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CONTAMINANTS OF EMERGING CONCERN: OCCURRENCE AND DISTRIBUTION IN AQUATIC ENVIRONMENTS

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ABBREVIATIONS

GENERAL ABBREVIATIONS

ADI: Acceptable Daily Intake

AF: Assessment Factor

APEO: Alkylphenol Ethoxylates

ARPA-ER: Agenzia Regionale per la Protezione Ambientale – Emilia Romagna

ATSDR: Agency for Toxic Substances and Disease Registry

BAF: Bioaccumulation Factor

BCF: Bioconcentration Factor

BMF: Biomagnification Factor

CER: Canale Emiliano Romagnolo

CRM: Certified Reference Material

CV: Coefficient of Variation

DEPA: Danish Environmental Protection Agency

DLLME: Dispersive liquid-liquid microextraction

DWTP: Drinking Water Treatment Plant

EC: European Commission

EC_{50}: Effective Concentration to 50% of test organisms

ECHA: European Chemical Agency

EDI: Estimated Dietary Intake

EEF:Estrogenic Equivalent Factor

EEQ: Estradiol Equivalent concentration

EFSA: European Food Safety Agency

EQS: Environmental Quality Standard

ESERI: Environmental Sciences for the European Refining Industry

ESI: Electrospray Ionization

EU: European Union

FDA: Food and Drug Administration

FL/TL: Fork Length/Total Length

FSA: Food Standards Agency

f_{OC}: percentage of organic carbon

GAC: Granular Activated Carbon

GC: Gas Chromatography

HC_5: Hazardous Concentration to 5% of test organisms

HD: Hazard Quotient

HPLC: High-Performance Liquid Chromatography

HREE: Heavy Rare Earth Elements

IPCS: International Programme on Chemical Safety

ISPRA: Istituto Superiore per la Protezione e Ricerca Ambientale

ISTAT: Istituto Nazionale di Statistica

K_d: sediment/water partition coefficient

K_{oc}: K_d normalized to the organic carbon content

K_{ow}: octanol/water partition coefficient

LC: Liquid Chromatography

LC_{50}: Lethal Concentration to 50% of test organisms

LLE: Liquid-liquid extraction

LLME: Liquid-liquid microextraction

LOI: Loss On Ignition

LREE: Light Rare Earth Elements

MEC: Measured Environmental Concentration

MDL (alternatively, **mLOD**): Method Detection Limit

MQL (alternatively, **mLOQ**): Method Quantification Limit

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

m/z: mass-to-charge ratio

NH_4Ac: ammonium acetate

NH_4OH: ammonium hydroxide

NIH: National Insitute of Health

NOEC: No-Observed Effect Concentration

OECD: Organisation for Economic Co-operation and Development

PAAS: Post-Archean Average Australian Shale

PAC: Powdered Activated Carbons

PC/BPA: Polycarbonate/Bisphenol A Group

PE: Population Equivalents

PEC: Predicted Environmental Concentration

PNEC: Predicted-No-Effect Concentration

POP: Persistent Organic Pollutants

Q: Quadrupole

QqQ: triple quadrupole

REE: Rare Earth Elements

RfD: Reference Dose

RQ: Risk Quotient

RSD: Relative Standard Deviation

SPE: Solid Phase Extraction

SPME: Solid-phase microextraction

SSD: Species Sensitivity Distribution

TDI: Tolerable Daily Intake

TFC: Turbulent Flow Chromatography

TGD: Technical Guidelines

TOC: Total Organic Carbon

UAE: Ultrasonic Assisted Extraction

UPLC: Ultra-Performance Liquid Chromatography

US EPA: United States Environmental Protection Agency

WFD: Water Framework Directive

WHO: World Health Organization

WWTP: Wastewater Treatment Plant

COMPOUNDS ABBREVIATIONS

BPA: Bisphenol A

CEC: Contaminants of Emerging Concern

EDC: Endocrine Disrupting Compound

E1: Estrone

E2: ß-estradiol

EE2: 17α-ethinylestradiol

Gd-DTPA: Gd-diethylenetriaminepentaacetate

NP: Nonylphenol

OP: Octylphenol

PFAS: Perfluoro Alkylated Substances

PFC: Perfluorinated compounds

PFCAs: Perfluorocarboxylic acids

PFPeA: perfluoropentanoic acid

PFHxA: perfluorohexanoic acid

PFHpA: perfluoroheptanoic acid

PFOA: perfluorooctanoic acid

PFNA: perfluorononanoic acid

PFDA: perfluorodecanoic acid

PFUdA: perfluoroundecanoic acid

PFDoA: perfluorododecanoic acid

PFBS: perfluorobutane sulfonate

PFHxS: perfluorohexane sulfonate

PFOS: perfluorooctane sulfonate

PFDS: perfluorodecane sulfonate

PFOSA: perfluorooctane sulfonamide

PFSAs: Pefluorosulfonates

PFASA: perfluorosulfonamide

SUMMARY AND THESIS LAYOUT

The main aim of this PhD project was to study Contaminants of Emerging Concern (CECs), and more specifically Endocrine Disrupting Compounds (EDCs), in order to assess their **occurrence**, **behavior** and **fate** in natural freshwater and saltwater environments, focusing on different environmental compartments.

Under this context, estrogens, perfluorinated compounds and phenolic compounds were analysed in the freshwater environment of the Romagna area, in the north-eastern part of Italy, which is a quite high urbanised area potentially affected by the surrounding industrial and domestic activities. To this purpose, surface waters, groundwaters, as well as river sediments were sampled and analysed. Two sampling campaigns were carried out, in two following years (July 2015 and July 2016). Sampling campaigns were performed only during summer period, which corresponds to the dry season in the study area, in order to study the worst scenario regarding EDCs occurrence in the environment. It is widely recognized, in fact, that contamination levels follow seasonal variations, with higher concentrations during the dry season, consequently to the high evaporation rates, and lower concentrations during the wet season, when the high rainfall rates and lower temperatures contribute to a pronounced dilution of contaminants. During sampling campaign in July 2015 both surface waters and groundwaters of confined aquifers of the Romagna area were analysed, in order to look for any relation between the two compartments, as regards EDCs contamination. Analysis on Gadolinium, another additional emerging contaminant useful to trace wastewater contamination, was also performed, in order to check for similarities in the occurrence of both types of contaminants. During sampling campaign of July 2016 the relation between water and sediment of river bodies of the Romagna region was examined.

Furthermore, the Ebro delta (NE Spain) was analysed, as well, to study PFCs behavior in the saltwater compartment and compare it with the freshwater system of the Romagna area, focusing on water, sediment and fish. The study on PFCs was broadened to the detection of 13 perfluorinated compounds, including short chain and long chain perfluorocarboxylic acids and sulfonates, as well as the perfluorooctane sulfonamide. Three sampling campaigns were carried out during autumn, winter and spring-summer, and fluctuations of PFCs concentrations were discussed.

Finally, ecological and human risk were assessed in both freshwater and saltwater environments in order to evaluate the level of risk that these contaminants can pose on biota of the two study areas.

STRUCTURE OF THE PhD THESIS

This work is divided into seven chapters.

The first chapter (**Chapter 1**) contains the introduction and the objectives of the PhD thesis.

Chapters 2, 3, 4 and 5 focus on the experimental work performed during the PhD research project.

In particular, **Chapter 2** deals with spatial distribution of Gadolinium anomaly in the freshwater environment of the Romagna area. Gd is an inorganic element considered as an emerging contaminant because of its use in medical applications; hence it is a highly useful tool to examine microcontamination related to the discharge of wastewater effluents into surface waters. This work represents a preliminary and introductory study on CECs contamination.

Chapter 3 deals with the occurrence of EDCs in surface and groundwaters of the Romagna area, and aims at analysing their spatial occurrence, identifying possible sources of contamination in the study area.

Chapter 4 further deepens the study in the freshwater system of the Romagna area, focusing on the interactions between water and sediment.

Chapter 5 presents the experimental work undertaken at the *Instituto de Diagnostico Ambiental y Estudios de l'Agua – Consejo Superior de Investigaciones Cientificas* (IDEA-CSIC) in Barcelona, Spain, during a six month research internship. In this chapter occurrence and behavior of 13 PFCs in water, sediment and biota of the transitional environment of the Ebro delta and their seasonal variation are assessed.

Chapter 6 contains ecological risk assessment conducted on water and sediment of both the freshwater and the saltwater environments, in order to evaluate if organisms in the different compartments can be at risk from exposure to the environmental EDCs concentrations. Moreover, human health risk is also evaluated, considering the potential exposure of local human population to EDCs from consumption of drinking water, which is supplied by surface and groundwaters in the Romagna area, and from Ebro River fish consumption.

In the final **Chapter 7** conclusions of this PhD work and future research needs are drawn.

Journal manuscripts prepared during the PhD project are enclosed in this PhD thesis and reported in the following chapters:

Chapter 2: Geochemical characterization and rare earth elements anomalies in surface- and groundwaters of the Romagna area (Italy). Pignotti E, Dinelli E, Birke M (2017) *Rendiconti Lincei* 28 (2): 265-279. DOI: 10.1007/s12210-016-0561-3

Chapter 3: Occurrence and distribution of six selected Endocrine Disrupting Compounds in surface- and groundwaters of the Romagna area (North Italy). Pignotti E, Farré M, Barcelò D, Dinelli E (2017) *Environmental Science and Pollution Research* 24 (26): 21153-21167. DOI: 10.1007/s11356-017-9756-0

Chapter 4: Distribution and partitioning of Endocrine Disrupting Compounds in water and sediment: case study of the Romagna area (Italy). Pignotti E, Dinelli E (in press) *Journal of Geochemical Exploration.* DOI: 10.1016/j.gexplo.2018.02.008

Chapter 5: Seasonal variations in the occurrence of perfluoroalkyl substances in water, sediment and fish samples from Ebro Delta (Catalonia, Spain). Pignotti E, Casas G, Llorca M, Tellbüscher A, Almeida D, Dinelli E, Farré M, Barcelò D (2017) *Science of the Total Environment* 607-608: 933-943. DOI: 10.1016/j.scitotenv.2017.07.025

The above scientific articles are reported in their original version, but adapted to the text format of this work.

Chapter 1

INTRODUCTION

1 CONTAMINANTS OF EMERGING CONCERN AND ENDOCRINE DISRUPTING COMPOUNDS

"Contaminants of Emerging Concern" (CECs) or, more simply, "emerging contaminants", are defined as compounds that are not currently monitored in the environment or regulated by legislation, but are thought to cause adverse effects on the ecosystem and human health. They encompass a wide variety of chemicals not necessarily new; in fact, they may have been used for decades, but have not been recognised until new detection methods were developed (Ragav et al. 2013).

These chemicals are mainly industrial products used in everyday life, such as pharmaceuticals, personal care products, plasticizers, pesticides, detergents, flame retardants and so on. Many of them are persistent in the environment, since they are highly resistant to degradation; others may not be persistent, but their continuous introduction makes them a potential threat to the environment.

The "emergence" related to these contaminants states the only recently growing awareness about the potential harmful effects they can pose on organisms and human health.

CECs enter the environment primarily through the discharge of municipal and industrial wastewater effluents. The conventional wastewater treatment plants (WWTPs) are inefficient in the complete removal of these compounds, with the consequence of releasing them in the aquatic compartment almost undegraded. Some of the CECs can undergo partial degradation; their metabolites, though, can be even more harmful than their parent compound (Pal et al. 2010; Naidu et al. 2016). Once in the environment, contaminants can migrate in other environmental compartments and reach groundwaters, or can be adsorbed onto sediments and soils, or be accumulated by the aquatic organisms and magnify through the food chain, ultimately reaching human beings. Landfill leachates, runoff from agricultural lands containing pesticides or from land-applied biosolids are additional secondary sources of contamination (Ragav et al. 2013).

Contaminants of emerging concern comprise numerous and various substances. For this reason, it is of common practice to group subcategories of CECs according to their use and characteristics. **Table 1.1** provides a summary of the main categories of CECs known to be present in the environment.

Table 1.1 Common classes of CECs (taken from Ragav et al. 2013)

Class of CECs	Example	Definition
Antibiotics	Tetracycline, erythromycin	Medications that fight bacterial infections, inhibiting or stopping bacterial growth
Antimicrobials	Triclosan	Biochemicals that kill or inhibit the growth of microorganisms including bacteria and fungi
Detergent metabolites	Nonylphenol	Chemical compounds formed when detergents are broken down by wastewater treatment or environmental degradation
Disinfectants	Alcohols, Aldehydes and oxidizing agents	A chemical agent used on non-living surfaces to destroy, neutralize, or inhibit the growth of disease-causing microorganisms
Disinfection by-products	Chloroform, Nitrosodimethylamine (NDMA)	Chemical substances resulting from the interaction of organic matter in water with disinfection agents such as chlorine
Estrogenic compounds	Estrone, Estradiol, Nonylphenol, Bisphenol A	Natural or synthetic chemicals that can elicit an estrogenic response
Fire or flame retardants	Polybrominated Diphenyl Ethers (PBDEs)	Any of several materials or coatings that inhibit or resist the spread of fire
Fragrances	Galaxolide	Chemical substances that impart a sweet or pleasant odor
Insect repellants	DEET (N, N-diethyl-meta-toluamide)	Chemical substances applied to skin or other surfaces to discourage insects from coming in contact with the surface
PAHs (Poly-Aromatic Hydrocarbons)	Benzo(a)pirene, Fluoranthene, Naphthalene	A large group of chemical substances usually found in the environment as a result of incomplete burning of carbon-coating materials like fossil fuels, wood or garbage
Personal Care Products	Para-hydroxybenzoate	Chemical substances used in a diverse group of personal items including toiletries and cosmetics
Pesticides or Insecticides	Permethrin, Fenitrothion, Bacillus thuringiensis israelensis (Bti)	Chemical substances or microbiological agents that kill, incapacitate or otherwise prevent pests from causing damage
Pharmaceuticals	Fluoxetine, Carbamezepine, Diphenhydramine	Chemical substances used in the prevention or treatment of physiological conditions
Plasticizers	Dioctyl Phthalate (DOP)	Chemical additives that increase the plasticity or fluidity of a material
Reproductive hormones	Dihydrotestosterone (DHT), Progesterone, Estrone, Estradiol	A group of chemical substances, usually steroids, whose purpose is to stimulate certain reproductive functions
Solvents	Ethanol, Kerosene	Chemical solutions, other than water, capable of dissolving another substance
Steroids	Cholesterol, Coprostanol, Estrone, Progesterone	A large group pf fat-soluble organic compounds with a characteristic molecular structure, which includes many natural and synthetic hormones
Surfactants	Sodium Lauryl Sulfate	Chemical substances that affect the surface of a liquid

Among these categories, Endocrine Disrupting Compounds (EDCs) represent a category of great concern because of the harmful effects they can exert on the endocrine system of organisms, like the term suggests. A clear definition of what these compounds are, has been provided by World Health Organization (WHO), which described Endocrine Disrupting Compounds as “any exogenous substance or mixture that alters

function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations" (IPCS 2002).

Even though limited information is available about the real effects of these contaminants on aquatic organisms and human health, there is growing concern because EDCs have been shown to cause adverse effects at very low concentrations. Hayes et al. (2010) reported hermaphroditism, testicular oogenesis and reduced testosterone and testicular volume in African clawed frogs (*Xenopus laevis)* when exposed to 100 ng/L of atrazine, an herbicide. Increase of vitellogenin in males, decreased fecundity, fertility and ratio of motile spermatozoa were also observed in medaka (*Oryzias latipes*) exposed to nonylphenol at concentrations higher than 11.6 µg/L (Hara et al. 2007). Crucian carp (*Carassius auratus*) exposed to WWTP effluents with steroidal and phenolic EDCs at concentrations <1 µg/L displayed a significant reduction in gonadosomatic index and increase in plasma vitellogenin; bioconcentration of contaminants was also observed (Liu et al. 2012).

