

Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE DELLA TERRA, DELLA VITA E DELL'AMBIENTE

Ciclo 35

Settore Concorsuale: 05/D1 - FISIOLOGIA

Settore Scientifico Disciplinare: BIO/09 - FISIOLOGIA

CONTAMINANTS OF EMERGING CONCERN IN DRINKING WATER:
ASSESSMENT OF THE EFFECTIVENESS OF REMOVAL BY DRINKING WATER
TREATMENTS AND POSSIBLE RISKS TO HUMAN HEALTH

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Esame finale anno 2023

Al mio papà, Nicola.

“It is the time you have wasted for your rose that makes your rose so important.”

(Antoine de Saint Exupéry)

ACKNOWLEDGMENTS

This work was financially supported by Romagna Acque Società delle Fonti. Without their financial support, the work presented here would not have been possible. I would like to dedicate this doctoral dissertation in memory of Dr. Andrea Gambi, who designed and supported this project. His person will be always appreciated for his great and visionary thinking, and for his support in the research. Sincere thanks are extended towards to Dr. Ivo Vasumini for his great advice in his fields of expertise and for the provided timely information. Thank you!

The work presented in this doctoral thesis would also never have been possible without the continual support and guidance of my supervisor and mentor Prof. Elena Fabbri. Her direction in all aspects of my graduate work was invaluable. Her dedication to science, critical thinking, and patience have motivated me throughout my Ph.D. work and I feel extremely fortunate to have had such an advisor.

I would like to especially thank Dr. Paola Valbonesi, for the various ways in which she assisted me in my work and for her enthusiastic patience in advising me on experimental designs. I would also like to thank my lab colleagues who enriched my experiences with thoughtful and insightful daily conversations and with whom I share unforgettable memories that I cherish.

Special thanks go to Prof. Henner Hollert, Dr. Andreas Schiwy, and colleagues at the Goethe University of Frankfurt, for creating a friendly atmosphere and an unforgettable experience abroad, and also for have been my ‘teachers’ of the English language.

I would also like to thank all participating DWTPs for their assistance in sampling and sample shipping.

Last but not least, a big thank you goes to Michele, my partner and best friend, for always being there for me, and for my family who always supported, encouraged, and sustained me!

Extended abstract

In the last few decades, contaminants of emerging concern have been detected in the water cycle at an alarming rate, compromising the quality of environmental and drinking waters. Among them, endocrine-disrupting chemicals (EDCs) are receiving growing attention as they may cause serious adverse health effects even at low concentrations. Only recently, the revision of the EU Drinking Water Directive has introduced for the first time EDCs in the drinking water legislation. However, drinking water treatment plants (DWTPs) are not designed to remove most emerging contaminants, thus the detection and removal of EDCs in DWTPs is still an important challenge.

The aim of this doctoral project was to investigate hormones and phenolic compounds as suspected EDCs in drinking waters across the Romagna area (Italy). The main objectives were (i) to assess the occurrence of considered contaminants in source water and drinking water from three DWTPs, (ii) to characterize the effectiveness of removal by different water treatment processes, and (iii) to evaluate the potential biological impact on drinking water and human health. Specifically, a complementary approach of target chemical analysis and effect-based methods was adopted to explore drinking water quality, treatment efficacy, and biological potential. Additionally, an *in vitro* investigation was conducted to compare the potential impact and mechanism of action of bisphenol A (BPA) and its analogue bisphenol S on a trophoblast cell line.

Chemical analysis through HPLC-MS/MS revealed that, among the tested micropollutants, nonylphenol (NP) prevailed in all the samples, followed by BPA. Sporadic contamination of natural estrogens was found only in source waters, whereas 17 α -ethynilestradiol was never detected. Although the measured EDC concentrations in drinking water did not exceed the threshold guideline values, emphasizing the high quality of produced water, attention should be drawn to the potential role of DWTP as an extra source of EDC contamination. A significant increase in BPA and NP levels was observed during pre-chlorination, flocculation, and ultrafiltration steps, thus the final quality of the water relied only on the last step of the DWTP. This contribution was also reflected by estrogenic and mutagenic responses detected in water samples after ultrafiltration. This highlights the need to monitor the water also during various treatment processes to improve the efficiency of DWTPs. The biological assessments on finished water did not reveal any bioactivity, except for few treated water samples that exhibited estrogenic responses. These effects were far below the available effect-based trigger values and in general agreement with the current scientific consensus on endocrine effects in drinking water. Overall, data emphasize the high quality of the produced drinking water and the value of applying integrated chemical analysis and *in vitro* bioassays for water quality assessment.

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Structure of the doctoral thesis

This thesis is written in a manuscript-style format and divided into seven chapters. **Chapter One** is a general introduction and literature review of emerging contaminants, more specifically endocrine-disrupting chemicals (EDCs), their occurrence and sources, known effects on human health. It also includes a description of the common water treatment processes applied by drinking water treatment plants (DWTPs) and an overview of the overall efficiency removal of EDCs. Background literature reported that many EDCs are ubiquitous in the environment including drinking water, and a low concentration severely impacts human health. Although water treatment techniques improve the water quality intended for human consumption, they are not designed to remove EDCs efficiently. (This chapter will be part of a review article in preparation).

Chapter Two provides a brief introduction to the analytical approaches used in this doctoral dissertation, by first discussing the context and research problem of chemical monitoring, its limitation, and the significance to implement biological screening. Finally, it provides the research aims and the overall objectives this thesis set out to address.

Chapters Three, Four, Five, and Six are organized as manuscripts for publication in peer-reviewed scientific journals, performed during the PhD project. As a consequence, there may be some repetition between the introduction, materials and methods, results, and discussion sections in each chapter. The above scientific articles are reported in their original version, but adapted to the text format of this work. Specifically:

Chapter Three: Contaminants of emerging concern in drinking water: Quality assessment by combining chemical and biological analysis. Valbonesi P., **Profita M.**, Vasumini I., Fabbri E. (2021). *Science of Total Environment* 758, 143624. <https://doi.org/10.1016/j.scitotenv.2020.143624>

Chapter Four: A three-year screening of suspected EDCs in three Italian drinking water systems combining chemical and effect-based tools. **Profita M.**, Valbonesi P., Vasumini I., Fabbri E. (manuscript in preparation).

Chapter Five: Effect-based screening for water quality and treatment efficiency of an Italian drinking water treatment plant. **Profita M.**, Schiwy A., Wollenweber S., Hollert H. (manuscript in preparation).

Chapter Six: Comparing effects and action mechanisms of BPA and BPS on HTR-8/SVneo placental cells. **Profita M.**, Fabbri, E., Spisni, E., Valbonesi, P., (2021). *Biology of Reproduction* 105, 1355–1364. <https://doi.org/10.1093/biolre/ioab139>.

Chapter Seven Overall conclusion and future research needs.

Chapter One

Introduction

1. Contaminants of emerging concern

Water is an essential resource for life and its quality represents a crucial component that can negatively/positively affect human and ecosystem health. The uncontrollable growth of anthropogenic activities has progressively led to alterations in the natural environment, with a global increase in water contamination. The increasing variety of human-made substances that have lately been discovered in water is known as contaminants of emerging concern (CECs) (Khan et al., 2022). “Emerging” is referred both new chemicals and existing chemicals or often unregulated and potentially hazardous compounds that are only now being detected in the environment (Glassmeyer et al., 2017; Lei et al., 2015). CECs belong to a large variety of chemicals and are characterized based on their chemical structure and mode of toxicity to humans and environment (Yadav et al., 2021). They include a divergent group of chemicals such as persistent organic pollutants, pharmaceuticals, personal care products, endocrine-disrupting chemicals (EDCs), their metabolites, and their transformation products, which enter, disseminate and persist in the environment (Mukhopadhyay et al., 2022). EDCs are among the most frequently detected anthropogenic contaminants in water (Kim and Zoh, 2016). The World Health Organization (WHO) defines EDC as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” and a potential EDC as “an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations” (WHO, 2002). Previously, the Environmental Protection Agency (EPA) defined EDCs as external agents that interfere with the formation, release, transport, attachment, activity, or displacement of the body's natural hormones that maintain homeostasis, development, reproduction, and behavior (EPA, 1997). Although their definitions vary widely, EDCs are commonly known to disrupt the endocrine system. There are three main classes of EDCs: estrogenic compounds (that mimic or block natural estrogens), androgenic compounds (that mimic or block natural androgens), and thyroidal compounds (with direct or indirect impacts on the thyroid and/or thyroid hormone-dependent pathways) (Snyder et al., 2003). Nevertheless, EDCs are also suspected to interfere with further pathways such as G protein receptor related pathways and insulin/glucagon pathways (Stahlhut et al., 2018; Ropero et al., 2008), and more

studies need to be conducted at this regard. EDCs are found in everyday products, including plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides.

The group of molecules identified as EDCs can be divided into:

- Synthetic chemicals from anthropogenic activities (i.e., 17 α -ethinylestradiol (EE2), pesticides, phthalates, alkylphenol ethoxylate surfactants, nonylphenol (NP), dioxins, coplanar polychlorinated biphenyls, parabens hydroxybenzoate derivatives, bisphenol A (BPA), polycyclic aromatic hydrocarbons, organotins),
- Natural substances derived from humans, animals, and plants (e.g., estrone (E1), 17 β -estradiol (E2), estriol, natural androgens, phytosterols, and isoflavonoids) (Pironti et al., 2021) (**Figure 1.1**).

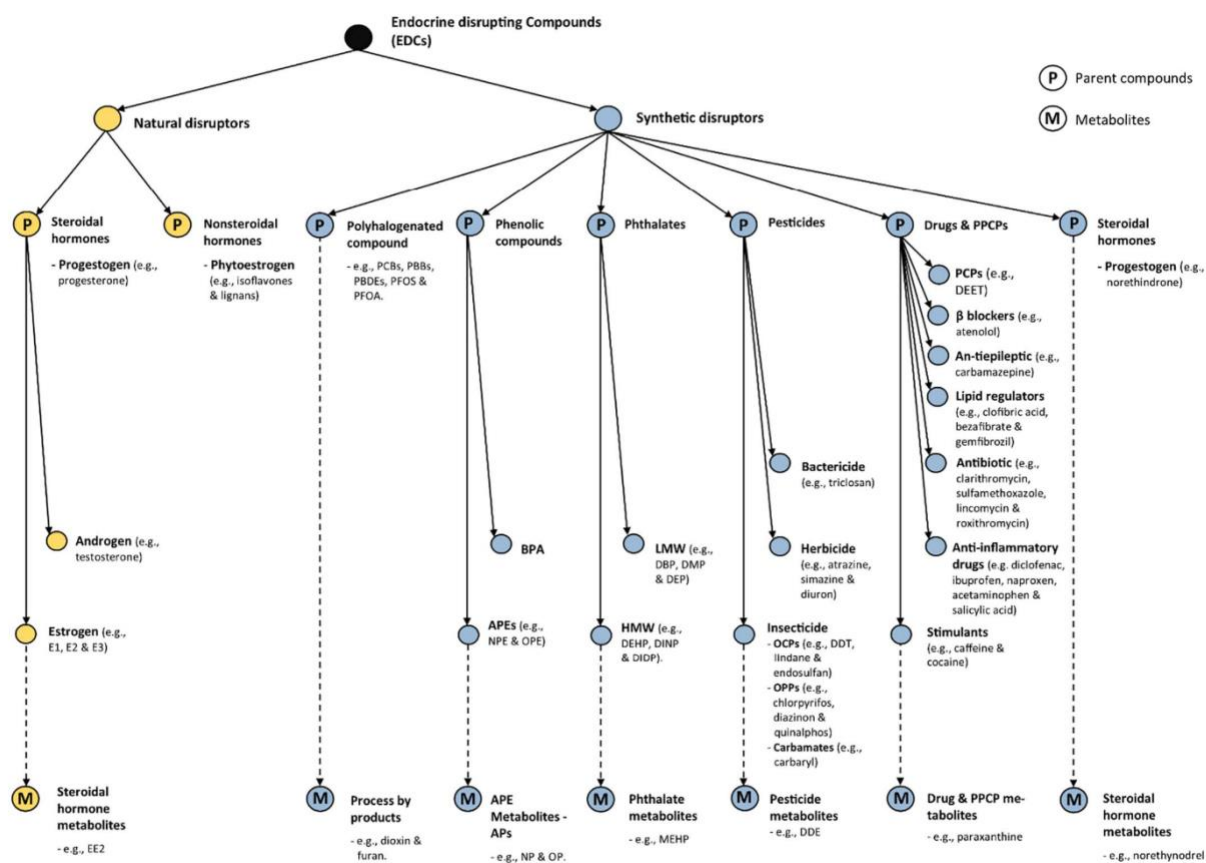


Figure 1.1: Hierarchy of EDCs (source: Wee e Aris, 2017).

The most recently developed analytical techniques can detect these contaminants at levels down to parts per trillion (nanogram/liter range; ng/L), which is equivalent to a drop of water in 20 olympic-sized swimming pools. Although most of these concentrations are far below the safety threshold level

expected to cause adverse effects, a small amount of some of these chemicals has the potential to have noticeable effects on human health (Escher et al., 2021).

1.1. EDCs exposure and human health impact

The endocrine system plays a major role in human survival as it influences a broad assortment of biological activities necessary for life. Hormones produced in the endocrine glands reach specific target organs through the circulatory system, acting as messengers to regulate specific physiological processes, including metabolism, growth and development, reproductive organ functions, immunity and behavior. Generally, hormones act by binding to nuclear-, cytosolic-, or cellular membrane receptors; the hormone-receptor complex carries out the hormone's instructions for specific biological processes in cells, tissues and organs (Leblebicioglu et al., 2013). The endocrine system can be modulated in two basic ways: 1) by agonists or antagonists of the respective receptors, and 2) by interference with hormone biosynthesis and metabolism (Autrup et al., 2020). Typically, hormones are present in the blood at physiologically functional concentrations and have both high affinities and high efficacies, thus working at extremely low doses (within and below the picomolar range). For example, the typical physiological levels of E2 are in the range of 10 – 900 pg/ml (Vandenberg et al., 2012). EDCs and potential EDCs may interfere with natural hormone systems, by mimicking or antagonizing the actions of naturally occurring hormones (Monneret, 2017). They are likely to have biological effects at the low levels documented for several contaminants (Varticovski et al., 2022). For example, the so-called weak estrogens like BPA can be equally potent as endogenous hormones in some systems, causing biological effects at picomolar levels (Vandenberg et al., 2012). A key characteristic of both endogenous hormone responses and EDC action is their non-linearity and display of non-monotonic dose-response curves (**Figure 1.2**), implying that stronger physiological effects can sometimes be seen at lower doses than at higher doses (Demeneix and Slama, 2019). Thus, EDCs go beyond the traditional “dose makes the poison” framework.

Non-Monotonic Curves

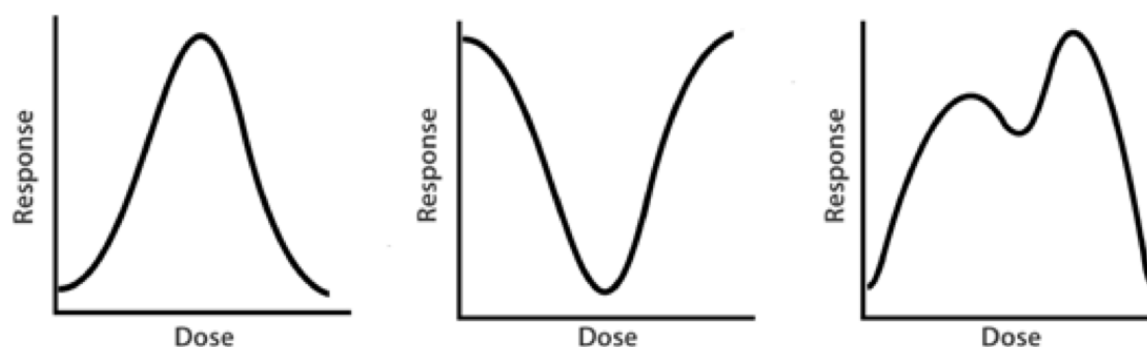


Figure 1.2: Examples of non-monotonic curves (Source: Landfried and Klein, 2017).

Human exposure to EDCs occurs mainly by ingestion and to some extent by inhalation and dermal uptake (Yilmaz et al., 2020). Potential sources of exposure include contaminated food and water, combustion sources, and contaminants in consumer products. Contamination of drinking water with EDCs has physiological consequences by simulating hormones or interfering with their production, release, and metabolic pathways (Kumawat et al., 2022). Sources of human exposure to EDCs have also been identified in indoor environments and air (Goralczyk, 2021; Annamalai and Namasivayam, 2015). When they enter the body, EDCs exert toxic effects by interfering with the physiology, synthesis, action, and metabolism of hormones which in turn causes developmental and fertility impairments (Yilmaz et al., 2020). Studies have shown that EDCs are found in the human body in the adipose tissue, as well as in body fluids, such as urine, amniotic fluid, serum, and breast milk (Xu et al., 2022; Chmielewski et al., 2021; Tang et al., 2020; Lee et al., 2018; Karalius et al., 2014). Many EDCs display estrogenic activity and interfere with normal estrogen signaling, which is mediated by estrogen receptors (ERs) (Shanle and Xu, 2011). Some EDCs, such as alkylphenols, BPA, dioxins, furans, heavy metals, and halogenated hydrocarbons, can cross the cell membrane and bind directly to intracellular ERs, either activating them by acting as agonists, or inhibiting them by acting as antagonists (Encarnação et al., 2019). EDCs have been associated with multiple reproductive disorders, such as infertility, endometriosis, polycystic ovarian syndrome, poor semen quality, and ovarian and testicular cancers (Gonsioroski et al., 2020; Piazza and Urbanetz, 2019; Sifakis et al., 2017; Gore, 2016; Giulivo et al., 2016). EDCs can alter developmental patterns and even cause some dangerous and hereditary diseases, including but not limited to cancer and cardiovascular diseases (Chen et al., 2022). Several compounds may also have a negative impact on energy metabolic homeostasis altering adipose tissue and promoting obesity, metabolic syndrome, and diabetes (Lauretta et al., 2019). Increasing evidence suggests that exposure to EDCs might be associated with

gestational diabetes, impaired glucose tolerance, obesity, and that these chemicals could be linked to type 2 diabetes (Kahn et al., 2020). New evidence supports the role of EDCs as possible carcinogens in breasts, prostate, thyroid, and ovaries (Modica et al., 2022). Industrial chemicals are recognized as the cause of neurodevelopmental disorders and subclinical brain dysfunction (Santoro et al., 2019; Street et al., 2018; Braun, 2017). Specific adverse health effects for selected EDCs will also be provided in further reading.

1.2. Source of EDCs in water

As a result of their extensive use, EDCs and their metabolites are continually discharged into the natural environment. It is observed that EDCs are present in the air that we breathe, the water that we drink, and even in the soil in which our food is cultivated (Kabir et al., 2015). EDCs in water bodies were first discovered as natural estrogens in wastewater treatment plant (WWTP) effluents in the United States in 1965 (Stumm-Zollinger and Fair, 1965). EDCs can enter the waterways through direct discharge from industries, municipal and home sewage, landfills, and farm effluents (Ismanto et al., 2022). Another possible source of emerging contaminants in the environment is stormwater runoff. Precipitation leaches contaminants from buildings, streets, land surfaces, or improperly disposed waste, and transports them into surface waters (Pironti et al., 2021). Runoff from agricultural areas is also a source of EDCs, particularly pesticides, antibiotics from animal feed processes, and steroids hormones from animals and livestock (Horak et al., 2021; Ismail et al., 2017). Once in the water body, EDCs undergo natural attenuation processes such as dilution, sorption, volatilization, and chemical or biological degradation. The fate and attenuation of contaminants in water often depend on the properties of each individual compound, each individual river or aquatic system, biotic and abiotic contaminant degradation, and its partition into solid components of the aquatic environment (Wilkinson et al., 2017). The reduction in the concentration of some EDCs is marked during the wet season, whereby the reduced occurrence of particular EDCs was due to elevated dilution during the wet season and the promotion of biodegradation at the warmer temperature of the wet season (Wee and Aris, 2017). Water discarded by municipal wastewater, industrial wastewater, stormwater runoff, rural runoff, and manure is collected by WWTPs to be treated and discharged into surface waters (Pal et al., 2014). However, wastewater effluents remain an important primary source of emissions for EDCs in the environment, as conventional treatment technologies are ineffective and insufficient for removing pollutants (Puri et al., 2022). Surface water and groundwater are the main sources of raw water intake by drinking water treatment plants (DWTPs) for the treatment and subsequent supply of drinking water in most countries (Katsanou and Karapanagioti, 2017). Reservoirs often serve as sources of water for drinking water treatment. Therefore, micropollutants in surface waters can enter

DWTPs, and their presence in drinking water can unconsciously expose consumers to them through tap water used for drinking, cooking, and bathing (Kim and Zoh, 2016). The occurrence of EDCs in water bodies is not merely due to the negative removal of treatment plants but also the dynamic sources of contaminants. Piping systems, specifically those with epoxy coating, are the main contributors to EDC loading in drinking water supplies (Wee and Aris, 2017). The leaching of EDCs from plastic materials has been shown to contribute to water contamination (Cantoni et al., 2021; Rajasärkkä et al., 2016).

1.3. EDCs and water quality legislation

In the context of safely managed water, the quality of drinking water supply free from priority chemical contamination, is a global concern. Achieving universal and equitable access to safe and affordable drinking water by 2030 is one of the 17 Sustainable Development Goals (SDG) of the United Nations Agenda (United Nations, 2015), namely SDG 6. Driven by growing concerns regarding EDCs and substantial research outcomes over the last few decades, several countries have developed a series of water quality regulations. The criteria for these pollutants are currently set in a limited number of countries, although the water quality standards are quite different. This suggests that the regulations for EDCs in drinking water are country-specific, without shared guidelines, revealing the lack of a solid scientific understanding of safe EDC concentrations for drinking water. The regulatory process must not be just at the national level; instead, a global agreement must be the goal, as some EDCs do not respect barriers or frontiers (Encarnação et al., 2019). Regulation of the maximum permissible limits of EDCs in drinking water is limited to only a few countries or regions through their environmental regulatory agencies. The EU, USA, UK, Australia, China, Israel, Japan, Korea, New Zealand, Oman, Philippines, Singapore, and United Arab Emirates have regulated drinking water standards for selected EDCs (E2, EE2, BPA, NP, di-2-(ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), di-butyl phthalate (DBP), di-ethyl phthalate (DEP), di-2-(ethylhexyl) adipate (DEHA), and per- and poly-fluorinated alkyl substances (PFAS)) (Puri et al., 2022). In response to the WHO guidelines for drinking water quality, the Ministry of Health, Labour and Welfare of Japan created a special committee to set new standards for all items that have the potential to cause adverse effects on human health. Currently, many EDCs are included in the drinking water regulations in Japan. However, comprehensive data on water quality in Asia are difficult to obtain. Chinese regulatory agencies have reinforced water quality measures, including BPA, in the relevant drinking water legislation, and NP to the list of restricted substances (Acir and Guenther, 2018). In the EU, the regulation that sets standard limits for BPA (2.5 µg/L) and PFAS (sum parameter of 0.5 µg/L) is the Drinking Water Directive 2020 (DWD, EU 2020/2184), while E2 and NP have been

included in the first “Watch List” mechanism, with levels of 0.001 µg/L for E2 and 0.3 µg/L for NP. In view of their endocrine-disrupting properties, Environmental Quality Standard (EQS) of 0.000035 µg/L for EE2 and 0.0036 µg/L for E1 have been established for surface water (EU WFD 2000/60/EC). Furthermore, BPA and NP already belong to Category 1 of the Endocrine Disrupter Priority List (2008/105/EC) for wildlife and human health, and NP is listed among the 33 priority substances in the Water Framework Directive. Switzerland follows similar regulations for several emerging contaminants (Gogoi et al., 2018). The regulated BPA concentrations in Japan, China, and the EU have been reported to be 100, 10, and 2.5 µg/L, respectively, indicating a significant concentration variation by a factor of 40 (Liu et al., 2021). In the USA, the Environmental Protection Agency (EPA) is responsible for nationwide drinking water quality standards. The US EPA has established Contaminant Candidate Lists (CCLs) for priority drinking water contaminants. Current CCL 5 includes EE2, BPA, NP, and PFAS. Hormones, such as E1, E2, and EE2, were monitored under the Unregulated Contaminant Monitoring Rule (UCMR), whereas monitoring for NP remained in method development (Wee et al., 2022). However, for freshwater, the threshold value for NP should not exceed 6.6 µg/L (USEPA, 2009). Furthermore, the Minnesota Department of Health has recently established drinking water advisories of 20 µg/L for BPA and NP, and 0.0002 µg/L for EE2. For surface waters of Canada, the Federal Environmental Quality Guideline sets a value of 3.5 µg/L for BPA and 1 µg/L for NP. In Latin America, the lack of regulations allows an increased and unregulated discharge of emerging pollutants, representing an important risk to environmental and public health impacts (Vargas-Berrones et al., 2020). Regulatory information from Africa is difficult to obtain, and it appears that only two African countries, Kenya and South Africa, have adopted environmental regulations (K’oreje et al., 2020). However, no information on EDC regulations is currently available. All parts of the water-related legislation are closely interconnected because of the water cycle: surface water quality is directly influenced by wastewater quality and, in turn, has a direct impact on the quality of drinking water. However, even if significant advances have been accomplished, chemical pollution remains a substantial risk in at least half of the countries monitored. In fact, currently available information is insufficient to determine the scientific basis of how these countries have adopted such significantly distinct regulated concentrations of EDCs.

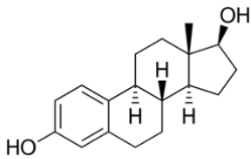
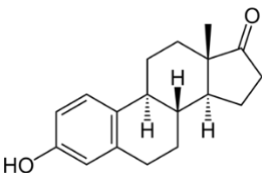
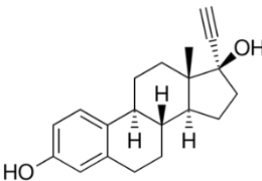
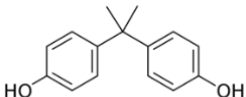
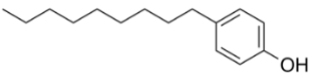
2. EDCs selected for this study

Over the past few decades, a multitude of new substances has been identified in aquatic environments, which has caused increasing concern regarding the world’s water supply. The US Food and Drug Administration has identified more than 1800 chemicals that disrupt at least one of the three endocrine pathways cited above. Over 800 EDCs are of great concern because of their “hormone-like action”,

which can alter cellular, molecular, and epigenetic functions of the endocrine system (Predieri et al., 2022). More than 300 chemicals screened according to the instructions of the European Commission showed evidence or potential evidence of endocrine disruption (Kahn et al., 2020).

For the purpose of the PhD project, this work focused on selected representative EDCs of global environmental concern: hormones such as E2, E1, and EE2, which have the highest estrogenic activity, in line with their main physiological function; and phenolic compounds such as NP and BPA, which have a much lower affinity for the estrogen receptors (ER) compared to estrogens but are generally detected in surface waters at a higher concentration than steroid hormones. The suspected EDCs selected for this study are listed in **Table 1.1**.

Table 1.1. Suspected EDCs, their characterization, and associated human health effects.

