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

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

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## ABSTRACT

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

## 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

hormones and pharmaceuticals (17-β-estradiol, E2; 17-α-ethinylestradiol, EE2; and DCF) were included (EU Commission, 2015).

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

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

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

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

## 2. Materials and methods

### 2.1. Chemicals and reagents

Table 1 shows the panel of CECs evaluated in this study. All non-labelled standards were purchased from Merck Life Science (Milan, Italy). Isotope-labeled compounds used as internal standards were purchased by Cambridge Isotopes Laboratories Inc. (Lab ServiceAnalytica Srl, Anzola dell'Emilia, Bologna, Italy) ($^{13}C_3$-Caffeine), CDN Isotopes (Quebec, Canada) (E2-$d_2$ and BPA-$d_6$), Wellington Laboratories Inc. (Guelph, ON, Canada) ($^{13}C_4$-PFOA), and Merck Life Science (Ibuprofen-$d_3$). Solvent reagents from Merck Life Science were of LC-MS analytical grade.

### 2.2. Sampling sites and sample storage

Two sampling campaigns per year were carried out during 2018 and 2019, in July and September/October, corresponding to the dry season with the purpose of analyzing the worst scenario regarding CECs in the study area, when rivers are drier and the expected concentration of pollutants is greater. Pre- and post- treatment water samples were collected from the three main Romagna's waterworks operated by the company Romagna Acque-Società delle Fonti (Figure 1). Capaccio (Forlì-Cesena) is fed by the large reservoir of Ridracoli, in the National Park of the Casentinesi Forests (high Tuscan-Romagna Apennines). Differently, NIP and Standiana receive water from areas with many anthropic activities, NIP (Bassette, Ravenna), receiving water mainly from the Lamone river (integrated, in particularly dry periods, from the Reno River) and from the CER (the Emilia-Romagna channel that branches off the Po river and brings its water in the Romagna area); Standiana (Standiana, Ravenna), active since 2015, using more advanced water treatment techniques, such as ultrafiltration through 0.04 μm 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-$d_2$, BPA-$d_6$, $^{13}C_4$-PFOA, Ibuprofen-$d_3$ at a concentration of 30 ng/L, and $^{13}C_3$-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 $cm^3$, 200 mg; Waters S.p.A., Sesto San Giovanni, Milan, Italy). Cartridges were eluted with 6 mL of methanol, evaporated under a $N_2$ 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

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

2.4. Chromatographic conditions and mass spectrometry detection

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

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

### 2.5. Quantification and method validation

Data related to quantification and method validation are reported in Table 3. Each water sample was analyzed in triplicate. Recovery and repeatability were tested in DWTP waters by mixing 3 L of entering and 3 L of exiting water (1 L of each DWTP in 2018 July campaign). From this amount, 3 L were spiked before the extraction procedure with 30 ng/L of the targeted analytes, with the exception of CBZ (5 ng/L). The remaining 3 L of unspiked samples were analyzed in the same batch to correct the final concentrations for the amount of analytes already present in DWTP waters. Recoveries and accuracy were calculated subtracting the concentration of each analyte in unspiked water to the measured concentration after spiking. Procedural blanks were prepared in parallel to samples in order to exclude any contamination during sample treatments. Three standard mixtures, containing all the CECs to be analysed, were prepared before each analytical run by diluting stock solutions to obtain six-point calibration curves (0–100/300 ng/mL), prepared in a mixture of water/methanol at the same initial conditions of samples. An instrumental blank containing only the labeled internal standards was used as control for analytical interference. To rule out any system contamination and check sensibility drifts, one point of the calibration curve (10 ng/mL) was run every six sample injections. Detection limits (LODs) of the methods were calculated as the amount of native standard (pg) loaded that yielded a signal to noise ratio of 3 and quantification limits (LOQs) of the methods corresponded 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% $CO_2$ 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 $10^{-10}$ M E2 were added to each assay.

223 2.8 Micronuclei test

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

### 2.9 Statistical analysis

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

## 3. Results and Discussion

### 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 µg/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

of plastic materials for the pipelines, which could release substances such as alkylphenols, bisphenol A (BPA), phthalates and PAH into drinking water (Achene et al., 2011).

BPA, one of the highest-volume chemicals produced worldwide, is used as a plastic monomer and plasticizer in the production of polycarbonate and epoxy resins. In turn, these materials are currently used as components of many consumer products, including reusable plastic bottles, household kitchenware, canned food items, and medical equipment (Prins et al., 2019). BPA exposure has been associated with serious endocrine-disrupting effects in humans and wildlife, thus it belongs to category 1 of the Endocrine Disruptor Priority List (EU Commission, 2007). The recent revision of the Drinking Water Directive (EU Council, 2020) represents the first regulation concerning BPA occurrence in water for human consumption, with the definition of the upper limit of 2.5 μg/L.