Considering the potential harmful effects that can be caused by exposure of such contaminants, EDCs occurrence in the environment has raised great concern among scientists. For this reason, in this PhD work it was decided to give primary attention to these contaminants. In the following sections additional information about their mechanism of action and their adverse effects is given.

1.1 Endocrine system and EDCs interference

The endocrine system is made of glands that produce hormones to be carried towards specific target organs through the circulatory system, acting as messengers to regulate specific physiological processes. Examples of endocrine organs are thyroid, hypothalamus, reproductive glands, pancreas, liver and kidney. Generally, hormones act by binding to nuclear- or cellular membrane receptors; the hormone-receptor complex switches on or switches off specific biological processes in cells, tissues and organs. Receptors are not present in all cells and are hormone-specific. This implies that hormone action is strictly specific and saturable, depending both on the affinity of the hormone with the receptor, and on receptor abundance itself (Zoeller et al. 2012).

Hormones exert their effects at very low concentrations, since they act on specific receptors with high affinity. Usually, hormones are characterized by a non-linear dose-response curve (**Figure 1.1**), in which small changes in hormone concentration at the low end of the dose-response curve produce greater differences in effects than concentrations at the high end of the curve. In this sense, dose-response curves in most cases have a sigmoidal pattern, but can also be more complex, such as U-shaped (with maximal responses detected at low and high doses) or inverted U-shaped form (with maximal responses observed at intermediate doses) (WHO 2012).

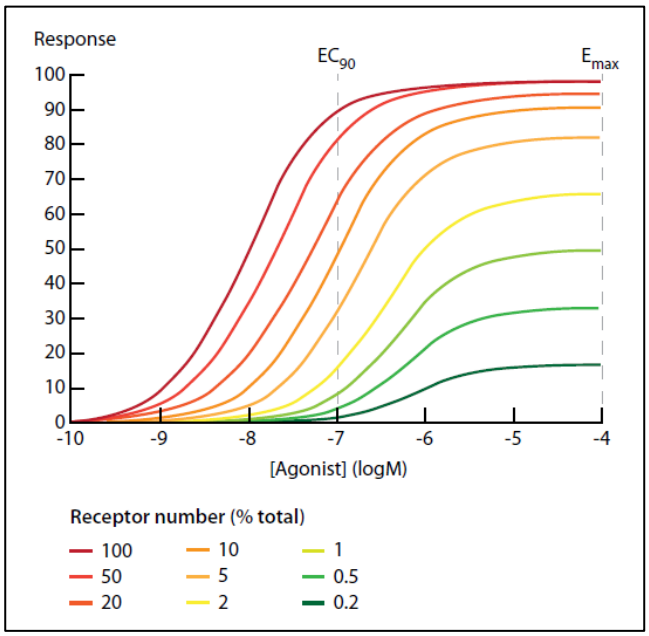


Figure 1.1 Sigmoidal dose-response curve for hormones, dependent on receptor abundance (taken from WHO 2012).

There are three main pathways of interference by xenochemicals. They can mimic the normal endocrine hormone functions, binding and activating endocrine receptors in a target tissue, and thus leading to an (unexpected) endocrine stimulation (*agonistic effect*). Another way of interference is related to the ability of certain EDCs to bind to the receptors without activating them, and preventing the natural hormone binding on the receptor, with the result of a decrease in the endocrine response (*antagonistic effect*). Moreover, EDCs can bind to the proteins in blood that are involved in the hormone transport and delivery to the target cells, interfering with the metabolic processes of the organism. Finally, they can also interfere with natural hormone synthesis and metabolism (Zoeller et al. 2012). In some cases, multiple receptor types can mediate hormone action. For example, estrogens exert their effects by acting on at least two major nuclear receptor types: Estrogen Receptor α (ERα) and Estrogen Receptor ß (Erß). However, estrogens can also induce rapid (seconds to minutes) extra-nuclear responses based on ER localized at the plasma membrane. A small pool of ERα and ERβ is indeed palmitoylated and localized at the plasma membrane in association with caveolin-1 (Acconcia et al. 2015). To date the ERα-mediated extracellular regulated kinase/mitogenactivated protein kinase (ERK/MAPK) and phosphatidyl-inositol-3-kinase/AKT (PI3K/AKT) pathways, as well as the ERβ-mediated p38/MAPK signalling, appear to be the unique molecular circuitries activated by E2 in different cell contexts. Several data support that bisphenol A (BPA)-dependent estrogenic activity flows through the ERα-mediated extra-nuclear signals activation that result in the ERK/MAPK and AKT phosphorylation. In contrast, testosterone effects are mediated by a single Androgen Receptor (AR) (WHO 2012).

Endocrine disruptors have a much lower affinity with the hormone receptors in comparison to the natural ligands. Notwithstanding the lower affinity, EDCs can still exert their interference at low doses, due to the

sigmoidal dose-response curve and the consequent greater impact of small changes in hormone action at low doses than at higher doses (WHO 2012).

The classical EDCs targets are nuclear receptors such as estrogen receptors (ER), androgen receptors (AR), mineralocorticoid receptors (MR), progesterone receptors (PR), glucocorticoid receptors (GR), thyroid receptors (TR) and peroxisome proliferator-activated receptors (PPAR) (Giulivo et al. 2016).

Disruption of the endocrine functionalities regards all hormonal systems, ranging from the development and function of reproductive organs to adult diabetes or cardiovascular problems. Up to now, the majority of studies have focused on disruption affecting only the reproductive system, but environmental chemicals can interact also with other endocrine systems, leading for example to metabolic alteration, with increased obesity, insulin resistance and type II diabetes mellitus (Giulivo et al. 2016). Several EDCs, including bisphenol A, alkylphenols, pesticides, phthalates, pharmaceuticals, dioxins and phytoestrogens have also shown thyroid disruption capability, causing growth and metabolic alteration, and eventually brain damage with increased mortality rate (Wee and Aris 2017).

Endocrine disruption is highly species-specific, and effects from invertebrates to mammalians can be very different, being dependent on the species physiology (Annamalai and Namasivayam 2015). For example, six different species of fish showed similar ERα sensitivity to 17ß-estradiol, but different responses to exposure to p,p'-dichlorodiphenyltrichloroethane (DDT) (Iguchi and Katsu 2008).

Moreover, endocrine disruption significantly differs accordingly to life-stage of individuals; in general, intrauterine, perinatal, juvenile or puberty periods are the most critical ones, since at these stages the endocrine system functionalities are not completely developed yet (Wee and Aris 2017). Exposure of children to phthalates, for example, has been evaluated to be higher than that of the mother, and reproductive dysfunctions were detected in children of workers exposed to pesticides (Kasper-Sonnenberg et al. 2012; Maqbool et al. 2016).

Toxicity and adverse effects exerted by chemicals are often evaluated considering the single chemical. As many studies have pointed out, however, natural environments are affected by a mixture of different chemicals that may be harmful for the most sensitive species. Even if xenoestrogens alone can be safe for organisms, combined with other chemicals at concentrations that on their own would not cause measurable effects, produce substantial estrogenic effects. When mixed with estradiol, for example, almost doubled estrogenic effects on organisms exposed to low levels of xenoestrogens have been registered (WHO 2012).

While numerous studies have been carried out on endocrine disrupting effects on wildlife, still little is known about the adverse effects on human health. Studies reported human endometrium physiology

altered by BPA and triclosan (Forte et al. 2016). Specht et al. (2014) highlighted an anti-androgenic action of di-2-ethylhexyl phthalate (DEHP), with decreasing testosterone levels, semen volume and sperm count at increasing exposure to the chemical and its metabolites. Still, even though some evidence of adverse effects on human health have been registered, the real mechanisms of EDCs disruption in humans remains unclear.

Human exposure occurs mainly through food ingestion and water consumption; non-dietary sources, including air and dust particles, and dermal contact (e.g. cosmetics, toys) are secondary sources of contamination, as well (Wee and Aris 2017; Giulivo et al. 2016). Fetuses and toddlers can also be exposed through placenta and breast milk.

1.2 EDCs regulation

Deriving risk assessment for ecological and human exposure to EDCs is quite complex, due to the differences in response by species and life-stage of organisms. Furthermore, the majority of toxicological studies have been carried out on high doses of contamination, but it is difficult to extrapolate from these data effects of exposure at low environmental doses, due to the non-linear dose-response curve of EDCs and to multiple receptors mediation on hormones action (Welshons et al. 2003; Ribeiro et al. 2017). For these reasons, there is still lack of regulation of many of these compounds by public authorities. Nevertheless, the growing concern about their potential adverse effects has led many organizations such as the United States Environmental Protection Agency and European Union to take provisional actions to regulate these contaminants.

US EPA periodically releases a Contaminant Candidate List (CCL), which is a list of unregulated chemicals and microbes that are known or anticipated to occur in public water systems and which may require regulation. This list provides the basis for a mandated US EPA regulatory action to decide if these contaminants are to be included or not in the Safe Drinking Water Action plan. US EPA's Final CCL-4, announced in 2016, includes 97 chemicals and 12 microbial contaminants.

In Europe EDCs, or CECs more in general, are not currently included in any drinking water act. However, in the recent years some action plans for surface and groundwater protection have been taken. Directive 2000/60/EC, commonly known as Water Framework Directive, was accepted by EU member states with the purpose of protecting and recovering water quality throughout the European territory, reaching a "good-quality status" of all European surface waters. With its amendment, the Directive 2008/105/EC, a list of 33 priority substances was established, including organic chemicals and some EDCs to be monitored in water analyses. For each of the 33 priority substances an Environmental Quality Standard (EQS) was set, as an environmental threshold not to be exceeded in order to safeguard surface waters. In 2013 a new

amendment was approved, the Directive 2013/39/EU, which added 12 more chemicals to the list of priority substances, with their relative EQS. Moreover, a watch-list of non-regulated substances was proposed, for which monitoring data are to be gathered throughout Europe with the purpose of supporting future prioritization exercises. The pharmaceutical diclofenac and the two estrogenic hormones 17ß-estradiol and 17α-ethinylestradiol have been included in this watch-list of monitoring substances.

2 EDCs SELECTED FOR THE PhD STUDY

So far, more than 600 chemicals have been identified as potential endocrine disruptors. For the purpose of this PhD work, different classes of EDCs were selected and analysed, in order to study a broad variety of chemicals. Selection of the compounds to be analysed was made considering the estrogenic potential of each group and their use in human products. Based on literature evaluation, three classes of EDCs were selected: **estrogenic hormones**, which are the compounds with the highest estrogenic activity, since their main physiological function is to determine estrogenic response in the organisms; **phenolic compounds** and **perfluorinated compounds**, which have much lower estrogenicity compared to estrogens, but because of their widespread use in man-made products, they are ubiquitous and continuously introduced in the environment. Moreover, Gadolinium (Gd) in surface waters was also analysed, as a new emerging contaminant considered a good tracer of wastewater contamination.

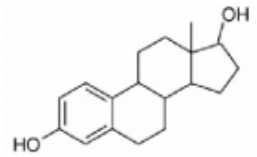
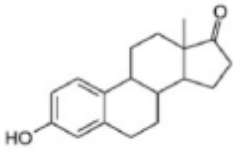
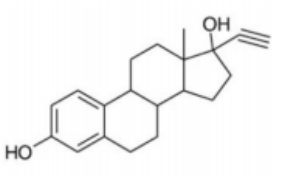
In the following section the three classes of contaminants will be discussed more in detail.

2.1 Estrogens

2.1.1 Chemical properties, occurrence and behavior in the environment

Estrogens are natural hormones produced by humans and animals and are involved in female sexual characterization. Among these, the most environmentally relevant ones are the natural hormones estrone (E1) and 17ß-estradiol (E2), and the synthetic hormone 17α-ethinylestradiol (EE2), used as medication and as component of birth control pills. **Table 1.2** summarizes the main physical and chemical properties of these compounds. The octanol-water partition coefficient ($\log K_{ow}$) higher than 2.5 and the low values of solubility state the hydrophobic characteristics of estrogens.

Table 1.2 Main physical-chemical characteristics of estrogens

	17ß-estradiol (E2)	**Estrone (E1)**	**17α-ethinylestradiol (EE2)**
Structure			
Molecular Weight (g/mol)	272	270	296
Solubility (mg/L)	3.6	13	4.8
logK_{ow}	3.94	3.43	4.15
pK_a	10.23	10.34	10.25

Estrogens are excreted by humans and animals mainly as conjugated forms with sulfates, glucuronide, or sulfoglucuronide that increase estrogen solubility and enhance their excretion through urine; a fraction of estrogens is also eliminated in their unconjugated forms through feces. Conjugated forms have very low estrogenic activity; nevertheless, once excreted, they can easily be broken down into the more estrogenic free forms by fecal flora (Combalbert and Hernandez-Raquet 2010). Estrogens excretion is highly dependent on sex and physiological state of humans. On average, it has been estimated that the total amount of estrogens released by humans into the environment is 4.4 kg/year/million inhabitants. Excretion of steroidal hormones by animals is highly dependent on species. Cattle and sheep excrete estrogens primarily via feces, while poultry and pigs through urine. Even though assessing estrogens excretion by animals is more difficult than for humans, a rough estimation of annual animal estrogens production of 49 tons in the USA and 33 tons in the EU has been proposed (Combalbert and Hernandez-Raquet 2010).

Estrogenic compounds mainly enter the environment through wastewater systems. Wastewater treatment plants are not always efficient to remove such microcontaminants from wastewaters, and in this way they are released in the receiving surface waters as parent compounds or only partially degraded molecules (Gorga et al. 2015; Tiedeken et al. 2017; Ting and Praveena 2017). In addition, natural estrogens can also reach surface waters through runoff from farming areas where animal manure is applied, or from grass lands with cattle breeding (Andaluri et al. 2012; Adeel et al. 2017). It has been estimated that livestock excreta represent the major source of estrogenic compounds in the aquatic environment (Pal et al. 2010). Estrogens have been detected in different environmental compartments, namely influents and effluents of municipal WWTPs, river waters, groundwaters, soils and sediments. For a better understanding of the environmental concentrations throughout the world, detection of estrogens taken from published works are reported in **Table 1.3**, and additional information are also given in *Chapters 3* and *4* of this PhD thesis.

Estrogens have a short life-time, being easily degraded under natural environmental conditions. Photochemical degradation is one removal mechanism that is primarily involved in their attenuation in the

aquatic environment. Ying et al. (2002) reported half-lives of 2-3 days for E1 and E2, and 4-6 days for EE2 in river waters. Compared to natural estrogens, the synthetic hormone EE2 has been recognized to be more resistant to degradation, requiring longer time for its removal (Aris et al. 2014). Studies on solar irradiation found that photodegradation enhanced estrogen removal from waters, lowering E1 half-life to 8 hours (Caupos et al. 2011) and 23 hours for EE2 (Zuo et al. 2013).