Suspected EDCs	Structure	Molecular Weight (g/mol)	Use	Human health impacts
17β-estradiol (E2)		272.4	Hormone replacement therapy	Cancer, immune and reproductive system disorders
Estrone (E1)		270.4	Hormone replacement therapy	Cancer, immune and reproductive system disorders
17α-ethinylestradiol (EE2)		296.4	Contraceptives (Synthetic steroid)	Impairs fetal growth, reproductive system disorders
Bisphenol A (BPA)		228.3	Plasticizers	Reproductive, metabolic, neurotoxic, estrogenic effects
Nonylphenol (NP)		220.3	Detergents and surfactants	Neuroimmune, reproductive, and estrogenic effects

2.1. Hormones

Estrogens are essential to human physiology (Nilsson and Gustafsson, 2011). They help regulate reproduction, cardiovascular function, bone strength, cognitive behavior, successful pregnancy, and the gastrointestinal system. E2 in particular is an alert hormone recruiting or recruited by other surveillance components that are essential for homeostasis (Patel et al., 2018). Natural hormones E1 and E2 and the synthetic hormone EE2 are used in pharmaceuticals in the hormone replacement therapy, to treat infertility, and as components of birth control pills. However, the occurrence of estrogens in water sources has become an emerging and serious concern, especially because of their increased release into the environment and their effects on human health. It is known that the primary sources of environmental estrogens are urine excretion and effluent from livestock feedlots (Du et al., 2020). These compounds reach rivers and estuaries either through the discharge of effluents from sewage treatment plants or directly from sewage that delivers their contents into waterways (Rocha and Rocha, 2015). In the environment, estrogens have a relatively short half-life, in the range of 0.2 – 9 days at 20°C for E2 and E1 (Benijts et al., 2002), and 4 - 6 days for EE2 in river waters (Ying et al., 2002). Generally, the environmental fate of these chemicals is vast and depends largely on their unique physicochemical properties. Their solubility in water depends on the temperature and pH (Varticovski et al., 2022). The synthetic steroid EE2 has lower solubility than natural hormones; therefore, it is reported to remain in the environment for longer periods, and it can be detected up to 100 km from the source along the river (Seibert et al., 2020). Some studies have reported that estrogens that contaminate surface waters can increase the risk of cancer and induce cardiovascular diseases in humans (Treviño et al., 2015; Wocławek-Potocka et al., 2013). Estrogens in food and water can also induce premature menopause and affect reproductive development (Adeel et al., 2017). High or low estrogen levels have been linked to chronic and acute diseases, including various types of cancer in females and males, disorders of the reproductive system, obesity, and diabetes (Patel et al., 2018). In addition to the information available on the relationship between estrogens and adverse reproductive outcomes, the role of estrogen contamination in different ecosystems is not well understood, yet. On this basis, the Joint FAO/WHO Expert Committee on Food Additives has established an acceptable daily intake of 0 – 50 ng/kg/body-weight (bw). This evaluation considers a no-observed-adverse-effect-level (NOAEL) of 0.3 mg/day (equivalent to 5 µg/kg bw/day) (JECFA, 2000). Thus, it is crucial to ensure that estrogens from both human and animal wastes are not consumed in food and water at levels above the accepted NOELs.

2.2. Phenolic compounds

2.2.1. Bisphenol A (BPA)

BPA, also known as 2,2-(4,4'-dihydroxy- phenyl) propane, is an organic synthetic chemical mainly used in the manufacture of polycarbonate, epoxy resins, and the paper industry. Even food and beverage cans, medical equipment, baby bottles, toys, and thermal receipts contain it. This compound can be found as colorless crystals or as a powder and can be released by plastic products into foods and drinks as a result of heating and acid or basic conditions (Cimmino et al., 2020). In fact, additives used in polycarbonate plastics are not 100% polymerized, consequently, they can easily leach out of the products into the environment (Encarnaç o et al., 2019). BPA is moderately soluble due to its two non-polar methyl groups (Puri et al., 2022). It is ubiquitous in aquatic environments and has been detected in rivers, groundwater, effluents from treatment plants (Gonsioroski et al., 2020), and bottled mineral water (da Silva Costa et al., 2021). One important source of human exposure to BPA is the diet, which may be associated with several chronic human disorders (Kumawat et al., 2022). The daily intake of BPA in humans is estimated to be approximately 1 µg/kg (Shanle and Xu, 2011). BPA has been reported to cause health impacts in humans, even at very low concentrations (Abraham and Chakraborty, 2020). It has been detected in 92.6% of the people tested in the US (Yilmaz et al., 2020). Owing to its hormone-like properties, BPA may bind to a significant number of hormone receptors, including nuclear and membrane-bound estrogen, androgen, thyroid, glucocorticoid, and peroxisome proliferator-activated receptors (PPARs) (MacKay and Abizaid, 2018). BPA can negatively affect several estrogen-related targets such as the thyroid, adipose tissue, liver, heart, and female reproductive system (Cimmino et al., 2020), and has been associated with the development of type-2 diabetes, obesity, cardiovascular diseases, breast and ovarian cancer, and altered liver function (Catenza et al., 2021; Dumitrascu et al., 2020; Sowlat et al., 2016). It may also affect metabolism and cancer progression, and impair male reproductive function by binding to androgen receptors (M n guez-Alarc n et al., 2016). BPA also induces oxidative stress (Zhang and Liu, 2020), and interferes with neuronal activity (Santoro et al., 2019). It also induces pro-carcinogenic signaling pathways (Khan et al., 2021). BPA has been detected in the human cord blood, serum, follicular fluid, placental tissue, and urine samples (Jalal et al., 2017), and a correlation between BPA and pregnancy complications has been reported (Pergialiotis et al., 2018). Recent evidence suggests that BPA affects the proliferation and migration of trophoblast cells (see Chapter Six), which are essential during the first-trimester of pregnancy. Given the potential risk to human health, the European Food Safety Authority (EFSA) in 2023 determined the reference dose for external oral exposure to BPA in humans

as 0.2 ng/kg/day (EFSA, 2023), replacing the previous temporary level of 4 µg/kg/day. The new tolerable daily intake is around 20,000 times lower than before.

2.2.2. Nonylphenol (NP)

NP is used as a formulant in pesticides, as a lubricating oil additive, as a catalyst in epoxy resin curing, in industrial laundries, and in the past was also used to produce nonylphenol ethoxylates (NPEs) for consumer products (e.g., surfactants, detergents, wetting agents, dispersants, defoamers, de-inkers, and antistatic agents) (Careghini et al., 2015). Its occurrence in the aqueous environment originates predominantly from the degradation of alkylphenol ethoxylates, which are used as nonionic surfactants in cleaning agents, disinfectants, and pesticide formulations. (Amiridou and Voutsas, 2011). NP has been detected in surface water, rivers, sewage sludge and effluents, sediments, and estuaries (Jie et al., 2017). Approximately 60% of NP and its derivatives manufactured worldwide are introduced into water supplies (Bhandari et al., 2021). Owing to its structural similarity with E2, NP can interact with the ER, altering estrogen-dependent signaling pathways (Soares et al., 2008). Most studies agree that cancer is one of the main outcomes, with positive associations between NP exposure and ovarian, uterine, pituitary, and testicular cancers (Noorimotlagh et al., 2020). Chronic exposure to NP may interfere with male and female reproductive systems, and induce oxidative stress and damage to the brain (Acir and Guenther, 2018). A recent finding indicated that NP may disrupt the decidualization of endometrial stromal cells and eventually affect the reproductive health of women (Ko et al., 2019). It has also been reported that NP may induce apoptosis, DNA damage, and other endocrine-related diseases through the production of reactive oxygen/nitrogen species (Noorimotlagh et al., 2018). Available *in vitro* and *in vivo* evidence suggests that NP exposure is associated with cognitive dysfunction, including depression- and anxiety-like behaviors, impaired learning and memory, inflammation, damage to synaptic plasticity, and interference with important signaling pathways that affect the synthesis and secretion of neurotransmitters (Lu et al., 2021).

3. Drinking Water Treatment Plants (DWTPs)

The treatment of drinking water is an ancient practice (Poleneni, 2020). Originally, water treatment focused on improving the aesthetic quality of water. Ancient Sanskrit and Greek writings recommended water treatment methods such as filtering through charcoal, exposing it to sunlight, boiling, and straining (Tischler, 2007). To clarify water, Egyptians reportedly used chemical alum to cause suspended particles to settle out of water (Poleneni, 2020). During the 1700s, filtration was established as an effective means of removing particles from water, and by the early 1800s, slow sand filtration began to be used regularly in Europe (U.S. EPA, 2000). During this period, scientists

discovered that turbidity was not only an aesthetic problem; particles in source water, such as fecal matter, could harbor pathogens. Thus, for the first time in 1853, at the International Congress of Hygienists in Brussels, an attempt was made to establish a standard for drinking water (Goncharuk, 2010). Further development of civilization required the creation of civilized systems of water supply and a complex of water disposal facilities. Consequently, the first DWTPs were established. By the early 1900s water treatment had moved beyond esthetic problems and into the realm of pathogens. In 1908, chlorine was used for the first time as a primary disinfectant for drinking water in the U.S. (Tischler, 2007). Conventional drinking water treatment for surface water sources involves coagulation, flocculation, sedimentation, sand filtration, and chlorine disinfection. Powdered and granular activated carbons have applications for taste and odor control, whereas ozone and membranes are in their early stages (Cotruvo, 2016).

Currently, DWTPs are constantly improved both in terms of easy operation and functionality to provide the best treatment efficiency; therefore the treatment processes are determined by prioritizing water quality in a more prominent way based on the type of water source to treat (Alver, 2019). The choice of water treatment depends on the characteristics of the water, the types of water quality problems, the costs of different treatments, and the regulations imposed in each country.

3.1. Drinking water treatment processes

Water treatment is a combination of processes that often include chemical, physical, and biological methods. Preliminary, main, secondary, tertiary and/or advanced water treatments are the most widely applied for drinking water treatment worldwide. In general, a combination of processes (**Figure 1.3**) is as follows:

Preliminary treatment: gross solid and grit are removed, and sometimes oil and grease are also removed if they are present in large amounts.

Main (primary) treatment:

- Coagulation: is often the first step in the water treatment process. Coagulation involves neutralizing the charges of colloidal materials and then forming a gelatinous mass to trap (or bridge) particles, thus forming a mass large enough to settle or be trapped in the filter (Engelhardt, 2010). The reaction occurs when a chemical, also known as a coagulant, is added to water. Powdered, granular or crystalline salts and solutions of iron and aluminum such as hydrated aluminum sulfate, liquid alum, ferric sulfate, and ferric chloride or polymers are widely used as coagulants. These chemicals have a positive charge that neutralizes the negative charges of particles in water. The reaction forms larger particles called flocs.

- **Flocculation:** Flocculation follows coagulation. When coagulants are added to water, gentle mixing occurs to favour the collision of particles and the gel to form a larger mass that will be settled or filtered from solutions (WHO, 1996). Additional chemicals may be added during this step to aid in the formation of the flocs. Both coagulation and flocculation are effective at removing fine, suspended particles that attract and hold bacteria and viruses on their surface (LeChevallier and Au, 2004). This mixing is often performed in a series of chambers.
- **Sedimentation:** is the final part of the flocculation. As flocs are formed, the larger size and weight of the flocs cause large particles to settle rapidly. These particles settle because they are heavier than the water. Some particles spontaneously settle at the bottom of the water supply.

Secondary treatment:

- **Filtration:** Filtration occurs as the water passes through filters that help to remove even smaller particles. This process usually removes fine suspended solid matter, as well as other particles, such as larger microorganisms. The filtration system consists of filters with varying pore sizes, and is often composed of sand, gravel, and charcoal. One of the oldest and simplest processes used to treat water is to pass it through a bed of fine particles, generally sand (Pakharuddin et al., 2021). The development of new synthetic materials has led to the application of a new range of filter materials and methods. In membrane filtration, water is filtered through tiny holes (pores) in the membrane wall rather than through a sand bed. The smaller the pore size, the more material is held by the membrane as water passes through. Two types of systems can be distinguished in membrane technology for drinking water treatment: low-pressure (microfiltration and ultrafiltration) and high-pressure (nanofiltration and reverse osmosis) systems (Hofman et al., 2004). Both types result in excellent water quality. Among the different types of membrane filtration processes, microfiltration, ultrafiltration and nanofiltration are the most widely used in water treatment (LeChevallier and Au, 2004).

Tertiary treatment:

- **Adsorption:** While coagulation, often combined with filtration, removes most of the troublesome contaminants from water, adsorption is used in drinking water treatment mainly to remove compounds that cause objectionable taste, odor, or color. This involves removing dissolved substances by chemically or physically binding them to the filter material. Many materials have been developed as adsorbents, including activated carbon, carbon nanotubes, graphene, fullerene, and zeolites (Chen et al., 2022). Specialized adsorbent materials such as activated carbon and ion exchange resins are used to remove certain soluble contaminants from water (Yargeau, 2012). Activated carbon is the absorbent that is by far the most widely

used in water treatment, as a powdered feed, powder activated carbon (PAC), or granular forms, granular activated carbon (GAC) (Azizi et al., 2022).

- **Disinfection:** Disinfection is an important step in ensuring that water is safe to consume, and its main objective is to control disease-causing microorganisms by killing or inactivating them (Sorlini et al., 2015). Without disinfection, the risk of waterborne diseases increases. The two most common disinfection methods include chlorination with chlorine dioxide, chloramines, oxidation with ozone, or irradiation with ultraviolet (UV) radiation.

Advanced treatment:

- **Advanced oxidation process (AOP):** Oxidation with chemicals, such as ozone or chlorine dioxide, is a common treatment technology in Europe. Strongly reactive chemicals such as ozone, are used to disinfect water and destroy soluble contaminants, such as algal toxins, taste and odor compounds. The three most common advanced oxidation technologies are Fenton, Ozonation, and Photocatalysis (Chen et al., 2022).

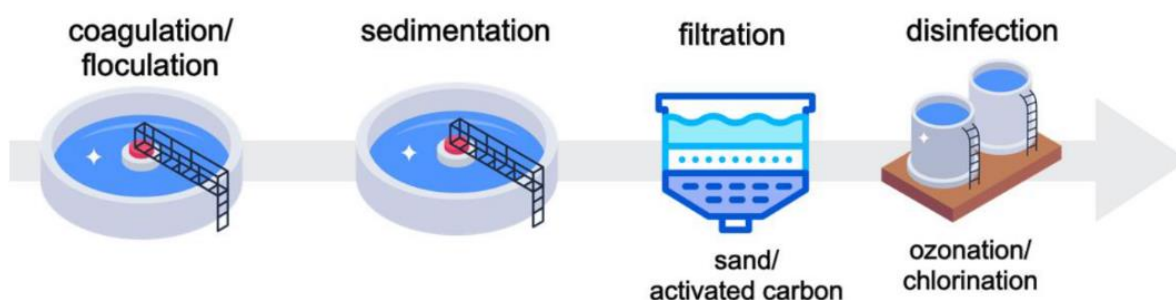


Figure 1.3: Schematic representation of the conventional DWTP (source: Papagiannaki et al., 2022).

3.2. Removal of EDCs during water treatment

Contaminants in drinking water may originate from source water entering the DWT facility, during drinking water treatment, and/or upon post-drinking water treatment, for instance, chemicals leached by materials in contact with drinking water (Cantoni et al., 2021; Cheng et al., 2016). The frequent detection and wide distribution of EDCs in the environment is of great concern. Efficient removal of EDC in DWTPs is a matter of growing interest when relatively high concentrations of EDCs in drinking water sources are discharged to the influents of DWTPs (Wee and Aris, 2017). Conventional WTP steps, such as coagulation, flocculation, oxidation, and disinfection, are known to be effective in treating surface water by eliminating microbial contaminants and the majority of chemical compounds (Katibi et al., 2021). However, their applications are not sufficiently effective to remove EDCs (Gou et al., 2016; Muhamad et al., 2016a; Chang et al., 2009).

Advanced treatment processes, such as adsorption, membrane filtration, and ion exchange, have demonstrated high EDC removal efficiencies (Chang et al., 2009). Integrated systems using GAC and/or membrane processes have been shown to remove hydrophilic and persistent EDCs (Nguyen et al., 2012). In the case of membrane filtration, it was reported that the nanofiltration membrane retained more EDCs than the ultrafiltration membrane, implying that retention is affected by membrane pore size (Yoon et al., 2006) and the physical nature of separation and membrane design (Werkneh et al., 2022). Furthermore, membrane separation is suitable for drinking water treatment because it does not utilize chemicals. Unlike biodegradation and chemical oxidation, which tend to create by-products or new metabolites, membrane filtration is environment friendly (Muhamad et al., 2016a).

Another widely used and potentially promising process for EDC removal is adsorption using activated carbon systems, which is highly effective for removing a wide number of target chemicals (Zamri et al., 2021). However, it has been reported that PAC is not an acceptable alternative for removing EDCs, with only 25 – 40% efficiency in the removal of BPA and NP. In comparison, GAC in combination with coagulation, ozonation, and/or ceramic membrane ultra-filtration was reported to be the most efficient (98%) (Surana et al., 2022).

Innovative water treatment technologies include AOPs. The most common AOPs used for drinking water treatment are ozonation and UV-based processes (Papagiannaki et al., 2022). However, despite the effectiveness of EDCs removal, the application of these advanced processes is a critical issue because of the potential formation of by-products and intermediates with unknown properties and harmful impacts.

Overall, updating DWTPs using a combination of conventional and innovative technologies can considerably reduce the occurrence of harmful substances in drinking water. There are many chemical inputs that can pollute drinking water, such as compounds originating from substances or reacting with substances in the water networks, chemicals that accumulate and migrate from deposits and scales, and compounds entering the water networks through defects and fractures (Mohammadi et al., 2022). The chemical and physical characteristics of DWTP materials could strongly affect water quality. Recent studies have reported that the materials used in the actual construction of water utility pipelines can contribute to EDC contaminations of water (Qi et al., 2022; Yan et al., 2022). Epoxy resins used for pipelining contain BPA, thus leaching from such materials is a potential source of BPA contamination in drinking water (Cantoni et al., 2021; Rajasärkkä et al., 2016). Similarly, NP (used as heat stabilizers for polyvinyl chloride (PVC) pipes) can be released into drinking water from household equipment, and may cause an increase in NP concentration in tap water (Jie et al., 2017). Both NP and BPA concentrations increased with contact time in PVC pipes, and the concentration

profiles during retention appeared to be more strongly affected by temperature (Cheng et al., 2016). However, the migration of these contaminants from pipes to drinking water and their health risk has not been fully investigated. Therefore, more studies are needed to measure EDCs, their occurrence, and their quantities in actual DWTPs, better understand their removal by treatment processes, and improve knowledge of the processes that can release these contaminants from pipelines into water. Thus, further research on these contaminants in drinking water treatment is required.

Chapter Two

Research approach and objectives of the doctoral study

1. Analytical approaches for EDCs assessment

Traditionally, chemical analysis is used to identify and quantify chemicals for which analytical methodologies, standards, and toxicological information are available. In environmental monitoring, the determination of EDCs is performed using conventional analytical methods such as chromatography techniques. High sensitivity and selectivity of methods are mandatory as these compounds exhibit endocrine activity even at very low levels. Up to now, gas and liquid chromatography coupled with high-efficiency tandem mass spectrometers (GC-MS and HPLC-MS) are recognized as the best analytical tools, achieving detection limits of 0.1 ng/L and showing high selectivity (Locatelli et al., 2016). However, results obtained through chemical analysis are often difficult to evaluate with regard to their potential hazards, as toxicological data of the emerging compounds are often not readily available or test have not been established, yet. Recently, legislation has begun to recognize and subsequently regulate water quality contaminants based on their ED properties, but only a few compounds are presently included in the regulatory frameworks. Furthermore, chemical monitoring does not account for the presence of unknown compounds such as transformation products, untargeted chemicals, or interactions among chemicals (e.g. synergism or antagonism) (Escher et al., 2021). Therefore, analyzing the vast amount of chemicals in complex environmental mixtures using chemical analytical methodologies is extremely difficult and time-consuming, and requires considerable effort and investigation. In other words, we cannot identify what cannot be measured.

Generally, several situations can occur analyzing CECs, such as EDCs, in environmental water samples:

- A contaminant is present at very low concentration, far below the analytical limit of detection (LOD). In some cases, the analytical limits are equal to or above the proposed standard limit values (i.e. EE2) (Kase et al., 2018), thus, this makes difficult the quantification of the contaminants;
- A contaminant is detected above the limit of quantification (LOQ), but there is limited information about health safety risks, thus it is not possible to extrapolate chemical guideline values or standards in the regulatory context to compare with;

- A contaminant can be present in a mixture. Typically, chemicals rarely occur alone in environmental samples, but instead, occur as mixtures. Complex mixtures of chemicals present in water are poorly addressed by individual chemical monitoring (Gómez et al., 2021; Kienzler et al., 2016);
- A contaminant is unknown, or more simply new or existing chemical that have not been recognized, yet.

Moreover, the biological responses induced by exposure to contaminants cannot be identified by chemical analysis. Thus, relying on the quantification of individual chemicals by instrumental analysis can underestimate the potential risks posed by these chemicals and some toxicologically important compounds could be overlooked (Hecker and Giesy, 2011). To overcome some of these limitations, bioanalytical approaches have been developed to supplement instrumental chemical analyses. A major advantage of bioanalytical tools is their ability to detect the toxicity of mixtures of known and unknown compounds, as the co-occurrence of different compounds may lead to additive/synergistic interactions, causing unexpected effects on human health and the environment (Neale et al., 2022; Touseva et al., 2017; Dulio and Pasanen-Kase, 2017; Escher et al., 2015). Therefore, combining effect-based methods (EBMs) with chemical screening tools is the most promising approach to evaluate the occurrence of EDCs and their overall toxic potential in drinking water samples (Könemann et al., 2018). EBMs can detect triggering molecular or cellular toxic events that occur at low environmentally relevant concentrations, often below the detection limits of chemical analysis and whole animal toxicity testing (Leusch and Snyder, 2015).

EBMs are helpful for:

- detecting the effects of mixtures of compounds in water resources and demonstrating their potential to affect aquatic organisms and human health,
- minimizing the risk of overlooking hazardous chemicals, transformation products and chemical mixtures,
- detecting hot spots of contamination for investigative monitoring,
- identifying risk drivers and prioritizing them for management measures,
- linking chemical and ecological status (Brack et al., 2019).

A limitation to the application of bioanalytical tools for regulatory purposes in the water quality field is because there are no effect-based water quality guidelines or trigger values available yet (Escher et al., 2015). Very recently, EBMs have been recommended in the context of the Common Implementation Strategy of the European Water Framework Directive to achieve good status for all

water bodies in Europe (CIS group, 2021). Therefore, effect-based trigger (EBT) values are required to establish limits of maximum tolerable (drinking) water concentrations for biological activity. Recent works developed and proposed EBT values for different types of bioassays (Been et al., 2021; Escher and Neale, 2021; Escher et al., 2018; Brand et al., 2013; Escher et al., 2013), important for water quality monitoring important for monitoring as they define the threshold between acceptable quality and low quality water (Escher and Neale, 2021). However, EBTs represent a newer field of science and are not included in the water regulatory frameworks, yet.

2. Objectives of the doctoral study

The presence of CECs in environmental waters continues to be a concern because of their well-characterized adverse effects on human health, especially when surface water is used as a source for human consumption. Therefore, the detection and removal of contaminating chemicals in DWTPs is an important challenge, because many compounds are ubiquitous and persistent, and not effectively removed by conventional drinking water treatment techniques. Furthermore, limited information is currently available on the concentrations of EDCs in source/treated water and their removal using drinking water treatment technologies.

The main aim of this Doctoral project was to conduct studies on CECs, and more specifically on suspected Endocrine-Disrupting Compounds (EDCs), in drinking waters across the Romagna area (Italy), in order to assess their **occurrence** in source/drinking water, the effectiveness of **removal** by different drinking water treatment techniques, and the potential **biological impact** on human health.

Under this context, the specific objectives were:

- 1) To assess the occurrence of the selected EDCs emanating from effluent water sources and characterize the contamination levels in drinking water. Specifically, to assess estrogens and phenolic compounds in influents and effluents from three main DWTPs serving the whole Romagna area (the southeastern part of the Emilia-Romagna region, a quite highly urbanized area), and managed by the company Romagna Acque Società delle Fonti SpA.
- 2) To determine whether treatment levels and seasonality influence the fate of EDCs and their efficiency of removal during water treatment. Since information on the fate of micropollutants in DWTP is limited and little is known about the effectiveness of the different treatment steps, the efficiency of the most advanced DWTP was investigated along the treatment performed within pre-chlorination, flocculation, and ultrafiltration processes.

- 3) To assess the potential biological activity of EDCs, specifically the potential endocrine and/or mutagenic responses, in finished drinking water evaluated as a mixture.
- 4) To investigate *in vitro* the potential human health impact of BPA.

Combining EBMs and chemical screening tools was the most promising approach adopted to evaluate the occurrence of suspected EDCs and their overall toxicological potential in drinking water samples. The analytical methods implemented for this doctoral research were a Solid Phase Extraction (SPE) process followed by chemical analysis using HPLC-ESI-MS/MS and Effect-based screening.

After the project started, decisions on BPA were taken at the EU level, and from January 2021 the compound has been added to DWD with a health-based parametric value of 2.5 µg/L. Thus, the possible risk of exceedance related to the newly revised regulation is now discussed in the thesis. The other considered substances do not have benchmark values specified in the latest version of DWD or are added to the first “watch list” mechanism (NP and E2).

Chapter Three

PREFACE

The aim of the work reported in this Chapter was to investigate the occurrence of a panel of CECs in surface water at the entrance of DWTPs and in treated water, ready for distribution in the network. This Chapter provides a historical perspective of trace EDC contamination across the Romagna area (Italy), representing a solid basis of the PhD project and highlighting the need to further investigate the occurrence of suspected EDCs in drinking water. Furthermore, this section provides detailed information about analytical methodologies, including sample collection, extraction, and measurement procedures also applied in the next Chapter Four. Based on the results obtained, Chapters Four and Five describe further investigation. This Chapter has been published in “Science of the Total Environment”.

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

1. Introduction

Contaminants of emerging concern (CECs) comprise a vast array of natural and anthropogenic substances introduced only recently in the environment or present for a long time, but only recently recognized as widely occurring and potentially dangerous. Not all of CECs are regulated or included in routine monitoring programs, and 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). 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 (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. Very 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 μ g/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, since co-occurrence of different compounds may lead to additive/synergistic interactions causing unexpected effects on human health and the environment. 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 3.1 shows the panel of CECs evaluated in this study. All non-labeled standards were purchased from Merck Life Science (Milan, Italy). Isotope-labeled compounds used as internal standards were purchased by Cambridge Isotopes Laboratories Inc. (Lab Service Analytica Srl, Anzola dell'Emilia, Bologna, Italy) ($^{13}\text{C}_3$ -Caffeine), CDN Isotopes (Quebec, Canada) (E2-d2 and BPA-d6), Wellington Laboratories Inc. (Guelph, ON, Canada) ($^{13}\text{C}_4$ -PFOA), and Merck Life Science (Ibuprofen-d3). Solvent reagents from Merck Life Science were of LC-MS analytical grade.

Table 3.1. 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)	

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 (**Fig. 3.1**). 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) receives 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, obtains 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 1 L-PE bottles and stored at 4 °C until analysis.

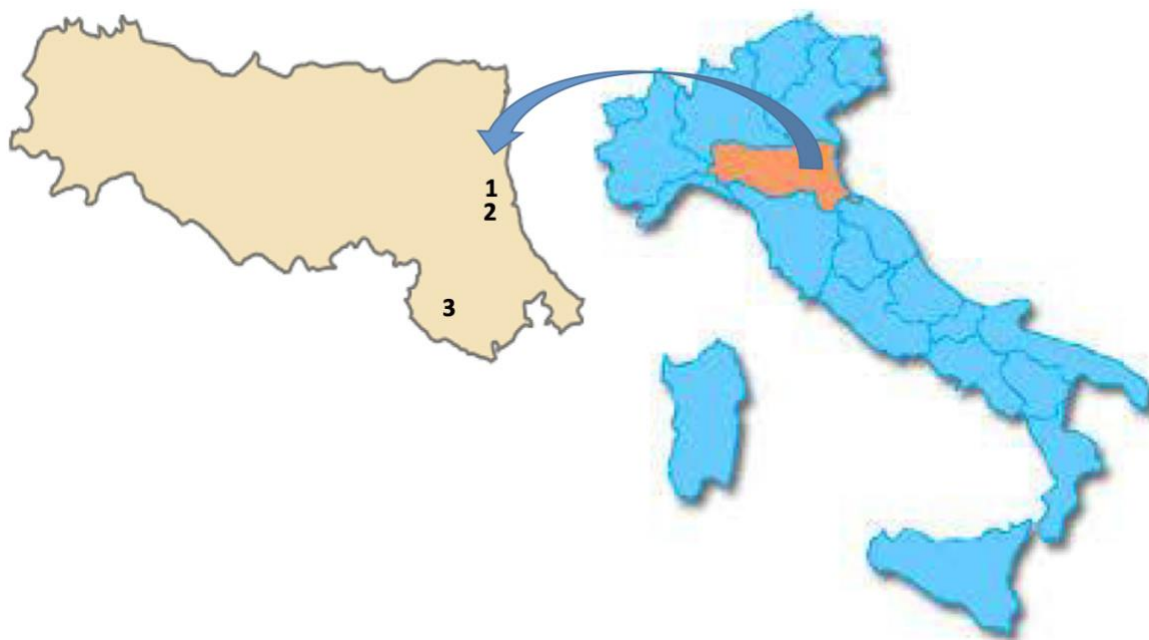


Figure 3.1. Geographical location of Emilia Romagna, Italy and the three DWTPs in Romagna, 1 (NIP) and 2 (Standiana): District of Ravenna; 3 (Capaccio): District of Forlì-Cesena.