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

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

*3.1.2. Perfluorinated substances*

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

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

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

As a comparison with other Italian data, occurrence of PFOA and PFOS in drinking water in the Veneto region dropped to maximum concentrations of 386 ng/L and 36 ng/L, respectively, after the abatement of an important water contamination detected in 2014, due to the draining of PFAS from a manufacturing company (WHO, 2016). PFOA and PFOS mean concentrations in tap water near the Maggiore lake were 2.4 ng/L and 8.1 ng/L, respectively (Loos et al., 2007), while PFOA in drinking water from an industrialized area in North of Milan reached 47 ng/L (Castiglioni et al., 2015). In 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-β-estradiol (E2), and 17-α-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

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

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

## 3.2. Biological analysis

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

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

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

399 2B indicates that the water samples analyzed did not show a PE different from control cells, with the
400 exception of the post-treatment water sampled in Capaccio in July 2019. This result was corroborated
401 by further analysis, which found the estrogenic activity also in pre-treatment water of the same
402 sampling campaign, and confirmed by TAM exposure test, which abolished the E-screen positive
403 response (data not shown). The estrogenic activity, quantified by the dose-response curve and
404 corrected for the concentration factor, corresponded to 24.6 and 9.06 pg/L EEQ in pre- and post-
405 treatment water, respectively. Similar results were found in drinking waters in 16 out of 35 Italian
406 cities, with a maximum of 13.6 pg/L EEQ, judged by the Authors as a low estrogenic activity
407 (Maggioni et al., 2013). Estrogenic activity was also observed in bottled water commercialized in
408 Europe, ranging from 1.9 to 12.2 pg/L EEQ (Wagner and Oehlmann, 2011).
409 The weak but significant estrogenic response was recorded in the Capaccio samples, although
410 estrogens (E1, E2, or EE2) and simil-estrogens (BPA or NP) were at concentrations similar to other
411 samples analyzed. Thus, a biological effect caused by either synergistic effects, or unidentified
412 chemicals present in the mixture was hypothesized.
413 Hu and coworkers demonstrated that when BPA reacted with high concentrations of chlorine,
414 derivatives were still present after 60 min and are more difficult to biodegrade than BPA; furthermore,
415 by-products were detected at the exit of the DWTPs showing an estrogenic activity greater than the
416 parent compounds at lower concentrations (Hu et al., 2002). The effects of by-products from
417 chlorination cannot be ruled out, because not analysed in our samples. However, due to the higher
418 estrogenicity found in the corresponding pre-treatment water, we suggest that an occasional peak of
419 contaminants in the water feeding the plant determined the estrogenic effects observed in the specific
420 samples.

421 *3.2.2. Evaluation of genotoxic activity by Micronuclei test*

422 A further issue relates to the occurrence of genotoxic chemicals, due not only to direct or indirect
423 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 μM
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.

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

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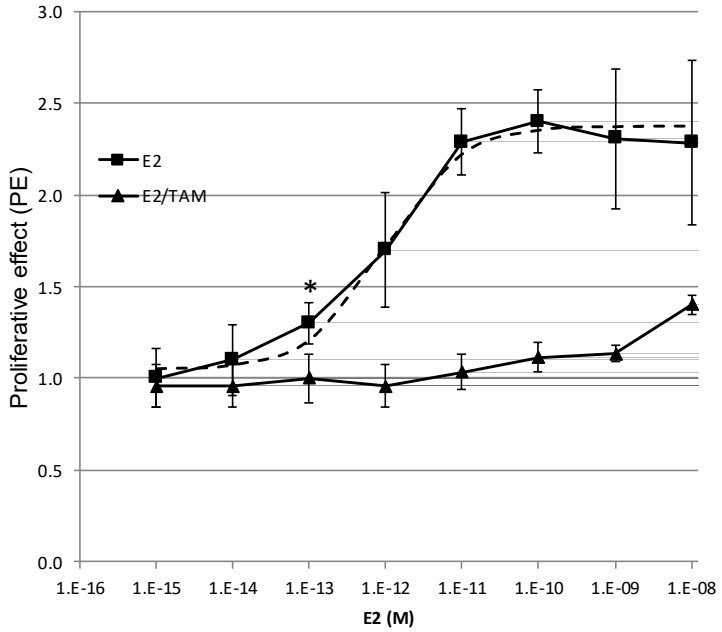
**Figure 1**. Geographical location of Emilia Romagna, Italy and the three DWTPs in Romagna, 1 (NIP) and 2 (Standiana): District of Ravenna; 3 (Capaccio): District of Forlì-Cesena.

