias, A., Faria, I., Cristovam, E., et al., 2019. Cytotoxic and  
643 genotoxic effects of environmental relevant concentrations of bisphenol A and interactions  
644 with doxorubicin. *Mutat. Res. - Genet. Toxicol. Environ. Mutagen.* 838, 28–36.  
645 <https://doi.org/10.1016/j.mrgentox.2018.11.009>

646 Riva, F., Castiglioni, S., Fattore, E., Manenti, A., Davoli, E., Zuccato, E., 2018. Monitoring  
647 emerging contaminants in the drinking water of Milan and assessment of the human risk. *Int. J.*  
648 *Hyg. Environ. Health* 221, 451–457. <https://doi.org/10.1016/j.ijheh.2018.01.008>

649 Skutlarek, D., Exner, M., Färber, H., 2006. Perfluorinated surfactants in surface and drinking  
650 waters. *Environ. Sci. Pollut. Res.* 13, 299–307. <https://doi.org/10.1065/espr2006.07.326>

651 Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., Lester, J.N., 2008. Nonylphenol in the  
652 environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters.  
653 *Environ. Int.* 34, 1033–1049. <https://doi.org/10.1016/j.envint.2008.01.004>

654 Sousa, J.C.G., Ribeiro, A.R., Barbosa, M.O., Pereira, M.F.R., Silva, A.M.T., 2018. A review on  
655 environmental monitoring of water organic pollutants identified by EU guidelines. *J. Hazard.*

656 Mater. 344, 146–162. <https://doi.org/10.1016/j.jhazmat.2017.09.058>

657 Togola, A., Budzinski, H., 2008. Multi-residue analysis of pharmaceutical compounds in aqueous  
658 samples. *J. Chromatogr. A* 1177, 150–158. <https://doi.org/10.1016/j.chroma.2007.10.105>

659 US EPA, 2017. How EPA Regulates Drinking Water Contaminants,  
660 <https://www.epa.gov/sdwa/how-epa-regulates-drinking-water-contaminants>.

661 Valcárcel, Y., González Alonso, S., Rodríguez-Gil, J.L., Gil, A., Catalá, M., 2011. Detection of  
662 pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain)  
663 and potential ecotoxicological risk. *Chemosphere* 84, 1336–1348.  
664 <https://doi.org/10.1016/j.chemosphere.2011.05.014>

665 Valcárcel, Y., Valdehíta, A., Becerra, E., López de Alda, M., Gil, A., Gorga, M., et al., 2018.  
666 Determining the presence of chemicals with suspected endocrine activity in drinking water  
667 from the Madrid region (Spain) and assessment of their estrogenic, androgenic and thyroidal  
668 activities. *Chemosphere* 201, 388–398. <https://doi.org/10.1016/j.chemosphere.2018.02.099>

669 Vulliet, E., Baugros, J.B., Flament-Waton, M.M., Grenier-Loustalot, M.F., 2007. Analytical  
670 methods for the determination of selected steroid sex hormones and corticosteroids in  
671 wastewater. *Anal. Bioanal. Chem.* 387, 2143–2151. [https://doi.org/10.1007/s00216-006-1084-](https://doi.org/10.1007/s00216-006-1084-z)  
672 [z](https://doi.org/10.1007/s00216-006-1084-z)

673 Wagner, M., Oehlmann, J., 2011. *Journal of Steroid Biochemistry and Molecular Biology*  
674 Endocrine disruptors in bottled mineral water : Estrogenic activity in. *J. Steroid Biochem. Mol.*  
675 *Biol.* 127, 128–135. <https://doi.org/10.1016/j.jsbmb.2010.10.007>