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-d₂, BPA-d₆, 13C₄-PFOA, Ibuprofen-d₃ at a concentration of 30 ng/L, and 13C₃- 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 cm³, 200 mg; Waters S.p.A., Sesto San Giovanni, Milan, Italy). Cartridges were eluted with 6 mL of methanol, evaporated under a N₂ gentle stream up to a volume of 250 µL, and split in two vials of 125 µL each. The first set of vials was additioned with 125 µL of water (finally 50:50 water/methanol) for the first set of LC-MS-MS analysis (group 1, **Table 3.2**). 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 3.2**). 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).

Table 3.2. MS-MS detection parameters for the 3 groups of compounds analyzed: cone voltage, precursor and product ions with the respective collision energy.

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)
IBU-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)
CFF-C ₁₃	37	197.9	139.9 (19)	

2.4. Chromatographic conditions and mass spectrometry detection

Chemical analyses 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 C18 3.5 μ m 2.1 $\times$ 150 mm column (Waters S.p.A.) and the volume injection was 20 μ L. Mass analyses were performed in multiple reaction monitoring (MRM) mode. **Table 3.2** 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 $^{\circ}$ 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 3.3**. 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 analyzed, 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 account the matrix effect. LOQ values were used as cut-off values for

quantification of the analytes. Intra- and inter-day precision were calculated by injection of one point of the calibration curve (10 ng/mL) and calculating the relative standard deviation (RSD, %) (n = 3). Concentrations below the LOQ were considered as half the LOQ.

Table 3.3. Quantification and method validation: detection limits (LOD), quantification limits (LOQ), recovery and reproducibility (RSD %), correlation factors of the calibration curves (r^2), precision (inter- and intra-day RSD %).

Compound	LOD (pg injected)	LOQ (ng/L)	Recovery ± RSD (%)	Correlation factor (<i>r</i> 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

2.6. Cell culture conditions

Human breast cancer cells MCF-7 were kindly provided by Prof. M. Marino (University Roma Tre, Rome, Italy). Cells were grown in a humidified atmosphere of 5% CO₂ in air at 37 °C, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mg/mL gentamicin and 1% of non-essential amino acids. Phenol red-free DMEM supplemented with 5% charcoal-dextran treated fetal calf serum was used as experimental medium, containing DWTP water extracts or mineral water extracts as laboratory blank samples. Cell culture reagents were from Merck Life Science.

2.7. *E-screen assay*

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

2.8. *Micronuclei test*

Genotoxic activity assessment was performed by the micronuclei test, as described by 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 μ M BPA as a positive control. After 48 h of exposure, the medium was replaced with experimental medium containing 2 μ g/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',6diamidino-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 $\times$ 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 $\pm$ standard error (SE). Results of MN test are reported as the mean $\pm$ 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

Tables 3.4 and **3.5** summarize the concentrations of compounds for industrial use recognized as endocrine disruptors and pharmaceuticals, respectively, investigated in the water entering and exiting the DWTPs.

Table 3.4. 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	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
Capaccio	July 2019	IN	0.99	0.66	2.05	0.08	0.07
		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

Table 3.5. Levels of pharmaceuticals (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: pharmaceuticals detected in OUT water samples.

			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
Capaccio	July 2019	IN	2.35	0.92	2.66	0.12	1.96	3.56	0.04	0.51
		OUT	<LOQ	<LOQ	<LOQ	57.96	<LOQ	<LOQ	<LOQ	<LOQ
	September 2019	IN	<LOQ	<LOQ	<LOQ	16.59	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	8.93	<LOQ	<LOQ	<LOQ	<LOQ
NIP	July 2019	IN	<LOQ	<LOQ	<LOQ	15.72	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	60.49	<LOQ	<LOQ	18.70	<LOQ
	September 2019	IN	<LOQ	<LOQ	<LOQ	16.81	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	178.79	5.22	4.20	26.46	<LOQ
Standiana	July 2019	IN	<LOQ	<LOQ	<LOQ	12.47	<LOQ	<LOQ	<LOQ	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	40.71	<LOQ	<LOQ	10.87	<LOQ
	September 2019	IN	<LOQ	<LOQ	<LOQ	18.61	<LOQ	<LOQ	1.20	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	67.80	<LOQ	<LOQ	17.84	<LOQ
		OUT	<LOQ	<LOQ	<LOQ	20.00	<LOQ	<LOQ	0.83	<LOQ

3.1.1. Surfactants and plasticizers

Alkylphenols, widely employed as surfactants in chemical industry, are frequently detected in the environment at concentrations in the order of $\mu\text{g/L}$; further they are toxic, persistent and able to bioaccumulate (Sousa et al., 2018). Among this group, nonylphenol (NP) and octylphenol (OP) belong to category 1 of the Endocrine Disruptor Priority List (EU Commission, 2007). Both compounds are among the 33 priority substances in the European water framework directive (EU, 2013) and are classified as priority hazardous substances. The document identifies the environmental quality standards (EQS) in the water column, corresponding to average values of 0.1 mg/L and 0.3 mg/L, for OP and NP, respectively. The same 0.3 mg/L concentration was initially proposed by the WHO as the upper limit for NP in drinking water (EU Commission, 2018); then, according with a following decision, the approved document includes NP in the Watch list (EU Council, 2020). In the present study, OP and NP showed concentrations below the above mentioned limits. In fact, OP has never been detected above the quantification limit (0.66 ng/L), neither in water entering or in water exiting the DWTPs. NP has been found in all plants and sampling campaigns, and ranged from 7.90 ng/L to 53.62 ng/L in the post-treatment water. NP measured in the water leaving 6 Italian plants was reported at concentrations up to 100 ng/L (Achene et al., 2011), and similar values have been published for drinking water from public fountains in 35 Italian cities, with NP highest concentrations of 84 ng/L (Maggioni et al., 2013). In European countries maximum value of 505 ng/L was reported in France (Colin et al., 2014), 16 ng/L in Germany (Kuch and Ballschmiter, 2001) and 126 ng/L in

Spain (Valcárcel et al., 2018). Interestingly, a higher amount of NP after DWTP treatment has been occasionally found in our investigation. Similar data were reported by the Italian Institute of Health concerning 6 Italian waterworks monitored between 2008 and 2009; the higher occurrence of NP in post treatment water was related to the use of plastic materials for the pipelines, which could release substances such as alkylphenols, 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 3.4**), 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 DWTPs, 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 occurred in all water samples analyzed 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 (Boiteux et al., 2012); in Germany, drinking water showed a maximum concentration of PFOA and PFOS of 519 and 22 ng/L, respectively (Skutlarek et al., 2006); in Spain the highest concentrations in drinking water corresponded to 2.40 and 1.81 ng/L, for PFOA and PFOS, respectively (Domingo et al., 2012). As from **Table 3.4**, while PFOS concentrations were always reduced by the treatment, PFOA levels were occasionally higher in post- with respect to pre-treatment waters. Rahman and coworkers reviewed PFAS fate in drinking water and noted the same PFOA behaviour, providing some explanations, such as the possible breakdown of certain precursor compounds to PFOS and PFOA during treatments, or the leaching from Teflon-coated components and desorption from GAC filters that had been in service for long periods of time without reactivation (Rahman et al., 2014).

3.1.3. *Pharmaceuticals*

This class of contaminants is synthetic or natural chemicals found in prescription medicines, over-the-counter therapeutics and veterinary drugs. Because drinking water limits for pharmaceuticals have not been established yet, and little has been published on safe long-term exposure levels, the

evaluation of drinking water quality is challenging. The need to collect monitoring data relative to pharmaceutical occurrence in water for human consumption was confirmed in 2015, when diclofenac (DCF), 17- β -estradiol (E2), and 17- α -ethinyl estradiol (EE2) were included in the Watch List of Decision for the compounds posing a significant risk to the aquatic environment, with insufficient monitoring data at European Union level (EU Commission, 2015). The first monitoring results, reported by Higher Institute for Environmental Protection and Research (ISPRA, 2017), showed that DCF was one of the most frequently detected pharmaceutical, found in 22 of the 35 Italian stations, at concentrations ranging from 5 to 683 ng/L. Due to its documented occurrence in the environment, bioaccumulation and adverse effects on the health of aquatic fauna, the EU Joint Research Centre removed DCF from the Watch list in the most recent update (Loos et al., 2018), and the definition of 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 3.5**) 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 has been found in each sample analyzed (**Table 3.5**). The highest concentration of CFF was detected in water entering NIP in both 2018 campaigns, when a value as high as 2.58 $\mu\text{g/L}$ 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). 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. 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 has been 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 nonmeasured 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 (**Fig. 3.2A**). 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 nonlinear regression ($r^2 = 0.987$, dotted curve), allows to quantify the PE in terms of equivalent estradiol (EEQ) concentration. **Fig. 3.2B** 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).

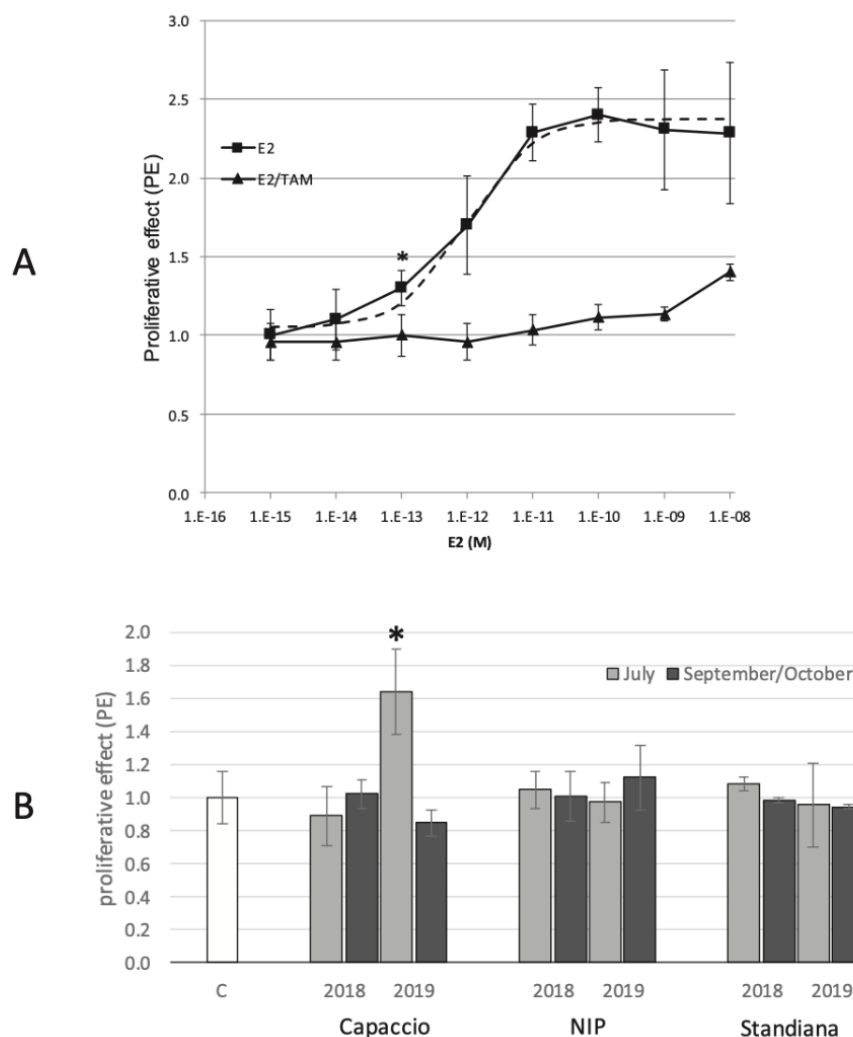


Figure 3.2. Evaluation of estrogenic activity. Data are expressed as the mean of proliferative effect (PE) $\pm$ SE of different experiments, each conducted in quadruplicate; (A) E-screen test sensitivity: dose-response curve to E2 of MCF-7 cells, in the presence (triangle) or not (square) of 10^{-7} 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).

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, and 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 similestrogens (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

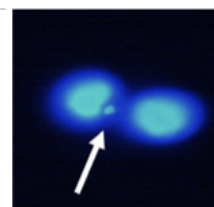
coworkers demonstrated that when BPA reacted with high concentrations of chlorine, derivatives were still present after 60 min and are more difficult to biodegrade than BPA; furthermore, byproducts were detected at the exit of the DWTPs showing an estrogenic activity greater than the parent compounds at lower concentrations (Hu et al., 2002). The effects of by-products from chlorination cannot be ruled out, because not analyzed 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, particularly when water is obtained from surface sources and then chlorinated. Thus, short-term genotoxicity tests predictive of carcinogenic activity have been suggested to assess the potential genotoxic activity of such complex mixtures in drinking water (Buschini et al., 2004; WHO, 2011; Ceretti et al., 2016). Many estrogen-like chemicals induce multiple effects *in vivo* that cannot be related only to estrogenic activity. For example, BPA is also a genotoxic compound, that leads to DNA damage, detectable by an increase of micronuclei (MN) number in exposed cells (Ramos et al., 2019). For carcinogenic compounds, the United States Environmental Protection Agency recommends zero level in drinking water (US EPA, 2017). Despite the risks associated with the presence of mutagenic/carcinogenic substances in water intended for human consumption, the current legislation does not provide for the application of mutagenesis tests. We presently used the MN test for its sensitivity and reliability. MN test has already been applied for the assessment of the quality of drinking water (Maffei et al., 2009), (Zeng et al., 2015) (Buchner et al., 2019). **Table 3.6** shows the frequency of MN evaluated in MCF-7 cells after 48 h treatment with the different sampled water extracts. None of the water extracts induced any statistically significant increase in the MN frequency compared to negative controls. The positive control BPA 0.1 μM showed a significant variation ($p < 0.05$), thus indicating the sensitivity of the test.

Table 3.6. 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 μ 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.

	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 μ M)				17.1 $\pm$ 2.7 37.2 *$\pm$ 4.8



4. Conclusions

The quality of drinking water and the efficacy of treatments in relation to CECs are a matter of concern, because the risk they pose to human health and the environment is not yet fully understood. A chemical and biological integrated approach is here proposed to evaluate the occurrence of selected CECs and the overall estrogenic and genotoxic potential of waters eligible for human consumption. The water analyzed in the present investigation met the criteria of good quality, according to the low concentration and/or regulation limit for each single contaminant. Chemical analysis indicated that NP, PFOA, BPA, CFF and CBZ were the most frequent contaminants in water samples, thus confirming that these substances are ubiquitous in the water cycle. While the waterworks treatment was generally effective in reducing BPA, CFF and CBZ, it was sometimes ineffective for NP and PFOA. For some of the studied CECs, occurrence in the incoming water was different among waterworks, which are fed by water coming from areas with lower (Capaccio) and higher (NIP and Standiana) anthropogenic impact. Water feeding Capaccio in fact was neither contaminated by pharmaceuticals nor by PFOS, while PFOA concentration was at least 5 times lower than in other plants. All CECs were instead detected in water entering NIP and Standiana. Interestingly, BPA and NP occurred in all plants at very similar concentrations, regardless the area of origin of the incoming water. Some of the chemicals investigated are included in the Watch list of substances for which EU-wide monitoring data need to be gathered to support future prioritization. Present data therefore provide information on the fulfilling of the purposes of EU Water Framework Directive (EU, 2013) and of the recently revised Drinking Water Directive (EU Council, 2020). Biological analyses were performed to ascertain the absence of cumulative estrogenic and genotoxic activities in the waters

from the DWTPs. Although previous reports are available on this possibility (Maggioni et al., 2013), no estrogenic or genotoxic activities were shown by the waters analyzed, with the exception of one sample. The recorded estrogenic activity remained an isolated phenomenon, of low entity and in line with estrogen concentrations previously reported in drinking waters. However, this may not always be the case, and high frequency monitoring are suggested for a comprehensive assessment of the risks associated with exposure to CEC mixtures. It is a recurrent suggestion that drinking waters do not represent a relevant source for human exposure to CEC as asserted for NP (Soares et al., 2008), (Colin et al., 2014), BPA (Arnold et al., 2013), PFAS (Domingo and Nadal, 2019) and pharmaceuticals (WHO, 2017). The above considerations, however, cannot bridge the knowledge gaps in terms of assessing the risks associated with long-term, low-level exposures, and possible combined effects of chemicals in the mixture. Overall, the present study points out the usefulness of an integrated chemical and biological approach as a screening tool for 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.

Chapter Four

PREFACE

In the previous Chapter, the levels of pharmaceuticals, industrial compounds, and hormones in drinking water from the Romagna area (Italy) were reported. The results were essential for determining the presence and concentrations of target contaminants, as well as for determine the biological potential in drinking water. Chapter Four addresses the main objective of this PhD study, focusing on three phenolic compounds (BPA, NP, and OP) and three natural and synthetic hormones (E2, E1, and EE2) as suspected EDCs, some of which were prioritized for inclusion in the EU regulations (Watch lists and Directives) for freshwater and drinking water. A three-year monitoring campaigns was designed to evaluate EDC contamination in source and drinking water from three full-scale DWTPs in the Romagna area (Italy). Combined chemical and biological approaches were adopted to determine the overall drinking water quality. Moreover, this study further evaluated the quality of the water after each single treatment step within the newest and most advanced DWTP to verify whether treatment processes and seasonality could affect the fate and removal of EDCs. Chapter 4 will be submitted soon for publication on an international peer reviewed Journal.

A three-year screening of suspected EDCs in three Italian drinking water systems combining chemical and effect-based tools

1. Introduction

Over the last few decades, an increasing variety of new compounds of anthropogenic origin has been reported in water bodies worldwide. The presence of contaminants of emerging concern (CECs) in drinking water, including endocrine-disrupting chemicals (EDCs), has drawn attention towards possible risks to human health. Specifically, EDCs can be found in a wide range of products used in everyday life, including natural chemicals (e.g. hormones), chemicals used as industrial additives and plastics (e.g. bisphenol A, BPA), alkylphenols, pesticides, fungicides, and pharmaceutical agents (De Jager et al., 2013). EDCs typically occur in water at trace concentrations, and their presence is largely attributable to their continuous release into source water (ground and surface water) from wastewater systems because of their incomplete elimination, and contamination from other sources (agricultural and industrial activities). The materials in contact with water supplies can be an additional source of EDC contamination. Recent studies have highlighted the leaching of substances such as alkylphenols

and BPA from plastic materials used in drinking water treatment plants (DWTPs) and pipelines (Cantoni et al., 2021; Cheng et al., 2016; Muhamad et al., 2016; Achene et al., 2011). DWTPs play a key role in supplying high-quality potable water. However, conventional drinking water technologies, including flocculation, precipitation, adsorption, membrane treatment, and other water treatment methods, are not specifically designed to remove CECs (Priya et al., 2022; Rodriguez-Narvaez et al., 2017; Kim and Zoh, 2016). Treatment processes have demonstrated different removal rates for EDCs, ranging from less than 30% to greater than 90% (Sorlini et al., 2019). This is because several EDCs are persistent and non-biodegradable, with strong chemical cohesion in the environment (Canle et al., 2017), and their removal efficiencies are dependent on the physical and chemical properties of the substances. To date, limited information is available on the concentrations of these substances in treated water, and data on their removal using drinking water treatment technologies are not easily comparable owing to different treatment conditions, variability in water characteristics, and waterworks design. Thus, understanding the potential chemical burden of water requires major attention, particularly when water is intended for human consumption.

Human health implications arising from EDCs exposure include adverse reproductive outcomes, developmental disabilities, endometriosis, and breast and testicular cancers (Kahn et al., 2020; Giulivo et al., 2016; Gomes and Lester, 2002). Contamination by synthetic and natural hormones has been linked to chronic and acute diseases, such as various types of cancer in females and males, disorders of the reproductive system, obesity, and diabetes (Predieri et al., 2022; Goralczyk, 2021; Patel et al., 2018). Phenolic compounds such as BPA and 4-nonylphenol (NP) have been found in human urine, blood, and reproductive tissues (Yilmaz et al., 2020; Jalal et al., 2017) and have the ability to disrupt the endocrine system at very low doses, causing a variety of human diseases (Catenza et al., 2021; Noorimotlagh et al., 2020; Cimmino et al., 2020). BPA may exert its toxicological effects at the genetic and epigenetic levels, influencing various cell signaling pathways, and altering the biological behaviors of cancer cells, notably proliferation, invasion, growth, survival, migration, and apoptosis (Khan et al., 2021; Profita et al., 2021; Dumitrascu et al., 2020; Murata and Kang, 2018; Le Magueresse-Battistoni et al., 2018; Sowlat et al., 2016; Ptak et al., 2014). Concerns about the potential impact of EDCs on humans and the environment have motivated the European Union to update the list of chemical parameters in the Drinking Water Directive (EU, 2020/2184), adding suspected EDCs for the first time, including PFAS and BPA. In view of their endocrine-disrupting properties, 17 β -estradiol (E2) and NP are included in the first “Watch List” mechanism (Council EU, 2020).

Chemical analysis is the most commonly used approach for monitoring drinking water quality. Analytical technologies include advanced, precise, and accurate methods for detecting environmental

contaminants at sub-trace levels. However, chemical analyses have some limitations, as they cover only a wide but finite number of substances, and do not account for the biological effects of chemicals or their mixtures. Moreover, in many cases, the analytical limit of detection (LOD) for some substances is above the proposed threshold guideline values (e.g. hormones like E2 and 17 α -ethinylestradiol, EE2) (Dulio and Pasanen-Kase, 2017), thus the occurrence of these contaminants cannot be determined. As such, the choice of combining chemical and bioanalytical tools has become of increasing interest and is recommended for water quality monitoring (Brack et al., 2019; Wernersson et al., 2015). The application of effect-based methods is relevant for drinking water, where chemicals are often present at low concentrations, potentially below the analytical LODs, nevertheless contributing to biological effects (Neale et al., 2022; Neale and Escher, 2019). Additionally, effect-based methods can provide valuable information regarding treatment efficiency. To comprehensively assess water quality, a battery of bioassays is recommended to include different health-relevant biological endpoints, such as those related to xenobiotic metabolism, hormone-mediated modes of action (e.g. endocrine activity), reactive modes of action (genotoxicity), and oxidative stress response (Escher et al., 2014).

In aquatic environments, estrogenic activity has primarily been ascribed to the natural steroids E2 and estrone (E1), and the synthetic estrogen EE2, used in contraceptives (Kinnberg, 2003). However, industrial xenobiotics alone or in mixtures may also contribute to overall estrogenic activity. The present study aimed to evaluate the occurrence of representative EDCs (hormones E2, E1, and EE2; and plasticizers BPA, NP, and 4-octylphenol (OP) (**Figure 4.1**), in the source and treated water of three DWTPs serving the Romagna area (the southeast part of the Emilia-Romagna region, Italy) and to monitor the potential bioactivity of drinking water. HPLC-ESI-MS/MS analysis were combined with cell-based assays to assess potential estrogenic activity (E-screen assay) and genotoxicity (micronucleus test) to ascertain the overall drinking water quality. The investigation was conducted in summer over three years (2020-2022). As one of the studied DWTPs applies more advanced treatment technologies, chemical monitoring was also conducted during the winter period throughout the water treatment steps to analyze their removal efficiencies and explore seasonal differences.

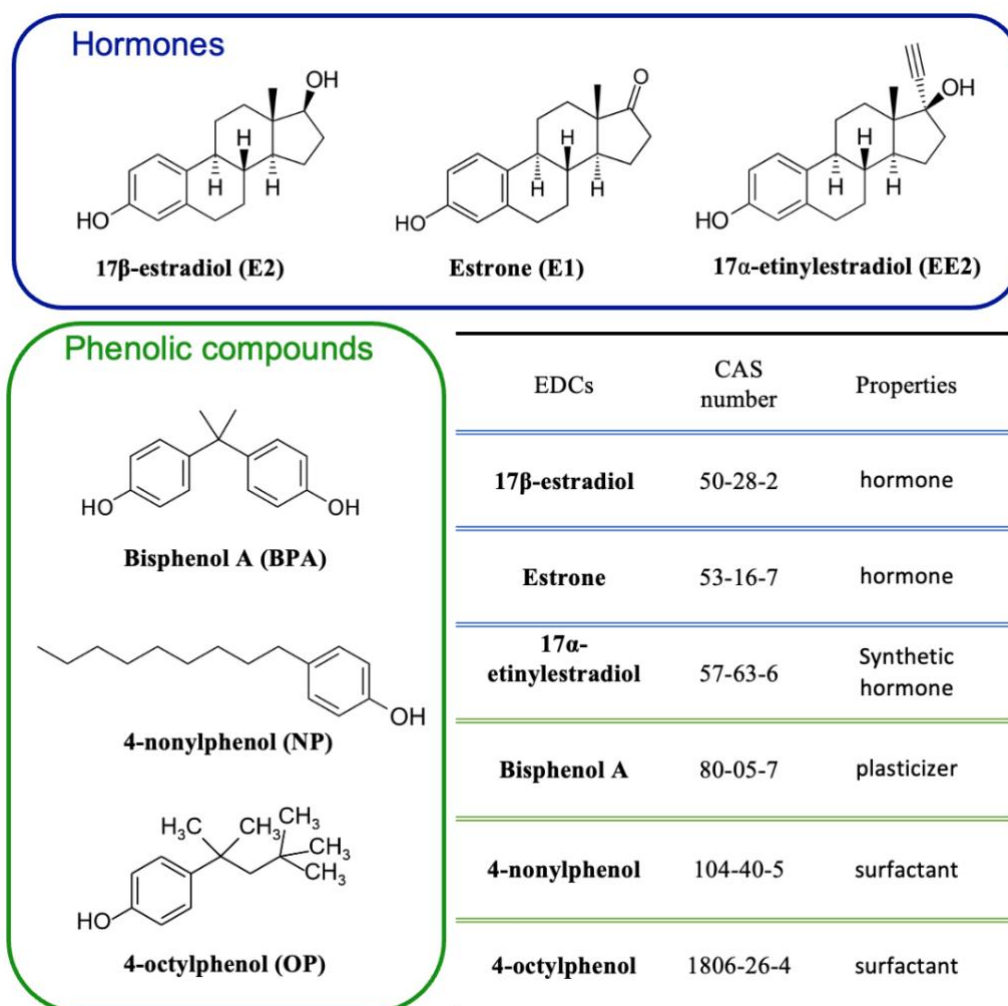


Figure 4.1. Targeted EDCs in the present study.

2. Materials and Methods

2.1. Chemicals

E2, E1, EE2, BPA, NP, and OP were selected as micropollutants for the study and were purchased from Merck Life Science (Milan, Italy). E2-d₂ and BPA-d₆ were used as labeled internal standards, purchased by CDN Isotopes (Quebec, Canada). Stock solutions and their internal standards were prepared in methanol and stored at -20°C. Mix working standards (1 mg/L), containing all EDCs, were also prepared in methanol by spiking each of the dissolved individual stock solutions. Thereafter, the mix working standard was diluted using water/methanol (50:50, v.v.) to the subsequent concentration needed. Solvent reagents from Merck Life Science were of LC-MS analytical grade. Solid phase extraction cartridges (Oasis HLB, 6cc) with a size of 200mg/6mL were purchased from Waters S.p.A. (Milan, Italy).