Table 1.3 Concentrations of E1, E2 and EE2 in different environmental compartments in the world

Environmental compartment	Location	E1	E2	EE2	Reference
WWTP influent[*]	French WWTP	18.8-170	5.1-37.9	<LOQ	Gabet-Giraud et al. 2010
WWTP effluent[*]	French WWTP	0.1-58	0.5-11.9	1.6-4.6	Gabet-Giraud et al. 2010
Duck-weed ponds influent[*]	The Netherlands	43.5	29.7	9.71	Shi et al. 2010
Duck-weed ponds effluent[*]	The Netherlands	2.45	1.38	0.59	Shi et al. 2010
Surface water[*]	Italy	<1.2-10	<1.0-175	<0.8-34	Pojana et al. 2007
	Portugal	2.4-4.0	4.9-10.1	4.6-9.14	Rocha et al. 2015
	China	22.4-66.2	<0.66-1.8	nd	Chen et al. 2007
Groundwater[*]	The Netherlands	9	31	nd	Lapworth et al. 2012
	Europe	1.1	<LOQ	<LOQ	Jurado et al. 2012
Drinking water[*]	Germany	0.20-0.60	0.20-2.1	0.15-0.50	Kuch and Ballschmiter 2001
	Spain	<LOQ	nd	<LOQ	Huerta-Fontela et al. 2011
Soil/sediment[**]	Spain	<LOQ – 3.5	<LOQ – 1.6	nd	Gorga et al. 2015
	Czech Republik	1.01-2.37	1.15-1.84	1.63	Matejicek 2011
	Tianjin area, China	0.98-21.6	nd-9.70	nd-9.26	Lei et al. 2009
	Luoma Lake, China	nd	0.52-1.21	0.61-1.48	Liu et al. 2017a
	Australia	0.16-1.17	0.22-2.48	<LOQ-0.5	Braga et al. 2005
Animal manure[***]					
Swine farrowing pits	U.S.A.	4800	1500	500	Combalbert and Hernandez-Raquet 2010
Poultry manure	U.S.A.	44.2	92.7	149.8	Andaluri et al. 2012
Cow manure	U.S.A.	16.1	6.2	16.6	Andaluri et al. 2012

nd: not detected; [*]: ng/L; [**]: ng/g dw; [***] µg/Kg

It is well known that estrogen degradation is highly enhanced under biotic conditions, being biodegradation the major removal mechanism (Khanal et al. 2006; Fan et al. 2007; Hamid and Eskicioglu 2012). Bacteria normally present in wastewater treatment plants have been found capable of totally removing estrogenic compounds. In particular, the most effective bacteria are the gram-negative *Rhodococcus zopfii* and *Rhodococcus equi*, capable of rapidly degrading steroidal hormones into non-estrogenic compounds (Khanal et al. 2006). Yu et al. (2007) further reported that, of the 14 phylogenetically E2-degrading wastewater strains, the *Sphingomonas* strain was the more effective one, leading to the complete degradation of E2 and E1 into non-estrogenic compounds.

Biodegradation can occur under two possible mechanisms: growth-linked (metabolic) and non-growth linked (co-metabolic). In growth-linked degradation, microorganisms use steroidal hormones as energy and carbon source for microbial growth. Wastewater bacteria like *Novosphingobium tardaugens*, *Sphingomonas* strain, *Sphingobacterium* sp., and *Pseudomonas aeruginosa* are responsible for E2 degradation through this mechanism. The co-metabolic degradation involves no carbon or energy benefits for microorganisms, which instead use their own enzymes to degrade steroidal hormones. E2 and EE2 are thought to be co-metabolically degraded by heterotrophic bacteria and by ammonia-oxidizing bacteria and nitrifying activated sludge. Since estrogen concentrations in wastewaters are not very high (range of ng/L), co-metabolic degradation is thought to be the major pathway for removing estrogens from contaminated waters (Yu et al. 2013).

Aerobic biodegradation is much faster than the anaerobic degradation. In anoxic environments, therefore, estrogens are more likely to accumulate and sorb in sludge or sediments, since their breakdown into inorganic compounds is very slow *(*Czajka and Londry 2006).

Hamid and Eskicioglu (2012) studied estrogens removal during wastewater treatment. They found that the common primary treatment in WWTP is quite ineffective, whereas the secondary treatment, mainly characterized by biological processes, plays the most significant role in their total degradation. Conventional activated sludge, which is the most common treatment of wastewaters, normally shows an efficient removal of E2 and estriol (90-99%, Hamid and Eskicioglu 2012 and references therein), lower degradation of E1, as a consequence of E2 partial degradation, and persistence of EE2, due to its recalcitrant nature. Activated sludge systems associated with nutrient removal processes heighten estrogens removal, with degradation of EE2 occurring only in nitrifying conditions, whereas natural estrogens can equally be degraded in both nitrifying and denitrifying conditions.

Sorption is the major abiotic factor that mostly accounts for steroidal hormones removal from waters. As aforementioned, estrogens have high $\log K_{ow}$ values, ranging from 3.4 to 4.2 (**Table 1.2**), suggesting hydrophobic behavior of these compounds that leads them to easily adsorb onto organic solid rather than remaining dissolved in solution. Carballa et al. (2008) reported sediment/water partition coefficient ($\log K_d$) of 2.18-2.77 for E1, 2.30-2.83 for E2 and 2.08-2.85 for EE2, and organic carbon-normalized values ($\log K_{oc}$) of 3.00-4.18 for E1, 3.13-3.69 for E2 and 2.90-4.16 for EE2, thus giving evidence of high affinity of these compounds for the solid phase and especially for organic carbon content.

Fan et al. (2007) stated that about 50-73% of E2 was associated with humic acids, which constitute the organic matter in soil, mainly through hydrogen or covalent bonds.

Partition of estrogens between sediment and water highly increases in salinity, as their solubility decreases, resulting in a higher accumulation in marine sediments (Pan et al. 2010).

Total decomposition of free estrogens from the solid phase occurs mainly by soil microbial degradation. Compared to degradation by sewage microbes, which is fast and complete, biodegradation by soil microbes is rather low and incomplete (Khanal et al. 2006). Therefore, estrogens can remain sorbed onto soil particles for longer time.

Mineral oxides, especially manganese oxides, have also been demonstrated to influence estrogens degradation in sediment. Sheng et al. (2009) reported E2 degradation into E1 driven by Mn-oxides which enhanced the oxidation of the hydroxyl group of E2 at position 17, transforming it into a carbonyl.

D'Alessio et al. (2014) in their batch experiment proved E2 and E1 sorption highly dependent on organic carbon content, with a higher sorption capacity of E1 compared to E2, thanks to the presence of a ketonic group in E1 molecule, which is a stronger H-acceptor if compared to the hydroxyl group of E2 and is more prone to form interactions with the phenolic hydroxyl groups of organic matter.

On the other hand, pH is not likely to influence estrogens sorption onto soil or sediment (D'Alessio et al. 2014), since under natural environmental conditions they are present in the water compartment in their neutral form (pK_a of around 10.3) and as such their sorption mainly occurs via hydrophobic interactions and hydrogen bonding rather than being influenced by pH changes.

2.1.2 Toxicology and health effects

Estrogens are essential for mammalian biology and physiology. They have a main role in sexual maturation and reproduction in females and males, and regulate pregnancy in females; they also participate in the modulation of growth, cardiovascular functions and energetic metabolism, and enhance bone strength (Adeel et al. 2017). However, if organisms are exposed to a high and continuous level of estrogens, they can suffer for adverse effects due to the prolonged exposition to external sources of estrogens.

Several studies pointed out that organisms exposed to elevated concentrations of estrogens were affected by altered growth and sexual behaviors. Rose et al. (2013) demonstrated that short-term exposure to low concentrations (2 ng/L) of EE2 in Gulf pipefish *(Syngnathus scovelli)* enhanced female reproductive success. However, at higher concentrations (5 ng/L), it caused a complete reproductive failure in males. In a Canadian watershed, exposure of male fish to wastewater sewages induced impairment in the capacity to produce testosterone. Rates of intersex were registered as well, downstream of two main municipal sewage discharges, of 33% and >60%, respectively (Tetreault et al. 2011). Kidd et al. (2007) experienced in a chronic exposure experiment of fathead minnow *(Pimephales promelas*) to low concentrations of EE2 (5-6 ng/L) a feminization of males through the production of vitellogenin mRNA and protein, intersex in males and altered oogenesis in females.

In relation to the use of estrogens during hormone replacement therapy, especially for menopausal women, the Joint FAO/WHO Expert Committee on Food Additives established an Acceptable Daily Intake (ADI) of 0-50 ng/kg bw/day based on the No-Observed-Effect-Level of 5 µg/kg bw/day for humans, as the maximum daily dose at which no adverse effects on human health should occur (Plotan et al. 2014). Chronic exposure of humans to estrogens has been argued to increase breast cancer incidence in females (Moore et al. 2016) and prostate cancer in men (Nelles et al. 2011), even though more information is needed.

2.1.3 Regulation

Until recently, no regulations have required monitoring of estrogens in surface waters. However, owing to the growing concern about their harmful effects on aquatic organisms, water public authorities have begun focusing attention on this issue. In Europe, Directive 2013/39/EU included 17ß-estradiol and 17α-ethinylestradiol in the watch-list of non-regulated substances, for which monitoring data are required with the purpose of future prioritization support (EU 2013).

On the other hand, US EPA included E2, E1 and EE2, and other estrogens as well, in the Drinking Water Contaminants Candidate List (CCL-4).

2.2 Phenolic compounds

2.2.1 Chemical properties, occurrence and behavior in the environment

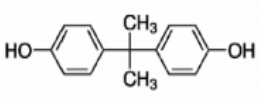
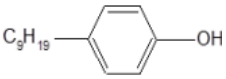
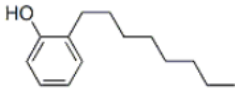
Bisphenol A (BPA) is an organic chemical widely used in industry primarily for the production of epoxy resins and polycarbonate plastics, as an antioxidant and inhibitor of end of polymerization in polyvinyl chloride plastics (PVC) and as a precursor for the synthesis of the flame retardant tetrabisphenol-A (Geens et al. 2012). Therefore, it can be found in many of the applications based on polycarbonates and epoxy resins, such as water bottles, compact discs, impact-resistant safety equipment, medical devices, dental materials, as well as food and drink packaging, food cans, and water supply pipes (NIH 2017).

Alkylphenols are a similar group of industrial chemicals synthesized through the alkylation of phenols. Among them, the main common compounds are octylphenol (OP, 8-carbon chain) and nonylphenol (NP, 9-carbon chain). They are non-ionic surfactants mainly used as precursors of alkylphenols ethoxylates, which are widely employed in detergents, emulsifiers, paints, pesticides, pharmaceuticals, cosmetics and plastics. They have been used in industrial production for more than 60 years (Zhu and Zuo 2013).

In **Table 1.4** the main physical and chemical properties of these compounds are reported. BPA has a moderate solubility (300 mg/L), while NP and OP are more hydrophobic (6.23 and 4.8 mg/L of solubility in water). Compared to NP and OP, BPA has a lower $\log K_{ow}$, which suggests a slight lower aptitude of BPA to

partition into an organic phase (e.g. sediment, soil or fish) rather than remain in the aqueous phase. The high pK_a values (9.6-10.3) indicate that at the environmental neutral pH range, these compounds are present in their neutral form.

Table 1.4 Main characteristics of the most abundant phenolic compounds

	Bisphenol A (BPA)	**Nonylphenol (NP)**	**Octylphenol (OP)**
Structure			
Molecular Weight (g/mol)	228.3	220.3	206.3
Solubility (mg/L)	300	6.23	4.8
logK_{ow}	3.4	4	4.6
pK_a	9.6	10.7	10.3

Phenolic compounds are considered persistent, toxic and bioaccumulating compounds; for these reasons, along with their widespread use in human products, they have raised concerns about their presence in the environment.

Table 1.5 provides an overview of the phenolic concentrations detected in different environmental compartments; additional information about their occurrence in the environment throughout the world is given in *Chapters 3* and *4*, as well.

As the majority of emerging contaminants, phenolic compounds occurrence in the environment is mainly related to the discharge of municipal and industrial WWTPs; other sources, such as landfill disposal, proximity of industrialized areas, and runoff from agricultural lands with pesticide application can contribute to their introduction in the aqueous compartment (Soares et al. 2008; Sanchez-Avila et al. 2009; Zhu and Zuo 2013; Careghini et al. 2015). Volatilization is also expected for these chemicals, given their semi-volatile properties. Once they reach the atmosphere, they can be transported to aquatic and terrestrial ecosystems by wet deposition (Soares et al. 2008).

NP and OP are frequently present in the environment as stable intermediates of alkylphenols polyethoxylates (APEOs) (Careghini et al. 2015). Low solubility and hydrophobic properties of the compounds indicate that they can be sorbed onto sediment or particulate matter, and can be accumulated in aquatic organisms. In fact, logK_{oc} values can range from 4.63 to 5.83 for OP; from 4.95 to 6.62 for NP and from 4.12 to 5.32 for BPA (Arditsoglou and Voutsa 2012). Bioconcentration factors have been reported to range between 21 and 1300 for NP, between 267 and 471 for OP and between 13.10 and 147.71 for BPA (Soares et al. 2008; Diao et al. 2017).

Table 1.5 Concentrations of BPA, NP and OP in different environmental compartments in the world

Environmental compartment	Location	BPA	NP	OP	Reference
Indoor air[*]	Tokyo	-	<4.5-680	<0.87-45.7	Saito et al. 2004
	France	<0.6-10	-	-	Blanchard et al. 2014
Outdoor air[*]	Tokyo	-	<4.5-53.1	<0.87-5.3	Saito et al. 2004
	North Sea	-	0.03-0.11	0.005-39	Xie et al. 2006
	China	0.23-1.26	-	-	Fu and Kawamura 2010
	Arctic	0.001-0.011	-	-	Fu and Kawamura 2010
WWTP influent[**]	Spain	2.40	102	66.6	Sanchez-Avila et al. 2009
	Australia	140	3,070	229	Tan et al. 2007
WWTP effluent[**]	Spain	0.62	21.9	53.8	Sanchez-Avila et al. 2009
	Australia	86.7	335	23.5	Tan et al. 2007
Surface water[**]	Spain	0.11-126	96-1,483	0.14-474	Esteban et al. 2014a
	Spain-Portugal	20-4,800	30-1,030	8-88	Salgueiro-Gonzalez et al. 2015
	Baltic Sea	31.6-713.9	<LOQ-3,660	<LOQ-329.5	Stanizewska et al. 2015
	Belgium	3-55	32-2,500	<LOQ	Loos et al. 2007
	Italy	36-175	460-700	11-111	Loos et al. 2007
	San Francisco, USA	-	<LOQ-72.9	-	Klosterhaus et al. 2013
	Miami River, USA	4.4-190	-	-	Singh et al. 2010
	Pearl River, China	7.72-311	64.8-1,550	2.38-16.85	Zhao et al. 2011a
	Songhua River, China	8.24-263	106-344	1.54-45.8	Zhang et al. 2014
Groundwater[**]	Spain	-	<10-5,280	<10-1,800	Tubau et al. 2010
	Europe	<1-2,299	-	-	Loos et al. 2010
Drinking water[**]	Italy	<0.73-102	<7.70-84	-	Maggioni et al. 2013
	France	-	<10	<2	Devier et al. 2013
	Guangzhou, China	2.3-317	196-1,070	-	Li et al. 2010
	China	2.1-128	8.1-558	-	Fan et al. 2013
Soil/sediment[***]	Italy	7-127	31.9-224.2	15.8	Viganò et al. 2015
	Spain	4.5-100	36-538	9.4-45	Gorga et al. 2014
	Greece	7.2-39	223-2,695	6.0-25	Arditsoglou and Voutsa 2012
	Spain-Portugal	4.3-130	21-4,460	9.3-74.5	Salgueiro-Gonzalez et al. 2015
	Baltic Sea	-	<LOQ-249.1	0.15-20.47	Koniecko et al. 2014
	San Francisco, USA	-	21.5-86.3	-	Klosterhaus et al. 2013
	Japan	1.88-23.0	-	-	Liao et al. 2012
	Pearl River, China	<LOQ-76.6	11.4-3,750	<LOQ-30.4	Zhao et al. 2011a
	Guangzhou, China	2.54-269	10.9-14,400	-	Peng et al. 2017
Biota[***]	Italy	36.6-1530	240-4,191	12.0-223.2	Viganò et al. 2015
	Baltic Sea	nd-273	nd-263.8	0.8-176.1	Stanizewska et al. 2017
	Finland	<LOQ-137.2	5.5-91.3	12.7-481.5	Nehring et al. 2017
	Turkey	<50	nd-52.73	<3	Yilmaz et al. 2016
	Pearl River, China	0.49-4.51	17.48-237.1	<0.11-0.47	Diao et al. 2017
	NE coast USA	-	122-2,380	-	Diehl et al. 2012
	Ohio, USA	-	6.6-110	-	Rice et al. 2003
	SE Asia	nd-13.7	nd-643	nd-14	Isobe et al. 2007

nd: not detected; [*]: ng/m^3; [**]: ng/L; [***]: ng/g

Adsorption processes into sediment and soil are mostly controlled by organic carbon content. In sediments free of organic matter, adsorption was also observed, suggesting sorption behavior driven also by hydrophilic interactions with mineral components (Soares et al. 2008). Biodegradation is a major factor influencing NP and OP occurrence in the solid phase, consisting in the deethoxylation of APEOs and release of NP and OP. Their formation is highly enhanced under anaerobic conditions. Lu and Gan (2014) reported a half-life of NP in sediments of 0.9-13.2 days under oxic conditions and 15.1-20.1 under slightly reducing conditions; under anoxic conditions, NP persistence highly increased, with registered half-life of more than 200 days. Fungal degradation activity has been registered only under aerobic conditions, while bacterial activity can be observed in both oxic and anoxic environments, even if deethoxylation of APEOs is favored under anaerobic conditions (Corvini et al. 2006). Among all the NP-degrading bacterial isolates, sphingomonads show the highest degradation capacity, using NP and OP as substrate for carbon and energy source; other minor bacteria co-metabolize the surfactants by microbial metabolic reactions (Corvini et al. 2006; Ying 2006). Considering these statements, Corvini et al. (2006) suggest the use of a combination of bacteria and fungii to remove NP from sludge or sediment, since the former assimilate the aromatic ring of the compound, and the latter attack the nonyl chain.