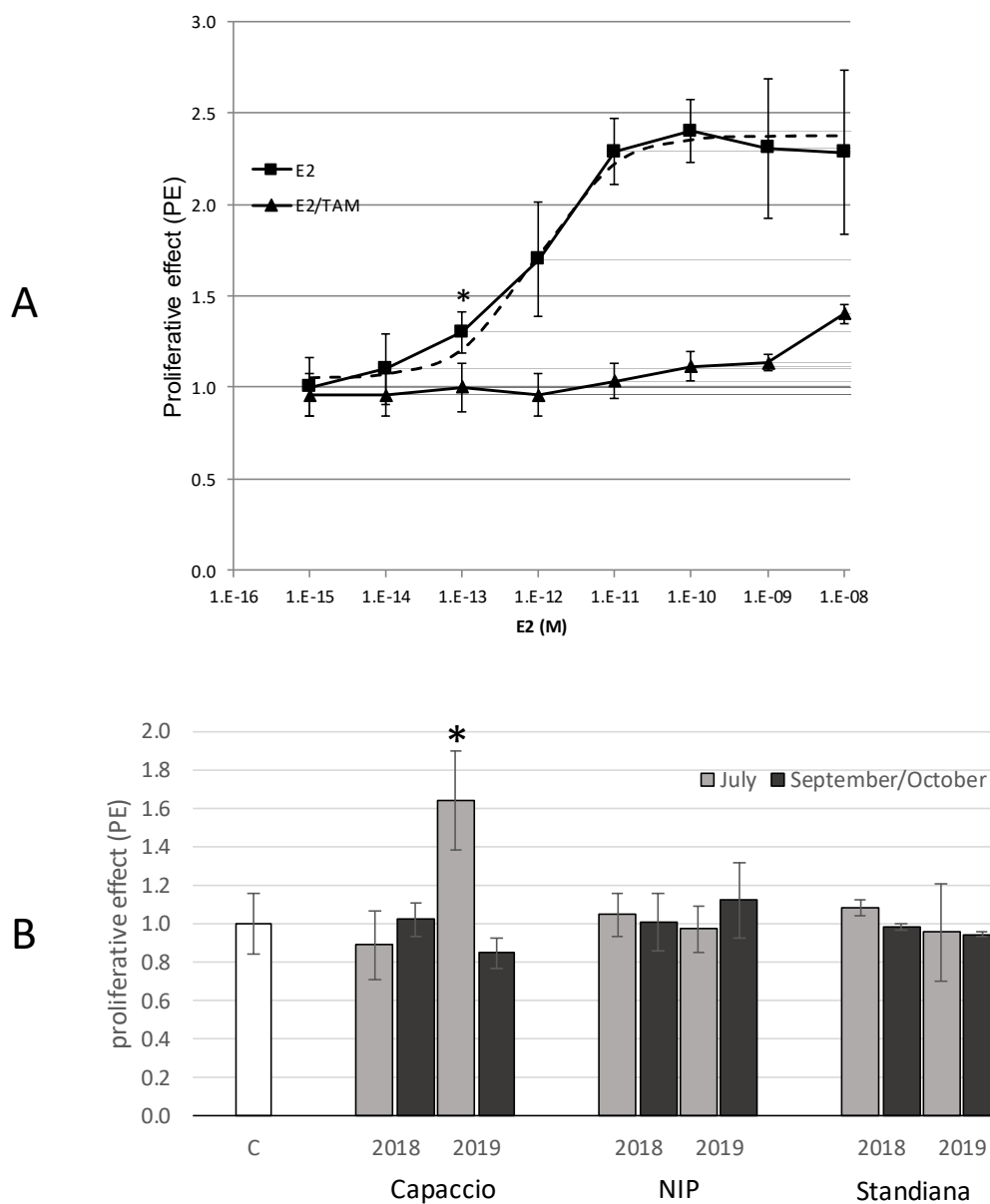
676 WHO, 2017. Guidelines for drinking-water quality: fourth edition incorporating the first addendum.  
677 WHO Libr. Cat. Data. [https://www.who.int/water\\_sanitation\\_health/publications/drinking-](https://www.who.int/water_sanitation_health/publications/drinking-water-quality-guidelines-4-including-1st-addendum/en/)  
678 [water-quality-guidelines-4-including-1st-addendum/en/](https://www.who.int/water_sanitation_health/publications/drinking-water-quality-guidelines-4-including-1st-addendum/en/) 1–541.

679 WHO, 2016. Keeping our water clean: the case of water contamination in the Veneto Region, Italy.  
680 [https://www.euro.who.int/en/publications/abstracts/keeping-our-water-clean-the-case-of-](https://www.euro.who.int/en/publications/abstracts/keeping-our-water-clean-the-case-of-water-contamination-in-the-veneto-region,-italy-2017)  
681 [water-contamination-in-the-veneto-region,-italy-2017](https://www.euro.who.int/en/publications/abstracts/keeping-our-water-clean-the-case-of-water-contamination-in-the-veneto-region,-italy-2017) 72.