2.2. *Drinking water treatment systems and sampling collection*

Water samples were collected from the main three DWTPs operating in the Romagna area (Italy) and managed by the company Romagna Acque Società delle Fonti. Schematic representation and geographical position of DWTPs are shown in **Figure 4.2**.

- DWTP Bassette (NIP), located in an industrial area of Ravenna, has been designed to treat surface water from areas with intense anthropic activities, mixing water from the Lamone and Reno Rivers, and from the CER (“Canale Emiliano-Romagnolo”, an artificial water transfer system that takes water directly from the Po River). Its instant production potential is 1.100 L/s for approximately 78.000 m³ water per day. The DWTP includes the following treatment processes: pre-chlorination using chlorine dioxide, clariflocculation using aluminum polyhydroxychloride in sludge recirculation basins called “Accelerator”, granular activated carbon (GAC) filtration, and final chlorination (using chlorine dioxide) before the water enters the distribution network.
- DWTP Standiana (STN), located near Ravenna and active since 2015, applies conventional and advanced processes to treat water from CER, that crosses a heavily anthropized area. STN DWTP instant production potential is 1.100 L/s. STN DWTP consists of micro-sieving followed by pre-chlorination (PRECL) treatment using chlorine dioxide, flocculation (FLOCC), ultrafiltration with membranes (ULTR), biologically activated carbon (BAC) filtration, and lastly dosing with chlorine dioxide for final disinfection.
- DWTP Capaccio (CAP), located near Forlì, receives surface water from the large artificial lake Ridracoli closed by a dam sited in the National Park of the Casentinesi Forests (an area with reduced anthropogenic impact). The Capaccio plant has a maximum production of approximately 220.000 m³ water per day. The DWTP currently employs conventional treatment with pre-chlorination (using chlorine dioxide), clariflocculation using aluminum polyhydroxychloride (the clariflocculation treatment of the water is carried out in 4 clariflocculators called Cyclofloc) followed by rapid filtration (the filter batteries consist of 16 units that can be fed individually), and disinfection with sodium hypochlorite.

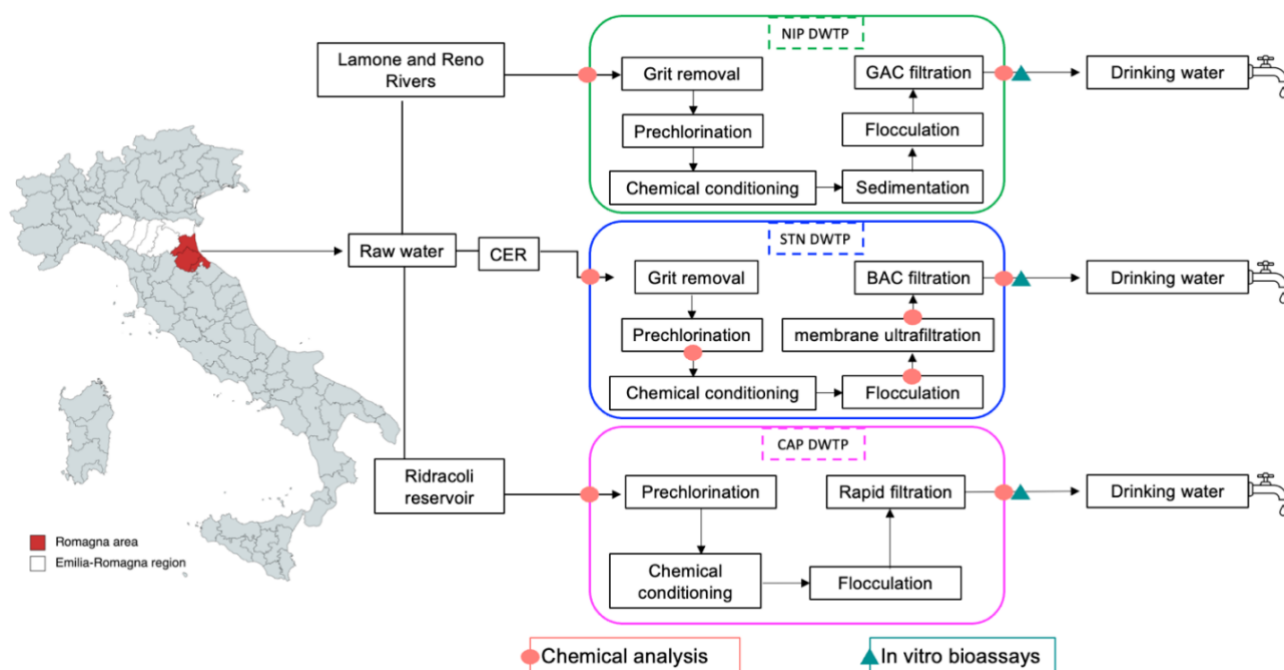


Figure 4.2. Schematic representation of treatment processes at the three studied drinking water treatment plants (DWTPs). The sampling points are indicated with dots and triangles. For each sampling point chemical or biological analyses further applied are indicated in the boxes at the bottom.

Raw (IN) and treated (OUT) water samples were collected from all three studied DWTPs. Furthermore, to investigate the effectiveness of removal by intermediate treatment of the advanced Standiana DWTP, three sampling points were also considered along the treatment processes: after pre-chlorination (PRECL), after flocculation (FLOCC), and after membrane ultrafiltration (ULTR). A total of 6 sampling campaigns were carried out in summer (July and September) of 2020, 2021, and 2022. The summer period was chosen for the above samplings because of the absence of rainfall and high evaporation rates in this area; thus, EDCs were expected to be more concentrated in water bodies. In addition, samples from the Standiana DWTP were collected in winter (January and February/March) over the same three years for a total of 5 additional campaigns. During the wet season, contaminants can be diluted because of the increase in rainfall and river flow rates.

The detailed weather conditions in the area during the 3-year sampling period are provided in **Figure S-1 (Supplementary material – 1)**. At each sampling point, three replicate water samples (1 L each) were collected in pre-cleaned PET- bottles with methanol, to avoid EDCs adhesion to the bottle surfaces. Then, all water samples were transported to the laboratory for storage in the dark at 4 °C and processed within 36 h.

2.3. Sample extraction

Before extraction, all water samples were separately spiked with a mixture of labeled internal standards (E2-d₂ and BPA-d₆) at a concentration of 30 ng/L. The samples were then filtered using glass microfiber filters (1.60 µm) and then with cellulose acetate filters (0.45 µm) to remove suspended particulate matter. The samples were then extracted using Oasis HLB cartridges as previously reported (Valbonesi et al., 2021). Briefly, cartridges were activated using 6 mL of methanol and 6 mL of deionized water. Then, the water samples were passed through the preconditioned cartridges at a max flow rate of 14 mL/min. Subsequently, the HLB cartridges were eluted with 6 mL methanol under gravity, after which the eluents were dried under a gentle stream of nitrogen. Finally, the extracts were re-dissolved with 0.5 mL methanol/water (50:50, v/v) for chemical analysis or dissolved into the assay medium for biological analysis. Thus, extracts were transferred into 2 mL vials using 0.22 µm membrane filters for analyses.

2.4. HPLC-MS/MS analysis

Chemical analysis of the selected substances in water samples was performed using HPLC-MS/MS, according to the previous study (Valbonesi et al., 2021). Generally, the selected EDCs and their corresponding internal standards were analyzed using high-performance liquid chromatography equipped with a triple quadrupole mass spectrometer (Waters, Milford, MA, USA) using a negative electrospray ionization source (ESI-) in multiple-reaction monitoring (MRM) mode. Separation of compounds was achieved through an XBridge C18 3.5 µm 2.1 × 150 mm column (Waters S.p.A.) and the volume injection was 20 µL. Detailed information and instrumental parameters of the contaminants are summarized in **Table 4.1**. Sample extracts concentrated 2000 times were measured along with calibration standards, which corresponded to a range of 0-300 µg/L for the phenols and steroids.

Table 4.1. Quantification and method validation: detection limits (LOD), quantification limits (LOQ), recovery and reproducibility (RSD %), correlation factors of the calibration curves (r^2), precision (inter- and intra-day RSD %).

<i>Chemical</i>	<i>LOD (pg injected)</i>	<i>LOQ (ng/L)</i>	<i>Recovery $\pm$ RSD (%) (n=18)</i>	<i>Correlation factor r^2</i>	<i>Precision (RSD %)</i>	
					<i>Intra-day (n=6)</i>	<i>Inter-day (n=6)</i>
E2	15	0.81	98 $\pm$ 10	0.9995	16	10
E1	9	0.92	100 $\pm$ 8	0.9994	19	14
EE2	41	2.66	101 $\pm$ 5	0.9993	15	14
BPA	9	0.99	105 $\pm$ 7	0.9998	9	8
NP	5	2.05	89 $\pm$ 20	0.9977	6	20
OP	13	0.66	101 $\pm$ 8	0.9973	14	19

2.5 Quality control (QC)/quality assurance (QA)

QC and QA measurements were performed for the entire experimental protocol. The apparatus and glassware were pre-cleaned by rinsing with tap water, deionized water, and methanol. An equal volume (1 L) of deionized water spiked with the same internal standard solution as water samples was simultaneously extracted and analyzed. It was extracted along with every batch of samples as a procedural blank sample to assess contamination during sample extraction and analysis. The limit of detection (LOD) was defined as the concentration in a sample with a signal-to-noise ratio of three while the limit of quantification (LOQ) was defined as ten. The LOD and LOQ values for each target compound are also provided in **Table 4.1**. Each calibration curve for the target compounds had a high correlation coefficient ($r^2 > 0.99$). Moreover, the standard mixture solution was injected in turn after the injection of every six samples during instrumental analysis as calibration of the data acquisition. HPLC-MS/MS performance and analyte identification were monitored throughout the retention time (within $\pm 2\%$) of the compound in the samples relative to those in the authentic standards. The performance of the method was evaluated by estimating the linearity, extraction recovery, sensitivity (both LOD and LOQ), precision (repeatability and reproducibility), matrix effects (ionization suppression or enhancement), and relative abundance (quantifier/qualifier transition ratio). Isotopically labeled compounds E2-d₂ and BPA-d₆ were used to correct analyte recovery and quantify analytes by regulating any possible ionization suppression or enhancement resulting from the matrix effect.

The removal efficiency of the treatment plant was calculated using the inlet and outlet water concentrations as follows:

$$\text{Removal efficiency} = \frac{C_{\text{inlet}} - C_{\text{outlet}}}{C_{\text{inlet}}} \times 100.$$

2.5. Cell-based methods

Treated water samples were assessed for estrogen receptor (ER) agonist activity using a cell proliferation assay (E-screen assay), cytotoxicity using a cell viability assay (MTT-assay), and genotoxicity using an *in vitro* micronucleus test (MN test). The human breast cancer cell line, MCF-7, kindly provided by Prof. M. Marino (University Roma Tre, Rome, Italy), was used for all bioassays. All *in vitro* assays have previously been used for water quality monitoring, with the methods fully described in (Valbonesi et al., 2021). All water samples were tested for cytotoxicity in a dilution series to ensure that experiments were carried out under noncytotoxic conditions. Only non-cytotoxic concentrations were evaluated for estrogenic/genotoxic activities. Positive controls were analyzed in parallel with the water samples: a standard curve with E2 for ER activity and 0.03 µg/ml of mitomycin C for genotoxicity.

2.6. Data evaluation

The concentrations of samples were expressed in units of relative enrichment factor (REF), which considers the combination of the extraction factor from the concentration step and dilution factor in the bioassay as reported by Escher et al. (2021). When incubated with the cells, extracts were diluted 40-fold with the cell medium to obtain a final concentration of 0.1% methanol. The resulting REF in the bioassays was 20, which means that the samples were 20 times enriched in the cellular medium. Cell-based bioactivities of the water samples and positive controls were normalized to the vehicle controls on each plate. For the E-screen assay, estrogenic activity was expressed as the Proliferative Effect (PE) compared to vehicle controls, set as 1. A dose-dependent cell proliferation curve was obtained by fitting the data to a four-parameter sigmoidal curve of E2, using a commercial graphical package (SigmaPlot software, ver 13, Systat Software Inc.). Results are expressed in terms of PE as the mean $\pm$ standard error (SE). A sample was defined as active if the tested REF value was significantly different from that of the vehicle control ($p < 0.05$). For all bioactive samples, the concentration was calculated as the same effect elicited by the standard compound and termed the E2 equivalent concentration (EEQ).

The results of the micronuclei (MN) test were expressed as the number of micronuclei/1000 binucleated cells scored on each slide and reported as the mean $\pm$ standard deviation (SD), obtained from three independent experiments.

Data groups were compared using one-way ANOVA and followed by Dunnett post-hoc test; the significance was calculated in comparison to the negative (untreated) control. A statistical difference was accepted when $p < 0.05$ (Sigma Stat, SPSS Science, Chicago IL, USA).

3. Results and Discussion

3.1. Chemical analysis

3.1.1. Occurrence of selected micropollutants in the source and treated water of the studied DWTPs.

The present work provides a full picture of the state of EDCs contamination in drinking water in the Romagna area over a three-year period. The EDCs detected at the inlet and outlet water of the studied DWTPs are shown in **Tables 4.2 – 4.3 – 4.4**.

Among the selected substances monitored by HPLC-ESI-MS/MS, NP was the most prominent compound in all samples (100% detection). The highest concentration of NP (99.93 ng/L) was reported in the inlet water of the NIP DWTP in September 2021. This substance was also detected in finished water samples from all the DWTPs, implying that NP persisted after water treatment. It is worth noting that a higher amount of NP was often measured in treated water, as is frequently observed in NIP DWTP. NP is widely used in industrial and household applications as detergents, emulsifiers, wetting agents, additives, and resins (Priac et al., 2014). The widespread use of NP leads to its occurrence at low concentrations in water (Bhandari et al., 2021). The NP concentrations detected in this study were similar to those determined in our previous investigation (Valbonesi et al., 2021) and were in line with those reported in drinking water from other findings. For instance, in drinking water from other Italian DWTPs, the highest concentrations of NP were 84 ng/L (Maggioni et al., 2013) and 90 ng/L (Russo et al., 2022). In drinking water from the United States, the NP level was 97 ng/L (Benotti et al., 2009), 72 ng/L in Canada (Chen et al., 2006), 34 ng/L in France (Leusch et al., 2018), 13 ng/L in Spain (Valcárcel et al., 2018), and 16 ng/L in Germany (Kuch and Ballschmiter, 2001).

BPA is primarily used as an additive in the production of polycarbonate plastics and epoxy resins to enhance the transparency, durability, flexibility, and longevity of plastics (Mohammadi et al., 2022). In this study, BPA occurred in all water samples entering NIP DWTP (2.96-13.87 ng/L), it was often detected in STN DWTP (1.64-4.15 ng/L), and occasionally found in CAP DWTP (1.67-3.52 ng/L).

Overall, BPA contamination was moderately to completely removed by the water treatment processes, with concentrations in the treated water often below the analytical LOQ. BPA levels reported in this work were in accordance with those reported previously (Valbonesi et al., 2021), but lower than those reported by Maggioni et al. (2013) (up to 102 ng/L), by Riva et al. (2018) (BPA median value 23.10 ng/L), and Russo et al. (2022) (BPA median concentration 46.17 ng/L).

The natural hormone E2 was detected only in one sample (5.51 ng/L) at the inlet water of CAP DWTP in September 2021, whereas the synthetic EE2 has never been reported to exceed its analytical LOQ. Sporadic OP contamination was found in the inlet water of NIP and STN DWTPs. OP and hormones were efficiently removed by water treatment at both DWTPs.

Table 4.2. Levels of suspected EDCs (ng/L) measured in water samples from NIP DWTP collected during six sampling campaigns in 2020, 2021 and 2022. IN: pre-treatment water, OUT: post-treatment water; LOQ: limit of quantification (ng/L). Bold numbers: EDCs detected in OUT water samples.

Sampling	NIP	BPA	NP	OP	E2	E1	EE2
July 2020	IN	13,355	9,010	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	5,631	11,970	< LOQ	< LOQ	< LOQ	< LOQ
September 2020	IN	7,367	7,500	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	6,286	2,430	< LOQ	< LOQ	< LOQ	< LOQ
July 2021	IN	3,796	8,210	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	5,219	36,730	< LOQ	< LOQ	< LOQ	< LOQ
September 2021	IN	3,550	99,925	1,868	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	4,580	< LOQ	< LOQ	< LOQ	< LOQ
July 2022	IN	2,960	6,280	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	22,910	< LOQ	< LOQ	< LOQ	< LOQ
September 2022	IN	13,870	5,970	1,47	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	14,820	< LOQ	< LOQ	< LOQ	< LOQ

Table 4.3. Levels of suspected EDCs (ng/L) measured in water samples from Capaccio DWTP (CAP) collected during six sampling campaigns in 2020, 2021 and 2022. IN: pre-treatment water, OUT: post-treatment water; LOQ: limit of quantification (ng/L). Bold numbers: EDCs detected in OUT water samples.

Sampling	CAP	BPA	NP	OP	E2	E1	EE2
July 2020	IN	3,346	22,270	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	1,912	3,190	< LOQ	< LOQ	< LOQ	< LOQ
September 2020	IN	3,519	9,050	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	3,367	2,190	< LOQ	< LOQ	< LOQ	< LOQ
July 2021	IN	< LOQ	12,760	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	3,310	< LOQ	< LOQ	< LOQ	< LOQ
September 2021	IN	< LOQ	6,134	< LOQ	5,514	< LOQ	< LOQ
	OUT	< LOQ	3,424	< LOQ	< LOQ	< LOQ	< LOQ
July 2022	IN	1,670	12,000	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	22,750	< LOQ	< LOQ	< LOQ	< LOQ
September 2022	IN	< LOQ	42,650	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	11,770	< LOQ	< LOQ	< LOQ	< LOQ

Table 4.4. Levels of suspected EDCs (ng/L) measured in water samples from Standiana DWTP (STN) collected during summer campaigns in 2020, 2021 and 2022. IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water; LOQ: limit of quantification (ng/L). Bold numbers: EDCs detected in OUT water samples.

Sampling	Standiana	BPA	NP	OP	E2	E1	EE2
July 2020	IN	3,042	18,263	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	1,550	27,506	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	3,617	16,507	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	5,347	14,951	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	3,478	8,668	< LOQ	< LOQ	< LOQ	< LOQ
September 2020	IN	4,146	12,722	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	2,031	16,727	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	4,392	22,880	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	5,365	1,497	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	2,024	6,831	< LOQ	< LOQ	< LOQ	< LOQ
July 2021	IN	3,706	4,580	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	< LOQ	2,600	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	< LOQ	5,680	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	2,480	132,610	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	12,760	< LOQ	< LOQ	< LOQ	< LOQ
September 2021	IN	3,810	83,430	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	< LOQ	14,126	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	< LOQ	7,736	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	1,500	5,305	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	10,130	< LOQ	< LOQ	< LOQ	< LOQ
July 2022	IN	1,640	8,730	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	1,990	53,430	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	2,050	14,360	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	3,400	14,830	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	4,950	< LOQ	< LOQ	< LOQ	< LOQ
September 2022	IN	< LOQ	30,530	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	1,420	143,300	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	1,880	14,950	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	< LOQ	12,150	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	6,040	< LOQ	< LOQ	< LOQ	< LOQ

Compared with the guideline values for drinking water quality established by the European Drinking Water Directive (EU, 2020/2184), BPA levels in drinking water were always below the 2.5 µg/L parametric value set by the regulation. No exceedances of drinking water guidance values proposed in the “watchlist” mechanism for NP and E2 (0.3 µg/L and 0.001 µg/L, respectively) were observed. The results obtained for the analyzed treated waters confirmed the suitability of the water treatment process applied to the waterworks investigated.

The removal efficiency of each waterwork was calculated as the concentration of finished water compared to the raw water, and the results are shown in **Figure 4.3**. Generally, the median overall

removal efficiency during summer samplings was as follows: Waterwork NIP: BPA 78.92%, NP 0%, and OP 100%. Waterwork STN: BPA 100%, NP 49.42%, and OP 100%. Waterwork CAP: BPA 42.85%, NP 73.23%, and E2 100%. The results of this study indicated a wide variability in the effectiveness of each water system among the detected EDCs.

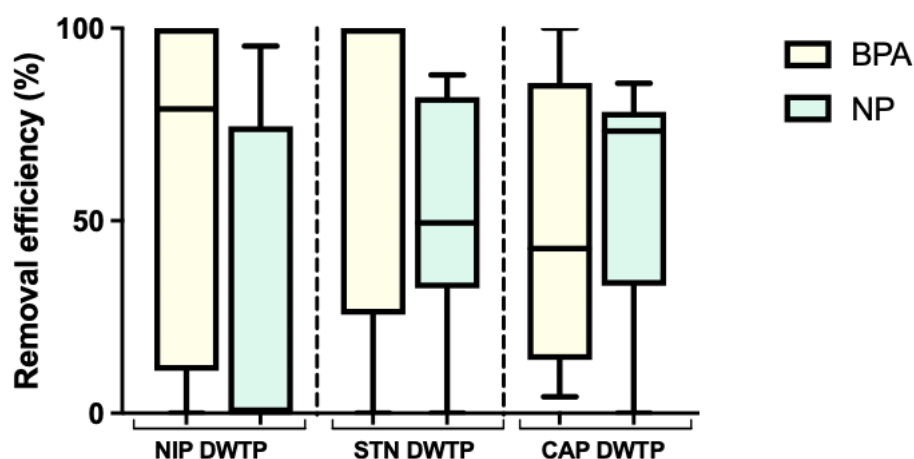


Figure 4.3. BPA and NP removal efficiency of three DWTPs (NIP; Standiana, STN; and Capaccio, CAP) (n=6).

3.1.2. Occurrence of micropollutants and their fate during water treatment processes of STN DWTP

To determine the fate and removal of micropollutants during water treatment performed by the STN DWTP, samples were collected from raw to finished water, including some intermediate processes, such as pre-chlorination, flocculation, and ultrafiltration. An overview of the occurrence of the considered EDCs along water treatments is shown in **Tables 4.4** and **4.5**.

Table 4.5. Levels of suspected EDCs (ng/L) measured in water samples from Standiana DWTP (STN) collected during winter campaigns in 2020, 2021 and 2022. IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water; LOQ: limit of quantification (ng/L). Bold numbers: EDCs detected in OUT water samples.

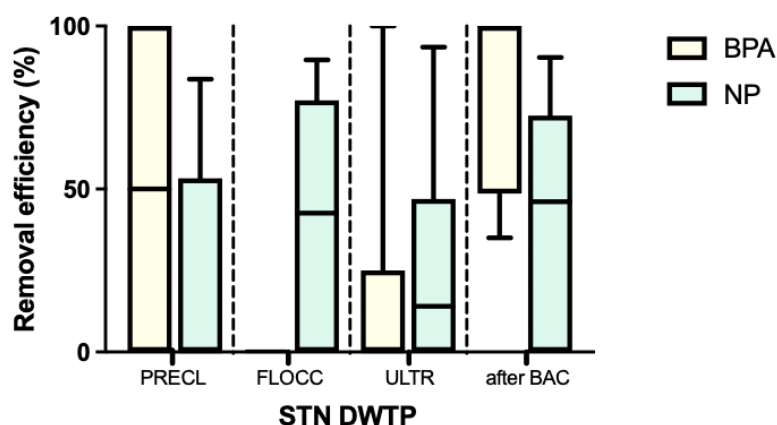
Sampling	Standiana	BPA	NP	OP	E2	E1	EE2
January 2020	IN	6,850	7,240	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	11,230	10,250	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	4,390	12,240	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	6,630	4,960	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	4,360	6,960	< LOQ	< LOQ	< LOQ	< LOQ
February 2020	IN	5,719	16,263	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	3,911	10,882	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	3,727	12,054	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	4,222	10,508	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	6,901	1,710	< LOQ	< LOQ	< LOQ	< LOQ
January 2021	IN	2,620	31,950	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	1,910	15,510	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	2,840	17,250	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	1,760	17,790	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	3,490	11,640	< LOQ	< LOQ	< LOQ	< LOQ
March 2021	IN	2,760	10,220	< LOQ	< LOQ	< LOQ	< LOQ
	PRECL	1,350	9,570	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	3,240	16,490	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	2,700	13,580	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	3,110	10,880	< LOQ	< LOQ	< LOQ	< LOQ
March 2022	IN	3,500	10,060	2,25	< LOQ	3,21	< LOQ
	PRECL	3,420	12,030	< LOQ	< LOQ	< LOQ	< LOQ
	FLOCC	4,930	12,910	< LOQ	< LOQ	< LOQ	< LOQ
	ULTR	3,040	14,890	< LOQ	< LOQ	< LOQ	< LOQ
	OUT	< LOQ	5,710	< LOQ	< LOQ	< LOQ	< LOQ

As previously reported (Snyder et al., 2007), no treatment alone can remove all contaminants. Some processes can significantly reduce the concentration, whereas others have little impact on their removal. Although the finished drinking water samples had relatively low concentrations of only two target compounds, the detected chemicals exhibited variable levels during the water treatment. This phenomenon was usually observed as an increase in BPA and/or NP levels corresponding to membrane ultrafiltration and after pre-chlorination and flocculation steps. In some cases, the NP level exceeded 100 ng/L, which was still lower than the threshold guideline value of 300 ng/L, but considerably greater compared with levels in the starting water. It may be hypothesized that the DWTP represents an extra source of EDCs contamination in water. This could be related to the leaching of organic chemicals from the plastic materials into the water. Various additives such as lubricants, plasticizers, antioxidants and other stabilizers, softeners, and coloring agents are used in

pipes, epoxy coating, valves, and other components of DWTPs to increase the life and stability of the materials (Gomes and Lester, 2002). Recent studies have demonstrated that emerging contaminants such as BPA, NP, PFAS and phthalates, widely used as additives in plastic materials, can be released by polymer pipes into drinking water (Mohammadi et al., 2022; Cantoni et al., 2021; Rajasärkkä et al., 2016; Cheng et al., 2016). Consequently, the migration of contaminants into water may adversely influence the quality of the produced drinking water. However, the leaching of contaminants from pipes and plastic materials into drinking water has poorly been addressed, and this also need to be considered in order to have a better understanding of the risk for human health. Furthermore, the question is overlapped to the fact that conventional treatments of WTPs are largely ineffective in removing EDCs (Priya et al., 2022; Katibi et al., 2021). It should be noted that the microporous sizes of ultrafiltration membranes are usually much larger than the size of some EDCs; thus, the sieving mechanism cannot contribute to their elimination (Katibi et al., 2021; Muhamad et al., 2016). In the present study, pre-chlorination accounted for 50% of the removal of BPA and was ineffective for NP. Flocculation and membrane ultrafiltration were usually ineffective in the removal of BPA and showed low removal efficiency of 42,6% and 14,1% for NP, respectively. Despite the increase in average concentrations of BPA and NP, as an indication of incomplete removal at the single treatment process, the results suggest that STN DWTP is able to remove EDCs to some extent. BAC filtration, corresponding to the last treatment step before water leaves the waterwork, showed more than 95% reduction in the concentration of BPA from the water reaching that previous step, whereas for NP the median removal efficiency was lower than 45% (**Figure 4.4A**).

Seasonal variations in the measured micropollutants in the STN DWTP were also investigated. The results revealed no significant seasonal differences in EDC levels in terms of concentration or removal between winter and summer samplings. However, flocculation treatment was ineffective for BPA in both seasons and less ineffective for NP in the winter season. Furthermore, despite the great efficiency shown during the summer, absorption removal by BAC filtration was ineffective in winter, especially for BPA (**Figure 4.4B**). Kim and Zoh (2016) suggested that low temperatures could decrease the adsorption removal of micropollutants, thus reducing the efficiency of activated carbon. Finally, the concentrations in drinking water were in the very low ng/L range, indicating that the considered micropollutants do not pose a risk to human health.