Contrarily to NP, BPA shows higher degradation rates under aerobic conditions, with an estimated half-life in soil or sediment between 3 and 37.5 days, although no degradation is observed in anaerobic soils within 70 days of experiment or in estuarine sediments during 120 days of experiment (Careghini et al. 2015). Degradation of BPA can undergo complicated metabolic routes that lead to the formation of different byproducts, though never reaching completely mineralization (Careghini et al. 2015).

Another way of BPA degradation is through photolysis, and advanced oxidation based on H_2O_2, UV light or ozone treatment have been proven to efficiently remove BPA from WWTPs (Im and Löffler 2016). Moreover, many studies have also shown that manganese oxides can play a significant role in BPA degradation. As a strong oxidant, MnO_2 serves as a mineral phase reactive toward many organic contaminants. Oxidative transformation of BPA by MnO_2 has been demonstrated under laboratory conditions, leading to the formation of 4-hydroxycumyl alcohol, which has much higher solubility and lower hydrophobicity (Lin et al. 2009a; Im et al. 2015).

Phenolic compounds sorption is enhanced in marine environments, as a consequence of the “salting-out effect”, which is due to the decrease in the aqueous solubility in the presence of salts, resulting in the compound being more attracted to the non-aqueous phase (Yang et al. 2016).

Sediment/water partition coefficients are also dependent on the concentration of the suspended particulate matter. Arditsoglou and Voutsa (2012) reported $\log K_d$ values generally decreasing with increasing suspended particulate matter, and this relation was mainly ascribed to the presence of dissolved

organic carbon in solution, which significantly increases solubility of organic compounds, affecting their partition into the solid phase.

2.2.2 Toxicology and health effects

Phenolic compounds have been recognized to have estrogenic activity on organisms, miming 17ß-estradiol activity. Soto et al. (1991) firstly reported that NP, employed in the manufacture of test tubes used in their experiments, induced proliferation in breast tumor cells, just as if estrogens were present in the experiment. NP and OP are also reported to have the ability of inducing the proliferation of vitellogenin in male fish. BPA is particularly harmful for fetus, infants, and young children (Careghini et al. 2015). The estrogenic potential of NP, OP and BPA relative to E2 has been estimated to be $6.3 \cdot 10^{-4}$, $9.3 \cdot 10^{-4}$ and $1.1 \cdot 10^{-4}$, respectively, thus suggesting weak estrogens mimic capacity (Zhao et al. 2011a).

The primary way of human exposure to phenolic compounds is through food consumption. Many authors have reported the migration of compounds from polymer packaging into food. Sun et al. (2006) reported BPA levels ranging from 32.8 to 164.5 µg/kg fw in canned food in Singapore, and Noonan et al. (2011) found <2-730 µg/kg fw of BPA in canned food from retail stores in Maryland, USA. Gyllenhammar et al. (2012) in Sweden detected BPA in fish (2.5-29 µg/kg fw), meat (6.9-13 µg/kg fw) and potatoes (2.2 µg/kg fw).

Based on BPA concentrations in food and food consumption, a daily dietary intake of BPA of 0.02-0.081 µg/kg bw/day for adults and 0.22-0.33 µg/kg bw/day for infants was estimated (Careghini et al. 2015). The European Food Safety Authority in 2015 lowered the Tolerable Daily Intake (TDI), that is the concentration below which harmful effects on human being through ingestion of contaminated food should not occur, from 50 µg/kg bw/day to 4 µg/kg bw/day, stating that current BPA exposure does not represent a risk for human health (EFSA 2015).

Significant NP concentrations have been detected in food, as well. High NP concentrations in seafood and various edible marine species in Asia, Europe and North America were reported to range between 122 and 2380 µg/kg fw (Diehl et al 2012). NP was also detected in edible marine species from Tyrrhenian Sea, Italy (5-1220 µg/kg fw), and in meat/seafood from market in Beijing, China (<0.05-55.98 µg/kg fw) (Careghini et al. 2015). Based on concentration in food and the expected consumption rates, the average daily intake for NP has been estimated to vary between 0.067 and 0.370 µg/kg bw/d (Careghini et al. 2015).

NP is an estrogenic agonist and highly toxic to fish, aquatic invertebrates and plants. A TDI value of 5 µg/kg bw/d was proposed for NP by the Danish Institute of Safety and Toxicology (DEPA 2000).

2.2.3 Regulation

The persistence of alkylphenols and their harmful effects on organisms have been recognized by public authorities. European Union on 13 January 2016 posed a restriction of nonylphenol ethoxylates in textile products to be applied from 3 February 2021, limiting the use of NPEOs at concentrations below 0.01% of weight of the article in all textiles sold in the EU (EU 2016).

Directive 2013/39/EU also sets maximum allowable concentrations of both NP and OP in surface waters (EU 2013). In particular, an annual average concentration of 0.3 μg/L for NP and 0.1 μg/L (0.01 μg/L in surface waters other than inland waters) for OP, and maximum allowable concentration of 2 μg/L for NP are established by EU Directive.

On the other hand, BPA is not included in any environmental monitoring. Though, its potential toxicity and estrogenicity have led to the definition of some restrictions in its industrial use by public authorities. For example, in December 2016 EU banned the use of BPA in thermal paper by January 2020. BPA is classified in EU as a substance with toxic effects, and by March 2018 all manufacturers, importers or suppliers of BPA will need to clarify and label mixtures containing BPA, according to the European Chemical Agency (ECHA) decision. BPA has also been banned by infant bottles in EU since 2011; in addition, France has banned BPA in all food packaging, containers and utensils. EU limited the use of BPA in toys for children, allowing a maximum of 0.1 mg/L of BPA that can leach out of toys for children under three years. In 2016, ECHA proposed to lower this limit to 0.04 mg/L, and this restriction should come into force in 2018 (ECHA 2016).

Outside EU, the US Food Drug Administration (FDA) declared that BPA "is safe at the very low levels that occurs in some foods", and "the use of BPA in food packaging and containers is safe", so there is no need in restriction. Health Canada in 2012 stated the safety of the current BPA concentration levels; nevertheless, the use of BPA in baby bottles has been banned since March 2010 (PC/BPA Group 2017).

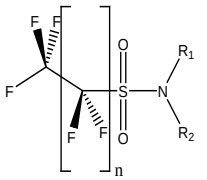
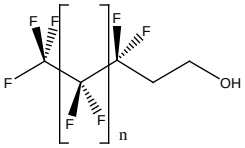
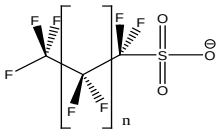
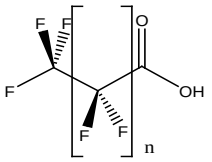
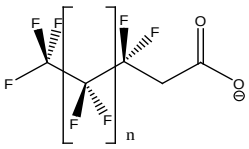
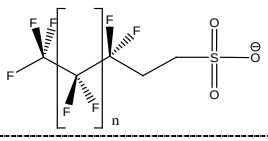
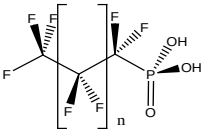
2.3 Perfluorinated compounds

2.3.1 Chemical properties, occurrence and behavior in the environment

Perfluorinated compounds (PFCs), or perfluoroalkyl substances (PFASs), are a group of chemicals made of a fully fluorinated hydrophobic carbon chain of different length, and a polar hydrophilic end group. Thanks to the strong C-F bonds that characterize these molecules, PFCs have extremely high thermal and chemical stability, and for these reasons they have been widely used in industrial and consumers products since the late 50s (Buck et al. 2011). In fact, they are widely used for the production of fire-fighting foams, paints, nonstick cookware, fiber textile, aircraft hydraulic liquids (Ahrens 2011; De Solla et al., 2012). Moreover, they have been broadly used as emulsifiers for the production of polytetrafluoroethylene (Teflon).

PFCs comprise a wide group of compounds varying in their structure, which gives them different properties, environmental behavior and toxicity. Though, they are all amphiphilic compounds and have in common a general high stability due to the strong C-F bond. The most common PFCs used in industry are summarized in **Table 1.6**.

Table 1.6 Chemical structure of perfluorinated compounds, divided by subgroups according to the functional group of their molecules (modified from Llorca 2012)

Class	Compound	Abbreviation	Formula	Chemical structure
Perfluorinated sulfonamides (PFASAs)	*N*-ethyl perfluorobutane sulfonamidoethanol	NEtFBSE	$F(CF_2)_4SO_2N(CH_2CH_3)CH_2CH_2OH$	
	Perfluorooctane sulfonamide	PFOSA	$F(CF_2)_8SO_2NH_2$	
	N-ethyl perfluorooctane sulfonamide	NEtFOSA	$F(CF_2)_8SO_2N(CH_2CH_3)$	
	N-ethyl perfluorooctane sulfonamidoethanol	NEtFOSE	$F(CF_2)_8SO_2N(CH_2CH_3)CH_2CH_2OH$	
Fluorotelomer Alcohols (FTOHs)	4:2 fluorotelomer alcohol	4:2 FTOH	$F(CF_2)_4CH_2CH_2OH$	
	6:2 fluorotelomer alcohol	6:2 FTOH	$F(CF_2)_6CH_2CH_2OH$	
	8:2 fluorotelomer alcohol	8:2 FTOH	$F(CF_2)_8CH_2CH_2OH$	
	10:2 fluorotelomer alcohol	10:2 FTOH	$F(CF_2)_{10}CH_2CH_2OH$	
	12:2 fluorotelomer alcohol	12:2 FTOH	$F(CF_2)_{12}CH_2CH_2OH$	
Perfluoro-sulfonates (PFSAs)	Perfluorobutane sulfonate	PFBS	$F(CF_2)_4SO_3^-$	
	Perfluorohexane sulfonate	PFHxS	$F(CF_2)_6SO_3^-$	
	Perfluorooctane sulfonate	PFOS	$F(CF_2)_8SO_3^-$	
	Perfluorodecane sulfonate	PFDS	$F(CF_2)_{10}SO_3^-$	
Perfluoro-carboxylic acids (PFCAs)	Perfluorobutanoic acid	PFBA	$F(CF_2)_4COOH$	
	Perfluoropentanoic acid	PFPeA	$F(CF_2)_5COOH$	
	Perfluorohexanoic acid	PFHxA	$F(CF_2)_6COOH$	
	Perfluoroheptanoic acid	PFHpA	$F(CF_2)_7COOH$	
	Perfluorooctanoic acid	PFOA	$F(CF_2)_8COOH$	
	Perfluorononanoic acid	PFNA	$F(CF_2)_9COOH$	
	Perfluorodecanoic acid	PFDA	$F(CF_2)_{10}COOH$	
	Perfluoroundecanoic acid	PFUdA	$F(CF_2)_{11}COOH$	
	Perfluorododecanoic acid	PFDoA	$F(CF_2)_{12}COOH$	
Fluorotelomer carboxylates (FTCAs, FTUCAs)	6:2 fluorotelomer carboxylate	6:2 FTCA	$F(CF_2)_6CH_2CO_2^-$	
	6:2 fluorotelomer unsatured carboxylate	6:2 FTUCA	$F(CF_2)_6CHCO_2^-$	
	8:2 fluorotelomer carboxylate	8:2 FTCA	$F(CF_2)_8CH_2CO_2^-$	
	8:2 fluorotelomer unsatured carboxylate	8:2 FTUCA	$F(CF_2)_8CHCO_2^-$	
	10:2 fluorotelomer carboxylate	10:2 FTCA	$F(CF_2)_{10}CH_2CO_2^-$	
	10:2 fluorotelomer unsatured carboxylate	10:2 FTUCA	$F(CF_2)_{10}CHCO_2^-$	
Fluorotelomer sulfonates (FTSs)	6:2 fluorotelomer sulfonate	6:2 FTS	$F(CF_2)_6CH_2CH_2SO_3^-$	
	8:2 fluorotelomer sulfonate	8:2 FTS	$F(CF_2)_8CH_2CH_2SO_3^-$	
	10:2 fluorotelomer sulfonate	10:2 FTS	$F(CF_2)_{10}CH_2CH_2SO_3^-$	
Perfluoro-phosphonic acids (PFPAs)	Perfluorohexaphosphonic acid	PFHxPA	$F(CF_2)_6PO_3H_2$	
	Perfluorooctaphosphonic acid	PFOPA	$F(CF_2)_8PO_3H_2$	
	Perfluorodecaphosphonic acid	PFDPA	$F(CF_2)_{10}PO_3H_2$	

Among all the PFCs, the two most commonly detected compounds in the environment are the perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). The two compounds have been widely produced by Dupont and 3M Companies since 1940. However, in 2002 the 3M Company decided to voluntarily stop the production of PFOS, because of the high levels encountered in human serum of exposed workers (3M 2003). In 2009 PFOS was included in Annex B of the Stockholm Convention as a Persistent Organic Pollutant (POP), and the 2010/2015 Stewardship Program of the US EPA established a complete elimination of PFOA production. Currently, the production of these persistent compounds has been substituted by short-chain ($<C_8$ chain) PFCAs and PFSAs, which have higher solubility and lower bioaccumulation potential (Onghena et al. 2012), while the production of fluorotelomers, which are PFOA and PFOS precursors, is still in use. Therefore, in spite of the phase out of PFOA and PFOS, the combined effects of chemical persistence and degradation of their precursors into the more recalcitrant forms, still causes the detection of these contaminants in the environment.

Due to the variety of this family of compounds, this PhD thesis will be addressed only to the study of PFCAs, PFSAs and PFASAs classes, with a major focus on PFOA and PFOS, since they are the compounds of most concern for environmental safety.

PFOA, as representative of the PFCA class, is highly soluble in water, with a solubility of 3400 mg/L. It is an organic acid (pk_a of 2.5; US EPA 2005), which means that in most environmental conditions it is present in its free anionic form. In aqueous solution, individual molecules of PFOA anion tend to associate on the surface water and partition between the air/water interface; as a surfactant, they can form micelles and hemi-micelles at high concentrations (Llorca 2012).