A




B

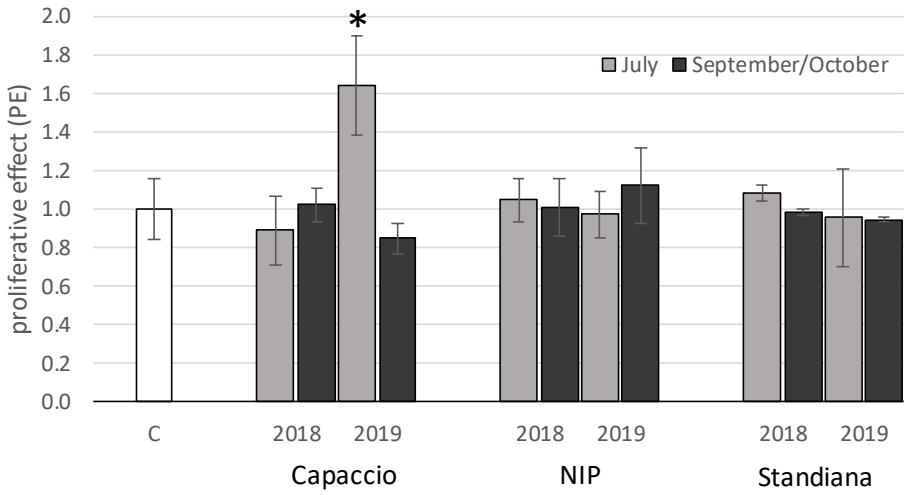



**Figure 2:** Evaluation of estrogenic activity. Data are expressed as the mean of proliferative effect (PE) ± SE of different experiments, each conducted in quadruplicate; (A) E-screen test sensitivity: dose-response curve to E2 of MCF-7 cells, in the presence (triangle) or not (square) of $10^{-7}$ 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).

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714

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

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717

| ***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) |

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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) |
|---|---|---|---|---|
| *Group 1 (ESI negative)* | | | | |
| 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-$d_3$ | 52 | 273.1 | 185.0 (40) | |
| BPA-$d_6$ | 36 | 233.0 | 215.0 (19) | |
| *Group 2 (ESI negative)* | | | | |
| 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 –$d_3$ | 20 | 208.0 | 164 (7) | |
| PFOA-$C_{13}$ | 14 | 417.1 | 372.2 (12) | |
| *Group 3 (ESI positive)* | | | | |
| 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-$C_{13}$ | 37 | 197.9 | 139.9 (19) | |

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

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732 **Table 4**. Levels of compounds for industrial use recognized as endocrine disruptors (ng/l) measured
733 in water samples from three DWTPs (Capaccio, NIP and Standiana) collected during 4 campaigns in
734 2018 and 2019. IN: pre-treatment water, OUT: post-treatment water; LOQ: limit of quantification
735 (ng/l). Bold numbers: CECs detected in OUT water samples.

736

737

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

| | | | | | |
|---|---|---|---|---|---|
| OUT | < LOQ | < LOQ | **23.36** | **6.57** | **0.42** |

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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

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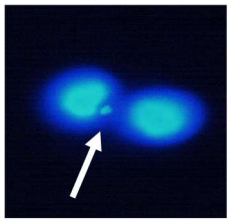
| | | | E2 | E1 | EE2 | CFF | IBU | ATE | CBZ | DCF |
|---|---|---|---|---|---|---|---|---|---|---|
| Capaccio | July 2018 | IN | 4.04 | <LOQ | <LOQ | 20.72 | <LOQ | <LOQ | <LOQ | <LOQ |
| | | OUT | <LOQ | <LOQ | <LOQ | **12.89** | <LOQ | <LOQ | <LOQ | <LOQ |
| | October 2018 | IN | <LOQ | <LOQ | <LOQ | 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 | **20.00** | <LOQ | <LOQ | **0.83** | <LOQ |

**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) ± 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 µM). * P <0.05 vs control. The picture shows an example of binucleated cell detected in the present study; the white arrow marks a micronucleus.

| | Capaccio | NIP | Standiana | control | |
|---|---|---|---|---|---|
| summer 2018 | 8.0 ± 4.8 | 13.3 ± 3.3 | 9.7 ± 4.1 | 12.0 ± 4.1 |  |
| october 2018 | 7.5 ± 2.5 | 13.0 ± 6.6 | 11.3 ± 6.0 | | |
| summer 2019 | 10.8 ± 4.5 | 9.6 ± 4.7 | 13.0 ± 2.6 | 11.3 ± 3.9 | |
| september 2019 | 12.8 ± 3.8 | 11.4 ± 3.2 | 9.7 ± 3.3 | | |
| | | | | | Positive control |
| Bisphenol A (0.1 µM) | | | | 17.1 ± 2.7 | **37.2 *± 4.8** |

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774 **Graphical abstract**


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