A.



B.

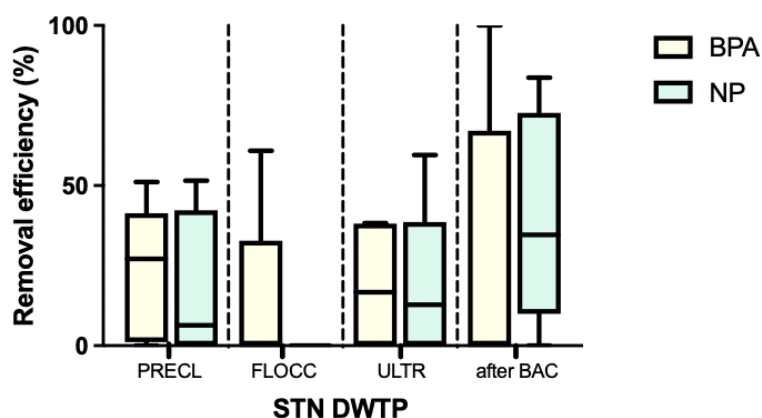


Figure 4.4. BPA and NP removal efficiency during PRECL (pre-chlorination), flocculation (FLOCC), ultrafiltration (ULTR), and after BAC treatment steps of Standiana DWTP (STN). [A] Removal efficiency during summer campaigns (July and September 2020, 2021, and 2022) (n=6). [B] Removal efficiency during winter campaign (January, February and/or March 2020, 2021, and 2022) (n=5).

3.2. Bioanalytical tools

Currently, water quality is mostly assessed by chemical analysis of targeted individual compounds. Chemical parametric values in legislation are based only on the toxicity of a single substance exposure at a time, or rarely on mixtures that are intentionally present in specific products (Kienzler et al., 2016). In environmental matrices, various substances coexist, establishing potential interactions, and mixtures are unintentionally formed. Furthermore, chemical analysis alone does not provide information on the synergistic/cumulative potential biological interactions of the mixtures that occur in drinking water. Thus, the complementation of bioassays and chemical analyses is an excellent tool to target the total biological effects and comprehensively assess water quality (Dingemans et al.,

2019). Given the evident relevance of ECDs in water and the importance of DWTPs in supplying high-quality water intended for human consumption, the present study investigated relevant endpoints for human health. E-SCREEN and MN assays were applied to determine the total estrogenic/genotoxic potentials in drinking water samples collected in summer over three years at the three DWTPs studied.

3.2.1. Cytotoxicity

The MTT assay was employed to determine the highest non-cytotoxic concentrations of the drinking water samples in the MCF-7 cell line. Cytotoxicity was defined as cell viability below 80% compared to the vehicle control (methanol concentration 0.1%). Initially, the concentrated water samples were tested at concentrations ranging from 10 to 80 REFs (**Supplementary material Figure S-2**). Due to the decreased cell viability observed in some samples above REF 40, drinking water extracts were then tested for their estrogenicity/genotoxicity at REF 20, to treat all samples in the same manner. Bioassays were then conducted under conditions in which cell viability was not compromised.

3.2.2. Endocrine activity

The E-screen assay using MCF-7 cell line is a sensitive and stable tool for quantitatively analyzing the estrogenic activity of environmental water samples (Kinnberg, 2003). The overall estrogenicity of the drinking water extracts is shown in **Figure 4.5**.

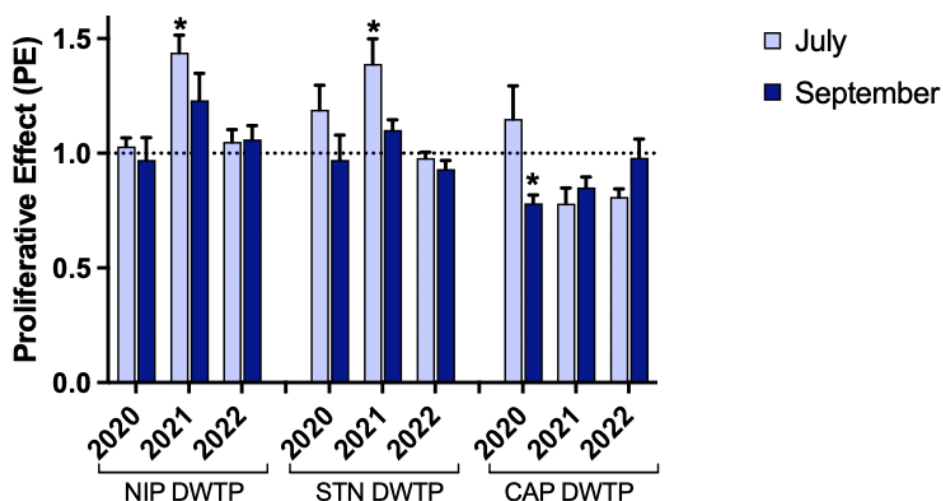


Figure 4.5. Estrogenic activity observed at REF 20 after exposure of MCF-7 cells to drinking water samples from three DWTPs (NIP; Standiana, STN; Capaccio, CAP) collected during six campaigns in 2020, 2021 and 2022 (n=4). Data are expressed as the mean of proliferative effect (PE) $\pm$ SE of different experiments, each conducted in quadruplicate. Activity is normalized to negative control, which was set at 1 (dotted line). *P < 0.05 vs control, cells exposed to negative control (0.1% methanol).

The estrogenic effect in the analyzed samples was compared to the activity of natural estrogen, E2, and calculated by interpolation from a dose-response curve in the range of 10^{-15} to 10^{-8} M (**Figure 4.6**).

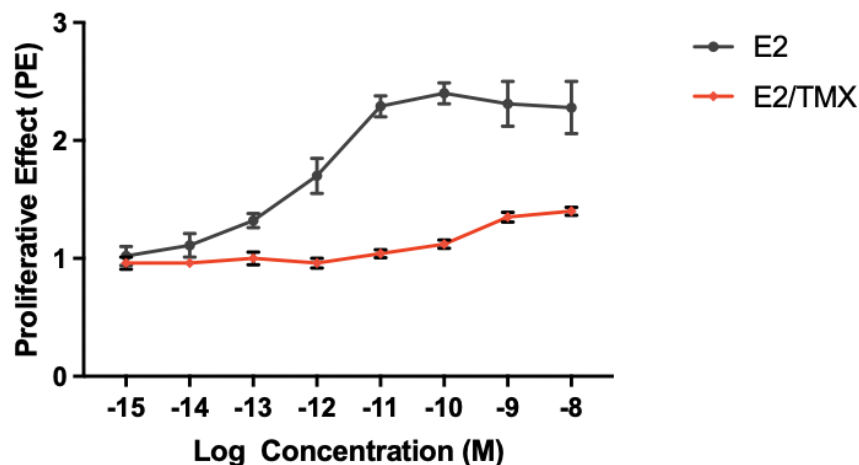


Figure 4.6. Dose-response curve to E2 (used as positive control) of MCF-7 cells, in the presence (black) or not (red) of 10^{-7} M tamoxifen (TMX) (n=10). Data are expressed as the mean of proliferative effect (PE) $\pm$ SE of different experiments, each conducted in quadruplicate.

The reference compound E2 showed an EC_{50} of 2.6 ng/L, which is in good agreement with previous studies reporting 2.2-2.4 ng/L (Macova et al., 2010; Korner et al., 1999). Notably, samples from CAP DWTP outlet waters often induced a slight reduction in cell viability, which was significant in September 2020. However, the observed cytotoxicity did not interfere with the measurement of ER activity because cell viability was never lower than 78% (slightly below the cut-off level). This tendency may have resulted from variations in the contaminant mixtures after the water treatment. Disinfectants used during water treatment may react with micropollutants to form transformation products, which may be more or less toxic than their parent compounds (Neale et al., 2020). However, it is unclear which chemicals induce the slight cytotoxic response, and it is not possible to speculate what may have contributed to this assay.

A significant increase in cell proliferation was observed in two drinking water samples (NIP and STN DWTPs) collected in July 2021, compared to the negative control. For these samples, the E2 equivalent concentration (EEQ), was 0.004 and 0.003 ng/L EEQ, respectively. The observed estrogenic activity was also analyzed in the presence of the estrogen receptor antagonist tamoxifen (TMX), which suppressed this response, confirming that the two water samples possessed ER agonistic activity (**Figure 4.7**).

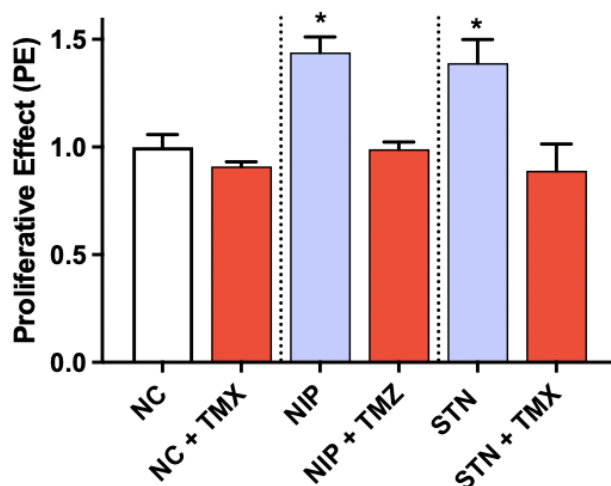


Figure 4.7. Estrogenic activity in drinking water samples from NIP and Standiana (STN) DWTPs in July 2021 (violet bars) compared in the presence of 10^{-7} M Tamoxifen (TMX) (red bars) (n=3). NC: negative control (0.1% methanol). Data are expressed as the mean of proliferative effect (PE) $\pm$ SE of different experiments, each conducted in quadruplicate. *P < 0.05 vs control, cells exposed to negative control.

Generally, the observed estrogenic activities of the samples were low, and within the similar range as previously measured (0.009 ng/L EEQ) (Valbonesi et al., 2021). It is, however, a weak activity compared to many other published studies, using different bioassays based on other mammalian cells (estrogenic activity in the range of 0.01–5 ng/L EEQ) (Oskarsson et al., 2021; Neale et al., 2020; Rosenmai et al., 2018; Shi et al., 2018; Conley et al., 2017; Gou et al., 2016; Maggioni et al., 2013). Overall, the detection of an effect does not necessarily indicate poor water quality (Been et al., 2021; Escher and Neale, 2021). To assess whether the detected effects were at an acceptable or an unacceptable level, the effect-based trigger (EBT) value for drinking water was derived according to the method described by Escher and coworkers (Escher et al., 2015), which recommended safety levels of 0.9 ng/L EEQ. In this study, drinking water samples showing estrogenic effect had activities considerably lower than the proposed EBT value, suggesting that they do not pose a risk by interfering with the estrogenic pathway. Compared with the acceptable daily intake (ADI) of 50 ng/kg/day (E2 equivalent) for humans, assuming complete absorption, a person would have to consume approximately 750,000 L of treated water to reach the ADI.

3.2.3. Genotoxicity

The quality of the drinking water is generally different from that of the DWTPs inlet water, since micropollutants and microorganisms present in source water can be removed by water treatment processes. However, various disinfection by-products can be generated after chlorination, as a result of the combination of disinfectants and naturally occurring inorganic and organic materials (Leusch et al., 2019; Lundqvist et al., 2019; Maffei et al., 2009). Such disinfection by-products exert toxic and

genotoxic/mutagenic effects on living organisms, including humans (Alias et al., 2022; Sui et al., 2022). Moreover, corrosion products, pipeline material leaching, and residual contaminants can pose a threat to the safety of water quality and to human health (Yan et al., 2022). In water quality assessment, genotoxicity tests provide a stronger basis for assessing the reactive toxicity of such complex mixtures (Escher et al., 2021; Ceretti et al., 2016). In this study, the MN test was applied to analyze damage as chromosome aberrations and disturbances in the mitotic cycle in water extracts after water treatment. The number of MN per 1000 nucleated cells was assessed as a measure of chromosomal abnormalities in MCF-7 cells exposed at concentration REF 20. None of the water samples induced a statistically significant increase in micronuclei frequency compared to the negative controls (**Figure 4.8**). The positive control mitomycin C (MTC) showed the expected significant variation ($p < 0.01$), confirming the sensitivity of the test (data not shown). These negative results suggest the absence of critical issues relating the drinking water quality.

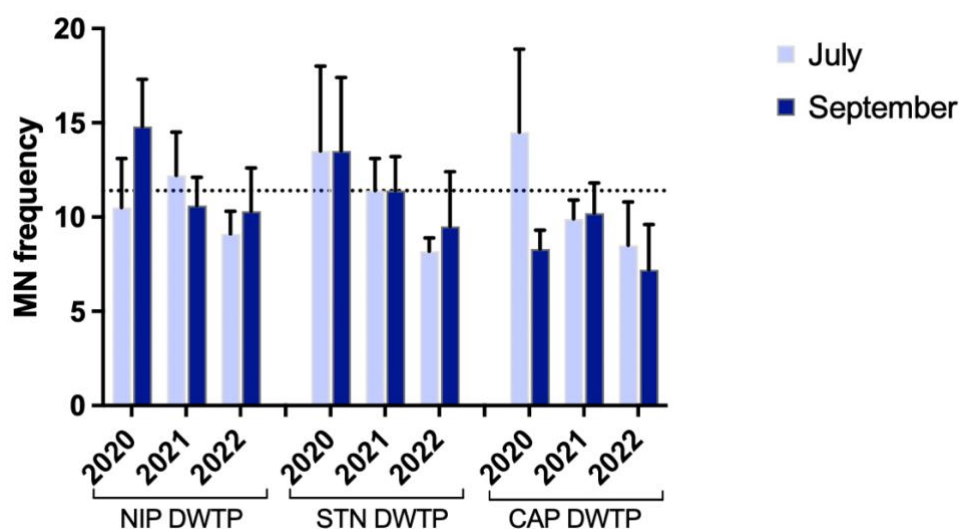


Figure 4.8. Genotoxic response of drinking water samples from three DWTPs (NIP; Standiana, STN; and Capaccio, CAP) collected during summer campaigns in 2020, 2021, and 2022. Data are expressed as the mean of micronuclei (n°/1000 binucleated cells) $\pm$ SD (n=3). Genotoxicity is compared to negative control (0.1 % methanol), which was set at 11.4 (dotted line).

4. Conclusions

This study integrated chemical and biological screening approaches to assess water quality, investigated the efficiency of treatment processes, and comprehensively evaluated the toxicity potential of drinking water across the Romagna area (Italy). The results showed that the final concentrations of the target EDCs in drinking water were considerably lower than the guideline

parametric values proposed by the DWD. However, NP and BPA overcame water treatments and remained in the outlet water of DWPTs. From a treatment perspective, conventional and advanced treatment processes showed high variability in EDC removal rates and were often ineffective in eliminating BPA and NP. This work highlighted the convincing role of DWTP as an additional source of EDC contamination in drinking water. This indicates that the quality of drinking water is not entirely dependent on the contamination level of the source water. Indeed, during water treatment, the leaching of harmful chemicals can adversely influence the quality of the treated water. As this phenomenon was observed with an increase in BPA and NP levels along the different steps of the treatment process, it is important to continue monitoring and identify improvements in implant materials. The results demonstrated that chemical analyses alone may result in bias when assessing the intrinsic safety of water samples. Drinking water samples showed no genotoxic potential after treatment, whereas the detected estrogenic activity in two of the produced waters was notably below the available EBTs. Although the water samples investigated were of good quality, the use of integrated chemical and effect-based approaches is recommended for assessing drinking water quality, in particular to counteract the risk associated with the presence of unknown substances and the mixture effects.

Supplementary Material

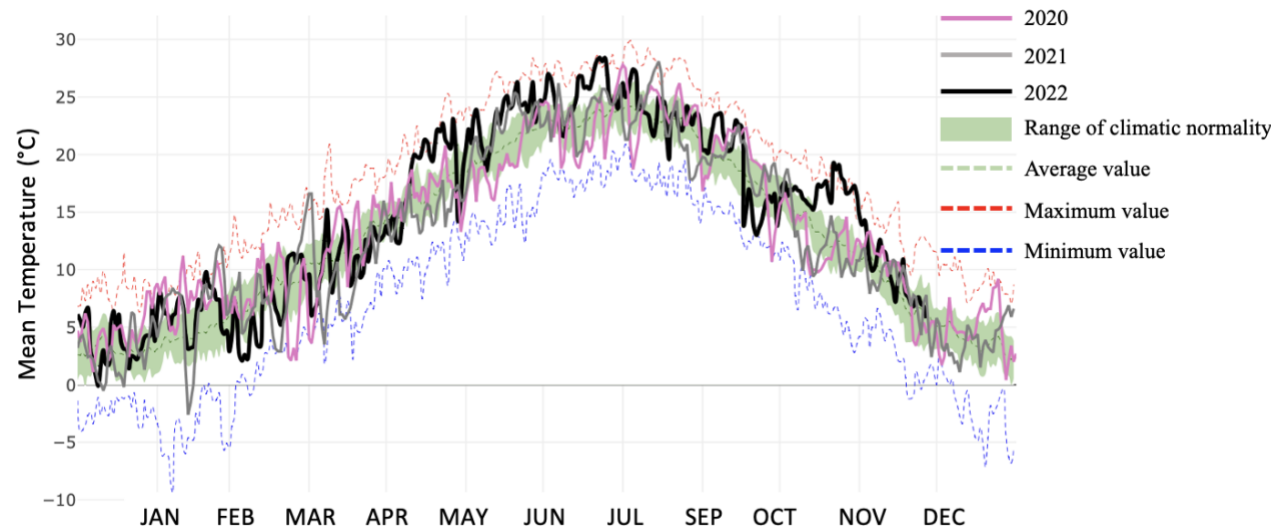


Figure S-1. Wheater condition of the Emilia-Romagna region (source: <https://www.arpae.it/it/temi-ambientali/clima>).

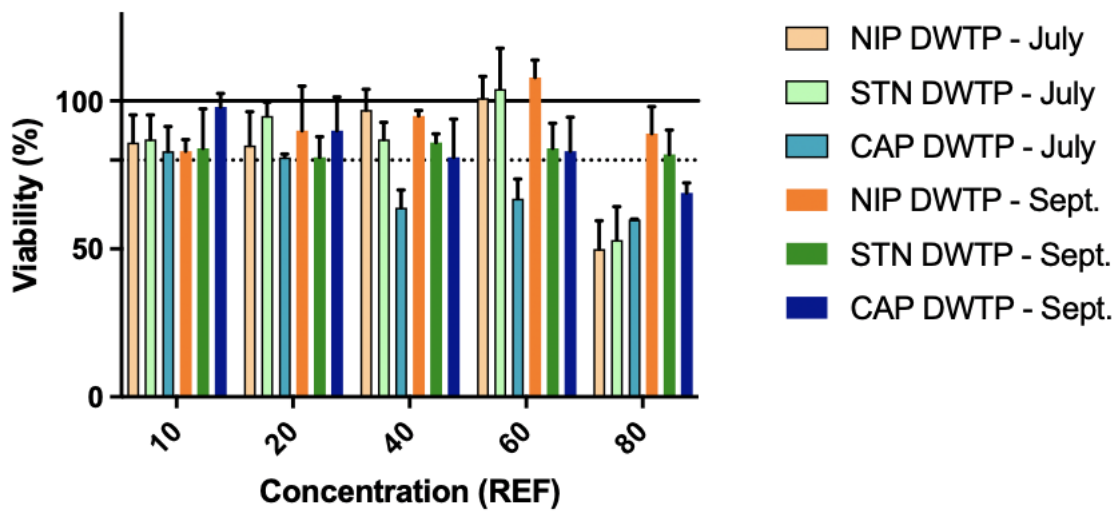


Figure S-2. Effect of different concentration (from 10 to 80 REFs) of drinking water extracts collected from three DWTPs (NIP, Standiana, STN; Capaccio, CAP) on cell viability in MCF-7 cells (n=3). Data are expressed as the mean $\pm$ SE of different experiments, each conducted in triplicate. Activities are normalized to vehicle control, which was set at 100% (solid line). The cut off level for a cytotoxic signal was set to 80 % viability (dotted line).

Chapter Five

PREFACE

In this case study, a battery of bioassays was applied to evaluate effective removal of target EDCs by conventional and advanced treatment processes of DWTP. Specifically, effect-based screening was conducted to assess whether the water treatment processes performed by the DWTP represent a significant source of EDCs with (anti-) estrogenic, (anti-) androgenic disrupting, and mutagenic potencies. Where possible, the observed effect was compared with available EBTs. Chemical analysis of the water samples analyzed in this study were provided in Chapter Four. Chapter 5 will be submitted soon for publication on an international peer reviewed Journal.

The research work presented in this Chapter was carried out during a research stay period of 3 months at the Department of Evolutionary, Ecology and Environmental Toxicology (E3T), Goethe University of Frankfurt (Germany). The research work was carried out with the valuable supervision of Dr. Prof. Henner Hollert, Dr. Andreas Schiwy, and Dr. Simone Wollenweber.

Effect-based screening for water quality and treatment efficiency of an Italian drinking water treatment plant

1. Introduction

Access to safe water is recognized as a human right that is fundamental to everyone's health, dignity, and prosperity (Sustainable Development Goal 6 of the United Nations Agenda) (United Nations, 2015). Contamination of drinking water by micropollutants is a critical issue worldwide (Khan et al., 2022; Mukhopadhyay et al., 2022; Meiburg, 2018). It has been postulated that the main cause of approximately 80% of human diseases is water pollution (Alver, 2019). A recent study reported that more than 700 contaminants may occur in drinking water systems (Wang, 2022). Many of these compounds have been identified as emerging contaminants (Tröger et al., 2021; Arman et al., 2021; Tang et al., 2019; Wee and Aris, 2017), including chemicals from both natural and anthropogenic activities (pharmaceuticals, personal care products, hormones, plasticizers, industrial compounds, and pesticides). Contamination of drinking water by emerging contaminants remains a global concern, because of their potential impact on human health and the environment. Emerging contaminants are associated with acute to chronic diseases, such as immune and metabolic effects, reproductive abnormalities, behavioral changes, diabetes, obesity, cardiovascular diseases, neurological disorders, disrupted fetal development and growth, and a wide variety of cancers (Goralczyk, 2021; Yadav et

al., 2021; Encarnação et al., 2019; Adeel et al., 2017; Wee and Aris, 2017). The occurrence of emerging contaminants and their by-products in water resources can be explained by municipal, hospital, industrial, and agricultural discharges in effluents intended for potable use, and by the insufficient removal achieved by the traditional treatments used in wastewater treatment plants (Morin-Crini et al., 2022). This occurrence, even at trace concentrations, can compromise the production and quality of drinking water (Tröger et al., 2021; Carere et al., 2015; Pal et al., 2014). It is important to note that surface water is an important source of drinking water in most countries (Katsanou and Karapanagioti, 2017). Generally, conventional water treatment techniques are effective in treating surface water by eliminating microbial contaminants and the majority of macropollutants (Katibi et al., 2021). Although drinking water treatment plants (DWTPs) are designed to ensure safe drinking water, they do not completely eliminate emerging contaminants that remain in treated water, leading to human exposure to hazardous compounds (Papagiannaki et al., 2022; Yu et al., 2021). Moreover, the quality of treated water is attributable not only to the quality of the starting water, but also to the conditions of the networks and the possible introduction of external contaminants. During drinking water treatment, contaminants can also form complex mixtures (e.g., chemical transformation) (Neale et al., 2020; Hebert et al., 2018; Maffei et al., 2009), or can be released by engineering systems (e.g. pipelines and plastic materials) (Qi et al., 2022; Yan et al., 2022; Cantoni et al., 2021; Muhamad et al., 2016b). Thus, incomplete removal of contaminants by water treatment contributes to their loading into the drinking water supply (Wee et al., 2021). Therefore, the combination of conventional and advanced treatment technologies can considerably reduce the occurrence of harmful substances in drinking water. To date, information on the fate of emerging contaminants in DWTP is limited and little is known about the efficiency of processes for the removal of micropollutants during drinking water treatment.

Routine chemical monitoring to test drinking water quality has been implemented continuously, as in the case of newly regulated chemicals. However, target chemical analyses do not consider the importance of multiple synergistic, antagonistic, or potentiating effects between components (Caporale et al., 2022; Altenburger et al., 2018; Howdeshell et al., 2017). Well-known chemicals can only explain as little as 0.1–5% of the observed toxicity, implying that up to 99.9% of the toxicity is caused by unknown chemicals or mixture effects (Lundqvist et al., 2019). Furthermore, chemicals may be present in a water sample below the limit of detection (LOD) of the analytical instrument, but it is possible that such concentrations still contribute to a biological effect (Neale et al., 2022; Kase et al., 2018). Alternatively, biological monitoring using effect-based methods (EBMs) has gained popularity over the last decade as a promising complement to existing water quality assessments (Brack et al., 2019; Neale et al., 2017; Leusch and Snyder, 2015; Escher et al., 2014). Such tools are

especially relevant for understanding unknown, new chemicals, and transformation products, without the chemical identification of compounds. Recent studies on drinking water quality have used *in vitro* bioassays to detect the biological effects of harmful chemicals in source and finished water (Oskarsson et al., 2021; Medlock Kakaley et al., 2021; Valbonesi et al., 2021; Leusch et al., 2018; Escher et al., 2014). Furthermore, the potential biological activity of drinking water during treatment of DWTPs has been only recently examined (Neale et al., 2020; Nivala et al., 2018; Rosenmai et al., 2018).

The aim of this study was to assess a full-scale DWPT in order to: 1) determine the potential biological effects elicited by typical water contaminants from sources to drinking water, and 2) verify the ability of water treatment processes to remove them. It has been reported that estrogenic and mutagenic/genotoxic effects are widely detected in water samples, and consequently, they are most commonly studied in drinking water (Neale and Escher, 2019). The CALUX (Chemically Activated LUciferase eXpression) assay is a frequently used bioassay to evaluate endocrine potential. This method allows the detection of estrogenic and androgenic compounds via human estrogen receptor alpha (ER α) and androgen receptor (AR). Here, CALUX assays were used to detect potential (anti-) estrogenic and (anti-) androgenic responses inside the DWTP from raw to treated water. Owing to the potential formation of mutagenic and/or genotoxic substances during the disinfection of drinking water (e.g., during chlorination), water was also evaluated to assess mutagenicity using the Ames fluctuation assay. In parallel, the occurrence of micropollutants in the water samples was analyzed and previously reported (Chapter Four).

2. Materials and Methods

2.1 Drinking water treatment plant (DWTP) in Ravenna, Italy

In the present study, water samples were collected from one main DWTP (Standiana) in the Romagna area (southeastern part of the Emilia-Romagna region, Italy), located near Ravenna (Italy) and operated by Romagna Acque Società delle Fonti SpA. The DWTP, which has been active since 2015, uses more advanced water treatment techniques, which obtain high-quality water starting from the CER (“Canale Emiliano-Romagnolo”), an artificial water transfer system that takes water directly from the Po River. The drinking water is treated using conventional water treatment techniques (mechanical retention of large solids, separation of sand and grit in aerated chambers, pre-chlorination with chlorine dioxide, chemical conditioning with ferrous chloride and poly aluminum chloride, flocculation, and chlorination with chlorine dioxide). To improve the efficiency of DWTP, a combination of ultrafiltration through 0.04 μ m membranes and biological activated carbon (BAC)

filters was designed to remove dissolved organic contaminants, micropollutants, and pathogenic organisms at the end of the treatment processes. The finished drinking water enters the distribution network, which consists of 40 km pipelines that serve residential, commercial, and industrial water users. An average of 1100 L/s and 20 million m³/year of drinking water was distributed from the DWTP in the Romagna area. A schematic representation and the sampling points of the DWTP are summarized in **Figure 5.1**.