PFOS, and PFSAs in general, have a lower solubility in comparison to the carboxylic acids (370 mg/L, OECD 2002). A pK_a of 3.3 has been estimated for this compound (EFSA 2008). Its anionic form can form strong ion pairs with cations in solution, with a resulting salting-out effect in waters which is higher with greater dissolved solids content. Consistently, PFOS solubility in seawater is much lower, of 12.4 mg/L (Llorca 2012).

Generally, solubility of both PFCAs and PFSAs tends to decrease with molecular weight, due to the progressively longer carbon chain length, which is hydrophobic (ESERI 2016).

Assessment of the octanol-water partition coefficient (K_{ow}) for PFCs, predictable of organic compounds capacity to bioaccumulate, is very complex, due to PFCs surfactant properties that lead to the formation of three different layers during partition between octanol and water (Giesy et al. 2010). However, $logK_{ow}$ is not suitable for predicting their bioaccumulation properties, since PFCs are more commonly bound to proteins rather than to lipids (Haukas et al. 2015).

PFCs are released into the environment during manufacturing processes, product use, and disposal of various industrial and consumer products (Ahrens 2011). In addition, once they enter the environment, they can be secondarily released, for example from ice smelting, precipitations, or sediment resuspension (Ahrens 2011). Thus, it is very important to understand their fate in the environment.

The primary affected compartment is the aquatic one, which can be interested by PFCs contamination through both point- and non-point sources, such as municipal and industrial wastewater treatment plants, landfill leachate, dry or wet atmospheric deposition, and soil runoff. Wastewater treatment plants are not always efficient in the complete removal of contaminants, which are thus released into the receiving water bodies as parent compounds or only partially degraded (Sanchez-Avila 2010; Lam et al., 2016). In addition, different studies reported higher concentrations of PFCs in effluents than in influents of WWTPs, as a result of the incomplete degradation of their precursors, such as polyfluoroalkyl phosphates and fluorotelomer alcohols, which can hence represent an additional source of introduction (Guo et al. 2010; Loos et al. 2010). Moreover, PFCs have been found also in the atmosphere and in rainwater, indicating that contamination can reach areas far from the point-source of emission (Liu et al. 2017b).

PFOA and PFOS are considered to be recalcitrant to degradation. Under aerobic conditions with activated sludge, no decomposition is observed; slightly higher rates of removal are more likely to occur under anaerobic conditions (Parsons 2008). On the contrary, their precursors, such as fluorotelomers, are more easily biodegraded and can be transformed into PFCAs and PFSAs under natural conditions. Regarding chemical transformation, oxidation processes or photolysis are not efficient in PFC breakdown. Molecular ozone and hydroxyl radicals, in fact, as well as chlorination treatments, have been found to be quite ineffective for the removal of these compounds from contaminated water (Rahman et al. 2014). Eschauzier et al. (2012) observed that only the use of Granular Activated Carbon (GAC) filters in drinking water treatment processes were effective for PFC removal, totally removing PFNA, PFOS and PFHxS, though they only partially eliminated PFOA (about 50% of its initial conditions), and were quite ineffective for short chain PFCAs. Du et al. (2014) confirmed the efficiency of GAC in PFCs removal, thanks to their non-polar surfaces with few functional groups, which make them suitable for removing hydrophobic pollutants. A even higher efficiency is performed by Powdered Activated Carbons (PAC), thanks to their smaller particle sizes resulting in higher surface area given the same volume of carbon, shorter internal diffusion distances, and additional available surface functional groups that increase the adsorption capacity of hydrophobic contaminants. PAC filters are more appropriate for a short-term removal of PFCs (e.g. in case of spills), while GAC filters can be suitable for long-term water treatment (Rahman et al. 2014). A similar removal efficiency is displayed by membrane filtration. Nevertheless, even if these treatment processes are efficient in the removal of perfluorinated contamination, great concern is related to the disposal of the saturated

filters or sludge. Burning them into incinerators or depositing in landfill sites, in fact, could represent a secondary source of emission (Ahrens 2011).

Due to their high resistance to biological and chemical degradation, PFCs have been detected in all environmental compartments, from surface waters, to groundwaters, sediment/soils, and even in remote areas (Llorca et al. 2012a). For detailed information about their occurrence in various environmental compartments, please refer to *Chapters 3*, *4* and *5* of this thesis, as they will be discussed later on.

PFCs behavior once released into the environment mainly depends on their perfluorocarbon chain length, which gives them hydrophobic characteristics, and on their functional group. On average, PFSAs are more strongly sorbed on sediment than do PFCAs (Higgins and Luthy 2006). Many studies pointed out that the main environmental parameter influencing transport of contaminants and sorption on soil or sediment is organic carbon content (Higgins and Luthy 2006; Ahrens et al. 2011), where sorption is based on a balance among attraction forces between the hydrophobic "tail" of PFCs and hydrophobic parts of organic matter in soil, and repulsion forces between the anionic PFCs and the negatively charged carboxylic groups present in soil organic matter. A common parameter used to estimate sorption of PFCs is the PFC- specific solid/water partition coefficient (K_d) and its corresponding value normalized to organic carbon content (K_{oc}). In **Table 1.7** the K_d and K_{oc} values (in their logarithmic form) taken from literature partitioning studies are reported.

Table 1.7 LogK_d and logK_{oc} values of the main PFCs detected in various studies

Compound	logK_d	logK_{oc}	References	Compound	logK_d	logK_{oc}	References
PFHxA	0.4	0.8	Ullberg 2015	**PFUdA**	3.8	4.3	Ullberg 2015
	0.8	-	Ahrens et al. 2015		-	3.47	Higgins and Luthy 2006
	0.8	0.8	Labadie and Chevreuil 2011	**PFHxS**	1.6	2	Ullberg 2015
PFHpA	0.9	1.2	Ullberg 2015		1.3	-	Ahrens et al. 2015
	1.97	3.4	Campo et al. 2016		1.68	3.37	Campo et al. 2016
PFOA	1.4	1.8	Ullberg 2015	**PFOS**	3.6	4.1	Ullberg 2015
	1.2	-	Ahrens et al. 2015		2.3	-	Ahrens et al. 2015
	3.36	5.03	Campo et al. 2016		2.45	4.21	Campo et al. 2016
	-	2.11	Higgins and Luthy 2006		2.4	3.7	Labadie and Chevreuil 2011
PFNA	2.2	2.7	Ullberg 2015			2.68	Higgins and Luthy 2006
	1.5	2.9	Labadie and Chevreuil 2011	**PFOSA**	4.1	4.6	Ullberg 2015
	-	2.5	Higgins and Luthy 2006		3.1	-	Ahrens et al. 2015
PFDA	3.5	4	Ullberg 2015	**PFUdA**	3.8	4.3	Ullberg 2015
	2.4	3.8	Labadie and Chevreuil 2011		-	3.47	Higgins and Luthy 2006
	-	2.92	Higgins and Luthy 2006				

Generally, longer chained PFCs (>C_8) adsorb more strongly to organic matter, with a linear relation between logK_{oc} and the perfluoroalkyl chain length, and different laboratory studies found that the soil/water

partition coefficient (K_d) increased linearly with increasing organic matter (Labadie and Chevreuil 2011; Milinovic et al. 2015).

However, due to the fact that all PFCAs, PFSAs, PFASAs and some precursors are strong or weak acids, as a consequence of the low pK_a values, they mostly exist as anions in natural waters. Therefore, surface sorption to charged mineral surfaces may be an additional significant mechanism controlling their behavior in the environment (ESERI 2016). The presence of cations and anions in water has been proven to influence PFC sorption. In their studies, Higgins and Luthy (2006) demonstrated that Na^+ in soil had little or no effect on PFC sorption, while Ca^{2+} enhanced sorption. The authors concluded that changes in sorption are not likely to be related to a difference in ionic strength, as the experiment was performed with similar ranges in ionic strength. On the contrary, they suggested that Ca^{2+} increase in soil or sediment has the effect of reducing the negative charge present on organic matter surface. This finding was confirmed by the study of Chen et al. (2009), which explained that the effect of Ca^{2+} on PFC sorption was to actually neutralize the negative surface charges through interactions of Ca^{2+} with the organic carbon in sediment and the anionic PFOS. Johnson et al. (2007) investigated PFOS sorption on different materials, and found that sorption followed the order Ottawa sand standard > high iron sand > kaolinite > goethite, suggesting that electrostatic attraction of PFOS to surfaces may be important in sediments with low organic carbon concentrations. In his study, Ullberg (2015) found an influence of cation concentrations on sorption of PFCAs (C_5-C_8) and PFHxS, with greater differences among cations as pH increased, following the order Al>Ca>Na. This result was explained by the fact that trivalent ions have higher capacity to neutralize the surface charge of organic matter than divalent ions; for this reason sorption was enhanced in the presence of Al^{3+} in sediments, rather than Ca^{2+}, even if at lower concentrations. Moreover, for most PFCAs (C_5-C_{13}) and PFOSA there was a general trend of decreasing $\log K_d$ with increasing pH, whereas for PFSAs no clear correlation was found. This was mainly due to a change in protonation/deprotonation of the soil sorbate as the pH changed, so that with the increase of pH, adsorbent surfaces become more negatively charged, leading to stronger electrostatic repulsion, and hence lower sorption. This effect was observed as a trend, regardless of perfluorocarbon chain length (Ullberg 2015).

The pH influence on PFCs sorption was confirmed by the study of Ferrey et al. (2012), which highlighted that at neutral pH, sorption of PFOA and PFOS was controlled by electrostatic attraction on ferric oxide minerals (goethite, magnetite, or Fe cations), rather than by sorption to organic carbon. This was owed to the net positive charge of aluminum hydroxides and iron oxides/hydroxides at pH values less than their Point of Zero Charge value. Being present in their anionic form, PFOA and PFOS would be sorbed to sediment particle surfaces to a lesser degree as sediment pH increases.

2.3.2 Bioaccumulation, toxicology and health effects

PFCs behavior in biological organisms is very similar to their behavior in soil/sediment, since bioconcentration and bioaccumulation strongly depend on the carbon chain length, with PFSAs more bioaccumulative than PFCAs, given the same carbon chain length (Conder et al. 2008). **Table 1.8** provides a summary of bioconcentration factors (BCF), calculated as the ratio between PFCs concentration in the organism and in water, and the biomagnification factors (BMF), obtained from the ratio of PFC concentration in the predator and in prey.

Table 1.8 Bioconcentration (BCF) and biomagnification factors (BMF) of perfluorinated compounds taken from literature

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFHxS	PFOS	PFOSA	Reference
Bioconcentration factors (BCF)										
Phytoplankton	-	-	-	1449	-	-	-	196	-	Lam et al. 2014
Zooplankton	-	-	-	-	-	-	-	3017-3450	-	
Carassius auratus (liver)	-	-	134	150	5957	1877	8	4572	-	
Carassius auratus (blood)	125	-	611	4686	34896	17328	342	11167	-	
Siniperca scherzeri (liver)	-	-	601	-	7238	-	-	24718	-	
Siniperca scherzeri (blood)	-	-	739	855	89216	-	-	73612	-	
Oreochrommic niloticus	-	-	1826	-	22952	142764	-	3551	-	Lam et al. 2017
Chana striata	-	-	-	-	-	-	-	894	-	
Eleotris fusca	-	-	-	138	1627	-	282	253	-	
Pangasius elongatus	-	-	293	-	-	-	131	-	-	
Esomus danricus	-	-	-	1093	-	-	-	1477	-	
Varuna litterata	-	1523	-	-	-	-	-	-	-	
Macrobranchium rosengbergii	-	-	12	100	102	-	111	156	-	
Pomacea canaliculata	-	-	-	64	-	-	1606	1117	-	
Corbicula fluminea	-	268	187	-	256	-	2781	2110	-	
Biomagnification factors (BMF)										
Dolphin/fish	-	-	1.3-2.6	-	-	-	-	7.7-63	-	Quinete et al. 2009
River dolphin/fish	-	-			4.4–9.9	9.3–2.5	-	12-33.7	-	Yeung et al. 2009
River dolphin/shrimp	-	-			15.1	13.8	-	113	-	
Dolphin/seatrout$_{SC*}$	-	-	1.8	2.1	2.4	2.5	3.3	0.9	1.3	Houde et al. 2006
Dolphin/seatrout$_{FL**}$	-	-					-	6.2	5.2	
Dolphin/striped mullet$_{SC*}$	-	-	13	5	2.9	1.9	4	2.6	8.3	
Dolphin/striped mullet$_{FL**}$	-	-	-	-	-	-	0.1	9.6	5.2	
Dolphin/sheephead$_{FL**}$	-	-	-	-	-	-	-	16	5.2	

SC*: South Carolina; FL**: Florida

As can be seen from the table, BCF can be very variable, depending mainly on the species considered. Fish are good indicator of the bioconcentration potential of PFCs, since they show high BCF values. Besides being species-specific, bioconcentration is highly related to the type of tissue analysed, as well, as in the

case of the study reported by Lam et al. (2014), which recorded higher concentrations in the blood than in liver. Overall, PFOS is generally the most detected compound among all the PFCs; among carboxylic acids, the bioconcentration potential is higher for the longer chain compounds, while PFHxA and PFHpA show lower BCF values. PFCs have been found to biomagnify through the food chain, as well, since predatory animals have been recorded with higher PFCs concentrations compared to their preys (BMF > 1).

Contrarily to the majority of organic contaminants, PFCs do not show to bioaccumulate in lipids, but have a higher affinity with proteins. The highest concentrations are usually detected in blood, liver, and kidney (Jensen and Leffer, 2008; Stahl et al. 2012; Llorca et al. 2016). In general, PFOA levels are lower than PFOS in blood, which in turn shows concentration ranges from sub-ppb to hundreds ppb levels (Ericson et al. 2007; Olsen et al. 2017). Even though concentration in muscle tissue is generally lower, it could be of great concern since it represents the main way of exposure to humans by consumption of contaminated fish and meat (Berger et al. 2009; Zhao et al. 2011b).

Exposure of PFCs is mostly related to ingestion of contaminated food and water. Consumption of fish, milk, meat and vegetables are considered the primary entrance of PFCs to human body (Llorca 2012). Wang et al. (2008) observed the presence of PFOS in all the analysed eggs, at concentrations from 45 to 87 ng/g ww. PFCs were also found in edible fish of Lake Maggiore (North Italy), at concentrations up to 46 ng/g ww for PFOS, which was the most abundant one (Squadrone et al. 2014). A Norwegian study conducted to assess human exposure to PFCs through diet revealed that the most abundant PFCs in food samples were PFOA, PFOS and PFHxS, with a median total intake of 5.6, 11 and 0.78 ng/day, respectively (Papadopoulou et al. 2017). Furthermore, since PFCs have been found also in air and dusts, organisms can be exposed to PFCs also by breathing air and contact with dusts (ATSDR 2015).

Infant exposure to PFCs is critical, since PFOA and PFOS can cross the placenta, being detected also in cord blood (Llorca et al. 2012b). Moreover, they are also detected in breast milk with concentrations of 28 - 865 ng/L for PFOS and 15 - 907 ng/L for PFOA (Llorca et al. 2010; Stahl et al. 2011).

PFOA and PFOS are hardly eliminated by human body. Studies reported a half-life in human body of 9 years for PFOS and 4 years for PFOA (Public Health England 2009). In general, PFCs blood half-lives are longer for sulfonates than for carboxylates and are dependent on the carbon chain length; moreover, they have been found to be dependent on species and gender, having shorter lifetime in females than in males (ESERI 2016).