682 Zeng, Q., Zhang, S., Liao, J., Miao, D., Wang, X., 2015. Evaluation of genotoxic effects caused by  
683 extracts of chlorinated drinking water using a combination of three different bioassays. J.  
684 Hazard. Mater. 296, 23–29. <https://doi.org/10.1016/j.jhazmat.2015.04.047>  
685  
686



**Figure 1.** Geographical location of Emilia Romagna, Italy and the three DWTPs in Romagna, 1 (NIP) and 2 (Standiana): District of Ravenna; 3 (Capaccio): District of Forlì-Cesena.



**Figure 2:** Evaluation of estrogenic activity. Data are expressed as the mean of proliferative effect (PE)  $\pm$  SE of different experiments, each conducted in quadruplicate; (A) E-screen test sensitivity: dose-response curve to E2 of MCF-7 cells, in the presence (triangle) or not (square) of  $10^{-7}$  M tamoxifen, an estrogen receptor-antagonist (N=10), \* first dose of E2 with  $P < 0.05$  vs control (PE = 1). (B) Evaluation of estrogenic activity in water samples from three DWTPs (Capaccio, NIP and Standiana) collected during 4 campaigns in 2018 and 2019 (N=4), \*  $P < 0.05$  vs control, cells exposed to ultrapure water (PE = 1).



713  
714  
715  
716  
717

**Table 1:** Contaminants of emerging concern investigated

***Pharmaceuticals***

atenolol (ATE)	anti-hypertensive
caffeine (CFF)	psychoactive
carbamazepine (CBZ)	anti-epileptic
diclofenac (DCF)	anti-inflammatory
ibuprofen (IBU)	anti-inflammatory
17-beta-estradiol (E2)	natural estrogen
estrone (E1)	natural estrogen
17-alfa-ethinylestradiol (EE2)	synthetic estrogen

***Surfactants and Plasticizers***

4-nonylphenol (NP)
4-octylphenol (OP)
bisphenol A (BPA)

***Perfluorinated substances***

perfluorooctane sulfonate (PFOS)
perfluorooctanoate (PFOA)

718  
719

720 **Table 2** MS-MS detection parameters for the 3 groups of compounds analyzed: cone voltage,  
 721 precursor and product ions with the respective collision energy  
 722

Compound	Cone voltage (V)	Precursor ion (m/z)	Product ion I (m/z) and collision energy (V)	Product ion II (m/z) and collision energy (V)
<i>Group 1 (ESI negative)</i>				
E1	54	269.2	145.0 (39)	159.0 (37)
E2	58	271.1	145.0 (44)	183.0 (38)
EE2	50	295.1	145.0 (38)	159.0 (42)
BPA	36	227.1	212.0 (18)	133.0 (24)
NP	34	219.1	132.9 (30)	147.0 (26)
OP	36	205.2	106.0 (20)	
E2-d <sub>3</sub>	52	273.1	185.0 (40)	
BPA-d <sub>6</sub>	36	233.0	215.0 (19)	
<i>Group 2 (ESI negative)</i>				
DCF	15	294.1	249.9 (13)	214.0 (20)
IBU	17	205.0	161 (7)	
PFOA	14	412.9	168.8 (20)	368.8 (10)
PFOS	59	498.8	79.9 (47)	98.9 (45)
Ibuprofen -d <sub>3</sub>	20	208.0	164 (7)	
PFOA-C <sub>13</sub>	14	417.1	372.2 (12)	
<i>Group 3 (ESI positive)</i>				
ATE	30	267.5	145.0 (28)	190.0 (18)
CFF	38	195.1	138.1 (19)	110.0 (24)
CBZ	29	237.1	194.0 (20)	192.0 (20)
Caffeine-C <sub>13</sub>	37	197.9	139.9 (19)	

723

724

725 **Table 3** Quantification and method validation: detection limits (LOD), quantification limits (LOQ),  
726 recovery and reproducibility (RSD %), correlation factors of the calibration curves ( $r^2$ ), precision  
727 (inter- and intra-day RSD %).