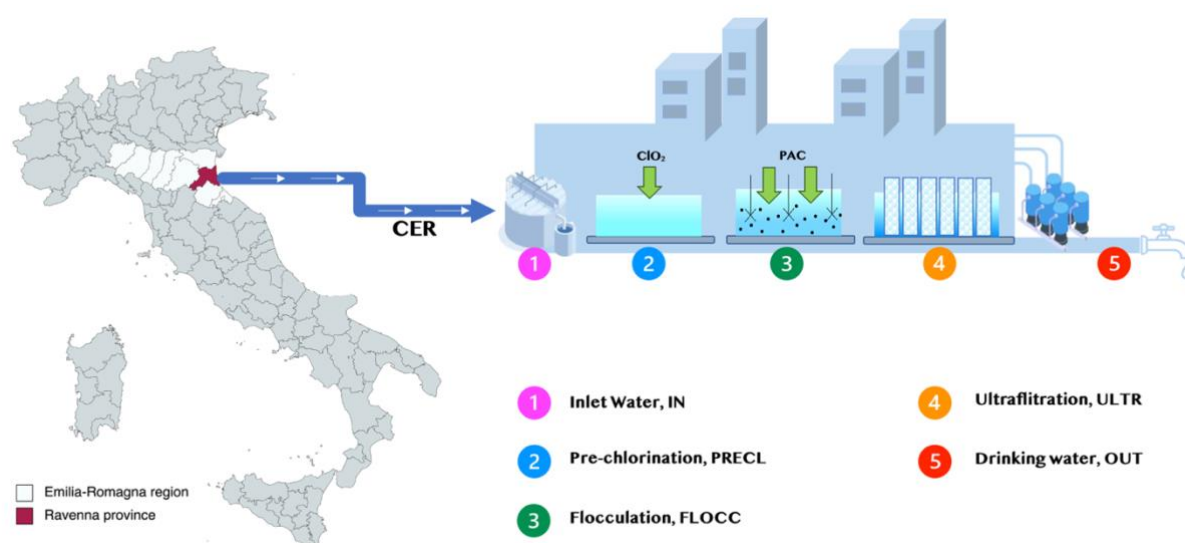


Figure 5.1. Schematic representation and sampling point of Standiana DWTP.

2.2 Sample collection and preparation

The sampling was carried out in March 2022. Grab samples were collected from 5 sampling sites representing the full treatment cycle of the drinking water production process: inlet water (IN), pre-chlorination (PRECL), flocculation (FLOCC), ultrafiltration (ULTR), and treated water (OUT). For each sampling site, 3 L of water samples were collected in 1-L PET bottles and transported immediately to the laboratory where they were stored at 4°C in darkness until sample preparation. The PET bottles have previously been rinsed with methanol to avoid EDCs adhesion on bottle surfaces. The water samples were first filtered with glass microfiber filters (1.60 µm) and then with cellulose acetate filters (0.45 µm) under vacuum. All samples (1 L) were extracted via solid-phase extraction (SPE) using Oasis HLB cartridges (6 cm³, 200 mg) (Waters S.p.A., Sesto San Giovanni, Milan, Italy). The sample extraction process consisted of: preconditioning with 6 mL of methanol and 6 mL of deionized water, loading of water sample volume under vacuum, extraction with 6 mL of methanol, followed by evaporation under gentle nitrogen stream up. All samples are re-suspended

with DMSO to obtain a final extract volume of 0.5 mL, finally sterilized with 0.20 µm syringe cellulose acetate filters. Each water sample was thus enriched by a factor of 2000. The concentration of the water samples in the cell cultures is expressed as the relative enrichment factor (REF), which is calculated by dividing the concentration factor of the SPE by the dilution factor in the cell culture medium, as reported below:

$$\text{REF} = \text{concentration factor SPE} / \text{dilution factor bioassay}$$

A REF > 1 implies that the water sample has been enriched, and a REF < 1 that the sample has been diluted compared to the initial water. REF = 1 is interpreted as the original sample.

2.3 Bioanalytical screening

In this study, were used a panel of effect-based methods targeting early molecular events in health-relevant toxicity pathways. The effect based-methods covered the effects of estrogen receptor activation and inhibition ((anti-)ERα-CALUX®), androgen receptor activation and inhibition ((anti-)AR-CALUX®), and mutagenicity (Ames fluctuation assay). All water extracts were analyzed in dilution series and vehicle controls, and a dilution series of the reference compound was included on each experimental plate.

2.4 CALUX® assays

The Chemically Activated Luciferase eXpression® assay, hereafter referred to as the ERα-CALUX and AR-CALUX assays were employed to determine the estrogenic and androgenic activity of water extracts. These reporter gene assays are based on the human osteoblastic cell line U2OS, which has been stably transfected with 3 ERE-TATA-Luc, pSG5-neo-hERα/3 × HRETATA-Luc or pSG5-neo-hAR reporter gene construct, obtained under license from Biodetection Systems B.V. (Amsterdam, the Netherlands). Receptor-mediated estrogenic or androgenic effects are highly specific in U2OS cells since they are the only available receptors after genetic modification. These modified cell lines are sensitive and highly responsible for (anti)- estrogenic/androgenic compounds. The U2OS cells were cultured using the methods recommended by the supplier and according to the ISO Guideline 19040-3 (2018). In brief, cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After 24-h incubation, the cells were exposed in triplicate to test substances, positive control (agonistic effect: 17β-estradiol, E2 / dihydrotestosterone, DHT; antagonistic effect: tamoxifen, TMX / flutamide, FLU) and solvent control (0.1% DMSO), for 24 h at 37°C and 5% CO₂. After the addition of 30 mL of lysis mix, luciferase activity was measured using a Tecan Spark plate reader. The

concentration-response curves of estrogen/androgen expressed by E2/DHT were fitted based on nonlinear regression. For agonistic activities, the maximum activity was defined as the highest activity recorded for the reference compound. For antagonistic activity, the maximum activity was defined as the activity recorded by control samples treated with TMX/FLU. For both agonistic and antagonistic CALUX® bioassays, the limit of the detection (LOD) within each tested plate was calculated as the average of the relative light unit (RLU) values within the blank of the standard row plus its three-fold standard deviation. The limit of quantification (LOQ) was calculated as the ten-fold LOD. Results are expressed as % of max activation/inhibition (with respect to the reference compound).

2.5 Ames fluctuation assay

The mutagenicity was assessed using the Ames fluctuation assay with the *Salmonella typhimurium* strains YG1041 and YG1042 with and without metabolic activation via S9, performed according to the ISO guideline 11350 (2012). *Salmonella typhimurium* strains are modified by a point mutation in the histidine gene. Repeated mutations in the histidine gene can cause reversion to the wild-type characterized by the own production of the growth-essential histidine. The strains YG1041 and YG1042 are sensitive to nitroarenes and aromatic amines (Hagiwara et al., 1993). In brief, both bacteria strains were cultured overnight for 10 h in a growth medium in a shaking incubator at 250 rpm and 37 °C in darkness. The overnight culture was diluted to 45 formazine attenuation units (FAU) in the assay medium. *Salmonella* strain was exposed for 100 min to extracted samples (2 % DMSO) in triplicate in 24-well microplates at 37 °C and 150 rpm. Nitrofurantoin (NF) or 2-Aminoanthracene (2-AA) served as a positive control. The negative control is DMSO both with and without S9 at 10 mg_{protein}/ml. After incubation, the exposure media was diluted by a reversion indicator medium with bromocresol purple as a pH indicator. Cultures were transferred to 384-well plates (16 wells per replicate) and incubated for 48 h at 37 °C. Due to the incubation in a histidine deficient medium, only bacteria that regained histidine prototrophy through mutation show growth. This growth leads to acidification of the indicator medium, which thereby changes color from purple to yellow. Wells with yellow color were considered revertant wells. Sample-induced reversion from purple to yellow was counted manually. Mutagenicity is expressed as the number of revertant wells of the sample.

2.6 Cytotoxicity

The cytotoxicity of the water extracts to U2OS cells for CALUX assays was assessed with the neutral red retention assay as originally developed by (Borenfreund and Puerner, 1985), with modifications. This colorimetric assay determines the accumulation of the neutral red dye (2-amino-3-methyl-7-

dimethyl-aminophenazoniumchloride) in lysosomes of viable, uninjured cells. Following 24-h exposure to the sample extracts, cells were incubated with neutral red dye (50 µl/ml) dissolved in free serum medium for 2-h. SDS was used as a positive control at a maximum concentration of 0.5%. Neutral red retention was measured at the optical density (OD) between 530 nm and 645 nm, with a Tecan Spark microplate reader. Results were expressed as % of the control value (see equation 1). Viability of <80% of the vehicle control was defined as cytotoxicity.

To assess the cytotoxicity in the Ames fluctuations assay, the AlamarBlue reduction assay was conducted according to Costa et al. (2020) with slight modifications. In brief, resazurin, the active ingredient of the AlamarBlue reagent, is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. YG1041 and YG1042 strains were exposed to water extract for 100 min, in presence of a 5% of AlamarBlue solution. The ability of bacteria to reduce resazurin into fluorescent resorufin was measured by spectrofluorimetry using a Tecan Spark microplate reader, with excitation set at 585 nm and emission set at 630 nm. The values obtained from the readings were expressed as Relative Fluorescence Unit (RFU). The cytotoxicity was determined by plotting the $RFU_{t=100}/RFU_{t=0}$, and calculated using eqn (1).

$$(1) \quad \text{Cytotoxicity} = \text{sample} / \text{control} * 100.$$

2.7 Data analysis

For each sampling point, 3 L of water were processed independently and each eluate was tested in independent experiments. The experimental data were obtained from the replication of at least three independent experiments. All the results were statistically evaluated using a one-way ANOVA followed by Dunnett's multiple comparisons test, a statistical difference was accepted when $p < 0.05$; performed in SigmaPlot v.13 (SigmaPlot software, ver 13, Systat Software Inc.). For all samples defined as active, effect concentrations were calculated. For agonistic activities, the effect concentration 10% (EC10) was calculated. EC10 is expressed as REF, i.e., the enrichment factor of the water needed to give an effect which is 10% of the maximum response of the reference compound. The EC value was translated into a bioanalytical equivalent concentration (BEQ), as described by Escher et al.:

$$BEQ = (EC_x) \text{ reference compound} / (EC_x) \text{ sample}.$$

For the estrogenicity assay, the reference compound is E2 and hence the BEQ is termed E2 equivalent concentration, (EEQ). The BEQ for the androgenic activity was expressed as a DHT- equivalent concentration (DHT-EQ), while for antagonistic assays, TMX/FLU equivalent concentration (TMX/FLU – EQ) was used.

For the Ames fluctuation assay, to meet the validity criteria according to ISO 11350, the negative control must have ≤ 10 positive wells and the positive control ≥ 25 positive wells in the Ames fluctuation assay (ISO, 2006; OECD, 2020). Environmental samples were considered mutagenic if the number of wells containing revertant was significantly increased compared to the average number of the negative controls ($p > 0.05$). Data were analyzed using ToxRat (Version 3.2, ToxRat Solutions GmbH, Alsdorf, Germany).

3. Results and Discussion

3.1. Cytotoxicity

All samples were tested for cytotoxicity in the dilution series. In CALUX assays the final concentrations tested were 0.10, 0.25, 0.50, 1, and 2 REFs, whereas in the Ames fluctuation assay were 10 and 20 REFs were tested. To the best of our knowledge, this is the first study in which the AlamarBlue reduction assay was applied to assess cytotoxicity in bacterial strains for the Ames fluctuation assay. Cytotoxicity was conducted as a pre-measurement to avoid false-negative or false-positive results. In all assays, none of the tested water samples exhibited cytotoxicity (**Supplementary Material, Fig. S-3 and S-4**) which demonstrated that the bioassays were conducted under conditions where the cell viability was not compromised. Therefore, the lack of masking effects due to cytotoxicity is expected to distort the results of specific assays.

3.2. Endocrine activity

Samples for endocrine activity were tested using CALUX[®] assays, in a dilution series starting at REF 2. Of the studied hormone receptor-mediated effects, the extracts were only active in the ER α CALUX assay, indicating estrogenicity (**Figure 5.2**). Significant estrogenic responses were detected at the inlet water and after the ultrafiltration step, with effects detected starting at 0.25- and 1-times enrichments, respectively. For these active samples, the BEQ values were calculated in the form of EEQ. EC₁₀ of the positive control E2 was corresponding to a BEQ value of 0.4 ng/L. The BEQ at inlet water was 1.7 ng/L EEQ, indicating that the sample needed to be diluted 0.25 times to cause 10% activation of ER, whereas BEQ at the ultrafiltration step was 0.5 ng/L EEQ. The inactive

samples, pre-chlorination, flocculation, and treated water, have not shown activity of 10% of assay maximum or more had a BEQ value < 0.4 ng/L E2 (Figure 5.2).

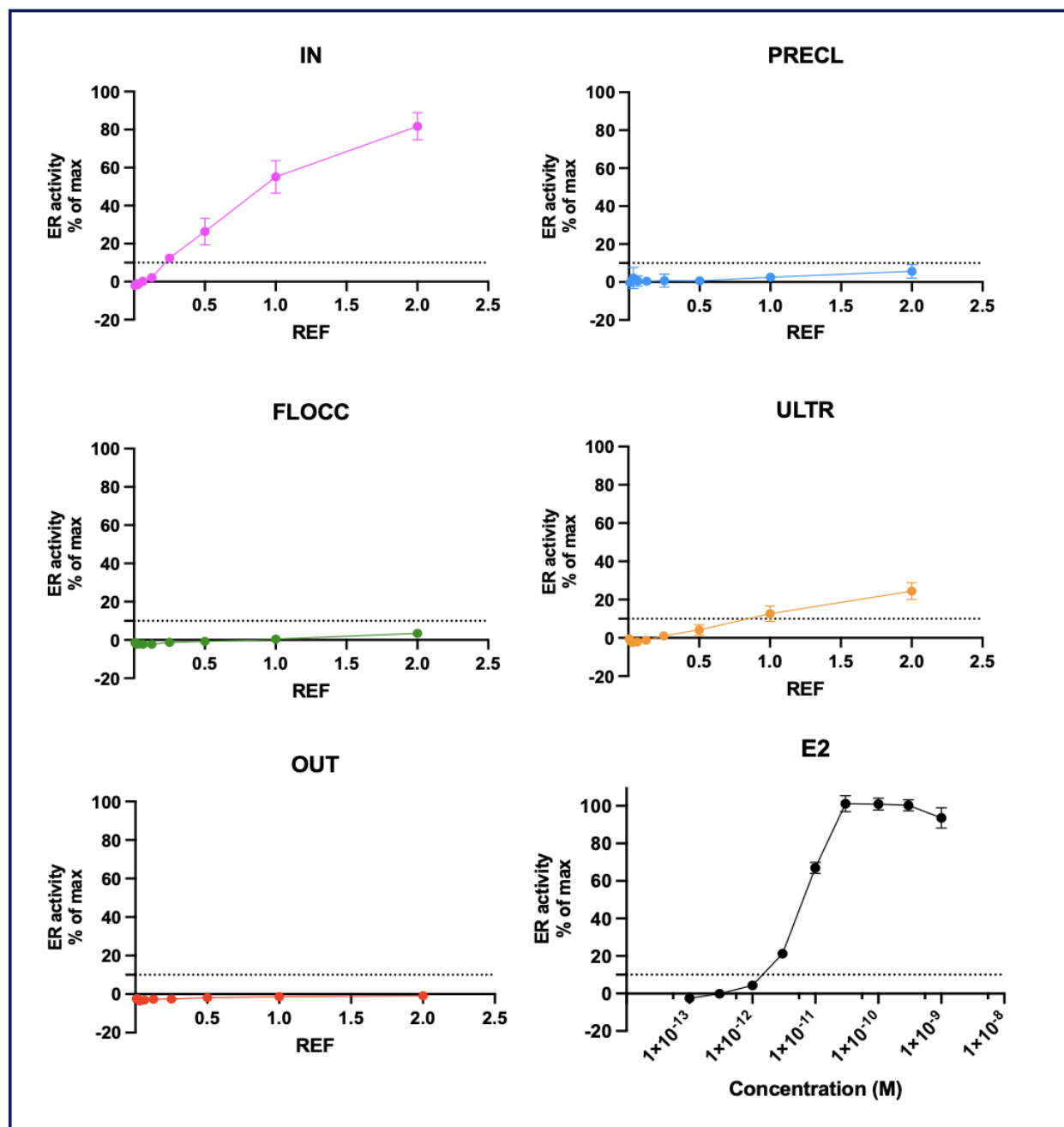


Figure 5.2. Estrogenic activity of drinking water samples detected in ER α CALUX assay. IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water. Samples were analyzed in a dilution series with REF 2 as the highest concentration. Each REF concentration represents mean values $\pm$ SD of three independent replicates. The response is expressed as % relative response to the reference agonist E2 (EC_{10} 1.6×10^{-12}). The black dotted line represents the activity level of 10% of the maximum response.

To determine whether the detected estrogenic effects in the inlet and ultrafiltration water samples were of any concern, the responses were compared with the available Effect-Based Trigger (EBT) for surface water (0.1 ng/L EEQ) (Escher et al., 2018), and for drinking water (0.2 ng/L EEQ) (Escher et al., 2015). The measured BEQ value of 1.7 ng/L EEQ in the drinking water source, exceeded 17 times that of the proposed EBT, indicating that contaminants are biologically active in surface water. Several studies have reported the estrogenicity of source water samples (Neale et al., 2020; Rosenmai et al., 2018; Leusch et al., 2018; Xiao et al., 2017; Conley et al., 2017). In the current study, the Standiana DWTP fed surface water from the CER, receiving freshwaters from the river Po (the most important Italian watercourse), which drains water from the most densely populated and productive regions of Italy. It transports inorganic nutrients, trace metals, and a multitude of contaminants, such as endocrine-disrupting substances, flame retardants, pharmaceuticals, and personal care products (Pignotti et al., 2017; Viganò et al., 2015). In a previous investigation of the same DWTP (Valbonesi et al., 2021), frequent contamination of bisphenol A, nonylphenol, caffeine, carbamazepine, PFOA, PFOS, and sporadic contamination of atenolol was observed in the inlet water of the Standiana DWTP. Many of these compounds are currently unregulated in water regulatory frameworks, and are known to be biologically active and associated with endocrine-disrupting effects in humans and wildlife. Their occurrence in the surface water can explain the high ER activity reported in the inlet water of the DWTP studied. In contrast to the first steps of treatment (pre-chlorination and flocculation) in which the ER activity was reduced below the cut-off level, after the ultrafiltration treatment the estrogenic activity increased. The measured effect exceeded of 2.5 times the trigger value of 0.2 ng/L EEQ for drinking water. Chemicals such as plasticizers, flame retardants, detergents and surfactants are traditionally used as constituents in the manufacturing of valves, pipes, and mechanical parts of DWTP. For instance, bisphenol A and phthalates are commonly used as polymers in equipment in pipes and water systems, nonylphenol is widely applied as an additive in epoxy resins, and PFAS have been widely applied in different household and industrial application (Mohammadi et al., 2022). Ultrafiltration membranes can also be fabricated from both polymers and inorganic materials (Wong et al., 2018). The available literature reports that these polymer compounds can leach from plastic materials and pipes into the water (Cantoni et al., 2021; Gunaalan et al., 2020; Cheng et al., 2016; Achene et al., 2011; Le et al., 2008), affecting the quality of drinking water. Therefore, the DWTP could represent a potential source of water contamination. Furthermore, the hydraulic condition of the membrane used during ultrafiltration can influence the removal of contaminants (Muhamad et al., 2016b). Considering all the above-mentioned situations, the present finding corroborated that bioactive contaminants released from treatment manufactures can contribute to the detected ER effects. Although the estrogenic activity was measured after the

ultrafiltration step, the final treatment process (corresponding to BAC filtration) was able to remove the overall estrogenic activity, with no detectable effect in the finished water, even at the highest REF. These data confirm previous results (Valbonesi et al., 2021), in which post-treatment water from the same DWTP did not exhibit any biological activity. The observed activities can also be compared to the benchmark value of 1 ng/L of E2, recommended in the first watchlist of the EU drinking water directive. It should be noted that the estrogenic activities observed in the studied water samples were all considerably below the benchmark value of 1 ng E2/L.

None of the samples showed a response in antagonist mode in the ER-CALUX and agonist/antagonist AR-CALUX assays (**Supplementary Material, Figure S-5**).

3.3. Mutagenicity

None of the source or produced water samples inhibited growth in the YG1041 and YG1042 strains; therefore, the Ames fluctuation assay was performed at REF up to 20. All the positive controls provided valid responses (data not shown). Several extract samples displayed a mutagenic activity in the bacterial strain YG1042 without or with a metabolic activation via S9 (**Table 5.1**). None of the samples showed any mutagenic response in the Ames *S. typhimurium* strain YG1041, with or without metabolic activation S9.

Table 5.1. Mutagenic potential of the water samples in the Ames fluctuation assay with *Salmonella typhimurium* strains YG1041 and YG1042 (+/-S9). IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water Data were reported only for positive response and expressed as % reversion (Step-down Cochran-Armitage test, $p < 0.05$) referred to the revertant number in the negative control. Data were obtained from three independent replicates ($n = 3$).

SAMPLE		% REVERSION (YG1042)		% REVERSION (YG1041)	
		-S9	+S9	-S9	+S9
IN	REF 20	14.6	-	-	-
	REF 10	-	-	-	-
PRECL	REF 20	-	-	-	-
	REF 10	-	-	-	-
FLOCC	REF 20	-	-	-	-
	REF 10	-	-	-	-
ULTR	REF 20	-	13.9	-	-
	REF 10	-	-	-	-
OUT	REF 20	-	-	-	-
	REF 10	-	-	-	-

In particular, inlet and ultrafiltration water samples induced mutations with or without metabolic activation S9, revealing the presence of both direct-acting mutagens and pro-mutagens, respectively. However, the measured mutagenic activity was a borderline effect only at the highest dose tested (REF 20). Overall, by considering the results obtained by Ames fluctuation assay, mutagenic effect in raw water was removed by the first treatment processes, pre-chlorination and flocculation steps, whereas after ultrafiltration was evidenced a slight mutagenicity (13.9% reversion). As reported above, this might be due to the characteristics of the material with which the plants are built, which can release contaminants with mutagenic induction into the water. As reported by Feretti et al. (2020) chemical interactions between disinfectant and substances in water can generate by-products contaminants during water treatment, contributing to the mutagenic/genotoxic effects. However, it is worth noting that these results should be interpreted with caution, as further studies are needed to confirm the findings, and it should be considered as a limitation of this study. Overall, final results will be added to the manuscript prior to submission to a journal for publication.

4. Conclusion

In conclusion, this study investigated the treatment efficiency of DWTP using effect-based methods indicative of endocrine and mutagenic pathways. Of the examined endocrine pathways, only estrogenic activity was detected in the source water, exceeding the proposed surface water EBT. Although the primary water treatment processes removed estrogenicity from the raw water, the bioactivity slightly increased after the ultrafiltration step, suggesting that the DWTP itself had some contribution to ER activity. Overall, removal by DWTP was found to be effective and there was no estrogenic response in the finished drinking water.

Parallel tests with *Salmonella typhimurium* strains YG1041 and YG1042 (Ames fluctuation assay) have not been concluded, yet. Further experiments will be carried out within a short time to fully understand the extent to which DWTP can produce mutagenic compounds and the potential health risks associated with exposure to these harmful compounds. Overall, the present work emphasizes the importance of monitoring the quality of water and efficiency of DWTP using effect-based methods in order to protect human health and the environment.

Supplementary Materials

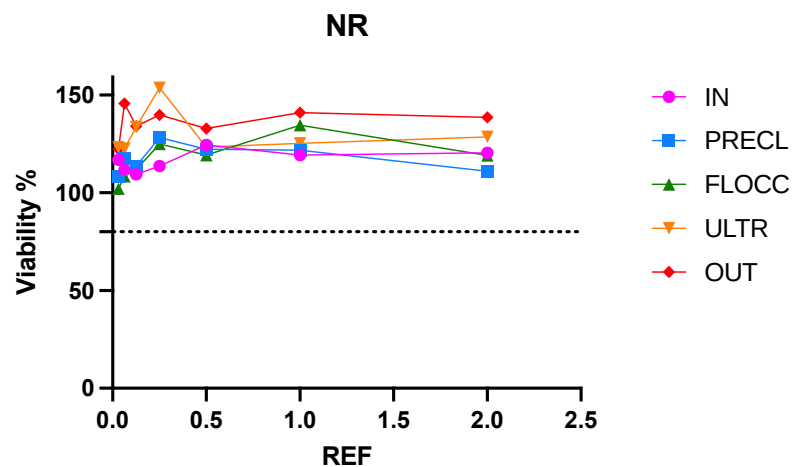


Figure S-3. Cell viability in U20S cell line, measured by Neutral Red assay at starting REF 2. IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water. The black dotted line represents a cell viability of 80% compared to negative control, which was used as the threshold for cytotoxicity. Negative Control: 0.1% DMSO.