Toxicity data about PFCs in humans are very limited. Nevertheless, an epidemiological study conducted on a cohort of mid-Ohio valley residents exposed to contaminated drinking water or who worked at the local fluorochemical plant discovered that exposure of PFOA was associated with kidney and testicular cancer (Barry et al. 2013). Other epidemiological studies suggested a link between PFCs blood serum levels and

low birth weight, infertility, early menopause in women, low semen quality of young men and thyroid disease in the US adult population (Rahman et al. 2014 and references therein). Another study found a positive association between thyroid-stimulating hormone levels in maternal blood with PFHxS and PFOS, and with PFNA in the blood of boys < 11 years (Ballesteros et al. 2017).

PFOS has been recognized to be more toxic than PFOA (DEPA 2013) and this is reflected in the decision of EFSA to set 150 ng/kg bw/d as threshold value for PFOS in food, and 1500 ng/kg bw/d for PFOA (EFSA 2008).

2.3.3 Regulation

Thanks to the growing awareness of PFC harmful effects on human body and the environment, PFOS has been listed as Persistent Organic Pollutant and included in the Stockholm Convention. Directive 2013/39/EU set Environmental Quality Standards (EQS) for the presence of PFOS in the environment, establishing an average annual concentration of 0.65 ng/L in surface water, with a maximum allowable concentration of 36 µg/L, considered as safe for the aquatic organisms, and a maximum allowable concentration of 9.1 µg/kg ww detectable in fish tissue (EU 2013). In adopting the European directive, the Italian government set an additional EQS for PFOA, which was not considered in the EU Directive, of 0.1 µg/L to be applied to surface waters from 2018 in order to achieve a good quality status in water bodies in 2027. US EPA (2016a) has included PFOA and PFOS in the fourth drinking water contaminant candidate list (CCL-4) of 96 compounds for further regulatory studies, and included other four PFCs (PFBS, PFHxS, PFHpA, PFNA) in the list of Unregulated Contaminants Monitoring Rule 3 (UCMR3), as unregulated contaminants to be monitored by public water systems (US EPA 2012). Moreover, provisional health advisory values were set at 200 ng/L (PFOS) and 400 ng/L (PFOA) in drinking water (US EPA 2009). Due to the ban of these two compounds, different industries are shifting in the production and substitution of PFOA and PFOS with the shorter- chain PFCs, which are thought to be less bioaccumulative and hence less toxic. Nevertheless, their toxicity and their effects on organisms are not well known yet.

2.4 Gadolinium

Gadolinium (Gd) in an inorganic chemical element with atomic number 64 belonging to the Rare Earth Elements (REE) class. Like all REEs, it occurs in natural environments in its trivalent form. It is associated in many mineral phases mainly as accessory element, substituting cations of similar size (e.g. Ca^{2+} in fluorides and phosphates). It is commonly found in gadolinite ($Be_2Fe(Y,Gd)_2Y_2Si_2O_{10}$), monazite ($(Ce,La,Nd,Gd,Th)PO_4$) and bastnaesite ($(Ce,La,Gd)CO_3(F,OH)$).

Gd has a very low mobility in the environment. If released as Gd^{3+} during weathering, it can be immobilised by sorption onto Fe-oxides (Reimann and Birke 2010). As a consequence, concentrations in surface water

are very low; Salminen et al. (2005) reported a median value for European surface waters of 0.01 ug/L, with concentrations higher in the Scandinavian peninsula than in Central and South Europe.

Since the late 90s, a widespread anthropogenic Gd anomaly has been reported in various river waters (Bau and Dulski 1996; Rabiet 2009; Kulaksiz and Bau 2013; Klave et al. 2014). These anomalous concentrations were related to the discharge of hospital sewages into the receiving water bodies. Gd, in fact, is used in various human products: in glass industry; for the production of magnets and garnets used in microwave applications; and in medical applications. Regarding medical application, it is common practice to use a highly stable form of Gd, the Gd-diethylenetriaminepentaacetate (Gd-DTPA) complex, as contrast agent in Magnetic Resonance Imaging (MRI) in hospitals. Thanks to the high stability of this complex, it is not metabolized by human body, and is excreted in few hours after administration. After excretion, the complex passes through the sewage system and persists even after wastewater treatments, finally reaching surface waters. According to Möller et al. (2000), considering that about 1 g of Gd-DTPA is administered per patient, at the natural concentrations of 10pmolal of Gd in water, in takes only 100 applications in hospitals to double the Gd level in 1 km^3 of surface water. Kummerer and Helmers (2000) estimated that 1 g of Gd results in the detection from 6 to 133 µg/L of the element in hospital sewages after only 2 hours.

Given its widespread anthropic use and consequent detection in surface waters, Gd has been considered in the last decades an emerging contaminant (Verlicchi et al. 2013; Tepe et al. 2014). The free form Gd(III) is highly toxic for animals, disturbing calcium homeostasis and affecting the nervous system and other Ca-related physiological processes (Migaszewski and Galuska 2015). Its chelated form (Gd-DTPA), however, does not cause adverse effects, since it is not metabolized by the organism. Therefore, the Gd-complex released in the river bodies is not considered to cause adverse effects on aquatic species and human health. However, many authors have pointed out the utility of Gd as a conservative tracer in hydrology studies, e.g. to investigate mixing of surface and groundwater or to follow infiltration of surface water, since it does not interact with mineral surfaces, colloids or organic matter (Möller et al. 2000; Williams et al. 2013). Furthermore, its release into the environment through the discharge of sewage waters makes it a good tracer of WWTP contamination. Several studies have reported detection of Gd anomalies downstream of the main cities and WWTPs (Loos et al. 2013a; Williams et al. 2013; Tepe et al. 2014; Barber et al. 2015). Considering that Gd and EDCs share similar sources of introduction in the environment, finding correlations between the two types of contaminants could be very useful to identify potential sources.

3 ANALYTICAL METHODS

The detection of organic contaminants at trace levels (ng/L – µg/L) requires sensitive and selective analytical methods able to obtain accurate identification, confirmation and quantification of these compounds. In the last decades many improvements have been reached for the detection of these contaminants, based on mass spectrometry (MS), coupled to liquid chromatography (LC) or gas chromatography (GC), depending on the target compounds (Chang et al. 2009; Agüera et al. 2013).

These analytical methods are very sensitive and robust; however, since EDCs mostly occur at very low concentrations in environmental matrices, sample pre-treatment, consisting of an extraction step, a clean up and a pre-concentration step, is needed to achieve better performance of the analytical method.

3.1 Sample pre-treatment

Sample pre-treatment is an important step in analytical chemistry, and is aimed to extract the target analytes from the matrix, enriching them in the extract and reducing matrix effect. Different extraction methods can be applied, according to the properties of the analytes of interest; the most common ones are liquid-liquid extraction (LLE), solid phase extraction (SPE) and solid phase microextraction (SPME).

3.1.1 Liquid-liquid extraction and Liquid-phase microextraction method

Liquid-liquid extraction (LLE) is a typical method for the analysis of organic solutes in water. The method consists of the extraction of a substance from one liquid (sample matrix) to another liquid phase, which is usually an immiscible organic solvent. It is based on the principle to separate compounds according to their solubilities in water and in the organic solvent. However, this extraction method is highly time-consuming and requires a large amount of solvents (150-200 mL) and water sample (500-1000 mL); moreover, an additional concentration step is then needed (Chang et al. 2009; Salgueiro-Gonzalez et al. 2017). Recently, LLE has been replaced by liquid-liquid microextraction (LLME) techniques that minimize these disadvantages. In contrast with LLE, LLME requires lower volume or organic sovents (<200 µL; Salgueiro-Gonzalez et al. 2017). Depending on the mechanism used to expose the extraction solvent to water samples, different LLME techniques can be distinguished. Single-drop microextraction (SDME) consist of a drop of extractant solvent exposed to the aqueous phase from a microsyringe needle. Authors have reported good recoveries using <4 µL of long alkyl chain alcohol (i.e. octanol and decanol) for the extraction of NP and BPA (Lopez-Darias et al. 2010; Jiang et al. 2015).

Dispersive liquid-liquid microextraction (DLLME) is another method increasingly used. DLLME is based on the extraction of analytes in water by an appropriate mixture of extraction solvent and dispersant agent producing a cloudy solution. While dispersant agent should be miscible with water (e.g. acetone,

acetonitrile, methanol), extraction solvent should be immiscible (e.g. chloroform or decanol) (Salgueiro-Gonzalez et al. 2017).

3.1.2 Solid-phase extraction method

Solid-Phase Extraction (SPE) method consists in using a solid phase as a selective sorbent to separate an analyte from the liquid sample by adsorption onto the sorbent surface. The solid-phase sorbent containing the analyte is then purified with a washing solution to remove other substances which can eventually be retained with the target analyte (clean-up step), and is then desorbed through elution with the specific organic solvent (extraction step). The solid phase sorbent is usually packed into small cartridges. Critical steps in SPE are the choice of the solid sorbent, as well as the washing and eluting solvents, according to the properties of target analytes (Chang et al. 2009).

In comparison with LLE technique, SPE allows the isolation and pre-concentration of target compounds in only one step, thus reducing the time of analysis.

For EDCs extraction, elution solvents mostly used are acetone, dichloromethane, methylbuthylether and methanol (Salgueiro-Gonzalez et al. 2017). Oasis HLB are one of the most used cartridges, thanks to their property to retain a large number of basic, neutral and acid compounds. These cartridges, hence, can be used for the simultaneous extraction of a broad group of both hydrophilic and hydrophobic analytes through Van der Waals interactions and hydrogen bonds with the sorbent surface, which is made of two monomers, namely the hydrophilic N-vinylpyrrolidine and the lipophilic divinylbenzene (Locatelli et al. 2016).

The main disadvantage of this type of extraction procedure is that a large amount of water sample is needed (250, 500, >1000 mL; Salgueiro-Gonzalez et al. 2017). In the recent years, the development of on-line methodologies has increased in order to overcome the time-consuming analysis and co-eluting interferences limits of the most common off-line SPE. The on-line SPE method is generally coupled to Liquid Chromatography (LC) instruments. In most of the published work, a polymeric pre-column (i.e. polystyrene divinylbenzene) is used to isolate the target compounds which are then eluted with LC mobile phases in the same step, general methanol or acetonitrile and water (Salgueiro-Gonzalez et al. 2017). Another possible on-line methodology is represented by the turbulent flow chromatography (TFC). Unlike SPE, which uses a laminar flow, the turbulent flow generates turbulence in the extraction column working at high flows (1.5-3.0 mL/min). Stationary phase columns of large porosity (>5 μm) allow the removal of high molecular weight molecules, such as proteins, whereas smaller molecules are trapped by the column pores (Llorca 2012). TFC column allows to separate the analytes of interest from their complex matrix, achieving an online clean-up step, minimizing the sample preparation and reducing the ion suppression due to higher specificity. Chromatographic separation is then achieved in a second analytical column. This dual column

technology is particularly suitable for the analysis of biological samples with high matrix effects, since it allows to achieve a better purification of samples. On-line methodology is reported to improve recoveries of analytes in complex samples (Gorga et al. 2014).

3.1.3 Solid-phase microextraction method

Solid-phase microextraction (SPME) method consists in the adsorption of analytes onto the surface of a coated silica fiber. Analytes are then desorbed at the injection port of the instrument, such as a Gas Chromatography (GC) instrument. The partitioning of an analyte between the aqueous sample and the stationary phase made of a thin silica fiber is the main principle of the SPME method. The amount of the analyte adsorbed onto the silica-coated fiber at equilibrium is directly related to its concentration in the sample. Sensitivity of the SPME method is based on the high affinity of the polymeric stationary phase and the organic analyte (Chang et al. 2009). Polydimethylsiloxane (PDMS) is the most common sorbent used in SPME for the extraction of compounds with $\log K_{ow} > 3\text{-}4$, but other polar phases are also used in order to improve extraction efficiency (Salgueiro-Gonzalez et al. 2017). SPME can also be coupled to LC instrumentation, but it usually shows lower efficiency in comparison with other techniques such as SPE because of the small amount of coating available (Salgueiro-Gonzalez et al. 2017).

3.2 Instrumental analysis

In the last decades, an increasing trend in the use of LC-MS/MS techniques for the detection of EDCs in environmental samples has been shown (Yeung et al. 2009; Stahl et al. 2012; Loos et al. 2013a; Gorga et al. 2014). LC-MS/MS instrumentation has been chosen over the GC- based techniques because of its higher versatility, as it overcomes the need for derivatization steps, highly improving the time of analyses (Omar et al. 2016).

There are several different types of mass analysers, depending on ion movement or storage. Those ones based on ion transport are quadrupoles (Q), time of flight (TOF) and their hybrid combinations. The analysers based on ion storage are ion traps (IT) and Fourier-transform ion cyclotron resonance (FT-ICR). The most commonly mass spectrometers used in EDCs analysis are triple quadrupoles (QqQ), due to their versatility, sensibility and robustness (Agüera et al. 2013). Quadrupoles work as a mass filter and are based on transport ions.

A QqQ consists of two quadrupole mass analysers in series (Q1 and Q3), with a non-mass resolving quadrupole between them (q2) that acts as a cell for collision-induced dissociation (**Figure 1.2**).

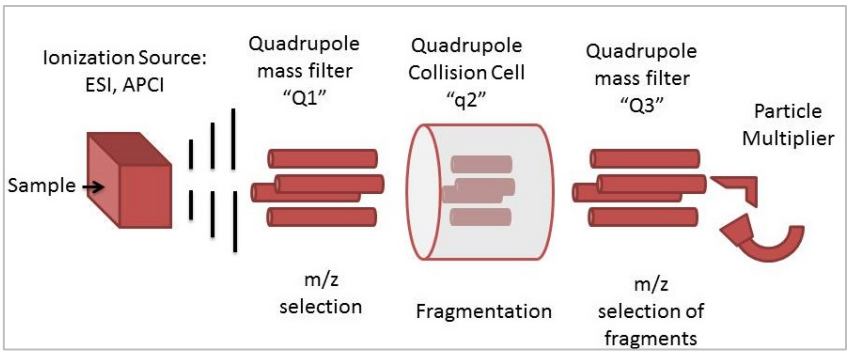


Figure 1.2 Schematic representation of a triple quadrupole mass spectrometer

The two mass filters Q1 and Q3 are made of four parallel, cylindrical metal rods. Both Q1 and Q3 are controlled by direct current (dc) and radio-frequency (rf) potentials, while the collision cell q2 is only subjected to rf potentials, that allow only ions that were selected for to pass through it.

For a successful detection of compounds, it is necessary to convert neutral compounds into molecular ions or ionized fragments in the gaseous state in the ion source, primarily that ion molecules reach the Q1. Several ionization methods can be employed, such as electrospray ionization (ESI), chemical ionization (CI), electron ionization (EI), atmospheric pressure chemical ionization (APCI) and matrix-based assisted laser desorption ionization (MALDI), all of which produce a continuous supply of ions. The most commonly interface between the LC and MS for the EDCs selected for the study is ESI, used both in negative or positive ionization modes. QqQ have excellent performance for quantitative analysis working in the selected reaction monitoring (SRM) mode, which allows the selection of two specific transitions for each compound, with subsequent accurate confirmation of the analyte in the sample (Agüera et al. 2013). The Q1 is the primary mass-to-charge-ratio (m/z) selector after the sample leaves the ionization source. Any ion with m/z greater than the one selected will not be allowed to enter Q1. Collision cell fragmentation of parent compounds occurs in q2 in the presence of an inert gas like Ar, He or N_2. A daughter ion is produced as a result of the collision of the inert gas with the analyte. After exiting the collision cell, the fragmented ions travel onto the Q3, when m/z selection on daughter ions occurs again.

3.2.1 Optimization of LC and MS parameters

Optimization of LC and MS/MS parameters is an important step in the development of an accurate and sensitive analytical method.