728

Compound	LOD	LOQ	Recovery $\pm$ RSD	Correlation	Precision (RSD %)	
	(pg injected)	(ng/L)	(%)	factor ( $r^2$ )	Intra-day	Inter-day
<i>Group 1</i>						
E1	9	0.92	90 $\pm$ 14	0.9994	15	10
E2	15	0.81	80 $\pm$ 5	0.9999	9	13
EE2	41	2.66	95 $\pm$ 10	0.9994	22	20
BPA	9	0.99	97 $\pm$ 15	0.9999	4	1
NP	5	2.05	104 $\pm$ 20	0.9997	3	7
OP	13	0.66	87 $\pm$ 21	0.9979	13	11
<i>Group 2</i>						
DCF	6	0.51	86 $\pm$ 18	0.9987	9	20
IBU	24	1.96	104 $\pm$ 3	0.9990	14	4
PFOA	1	0.07	103 $\pm$ 13	0.9984	3	3
PFOS	2	0.08	75 $\pm$ 7	0.9996	5	9
<i>Group 3</i>						
ATE	2	3.56	111 $\pm$ 9	0.9974	3	11
CFF	1	0.12	96 $\pm$ 17	0.9997	1	9
CBZ	0.03	0.04	105 $\pm$ 7	0.9991	2	8

729

730

731

**Table 4.** Levels of compounds for industrial use recognized as endocrine disruptors (ng/l) measured in water samples from three DWTPs (Capaccio, NIP and Standiana) collected during 4 campaigns in 2018 and 2019. IN: pre-treatment water, OUT: post-treatment water; LOQ: limit of quantification (ng/l). Bold numbers: CECs detected in OUT water samples.

			BPA	OP	NP	PFOA	PFOS
Capaccio	July 2018	IN	8.57	< LOQ	32.30	1.04	< LOQ
		OUT	<b>3.56</b>	< LOQ	<b>33.97</b>	<b>1.03</b>	< LOQ
	October 2018	IN	9.25	< LOQ	60.83	0.24	< LOQ
		OUT	<b>4.18</b>	< LOQ	<b>53.62</b>	<b>0.33</b>	< LOQ
NIP	July 2018	IN	9.77	< LOQ	42.94	5.52	0.33
		OUT	<b>6.27</b>	< LOQ	<b>22.83</b>	<b>2.47</b>	< LOQ
	October 2018	IN	7.84	< LOQ	42.71	9.74	0.95
		OUT	<b>5.84</b>	< LOQ	<b>21.45</b>	<b>1.83</b>	< LOQ
Standiana	July 2018	IN	11.18	< LOQ	49.49	7.82	0.85
		OUT	< LOQ	< LOQ	<b>21.26</b>	<b>12.66</b>	<b>0.81</b>
	October 2018	IN	17.98	< LOQ	31.52	7.73	0.65
		OUT	<b>2.34</b>	< LOQ	<b>14.89</b>	<b>5.50</b>	<b>0.08</b>
	LOQ		0.99	0.66	2.05	0.08	0.07
Capaccio	July 2019	IN	3.81	< LOQ	14.70	< LOQ	< LOQ
		OUT	< LOQ	< LOQ	<b>7.90</b>	< LOQ	< LOQ
	September 2019	IN	1.81	< LOQ	18.68	0.14	< LOQ
		OUT	< LOQ	< LOQ	<b>18.31</b>	<b>0.16</b>	< LOQ
NIP	July 2019	IN	5.85	< LOQ	9.74	4.79	0.46
		OUT	<b>1.93</b>	< LOQ	<b>18.51</b>	<b>0.75</b>	< LOQ
	September 2019	IN	4.03	< LOQ	23.52	5.99	0.97
		OUT	< LOQ	< LOQ	<b>16.89</b>	<b>0.84</b>	< LOQ
Standiana	July 2019	IN	2.56	< LOQ	13.78	5.50	1.06
		OUT	< LOQ	< LOQ	<b>16.46</b>	<b>5.05</b>	<b>0.18</b>
	September 2019	IN	< LOQ	< LOQ	15.89	7.09	1.43

738  
739  
740  
741  
742

OUT	< LOQ	< LOQ	<b>23.36</b>	<b>6.57</b>	<b>0.42</b>
-----	-------	-------	--------------	-------------	-------------

743

744 **Table 5.** Levels of pharmaceuticals (ng/l) measured in water samples from three DWTPs (Capaccio,  
 745 NIP and Standiana) collected during 4 campaigns in 2018 and 2019. IN: pre-treatment water, OUT:  
 746 post-treatment water; LOQ: limit of quantification (ng/l). Bold numbers: pharmaceuticals detected in  
 747 OUT water samples.