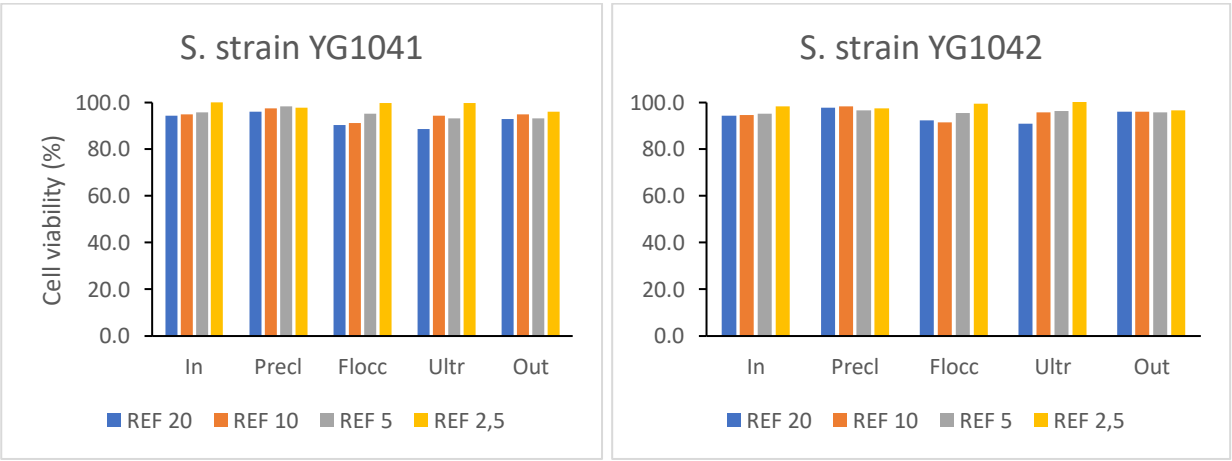
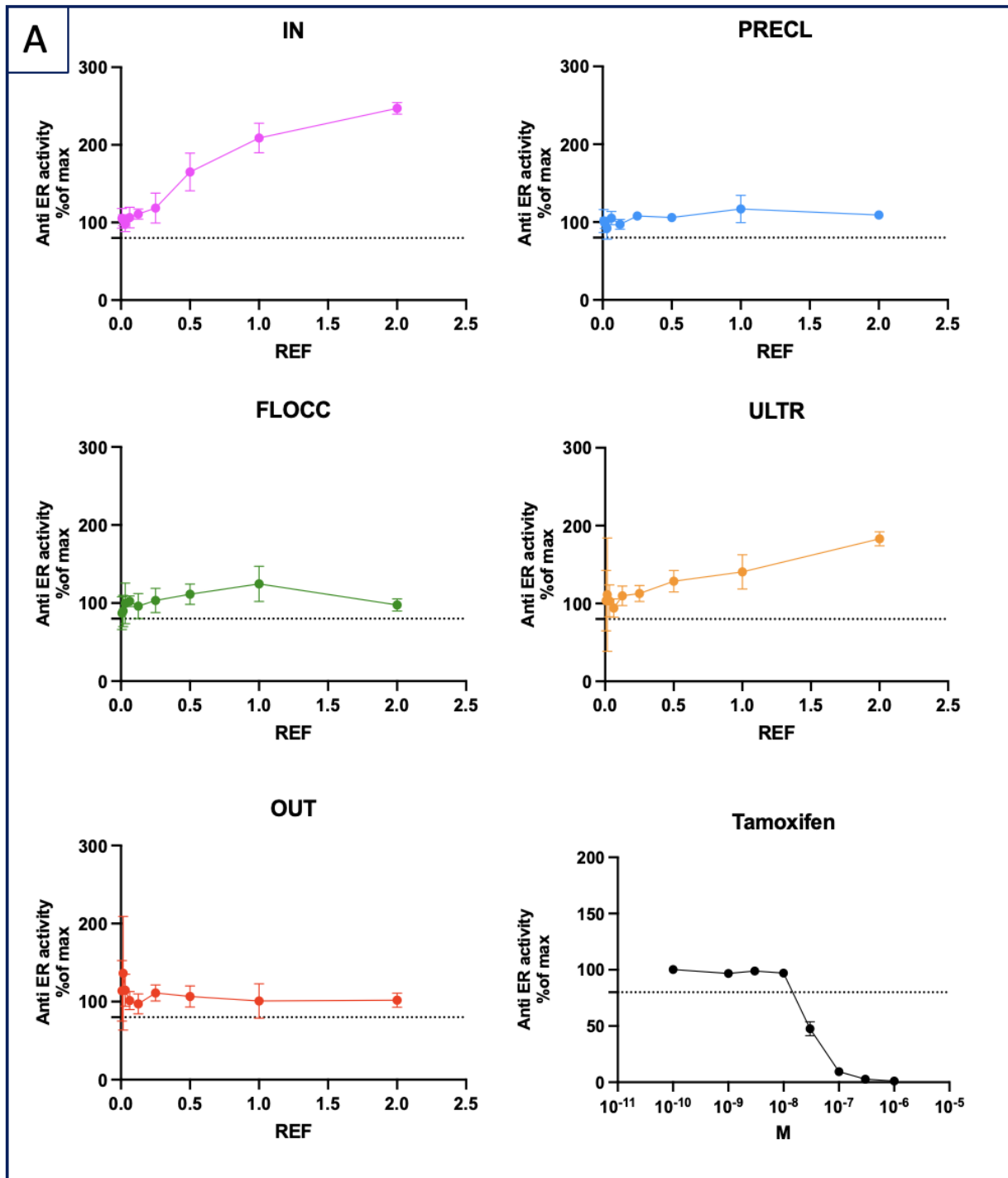
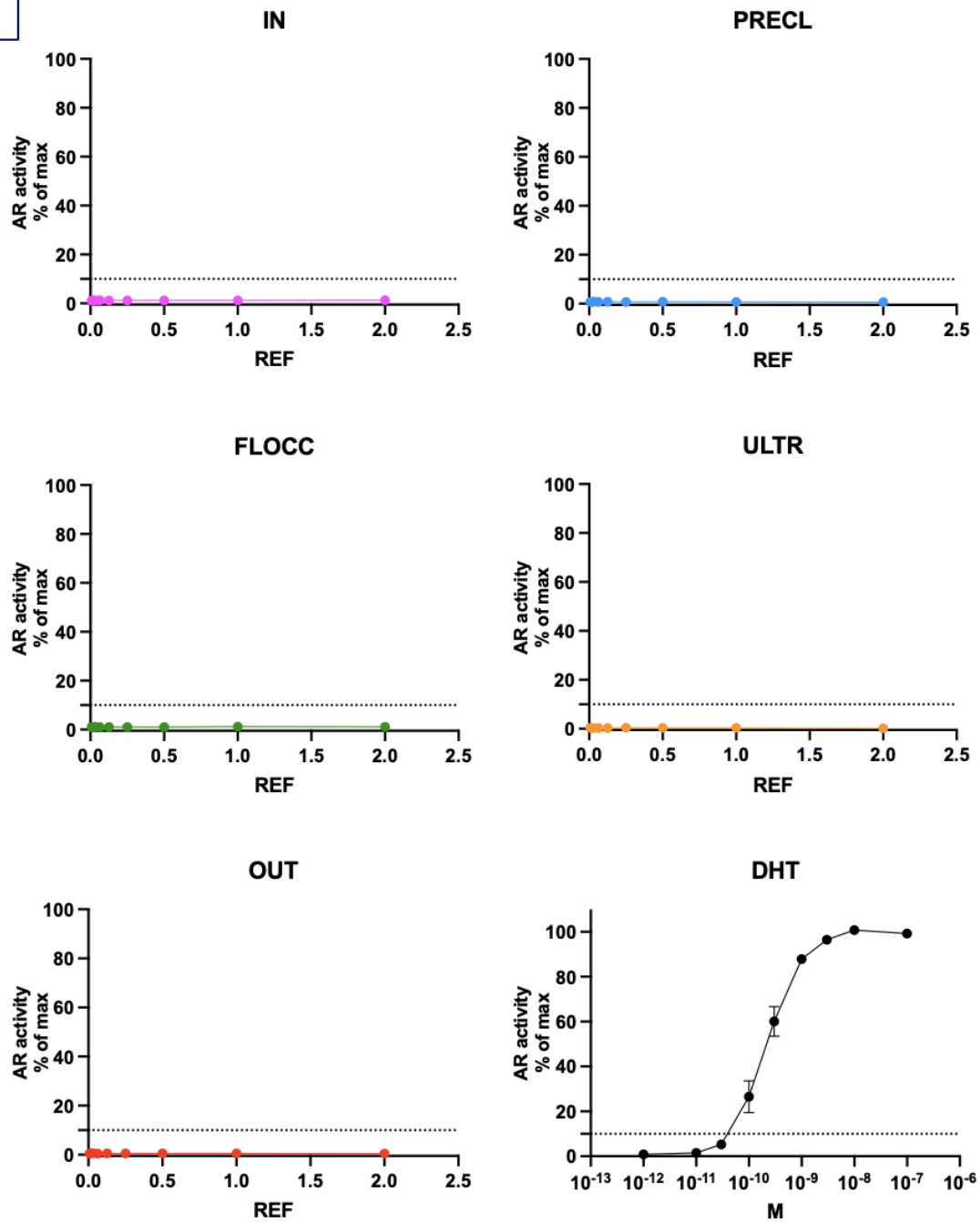


Figure S-4. Cell viability in the Ames fluctuation assay with *Salmonella typhimurium* strains YG1041 and YG1042 at starting REF 20. IN: pre-treatment water, PRECL: pre-chlorination step, FLOCC: flocculation step, ULTR: ultrafiltration step, OUT: post-treatment water. A cell viability of <80% was considered as cytotoxic effect, compared to negative control. Negative control: DMSO 0.1%.



B

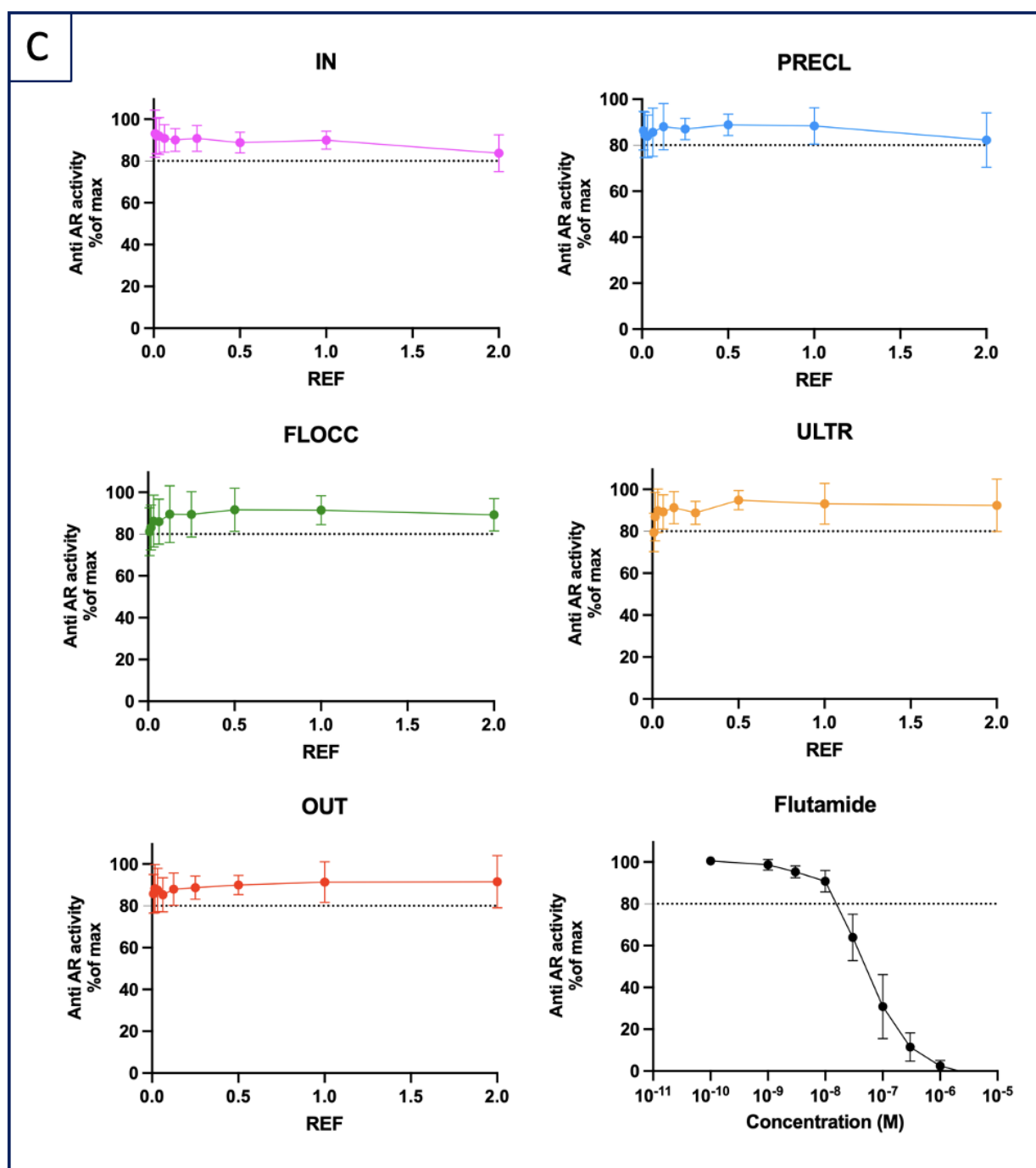


Figure S-5. [A] Antiestrogenic activity assayed with ER U20S cell line. An antiandrogenic effect, if present, should have been observed as a decrease in activity. Samples were analyzed in a dilution series with REF 2 as the highest concentration. Each concentration was analyzed in triplicate. The black dotted line represents the activity level corresponding to a 20% inhibition compared to the maximum response. **[B]** Androgen receptor activity assayed with AR U20S cell line. Samples were analyzed in a dilution series with REF 2 as the highest concentration. Each concentration was analyzed in triplicate. The black dotted line represents the activity level of 10% of the maximum responses. **[C]** Antiandrogenic activity assayed with AR U20S cell line. An antiandrogenic effect, if present, should have been observed as a decrease in activity. Samples were analyzed in a dilution series with REF 2 as the highest concentration. Each concentration was analyzed in triplicate. The black dotted line represents the activity level corresponding to a 20% inhibition compared to the maximum response.

Chapter Six

PREFACE

To conclude, since EDCs are ubiquitous and given the potential risks posed by BPA on human health and reproduction, this doctoral dissertation included an in-depth investigation of biological activities such as proliferation, migration, signal transduction pathways, and activation of the inflammatory response induced by bisphenol A (BPA) and bisphenol S (BPS, an analog of BPA used as a substitute in many products) on human trophoblast first-trimester cells, HTR-8/SVneo. This Chapter has been published in “Biology of Reproduction”.

Comparing effects and action mechanisms of BPA and BPS on HTR-8/SVneo placental cells

1. Introduction

The increasing abnormalities in the female reproductive functions over the years highlighted a strong link between the exposure to industrial chemicals and the effects on human health (Piazza and Urbanetz, 2019; Giulivo et al., 2016; Caserta et al., 2007). Within this relationship, the concept of endocrine disrupting chemicals (EDCs) has taken a central role in the field of reproduction. EDCs comprise many different substances from industrial, agricultural, and domestic sources. They have been related with adverse effects on female reproductive system and may cause the development of gynaecological pathologies (Brehm and Flaws, 2019). Further, exposure to EDCs may interfere with normal growth and development of the fetus and render the placenta unable to support the requested physiological functions (Yang et al., 2019). Bisphenol A (BPA) is one of the most investigated substances as a suspected EDC. BPA is a monomer that was first developed as a synthetic estrogen in the 1890s and reported to have the efficacy of estrogens in stimulating the female reproductive system in rats in the 1930s (Dodds and Lawson, 2019). Later, BPA has widely been used for its cross-linking properties in the manufacture of polycarbonate plastics and epoxy resins (Björnsdotter et al., 2017). As an additive of plastics (Gunaalan et al., 2020), BPA is released in food and water from plastics devices and from food packaging materials, as well as from personal care products and thermal receipts/papers, which constitute the main source of oral and dermal intake of BPA in humans. Epidemiological studies have reported that up to 95% of adults have detectable levels of BPA in their serum, saliva, and urine (Lee et al., 2018), within the nM range. Recent *in vivo* studies showed that BPA exposure is a known risk factor for the development of type 2 diabetes (Sowlat et al., 2016), obesity (Vom Saal et al., 2012), earlier puberty (Leonardi et al., 2017), cardiovascular

diseases (Olsén et al., 2012), breast and ovarian cancer, altered liver function, oxidative stress and inflammation (Dumitrascu et al., 2020). As to reproductive tissue, BPA has been found in maternal serum (0–154 ng/ml), amniotic fluid (0–8.38 ng/ml), cord serum (0–62.8 ng/ml), and placental tissue (0.104–9 ng/g) (Zbucka-Kretowska et al., 2019; Lee et al., 2018; Jalal et al., 2018; Vandenberg et al., 2007). Due to its high lipophilic property, BPA may permeate the placenta (Corbel et al., 2014). Given the potential risks posed by BPA on human health and reproduction, attempts are being made to replace it with the analog Bisphenol S (BPS). Structurally similar to BPA, BPS was first synthesized in 1869 as a dye, and is currently used in a variety of industrial applications and introduced into many commercial products available on the market as “BPA-free” (Usman and Ahmad, 2016; Liao et al., 2012b). Human exposure to BPS occurs through ingestion, inhalation, and dermal contact (Wu et al., 2018). BPS was found in human urine, generally at concentrations and frequencies comparable to BPA (Liao et al., 2012a). However, the information available on adverse effects by BPS, in particular as a potential EDC, is rather poor. As a consequence of its structural similarity to BPA, BPS could potentially lead to similar endocrine-disrupting capacity and effects on the reproductive system (Siracusa et al., 2018). BPS was found in maternal (0.03–0.07 ng/ml) and cord serum samples (0.03–0.12 ng/ml) (Santoro et al., 2019; Liu et al., 2017), suggesting its possible ability to cross the placental barrier (Grandin et al., 2019). BPS was also detected in urine samples from the at-risk population, such as pregnant women (Huang et al., 2019; Lehmler et al., 2018). Emerging evidence suggest that BPS is capable of imitating properties of hormones (Zalmanová et al., 2016), interacting with various physiological receptors, including estrogen receptors (ERs), androgenic and aryl hydrocarbon receptors (Rochester and Bolden, 2015). Some *in vitro* studies observed that BPS affects estrogenic and antiandrogenic activities on cells derived from human ovarian and breast cancer, in a similar manner and potency of BPA (Molina-Molina et al., 2013; Grignard et al., 2012). Considering its complexity, knowledge on potential EDC effects on placental function is still limited, especially regarding BPA and even more BPS. The development of the human placenta depends entirely on normal differentiation, proliferation, and invasion of trophoblast cells (Carter et al., 2015). Impaired function of trophoblasts can lead to severe pregnancy complications (Turco and Moffett, 2019; Chen et al., 2012). The proliferation and invasion of trophoblast cells represent a complex process, strictly regulated by many factors, including hormones, prostaglandins, cytokines and hypoxia, which either promote or inhibit proliferation and/or invasion (Hemberger et al., 2020). Numerous signalling cascades/proteins are involved in the regulation of trophoblast cell activity. Among them, mitogen-activated protein kinase (MAPK) pathways play a critical role in a wide range of biological processes (Mebratu and Tesfagzi, 2009; Vaillancourt et al., 2009). Further, cytokines and growth factors are secreted by trophoblast cells, and regulate the functional activity of

the trophoblast via paracrine and autocrine mechanisms, control placenta development, and maintain immunological tolerance in the mother-fetus system (Sokolov et al., 2015; Garcia-Velasco and Arici, 1999). The present study aims to elucidate and compare the effects of BPA and BPS on the proliferation and migration ability of extravillous trophoblast HTR-8/SVneo cells, as well as on the secretion of inflammatory mediators. These cells preserve well-known factors controlling proliferation, migration and invasion, thus representing a good model for the *in vitro* study of molecular mechanisms at the basis of placentation, as well as of early events modulating placental development (Msheik et al., 2020). To provide insights into the molecular pathways implicated in cellular responses, involvement of the ERs and modulation of the ERK1/2 dependent pathway by BPA and BPS were investigated.

2. Materials and methods

2.1. Chemicals

Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-rabbit conjugated with horseradish peroxidase immunoglobulin G (IgG), and U0126 (MEK1,2 inhibitor) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Roswell Park Memorial Institute RPMI 1640 medium, Fetal Bovine Serum (FBS, EU origin), Charcoal Stripped Fetal Bovine Serum (CS-FBS), glutamine, penicillin/streptomycin, anti β -tubulin, anti-mouse IgG conjugated with horseradish peroxidase, Bisphenol A (BPA), Bisphenol S (BPS), Tamoxifen (TAM) and all other reagents were from Merk Life Science (Milan, Italy). Stock solutions were prepared in DMSO as follows: 0.1 M BPA, 0.1 M BPS, 0.01 M U0126 and 0.01 M TAM.

2.2 Cell culture and treatments

The HTR-8/SVneo human trophoblast cell line was kindly provided by Dr. Charles H. Graham (Queen's University, Ontario, Canada (Graham et al., 1993). HTR-8/SVneo cells originated from an explant culture of the human first-trimester placenta; they exhibited the following intrinsic mechanisms: adhesion, migration and invasion (Hannan et al., 2010). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 2% penicillin/streptomycin, and were maintained at 37°C in a standard atmosphere containing 5% CO₂. For the experiments, cells were treated with trypsin and removed from culture flasks, then seeded in 6-well, 12-well or 24-well culture plates at a density of 1.4×10^4 cells/ml, and cultures were incubated overnight. After 24 h culture, cells were exposed to bisphenols (BPA or BPS, dissolved in DMSO) at different concentrations for 3 days. When indicated, cells were incubated with 10 μ M U0126 (ERK1/2

inhibitor) or 0.1 μ M TAM (ER antagonist). Because of the hormonal activity of phenol red and FBS (Welshons et al., 1988), experiments were performed in phenol-red-free RPMI, supplemented with 5% CS-FBS. Dextran treated charcoal is used to selectively remove hormones without nonspecific loss of other serum components. Final DMSO concentration in the medium never exceeded 0.1%. In each experiment, control cells were exposed to the vehicle alone to ascertain absence of effects.

2.3 E-SCREEN assay

The E-screen assay was originally developed to evaluate the proliferative effect of estrogen-like compounds on MCF-7 cells, a human breast cancer cell line, that endogenously overexpresses ERs (Soto et al., 1995). Since HTR-8/SVneo cells do possess both nuclear- and membrane localized- ERs (Patel et al., 2015), we verified the possibility to use the E-screen assay in the present investigations. Preliminary trials demonstrated that HTR-8/SVneo cells incubated with increasing amounts of 17 β Estradiol (E2) are induced to proliferate, starting from the concentration 10^{-12} M, and reaching the maximum response at 10^{-8} M (**Supplemental Figure S-6**). The E-screen assay is then used for the first time on HTR-8/SVneo, with a modification with respect to the original method, i.e. cells are exposed to potential estrogenic compounds for three days instead of five days. In fact, previous observations by our laboratory indicated that HTR-8/SVneo cells replicate at higher rate ($\sim$ 22 h) than MCF-7 cells ($\sim$ 32 h). Cell proliferation was then determined by a colorimetric assay based on the reduction of a tetrazolium salt, MTT (3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by metabolically active cells (Mosmann T., 1983). Optical density was assessed at 570 nm with background correction at 650 nm, and results are expressed as Proliferative Effect (PE), the ratio of the cell number achieved after BPA or BPS exposure, and the cell number in the control cells.

2.4 Western blotting analysis

Western blotting procedures were carried out as previously reported (Valbonesi et al., 2008). After the experimental treatments, cells were lysed in icecold lysis buffer containing phosphatase inhibitors (1 mM sodium ortovanadate and 50 mM sodium fluoride) and proteins were electrophoresed, transferred to nitrocellulose membrane and probed with specific antibodies. MAPK activity was assessed by using rabbit anti-phospho-ERK1/2 or anti-total-ERK1/2 as primary antibodies (1:1000) and horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:2000). Immunoblots were developed by enhanced chemiluminescence reagent, and a densitometric analysis of the films was performed by Image Master equipped with TotalLab software ver. 1.0 (Amersham-Pharmacia). Given the inherent variations in film development, densitometry data were normalized to an external reference sample

(REF) (unexposed cells) loaded in each immunoblot. MAPK activity was evaluated as phospho/total ERK1/2% of phosphorylation with respect to control. β -tubulin immunodetection was used as the control to assess equal protein loading.

2.5 Transwell cell migration assay

Transwell inserts (PET membrane, 8- μ m pore size, Millicell Hanging, Merck Life Science, Milan, Italy) were used for the cell migration assay, according to manufacturer's instructions. Briefly, after three days of treatment with BPA or BPS, HTR-8/SVneo cells were trypsinized, resuspended in RPMI with 1% FBS and seeded in the upper chamber of each insert at a density of 3×10^4 cells/well; the lower chambers contained 750 μ L RPMI supplemented with 10% FBS. After 24 h of incubation at 37°C the non-migrated cells on the upper surface of the membrane were removed by gentle swabbing. Cells that had reached the lower side of the filter were fixed with methanol for 30 min, stained for 10 min with crystal violet 0.3%, and quantified by visual counting of ten randomly selected fields from each filter under a light microscope. Data were normalized against cell count. Migration capacity was calculated as the percentage of cells that passed through the membranes in the treated- with respect to control- cells.

2.6 Luminex assay

Analysis of inflammatory cytokines and cytokine-induced vascular endothelial growth factor (VEGF) was performed in the medium of trophoblast cell culture, where soluble factors can be released after bisphenols exposure. After the experimental treatments, the medium was collected in 2 mL tubes, centrifuged at $1000 \times g$ for 10 min at 4°C, and supernatant was kept at -80°C until analysis. Secretions of IL-6 (interleukin 6), IL-8 (interleukin 8), IL-1 β (interleukin 1 β), TNF- α (tumor necrosis factor- α), chemokine CCL2 (CC Motif Chemokine Ligand 2), and VEGF were analysed using the immunological kit for Human Magnetic Luminex Screening Assay (Bio-technie s.r.l. Milan, Italy). The analysis was performed using the BioPlex200 equipment (BIO-RAD Laboratories srl, Milan, Italy). Sample concentrations of the different compounds were estimated through a standard curve using a 5-order polynomial curve and expressed pg/mL (Bio-Plex Manager software 5.0). Data were normalized against cell count.

2.7 Data analysis

Data groups were compared using one-way ANOVA with SigmaPlot software (ver 13, Systat Software Inc.), and followed by Dunnet post-hoc test; a statistical difference was accepted when $P < 0.05$. All experiments were independently repeated at least three times.

3. Results

3.1. BPA and BPS exposure increases HTR-8/SVneo cell proliferation

Cells were exposed to increasing concentrations of BPA or BPS (from 10^{-13} M to 10^{-3} M) for three days. As reported in **Figure 6.1**, both BPA and BPS affected the proliferation of HTR-8/SVneo cells. At concentrations ranging from 10^{-11} M to 10^{-7} M BPA or BPS, cell proliferation was significantly increased compared to the control cells. The most active concentration was 10^{-7} M for BPA and 10^{-9} M for BPS, reaching the maximum proliferative effect of 1.89 and 1.62, respectively. BPA at 10^{-5} M concentration significantly increased HTR-8/SVneo cell proliferation, whereas no effect was observed at an equimolar concentration of BPS. At the highest concentration tested (10^{-3} M) of BPA or BPS, the proliferative effect was significantly lower than 1. This result can be ascribed to toxic effects of the compounds on HTR-8/SVneo cells.

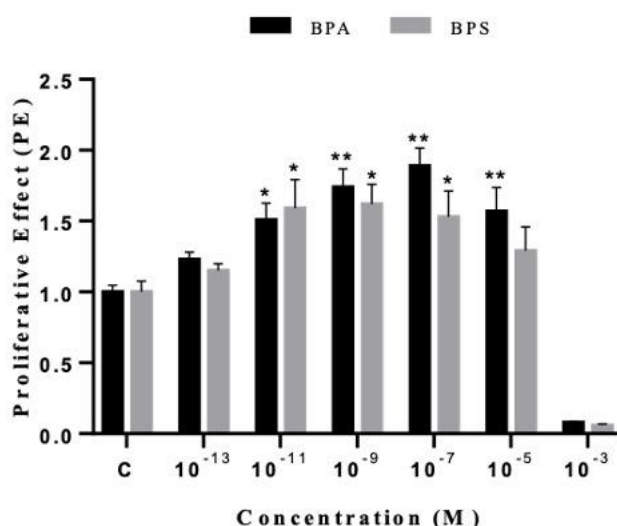


Figure 6.1. Effect of BPA or BPS on HTR-8/SVneo cell proliferation. Data are expressed as PE and reported as means $\pm$ SE of 5 different experiments, each performed in quadruplicate. * $P < 0.05$, ** $P < 0.01$ related to control (C, control cells exposed to vehicle alone, PE = 1).

3.2. MAPK (ERK1/2) pathway is involved in the stimulatory effects induced by BPA and BPS

The ability of BPA and BPS to modulate the MAPK pathway, and its involvement in the proliferative effect, were investigated by assessing ERK1/2 phosphorylation through western blot analysis. As reported on **Figure 6.2**, BPA triggered ERK1/2 phosphorylation in the 10^{-9} – 10^{-5} M concentration range. A significant increase of ERK1/2 phosphorylation was observed at 10^{-9} M and 10^{-7} M BPS. In the presence of U0126, a selective MAPK inhibitor, BPA and BPS effects on ERK1/2 phosphorylation were significantly reversed.