LC parameters, such as mobile phase and chromatography column for compounds separation, and MS parameters such as ion source temperature, collision energy, desolvation temperature, and source temperature need to be optimized to obtain good peak separation and optimum peak intensity.

Common mobile phases include any miscible combination of water with various organic solvents. Acids, salts or buffers (e.g. formic acid, trifluoroacetic acid, ammonium hydroxide, ammonium acetate) can be added to the mobile phases as ionization additives to increase the peak intensity of compounds in MS detectors (Sodré et al. 2010). However, the use of chemical additives needs to be evaluated according to the analytes properties and modes of operations of MS. Several authors, for example, reported a variation in the peak intensity working in positive and negative mode, using the same chemical additive (Omar et al. 2016, and references therein).

Collision energy is also another important parameter to be optimized in order to achieve better detection of compounds. The amount of energy applied to the collision cell influences the formation of fragmentation ions, resulting in higher peak intensity (Omar et al. 2016).

3.3 Sample storage, sample contamination and interferences

A wide variety of laboratory materials can contain fluoropolymers or other plastic materials which can affect perfluorinated compounds and alkylphenols detection; hence, the use of these materials should be avoided. When possible, glass materials should be preferred; polypropilene (PP) or polyethylene (PE) bottles can be used, as well (Achene et al. 2011a; Llorca 2012) for liquid sampling. For solid samples, foil containers are commonly employed (Llorca 2012). Prior to use, containers should be rinsed with methanol, in order to remove any trace of contaminants. Samples should be stored in darkness at 1-10 °C until analyses, which need to be performed within 15 days (Achene et al. 2011a). Another possibility to store samples is to pass the sample through a SPE cartridge and store it at -18 °C before the elution step (Locatelli et al. 2016).

Analytical instrumentation can be another important source of contamination: contamination of mobile phases or system tubing, injection and degasser valves can lead to false-positive detection of chemicals such as PFCs and alkylphenols. To avoid this, an instrumental blank made of pure mobile phases should be analysed, and eventual concentrations of chemicals should be subtracted. Procedural blanks should be analysed, as well, and should be prepared in parallel to samples treatment and extraction, in order to rule out any type of contamination during the extraction procedure.

Use of high purity standards, as well as high purity solvents, is required to avoid any further interference with the environmental samples.

Detailed and more specific information about the extraction methods, analytical instruments and method optimization used in this PhD thesis are provided in *Chapters 3, 4* and *5*.

4 OBJECTIVES OF THIS PhD THESIS

The global aim of this PhD thesis was to study Contaminants of Emerging Concern, and more specifically Endocrine Disrupting Compounds, in order to assess their **occurrence**, **behavior** and **fate** in natural freshwater and saltwater environments, focusing on different environmental compartments.

To this purpose, the specific objectives were:

1. To analyse EDCs occurrence in the aquatic environment of freshwater and saltwater systems, identifying possible sources of contamination. To this purpose, a major focus was addressed on the relation between EDCs and Gd in freshwaters of the Romagna area, using Gd as tracer of WWTP contamination.

2. To analyse EDCs partitioning in water and sediment of both freshwater and saltwater compartments, in order to understand their behavior in natural environments and identify which are the main factors that mostly control EDCs distribution in the different compartments.

3. To assess PFCs bioconcentration in both freshwater and seawater fish species.

4. To finally assess EDCs behavior, fate and migration in the different environmental compartments.

Chapter 2

GEOCHEMICAL CHARACTERIZATION AND RARE EARTH ELEMENTS ANOMALIES IN SURFACE- AND GROUNDWATERS OF THE ROMAGNA AREA (Italy)

1 INTRODUCTION

Rare Earth Elements (REE) are a group of elements that include lanthanides, from La (atomic number of 57) to Lu (atomic number of 71). This group of elements is characterized by an exclusively trivalent oxidation status (the only exception being Ce and Eu, that are redox sensitive) and a similar ionic radius, that decreases systematically with increasing atomic number (Liplin and McKay 1989). Thanks to these properties, they are characterized by a coherent behavior in natural waters, with little fractionation within siliciclastic material during weathering processes or transport as colloidal materials (Piper and Bau 2013). In the last decades they have been used as a powerful tool in geochemical studies to investigate the interaction processes between water bodies and rocks. A common practice in geochemistry is to represent REE concentrations in rivers normalized with rock reference standards, in order to identify possible anomalous behaviors of elements (Piper and Bau 2013). Recently, different studies have pointed out the presence of very anomalous concentrations of Gd in river waters related to anthropogenic activity (Bau and Dulski 1996; Knappe et al. 2005; Kulaksiz and Bau 2013). Gd, as a Gd-chelated substance, is widely used as contrast agent in diagnostic magnetic resonance imaging (MRI), thanks to its paramagnetic properties (Möller et al. 2000). The chelated forms of Gd are administered to patients in high doses (1.1-1.3 g per average adult, Aime and Caravan 2010) and are rapidly excreted through urines. Once released into wastewater effluents, Gd chelates can easily reach river waters because of their high resistance to degradation and of the inefficiency of the most common treatment plants to remove these microcontaminants from effluents (Lawrence 2011).

In this work rivers and groundwaters of the Romagna area were investigated in order to assess the degree of contamination by REEs. In addition, samples taken at the entrance and exit of the most important drinking water treatment plants of the study area were analysed to assess the efficiency of the treatment plants in the removal of micropollutants from contaminated waters. Furthermore, an extensive analysis on trace elements was carried over for a complete geochemical characterization of the studied waters, useful to better assess water quality of the area.

2 MATERIALS AND METHODS

2.1 Study area

The study area is located in the eastern area of the Emilia-Romagna region, in Italy. From a physiographic point of view, the area can be subdivided in two portions: the south-western part, characterized by the Romagna Apennines Mountains, and the north-eastern one, dominated by the Adriatic coastal plain south of the Po delta, formed by alluvial deposits of both Po river and Apennines rivers provenance (Amorosi et al. 2002; 2008). About 1.12 million inhabitants live in the area and are mainly concentrated in the plain section and along the coastline. Major towns include Ravenna, at the closing section of Lamone and Fiumi Uniti river bodies; Forlì, at the end of the mountain course of Montone and Ronco rivers; Faenza, located at the end of the mountain course of the Lamone river and Rimini at the mouth of the Marecchia river (**Figure 2.1**). Main human activities are concentrated in the coastal plain, as well, and are based on crops and livestock farming, requiring a high demand of water supply. Industries are also located in proximity of the main cities; in Ravenna a petrochemistry pole is located very close to the harbor, with manufacturing of plastics and oil by-products as main activities. Summer tourism along the coastal area is a further pressure on drinking water consumption. Water-demanding agricultural activities and stockbreeding represent additional activities affecting water quantity and quality in inland areas.

The geological setting of the north-western part of the area (Lancianese and Dinelli 2015; 2016), in which Lamone and Fiumi Uniti river basins are settled, is mainly characterized by Miocene and Pliocene sedimentary rocks. The main rock outcrops belong to the *Marnoso-Arenacea Formation* (alternation of sandstones, marls and clays), the *Gessoso-Solfifera Formation* (gypsum evaporites) and *Argille Scagliose unit* (scaly clay deposits). In the Marecchia and Conca river basins are common clays and argillites, alternation of calcareous marlstones and limestones of the *Ligurian-Epiligurian dominion* and the *Umbro-Marchean Succession* (Marnoso-Arenacea Formation). In the plain section, the rivers flow within alluvial deposits of the same rivers over. The hydrogeological setting of the plain section involves a Quaternary succession made up of continental gravels and sands located at the Apennines foothills (Martinelli et al. 2014).

The rivers selected for this study (**Figure 2.1**) flow from the Apennines Mountains to the Adriatic Sea and are characterized by a torrential behavior mainly dependent on rainwater inputs. The Lamone river (530 km^2 basin extension, 8 m^3/s river flow in winter and 1.25 m^3/s in summer) is the northern river studied. It is characterized by poor water quality as a consequence of drought during summer periods and concurrent high farming water demand (ARPA-ER 2013a). The Fiumi Uniti system (1240 km^2 basin extension, 14 m^3/s river flow) spreads out into the Adriatic Sea, south of the Lamone river (**Figure 2.1**). It is composed of two rivers, Ronco and Montone, which converge in proximity of the city of Ravenna to form a unique river body.

The south-eastern portion of the area includes Marecchia and Conca rivers. The Marecchia river catchment covers 665 km^2, with a flow of 7-8 m^3/s; Conca river has an average discharge of 1.5 m^3/s and its catchment covers 173 km^2.

To better assess surface water quality of the Romagna area, CER canal was also taken into consideration. It is an artificial irrigation canal that branches off the Po river and brings its waters in the Romagna area, crossing all the study area. The CER canal is a relevant water resource: it supplies water to a highly urbanized area for farming activities and tourism, especially at increasing water demand in the summer season.

Groundwaters selected for this study come from those confined aquifers in the cities of Forlì, Cesena and Rimini used for drinking water supply. All aquifers can be classified as confined alluvial conoid deposits, belonging to the Quaternary succession. In addition, waters from two drinking water treatment plants (DWTPs) in the city of Ravenna (samples R6 and R8, **Figure 2.1**) receive surface waters from the Lamone river and CER canal. The Drinking Water Treatment Plant (DWTP) R8 has been recently built (in 2015) and uses more advanced water treatment techniques (such as ultrafiltration through 0.4 μm membranes and activated carbons membranes) in comparison to the other DWTP R6.

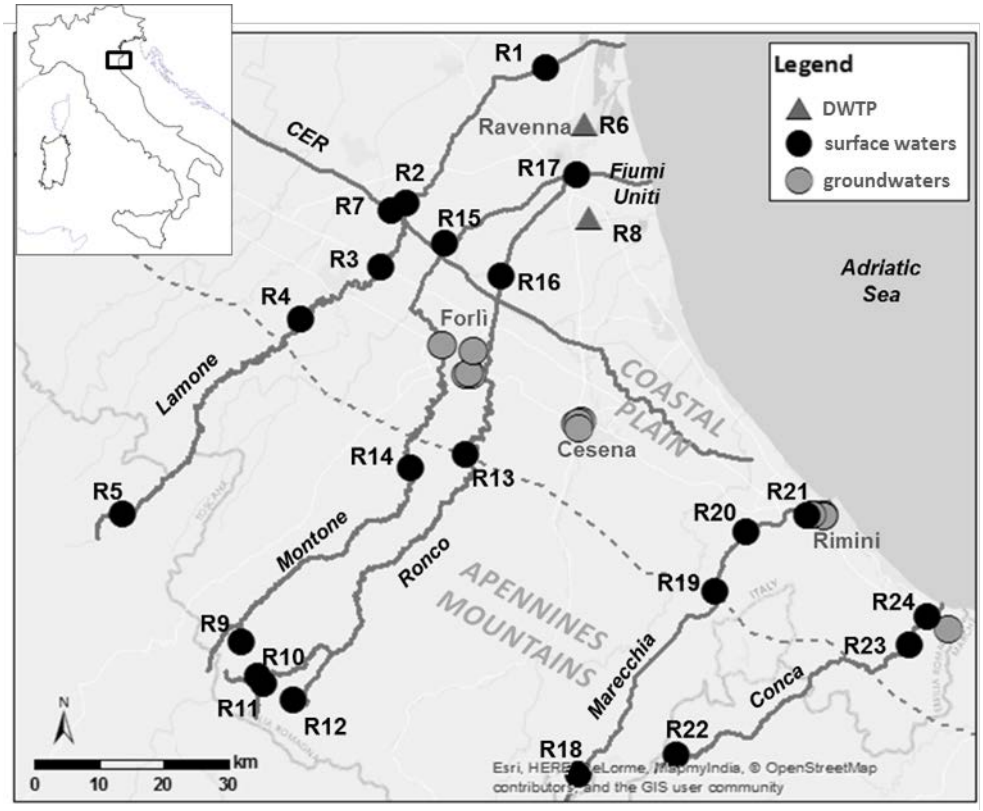


Figure 2.1 Surface waters, groundwaters and drinking water treatment plants sampling points

2.2 Sampling and chemical analyses

Surface water sampling points (**Figure 2.1**) were located along the main river channels in order to have a homogeneous distribution from the headwaters to the mouth. The presence of possible sources of contamination, such as hospitals and wastewater treatment plants (WWTPs), was also taken into consideration in the sampling plan. Only one sampling point was placed in the CER canal (sample R7) since it does not have exchanges with other river bodies during its flow, thus its chemical composition cannot be affected by external water inputs. Groundwaters were sampled at different depths (Forlì: 35-155 m; Cesena: 35-98 m; Rimini: 20-113 m) in those wells of confined aquifers used to produce drinking water in the cities of Forlì, Cesena and Rimini. Samples at the drinking water treatment plants were taken both at the entrance and at the exit of the plants. The sampling campaign took place in July 2015.

Samples were collected in 250 mL PE bottles after being filtered (0.45 µm) and acidified to pH 2.0 with ultrapure HNO_3. Temperature, pH, alkalinity and conductivity were measured immediately in field before collection. All chemical analyses were conducted at the Federal Institute for Geosciences and Natural Resources (BGR) in Hannover: major elements were quantified using a Spectro Ciros ICP-AES and a Dionex ICS 3000 IC instrument; trace elements (Ag, Al, As, B, Ba, Be, Cd, Co, Cr, Cs, Cu, Fe, Ga, Ge, Li, Mn, Mo, Ni, Pb, Rb, Sb, Se, Sr, Th, Ti, Tl, U, V, W, Y, Zn, Zr) and REEs by an Agilent 7500ce ICP-MS instrument. Details on the analytical instrumentation and methods can be found in Birke et al. (2010). To assess the analytical quality control, the certified reference material (CRM) for river waters SLRS-4 (National Research Council, Canada) was used. Measures of the reference material were carried over during sample analyses at regular intervals for a total amount of 173 measurements. **Table 2.1** provides a comparison among mean values and coefficient of variation of the analysed elements and SLRS-4 certified and published values. For the majority of the elements results are in agreement with the certified values, with little CV, mostly below 40%.

2.3 Data processing

Element concentrations which were below the detection limit were substituted with a value corresponding to half the detection limit. All variables were tested for normality applying the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was used to test equality of medians between surface and groundwaters. Boxplots were used for the analysis of the distribution of each variable. All statistical analyses were performed with R software. AquaChem software was used to create Piper diagram for water geochemical analysis; REE pattern in rivers and groundwaters was graphically analyzed with Microsoft Excel, while maps were created by ArcGIS 10.1 software.