748

749

			E2	E1	EE2	CFF	IBU	ATE	CBZ	DCF
Capaccio	July 2018	IN	4.04	<LOQ	<LOQ	20.72	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	12.89	<LOQ	<LOQ	<LOQ	<LOQ
	October 2018	IN	<LOQ	<LOQ	<LOQ	56.56	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	25.92	<LOQ	<LOQ	<LOQ	<LOQ
NIP	July 2018	IN	2.61	<LOQ	<LOQ	1390.15	<LOQ	2.39	26.76	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	66.33	<LOQ	<LOQ	0.17	<LOQ
	October 2018	IN	<LOQ	<LOQ	<LOQ	2579.60	15.57	8.55	34.57	15.91
		OUT	<LOQ	<LOQ	<LOQ	54.82	<LOQ	<LOQ	<LOQ	<LOQ
Standiana	July 2018	IN	<LOQ	<LOQ	<LOQ	78.63	<LOQ	4.21	13.11	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	62.64	<LOQ	<LOQ	0.58	<LOQ
	October 2018	IN	<LOQ	<LOQ	<LOQ	59.37	4.31	<LOQ	17.40	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	38.06	<LOQ	<LOQ	0.20	<LOQ
LOQ			2.35	0.92	2.66	0.12	1.96	3.56	0.04	0.51
Capaccio	July 2019	IN	<LOQ	<LOQ	<LOQ	57.96	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	16.59	<LOQ	<LOQ	<LOQ	<LOQ
	September 2019	IN	<LOQ	<LOQ	<LOQ	8.93	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	15.72	<LOQ	<LOQ	<LOQ	<LOQ
NIP	July 2019	IN	<LOQ	<LOQ	<LOQ	60.49	<LOQ	<LOQ	18.70	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	16.81	<LOQ	<LOQ	<LOQ	<LOQ
	September 2019	IN	<LOQ	<LOQ	<LOQ	178.79	5.22	4.20	26.46	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	12.47	<LOQ	<LOQ	<LOQ	<LOQ
Standiana	July 2019	IN	<LOQ	<LOQ	<LOQ	40.71	<LOQ	<LOQ	10.87	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	18.61	<LOQ	<LOQ	1.20	<LOQ

September 2019

IN	<LOQ	<LOQ	<LOQ	67.80	<LOQ	<LOQ	17.84	<LOQ
OUT	<LOQ	<LOQ	<LOQ	<b>20.00</b>	<LOQ	<LOQ	<b>0.83</b>	<LOQ

750

751

752

753

754

755

756

757

758

759

760

761

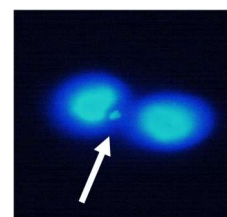
**Table 6:** Evaluation of genotoxic activity in water samples from three DWTPs (Capaccio, NIP and Standiana) collected during 4 campaigns in 2018 and 2019. Data are expressed as the mean of micronuclei (n°/1000 binucleated cells)  $\pm$  SD of 4 different experiments (N=4). Control: cells exposed to ultrapure water. Positive control: evaluation of genotoxic activity in cells exposed to Bisphenol A (0.1  $\mu$ M). \* P <0.05 vs control. The picture shows an example of binucleated cell detected in the present study; the white arrow marks a micronucleus.

768

769

770

	Capaccio	NIP	Standiana	control
summer 2018	8.0 $\pm$ 4.8	13.3 $\pm$ 3.3	9.7 $\pm$ 4.1	12.0 $\pm$ 4.1
october 2018	7.5 $\pm$ 2.5	13.0 $\pm$ 6.6	11.3 $\pm$ 6.0	
summer 2019	10.8 $\pm$ 4.5	9.6 $\pm$ 4.7	13.0 $\pm$ 2.6	11.3 $\pm$ 3.9
september 2019	12.8 $\pm$ 3.8	11.4 $\pm$ 3.2	9.7 $\pm$ 3.3	
				Positive control
Bisphenol A (0.1 $\mu$ M)				17.1 $\pm$ 2.7 <b>37.2 * <math>\pm</math> 4.8</b>



771

772

773