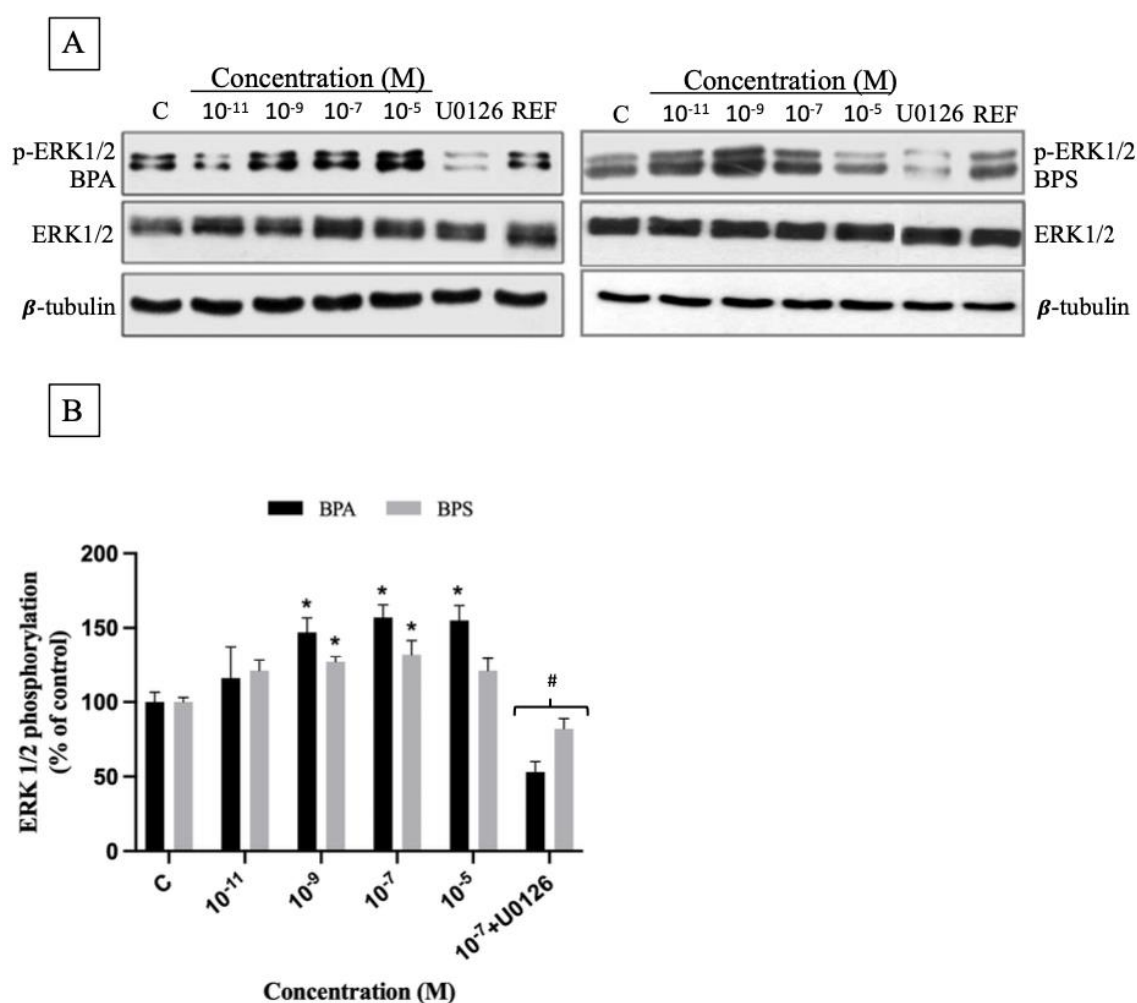


Figure 6.2. Effect of BPA or BPS on ERK1/2 phosphorylation in HTR-8/SVneo cells. [A] Representative immunoblots relative to phosphorylated and total ERK1/2. U0126 = BPA or BPS 10^{-7} M treatment with the ERK1/2 inhibitor U0126. REF = external reference sample, loaded in each immunoblot. β -tubulin levels were also measured to assess equal loading of proteins. [B] Densitometric analysis of ERK1/2 phosphorylation, evaluated as the ratio between the phosphorylated and total forms, expressed as % of control (C) and reported as means $\pm$ SE of 4 different experiments. * $P < 0.05$ related to C (control cells exposed to vehicle alone), # $P < 0.05$ related to 10^{-7} M BPA or BPS.

3.3. BPA but not BPS impairs HTR-8/SVneo cell migration

In vitro effects on the migration capacity of HTR-8/SVneo cells were examined after incubation at different concentrations of BPA or BPS (from 10^{-13} M to 10^{-5} M) for three days. A significant reduction of trophoblast cell migration was observed after treatment with BPA at 10^{-7} M and 10^{-5} M concentrations (–22% and – 53% versus control) (**Figure 6.3**). BPS exposure did not induce any statistically significant change on HTR-8/SVneo cell migration, at any concentration tested.

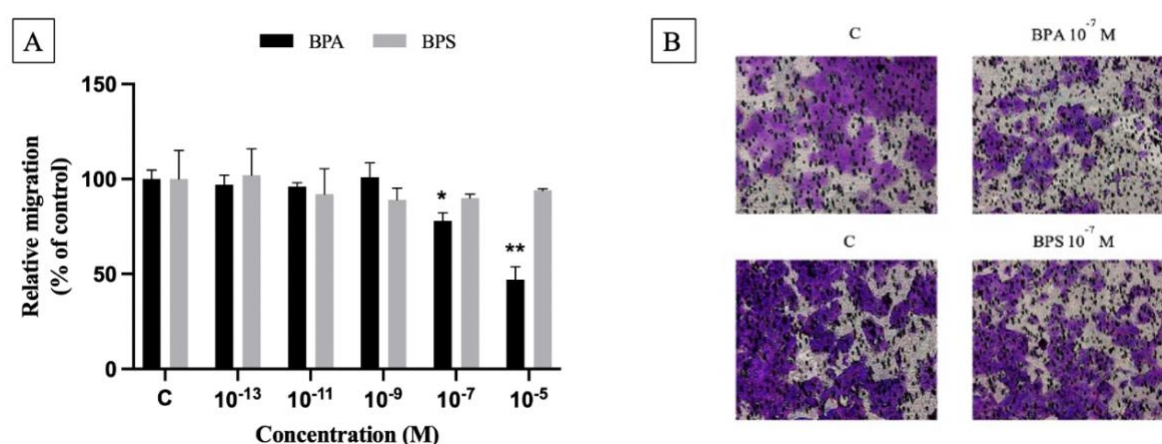


Figure 6.3. Effect of BPA or BPS on HTR-8/SVneo cell migration. [A] Densitometric data relative to cell migration, evaluated as the number of cells reaching the lower side of the membrane in Transwell inserts. [B] Representative light microscope images of Transwell migration assay (10 ×). Data are expressed as a percentage of the control (C) and reported as means $\pm$ SE of 4 different experiments. *P < 0.05, **P < 0.01 related to C (control cells exposed to vehicle alone).

3.4. The effects of BPA and BPS on cell proliferation and migration are mediated by ER and MAPK signalling pathways

To reveal the involvement of ER and ERK1/2 signalling pathways in mediating the effects induced by BPA and BPS on HTR-8/SVneo cell proliferation and migration, further experiments were performed in the presence of TAM, a specific antagonist of ER, and U0126. The effects of TAM or U0126 were tested using one of the peak active concentrations of BPA or BPS. Blocking ER with TAM, the effect of both BPA and BPS on cell proliferation (**Figure 6.4A**), and the effect of BPA on HTR-8/SVneo cell migration (**Figure 6.4B**) were abolished. The proliferative effects induced by BPA and BPS were fully reversed by U0126, which decreased to levels lower than control (**Figure 4A**). Treatment with U0126 also recovered the migration ability of trophoblast cells, which was inhibited by BPA (**Figure 6.4B**).

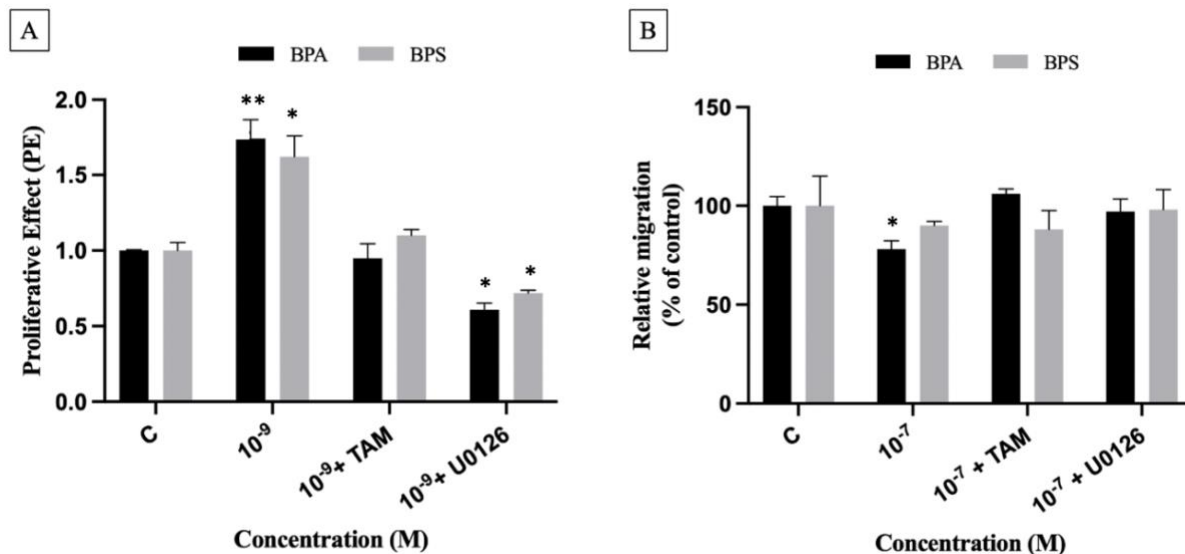


Figure 6.4. Effect of Tamoxifen and U0126 on HTR-8/SVneo cells exposed to BPA or BPS. [A] Effect of 10^{-9} M BPA or BPS with or without TAM or U0126 on cell proliferation. [B] Effect of 10^{-7} M BPA or BPS with or without TAM or U0126 on cell migration. Data are reported as means $\pm$ SE of 4 different experiments. * $P < 0.05$, ** $P < 0.01$ related to C (control cells exposed to vehicle alone).

3.5. BPS but not BPA affects inflammatory cytokines secretion in HTR-8/SVneo cells

Cells were incubated with different concentrations of BPA or BPS (from 10^{-11} M to 10^{-5} M) for three days, then secretion of different inflammatory proteins was measured: IL-6, IL-8, TNF α , IL-1 β , CCL2, and VEGF. Interestingly, IL-6 and IL-8 secretion was affected by BPS exposure (**Figure 6.5A and B**), with significant increases at 10^{-11} M that were counteracted by treatment with the U0126 inhibitor. No effect of BPA was detected on interleukin secretion, with respect to the basal levels. Stimulation of HTR8/SVneo cells with BPA or BPS treatment did not affect CCL2 secretion (**Figure 6.5C**). In our experimental conditions, basal levels and secretion of IL-1 β TNF- α and VEGF from HTR-8/SVneo cells were always below the sensitivity of the assay.

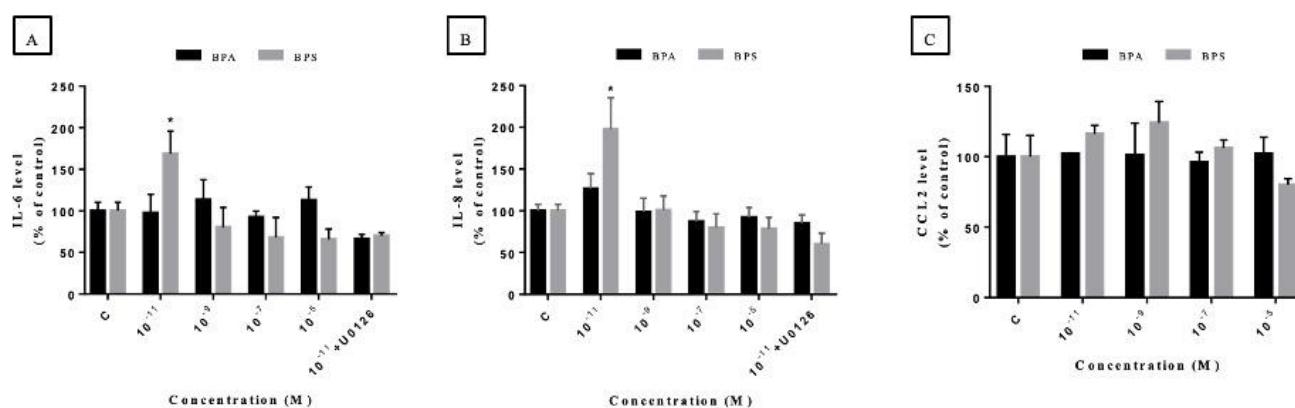


Figure 6.5. Effect of BPA or BPS on IL-6, IL-8, and CCL2 secretion by HTR-8/SVneo cells. [A] IL-6 secretion (C = 23.86 pg/ml); [B] IL-8 secretion (C = 27.70 pg/ml); [C] CCL2 secretion (C = 11.72 pg/ml). Data are expressed as % of control (C) and reported as means $\pm$ SE of 3 different experiments. *P < 0.05 related to C (control cells exposed to vehicle alone).

4. Discussion

BPA and its analog BPS are ubiquitous emerging contaminants of increasing concern. The real-world exposure to BPA and BPS occurs daily at low concentrations, but relevant for human health risk (Nevoral et al., 2018). Emerging evidence suggest high potential of adverse biological effects on reproductive functions after exposure to these chemicals (Catenza et al., 2021; Pergialiotis et al., 2018; Rochester, 2013). The aim of this work was to elucidate and compare the effects of BPA and BPS on biological activities of HTR-8/SVneo cells, derived from extravillous trophoblast of first-trimester pregnancy. A wide range of concentrations of BPA and BPS (from 10^{-13} M to 10^{-3} M) were used to perform *in vitro* experiments, which comprises the nM range often documented in human fluids. To the best of our knowledge, these are the first data showing that BPS affects trophoblast functions through ER/MAPK mediated pathways. Due to the observed effects on humans, particularly those related to hormonal regulation of reproductive processes (Tomza-Marciniak et al., 2018), BPA has been qualified as a xenoestrogen (Mhaouty-Kodja et al., 2018). Based on the chemical similarity to the natural hormone E2, BPA can bind with ERs (ER α and ER β), although displaying 1000 to 2000-fold less affinity with respect to E2 (Le Magueresse-Battistoni et al., 2018). BPS has been reported to have activity and potency similar to those of BPA (Grignard et al., 2012). Since the ERs signalling pathways are involved in the cell proliferation equilibrium, we investigated the possible physiological consequence of BPA and BPS exposure in triggering estrogen-like proliferation on extravillous trophoblast cells. The findings demonstrated that both BPA and BPS increase HTR-8/SVneo cells proliferation, starting from the concentration 10^{-11} M (**Supplemental Figure S-7**), one order of magnitude higher than natural estrogen E2, whose effect on HTR-8/SVneo cells is significant

at 10^{-12} M (**Supplemental Figure S-6**). Similar to other EDCs, BPA and BPS displayed non monotonic dose–response curves. More specifically, they showed an inverted U-shaped curve, inducing a significant biological effect at low concentrations, reaching the maximum at 10^{-9} M and 10^{-7} M, and causing cytotoxic responses at the highest doses. The proliferative effect of both BPA and BPS was abolished by the treatment with the ER-blocker TAM, which confirms that both bisphenols mimic the effect of E2. Similar findings, obtained after exposure of cells at different BPA concentrations, were observed on the human choriocarcinoma cell line BeWo (Ponniah et al., 2015; Wang et al., 2015), JEG-3 cells (Mannelli et al., 2015), and human ovarian carcinoma cell line OVCAR3 (Ptak et al., 2014). To the extent of our knowledge, no studies are currently available on BPS effects on reproductive tissues. MAPKs are responsible for converting many cellular and extracellular stimuli into specific responses controlling cell proliferation, differentiation, apoptosis, embryogenesis, and regulation of inflammatory and stress responses (Lavoie et al., 2020). Transduction pathways mediated by phosphorylated ERK1/2 play an essential role in the placenta development (Hatano et al., 2003) and is reported to facilitate trophoblast differentiation (Daoud et al., 2005). Several data support that BPA-dependent estrogenic activity flows through the ER-mediated extranuclear signals activation, that results in the ERK1/2 phosphorylation (Acconcia et al., 2015). Previous studies have revealed that BPA activates ERK1/2 phosphorylation in human adrenal and breast (Song et al., 2015), placenta (Chu et al., 2018), and also HTR-8/SVneo cells (Li et al., 2018). Present data demonstrate that phosphorylation of ERK1/2 in trophoblast cells is activated by treatment with both BPA and BPS. Since TAM prevented their effect, we concluded that the ERK1/2 stimulation was induced through the interaction of bisphenols with ERs. Further, ERK1/2 activation is involved in the effect of BPA and BPS on trophoblast proliferation, as the effect was abolished in the presence of the U0126. Overall, BPA and BPS, similarly to E2, activate the ER-dependent signals that culminate with the activation of rapid extra-nuclear pathways in HTR-8/SVneo cells. We conclude that bisphenols effects on trophoblast cells proliferation and migration require the activation of two consequential signals, ERs and ERK1/2 pathways; by blocking one of them the downstream effects are abolished. The present study shows for the first time an ER and MAPK mediated effect of both BPA and BPS on trophoblast cell proliferation. Previous studies reported that BPA did not affect cell proliferation (Li et al., 2018; Lan et al., 2017; Spagnoletti et al., 2015). In a further study (Basak et al., 2018) a concentration-dependent decrease in the proliferation of HTR8/SVneo cells exposed to BPA or BPS was observed. Therefore, additional work is advisable for a better understanding of these modulations. The trophoblast is an embryonic tissue that exerts a crucial role during implantation and placentation, and its migration and invasion capacity represents a pre-requisite for normal embryo implantation. Numerous studies indicated that exposure to BPA might be associated with severe

pregnancy-related complications affecting both mother and fetus (Filardi et al., 2020). BPA can affect placentation, a significant factor determining pregnancy outcome. It has been associated with implantation failure, miscarriage, premature delivery, and it may also contribute to infertility and subfertility (Tang et al., 2020; Konieczna et al., 2015). Few studies about the effect of BPA on the trophoblast migration were performed (Wei et al., 2020; Li et al., 2018; Lan et al., 2017; Spagnoletti et al., 2015), while no information is available yet on the effect of BPS on this process. Present results indicate that only BPA affected cell migration ability. The inhibitory effect of BPA was abolished by TAM, suggesting an estrogen-like effect mediated through ERs. Besides, the migratory ability of cells was recovered by treatment with the inhibitor U0126, demonstrating that ERK1/2 pathways are relevant for BPA reduction of cell migration. In this study, BPS exposure, unlike BPA, never affected migration of HTR-8/SVneo cells. Similar to cell proliferation, also data on BPA effect on HTR8/SVneo cell migration are conflicting. Some studies (Wei et al., 2020; Li et al., 2018; Spagnoletti et al., 2015) observed that migration and invasion were reduced following BPA exposure, while others (Lan et al., 2017) assessed that BPA enhances cell migration through ERK1/2 stimulation. The present work reports a reduction of migration at micromolar BPA concentrations. The discrepancy among the above studies may be related to the complexity of the migration process modulation. In fact, the control of trophoblast functions is strictly dependent on the balance of various factors (Lunghi et al., 2007), which can be influenced by BPA or BPS exposure thus interfering with the intricate role of the trophoblast in human placentation. Among other examples, a very recent investigation reports the important modulation by nutrient transfers, which is affected by BPA (Benincasa et al., 2020). Therefore, further work is advisable to clarify BPA and BPS effects on physiological response of trophoblast cells, possibly performed on ex-vivo preparations thus avoiding possible influence by culture conditions. Previous studies indicated that cytokines may have a critical role in the functions of trophoblast cells; this is particularly true for interleukins (Du et al., 2014; Zhang et al., 2013; Red-Horse et al., 2004) as key molecules involved in the biological processes related to migration and invasion of trophoblast cells (Sharma et al., 2016). IL-6 is widely expressed in the endometrium at implantation, and increased levels of placental IL-6 have been associated with preeclampsia, recurrent miscarriage, and infertility (Champion et al., 2012). Previous studies provided some evidence that IL-6 enhances migration of HTR8/SVneo cells (Jovanović et al., 2010). In addition, the HTR-8/SVneo cell line secretes IL-8, which is reported to increase cell migration and invasion (Jovanović et al., 2009). However, the critical role of the cytokines on the embryo implantation is not yet understood. Herein, we investigated the effect of BPA and BPS on cytokines secretion by HTR-8/SVneo cells. BPS, only at 10^{-11} M, upregulated interleukins IL-6 and IL-8 secretion. We also assessed that IL-1 β , CCL2, TNF- α and VEGF levels were not affected. The effect

of BPS on IL-6 and IL-8 protein levels was reversed by treatment with U0126. Thus, BPS induction of both IL-6 and IL-8 is mediated by the ERK1/2 signalling. BPA did not cause any effect on cytokines, although a slight but not significant stimulation was observed only at 10^{-11} M BPA on IL-8 secretion. These data are not sufficient to correlate cytokine secretion by HTR-8/SVneo with cell proliferation/migration modulated by BPA or BPS.

5. Conclusion

Our results indicate that BPA, already at sub-nanomolar concentrations, affects both proliferation and migration of HTR-8/SVneo cells through ER/MAPK mediated processes. BPS, at the same concentration range, enhances proliferation, also acting through ER/MAPK pathways. BPS has no effect on HTR-8/SVneo cell migration. Thus, BPA and BPS share estrogen-like activity and modulation of ERK1/2 signaling, while targeting different biological processes in trophoblast cells. Furthermore, BPS while not BPA, increases interleukins secretion through ERK1/2 phosphorylation. The physiological significances of this effect, observed only at a subnanomolar concentration, needs to be further elucidated. In the current state of knowledge, the replacement of BPA with BPS does not seem to ensure the safety of human health.

Supplementary material

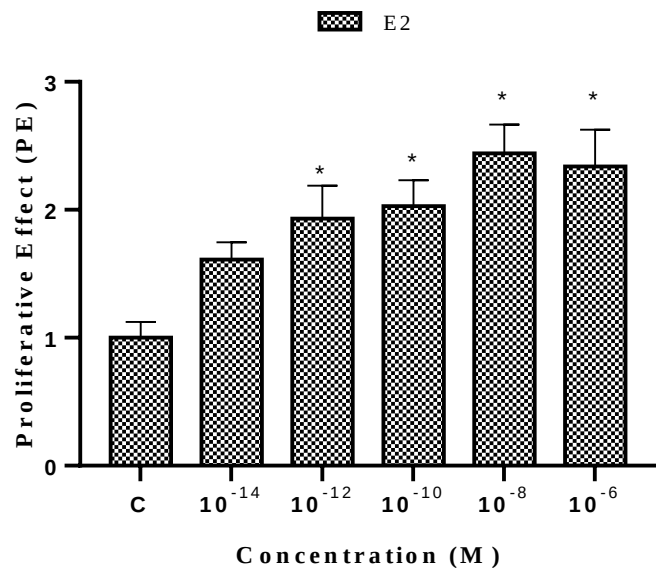


Figure S-6: Proliferative effect of E2 on HTR-8/SVneo cells. Data are expressed as PE and reported as means $\pm$ SE of 5 different experiments, each performed in quadruplicate. * $p < 0.05$ (C, control cells exposed to vehicle alone, PE=1).

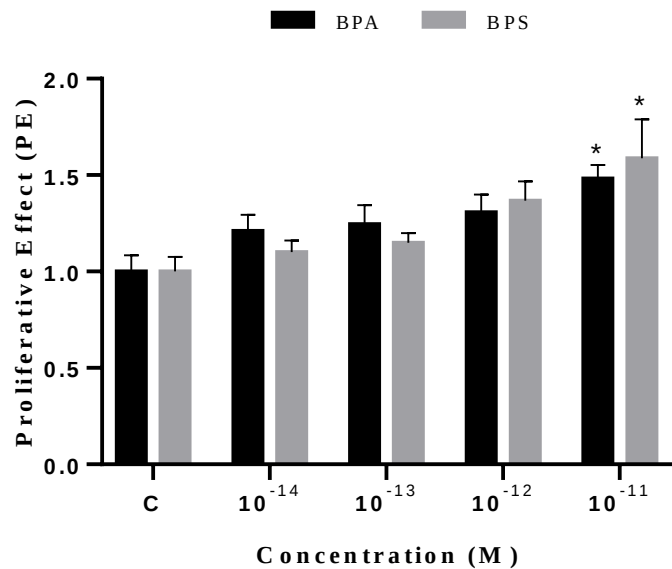


Figure S-7: Effect at low concentrations of BPA or BPS on HTR-8/SVneo cell proliferation. Data are expressed as PE and reported as means $\pm$ SE of 5 different experiments, each performed in quadruplicate. * $p < 0.05$ (C, control cells exposed to vehicle alone, PE=1).

Chapter Seven

Overall conclusion

The present doctoral dissertation has been focused on the occurrence of suspected EDCs (BPA, NP, and hormones) in drinking water across the Romagna area (Italy). The results of the investigation have allowed to fulfil the main objectives of this thesis: assess the EDC contamination levels in source water and treated water from three DWTPs, determine their fate and removal efficiency during water treatment, and evaluate the potential biological impact of drinking water samples with reference to human health. The main conclusions drawn from the investigation are given below:

- NP and BPA were the most frequent contaminants in drinking water, thus confirming that these substances are ubiquitous in the water cycle, regardless the area of origin of the source water and treatment systems adopted. Despite contamination levels in source waters were at low ng/L range, sometimes EDCs remained in the finished water, indicating that they were not well removed by water treatment processes. However, the concentration of these contaminants in treated water were extremely low. Chemical data also showed that estrogens rarely occurred in source water, and were the most efficiently removed by the water treatment. The large difference between all reported concentrations and the guideline values emphasizes the high quality of the drinking water produced by the examined DWTPs.
- During water treatment, materials in contact with the water could contaminants of emerging concern such as BPA and NP, compromising the quality of the final water. This phenomenon was found after pre-chlorination, flocculation, or ultrafiltration processes. Thus, it is imperative to be aware of the type of materials used in the DWTPs. Furthermore, this extra contribution was also reflected by biological effects observed during water treatment using bioanalysis. Estrogenic responses were detected after ultrafiltration step, as a results of the potential formation of bioactive compounds. However, these bioactivities were close to the detection limits and no longer present in the outlet water, confirming the good quality of the drinking water produced.
- Even though drinking water seems to represent a minor source of EDCs for humans, exposure to BPA is a concern because of its possible health effects. The results of the *in vitro* research demonstrated that BPA and its substitute BPS, even at low concentrations, share estrogen-like

activity and modulation of signaling pathways, though affecting different biological processes in trophoblast cells, essential for the early events of pregnancy.

- Assessment of emerging contaminants in treated water through chemical analysis alone is not a sufficient indication of water quality. The overall good quality of the water examined in the present work did not allow to reveal significant estrogenic or genotoxic effects of the mixture. Nevertheless, complementary biological approaches focused on the most health-relevant endpoints are recommended, and supported by other laboratories, to ensure the safety of distributed drinking water.

In general, this research successfully demonstrated that targeted chemical analysis combined with *in vitro* bioassays are valuable tools to pinpoint specific chemicals and characterize the overall toxic potential in drinking water. The results of this study will hopefully contribute to the growing body of research supporting the need to implement regulatory frameworks for water quality monitoring with bioanalytical tools. Further, this study calls attention on the role of DWTP as an additional source of EDC contamination. Among these BPA is included, of which we demonstrated the negative impact on human cells. Thus, it is important to develop monitoring strategies from source to distribution networks, including water treatment trains, to verify treatment efficiency in order to deliver safe water eligible for human consumption. Overall, the levels of drinking water contamination highlighted in the study, although representative of a regional area and limited to some compounds, are substantially in line with the range of values found at European level and did not reveal significant risks correlated to water consumption.

Limitation, recommendations, and future needs

While the contaminants selected in this PhD project are a representative group of (suspected) EDCs that can occur in drinking water, there are many other emerging pollutants that were not considered and may be responsible for detrimental effects. A limited number of CECs is currently prioritized by water legislation, possibly due to the knowledge gap in terms of risks associated with human health. Therefore, there is a need to globally implement the list of new contaminants in the regulatory frameworks and harmonize the limit levels worldwide.

It has been recognized that classic approaches relying on the analytical determination alone tend to assess drinking water quality incompletely. Unfortunately, combined chemical/biological approaches

are not used in current daily life to evaluate drinking water quality and the performance of DWTPs, yet. As widely recommended also in literature, the suggestion is to work with integrative approaches, including effect-based monitoring with a greater diversity of biological endpoints.

Water treatments will remain a major field of scientific research and technological approach in the coming years, also due to the increasing number of contaminants of emerging and need for removal. The importance of effect-based tests will be even more enhanced by the increasing production of new chemicals, that cannot be targets of chemical analysis because not known and at the same time may be able to change the overall biological activity of the water mixture.

Despite major advances in the field of water quality assessment, there is still a knowledge and legislation gaps regarding leaching from materials in contact with water; therefore, more research efforts are needed to better understand the factors that promote the release of contaminants into water, and where needed, a precautionary approach is a must to minimize public health risks.

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