Table 2.1 ICP-MS analytical results on the CRM SLRS-4 made during sample analyses (n=173) and comparison with the certified and published values of the reference material

	Mean (µg/L)	**SD** (µg/L)	**CV** (%)	**SLRS-4 certified value**[a]	**SLRS-4 published value**[b]
Ag	0.001	$3 \cdot 10^{-4}$	30.2	-	0.035
Al	56.6	4.9	8.6	54	53.5
As	0.70	0.04	6.4	0.68	0.68- 0.7
B	6.2	1.0	15.7	6	5.7- 6.3
Ba	13.6	0.7	5.4	12.2	12.2- 12.6
Be	0.008	0.002	19.8	0.007	0.007
Bi	0.004	0.002	42.5	-	0.002
Ca	5854	449	7.7	6200	5200- 6200
Cd	0.013	0.002	15.7	0.012	0.012
Ce	0.380	0.021	5.5	0.360	0.360
Co	0.038	0.005	13.8	0.033	0.033- 0.048
Cr	0.336	0.052	15.4	0.33	0.312
Cs	0.008	0.002	21.2	0.009	0.007- 0.009
Cu	1.95	0.15	7.9	1.81	1.86
Dy	0.025	0.002	8.3	0.024	0.024
Er	0.014	0.001	7.5	0.013	0.013
Eu	0.009	0.001	11.8	0.008	0.008
Fe	107.0	6.3	5.9	103	95- 117
Ga	0.015	0.005	36.2	-	0.012
Gd	0.038	0.004	11.0	0.034	0.034
Ho	0.005	0.000	8.8	0.004	0.004
K	610	57	9.3	680	597- 712
La	0.301	0.016	5.3	0.287	0.287
Li	0.55	0.09	15.8	0.54	0.54
Lu	0.002	0.000	12.6	0.0019	0.0019
Mg	1585	112	7.1	1600	1600- 1624
Mn	3.48	0.24	6.8	3.37	3.37
Mo	0.21	0.07	32.2	0.21	0.21
Na	2250	156	6.9	2400	2400- 2500
Nb	0.006	0.003	46.9	-	0.004
Nd	0.275	0.015	5.5	0.269	0.269
Ni	0.74	0.09	11.8	0.67	0.67- 0.82
Pb	0.09	0.01	10.6	0.086	0.084- 0.086
Pr	0.072	0.004	5.5	0.069	0.069
Rb	1.61	0.17	10.4	1.53	1.53
Sb	0.25	0.02	6.1	0.23	0.23-0.27
Se	0.11	0.06	51.3	0.23	0.23
Sm	0.061	0.005	7.5	0.057	0.057
Sr	28.4	2.0	6.9	26.3	26.3- 28.2
Ta	0.001	0.001	65.7	-	0.003
Tb	0.004	0.0001	7.9	0.004	0.004
Te	0.008	0.004	44.9	-	0.004
Th	0.017	0.005	29.9	0.018	0.019- 0.022
Ti	1.08	0.16	15.1	1.4	1.31- 1.56
Tl	0.011	0.006	55.3	0.008	0.008
Tm	0.002	0.0003	13.4	0.002	0.002
U	0.052	0.004	6.9	0.050	0.047- 0.053
V	0.37	0.07	18.1	0.32	0.32- 0.35
W	0.019	0.013	68.8	0.013	0.013
Y	0.143	0.008	5.6	0.146	0.146
Yb	0.013	0.001	9.7	0.012	0.012
Zn	1.22	0.25	20.7	0.93	0.93- 1.24
Zr	0.100	0.016	15.9	0.12	0.12

[a] *National Research Council Canada River Water Reference Material for Trace Metals;* [b] *http://georem.mpch-mainz.gwdg.de/*

2.4 Quantification of anomalies

It is common practice to normalize the REE concentrations against a reference standard in order to reduce the Oddo-Harkins Rule effects (dependent on the fact that even-numbered elements are more abundant than odd-numbered elements; Piper and Bau 2013), and plot them on a logarithmic scale. The reference standard used in this study is the Post-Archean Average Australian Shale (PAAS) (McLennan 1989).

Detection of anomalies is based on the presence of individual elements that are higher or lower than the corresponding shale-normalized patterns. Gd anomaly was quantified calculating a Gd local background concentration (Gd^*), which was obtained interpolating Sm and Tb concentrations (the neighbor REE elements of Gd). The formula suggested by Rabiet et al. (2009) was used:

$$Gd/Gd^* = Gd_N/(Sm_N^{0.33} \cdot Tb_N^{0.67})$$

where Gd^*: Gd local background concentration; Gd_N: shale-normalized Gd concentration; Sm_N: shale-normalized Sm concentration; Tb_N: shale-normalized Tb concentration.

The amount of anthropogenic Gd (Gd_{anthr}), expressed in ng/L, was then assessed (EU 2012):

$$Gd_{anthr} = \left[\frac{Gd_{anomaly}\text{-}1}{Gd_{anomaly}}\right] \cdot Gd_{measured}$$

For rivers where La anomalies appeared from the graphical representation, the formula suggested by Klaver et al. (2014) was used for the anomaly quantification:

$$La/La^* = La_{measured}/(Pr_N \cdot La_{PAAS})$$

where La^*: La local background concentration; Pr_N: Pr shale-normalized concentration; La_{PAAS}: La concentration in PAAS shales.

La anthropogenic input (ng/L) was calculated as follows (Klaver et al. 2014):

$$La_{anthr} = La_{measured} - La^*$$

Values of the ratio Gd/Gd^* and La/La^* greater than 1.5 were considered to be anthropogenetically influenced (Bau et al. 2006; Kulaksiz and Bau 2011), and situations with value greater than 5 were stressed.

Moreover, Eu and Ce anomalies were also quantified, according to Noak et al. (2014), in which the geogenic value of each element was obtained by interpolation of the neighbor normalized REE elements of Ce and Eu:

$$Ce_N^* = 2 \cdot Ce_N/(La_N + Pr_N)$$

$$Eu_N^* = 2 \cdot Eu_N/(Sm_N + Gd_N)$$

It is worth to be noted that these last two indexes could be influenced in some situations by anomalies in La and Gd; nevertheless, their calculation can be useful to discriminate between the different types of sampled waters.

3 RESULTS AND DISCUSSION

3.1 Water type classification and major ion content in waters

Physicochemical parameters and the complete list of results of major element composition of both surface and groundwater are reported in **Table S2.1** (surface waters) and **Table S2.2** (groundwaters) of the *Supplementary Material*. All surface waters are slightly alkaline (pH range: 7.5-8.2), while groundwaters are characterized by more neutral pH (range: 6.7-7.7). Temperature variability for surface water is high (from 16.3 °C to 35.2 °C), the lowest generally observed in the headwater area, depending on the sampling time and location. Groundwater temperatures record a lower variability (15.9-20.9 °C). Surface water show lower values of electrical conductivity (271-865 µS/cm) than groundwaters, with a mean value of 491±153 µS/cm. The lowest values occur at the headwaters, while maxima are spread all along the river bodies. This pattern is not surprising, since interactions between waters and rocks along the river enhance weathering processes, leading to a progressive dissolution of minerals and thus increasing the amount of ions in solution. Groundwater values for electrical conductivity vary from 679 to 1233 µS/cm, with mean value of 953±179 µS/cm, reflecting a stronger interaction between water and rocks than in rivers, as it should be expected in groundwaters, where higher water residence time and slow flows enhance water-rock interactions.

Major chemical composition of surface and groundwater were studied through Piper diagram (**Figure 2.2**), useful for water chemical classification.

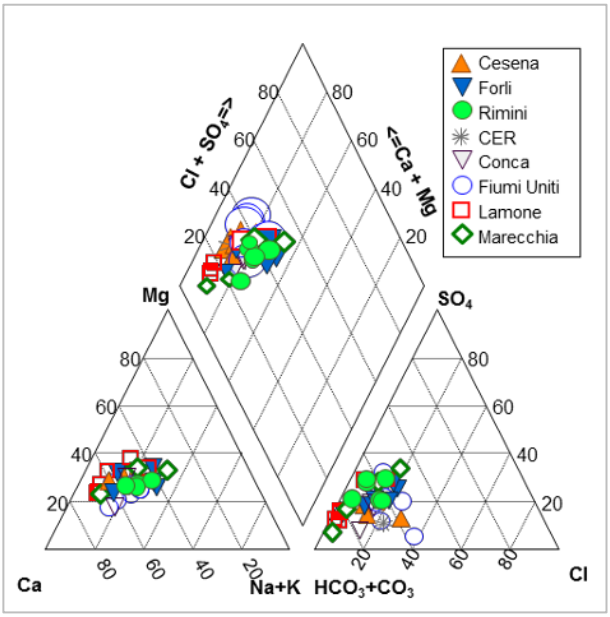


Figure 2.2 Piper diagram showing water type of groundwater wells (full dots) and surface waters (empty dots). Symbols size in the upper diamond is proportional to the electrical conductivity

From the diagram it can be noticed that all sampling points are concentrated in the left quadrant of the upper diamond. Based on Kumar classification (Kumar 2013), all waters belong to Ca-HCO_3 type. Analysing separately the cationic and anionic contributions to the overall water chemistry (the two ternary plots), it is worth to be noted that the carbonatic content found in rivers and groundwaters is mainly due to the weathering of calcite rather than dolomite (Ca range of 40-80 %meq; Mg range of 30-60 %meq). However, a few samples of both surface and groundwaters show a Ca/Mg ratio close to 1, thus dolomite contribution can not totally be excluded (**Figure 2.2** – ternary plot on the left). The anionic component (**Figure 2.2** - ternary plot on the right) is mostly dominated by carbonates and bicarbonates content, with minor contribution of Cl and SO_4. Lamone river, above all, shows the major contribution of carbonates and bicarbonates as prevailing anions (HCO_3 and CO_3 of 80-95 %meq; SO_4 less than 30 %meq, Cl about 10 %meq). The other water samples show a slight increase of SO_4 content in water (Forlì and Rimini wells), possibly indicating contribution from gypsum outcrops, or a slight increase in Cl content, expecially in those samples of Marecchia and Fiumi Uniti nearer to the river mouth, reflecting contribution of seawater on river chemistry. This major element composition is consistent with data from Petrini et al. (2014), Mollema et al. (2013) and comparable to the Po River (Marchina et al. 2015).

Table 2.2 reports major ions and trace element summary statistics, along with median values representing Italian surface waters from the FOREGS database (Salminen 2005) which can be useful for a general comparison, although the database includes samples from different geological environments. Kruskal-Wallis test was carried out in order to assess whether the analysed surface and groundwater samples belong to the same population or have a different origin. Results of the test and associated *p*-value (α=0.05) for each element are also reported in **Table 2.2**.

Even though from the graphical representation provided by Piper diagram (**Figure 2.2**) surface and groundwaters have similar characteristics, the Kruskal-Wallis test of medians outlined significant differences between the two groups of waters (**Table 2.2**). All major ions, except for K, F and SO_4, have median values greater in groundwaters than in surface waters. This is due to the slower water movement in aquifers which enhances rock-forming minerals dissolution, such as plagioclase in the case of Na and carbonates in case of Ca, Mg and HCO_3, due to the longer interaction times between waters and rocks.

Different concentration ranges were obtained for nitrates in the two water systems, with median value of 2.51 mg/L in rivers (range 0.27-12.21 mg/L) and 38.46 mg/l (range 0.35-69.87 mg/L) in groundwaters (**Table 2.2**). Concentrations in river waters are comparable to those of the FOREGS database, whereas there is high variability in groundwaters. Some of the samples of Forlì, Rimini and Cesena wells (GW30, GW36, GW39, GW43, GW44, see **Table S2.2** of the *Supplementary Material*) exceed the threshold limits of 50 mg/L (Legislative Decree No. 30/2009). NO_3 enrichments in waters are likely related to anthropogenic sources such as fertilizers used in agriculture and leakages from sewage systems, and from organic matter

degradation, as well. Nitrates are very soluble and are barely incorporated in soil particles; once released into the environment, they can thus easily reach the aquifer (ISPRA 2015). The Emilia-Romagna Environmental Agency has already reported high N loads affecting groundwater quality both in the freatic aquifers and in some confined alluvial aquifers of the Emilia-Romagna region (ARPA-ER 2013b). The Marecchia-Conca conoid is one of the aquifer bodies most affected by nitrates contamination (ARPA-ER 2013b).

Table 2.2 Summary statistics of the analysed elements in surface and groundwaters. Values representing Italian surface waters from the FOREGS database (Salminen 2005) are also reported. The table includes the results from the Kruskal-Wallis test and associated p-value (α=0.05). Measure units are: T (°C), EC (µS/cm), major ions (mg/L), trace elements (µg/L)

	Surface waters *(n=24)*					**Groundwaters *(n=17)***					
	Min	**Max**	**Mean**	**Median**	***FOREGS***	**Min**	**Max**	**Mean**	**Median**	**Kruskal-Wallis test**	***p*-value** *(α=0.05)*
T	16.3	35.2	25.4	24.9	---	15.9	20.9	17.7	17.6	17.391	**$3.04e10^{-5}$**
pH	7.5	8.2	8.0	78.0	***8.1***	6.7	7.7	7.3	7.4	23.365	**$2.82e10^{-7}$**
EC	271	865	491	476	***460***	679	1233	952	947	26.902	**$2.14e10^{-7}$**
major ions											
K	1.4	17.1	5.1	4.4	***1.8***	2.4	7.8	3.8	3.5	0.511	**0.475**
Na	7.5	68.1	28.4	28.1	***10.6***	30.3	93.4	49.8	49.0	13.725	**$2.1e10^{-4}$**
Mg	9.6	36.1	21.8	19.2	***15.0***	24.4	56.3	40.0	41.9	18.609	**$1.6e10^{-5}$**
Ca	40.2	89.2	60.2	58.4	***65.8***	95.3	197.0	146.0	156.0	29.148	**$6.7e10^{-8}$**
Cl	4.5	87.2	29.5	23.8	***13.7***	45.8	150.0	81.3	71.7	22.190	**$2.5e10^{-6}$**
SO_4	16.9	138.0	75.0	75.8	***26.4***	28.9	267.0	114.0	101.0	3.632	**0.056**
HCO_3	158	439	276	262	***213***	372	610	501	536	26.464	**$2.7e10^{-7}$**
NO_3	0.27	12.21	3.68	2.51	***2.85***	0.35	69.87	33.45	38.46	12.387	**$4.3e10^{-4}$**
F	0.044	0.257	0.130	0.120	***0.085***	0.054	0.249	0.110	0.100	1.449	**0.228**
trace elements											
Ag	0.003	1.950	0.260	0.140	***0.001***	0.002	0.114	0.010	0.003	21.620	**$3.3e10^{-6}$**
Al	1.30	13.70	5.06	4.35	***17.70***	3.30	30.20	6.96	5.20	2.565	0.109
As	0.11	1.64	0.63	0.48	***0.63***	0.07	0.82	0.17	0.12	17.13	**$3.5e10^{-5}$**
B	25.5	295.0	131.9	131.0	***15.6***	68.4	228.0	125.3	122.0	0.928	0.92
Ba	31.3	233.0	68.	59.5	***24.9***	35.1	1075.0	142.8	60.3	0.955	0.328
Be	<0.005	0.008	0.006	0.006	***0.009***	<0.005	0.007	0.003	0.003	15.749	**$7.3e10^{-5}$**
Cd	0.006	0.091	0.03	0.018	***0.01***	0.013	0.057	0.03	0.022	1.577	0.217
Co	0.02	0.37	0.09	0.06	***0.16***	<0.01	0.23	0.05	0.04	4.387	**0.036**
Cr	<0.10	0.25	0.13	0.12	***0.38***	<0.10	0.18	0.07	0.06	15.286	**$9.1e10^{-5}$**
Cs	0.004	0.109	0.01	0.009	***0.006***	<0.002	0.01	0.001	0.004	16.467	**$4.9e10^{-5}$**
Cu	0.70	4.56	2.04	1.99	***0.88***	0.20	5.40	1.52	1.36	4.818	**0.028**
Fe	1.7	14.6	6.3	6.2	***67.0***	4.6	1213.0	212.3	20.2	18.781	**$1.5e10^{-5}$**
Ga	0.006	0.054	0.009	0.009	***0.011***	<0.005	0.023	0.006	0.006	7.722	**0.005**
Ge	<0.03	0.05	0.03	0.03	***0.009***	<0.03	0.08	0.03	0.03	1.305	0.253
Li	2.8	38.1	17.8	19.2	***2.1***	6.8	24.9	13.9	13.2	1.220	0.269
Mn	0.3	5.3	1.5	1.1	***15.9***	0.9	392.0	65.4	2.7	10.450	**0.001**
Mo	0.31	2.02	0.94	0.94	***0.22***	0.19	0.92	0.43	0.37	13.996	**$1.8e10^{-4}$**
Ni	0.15	4.47	1.53	1.51	***1.91***	0.36	18.60	2.46	1.63	0.088	0.766
Pb	0.07	1.05	0.27	0.26	***0.09***	0.27	0.89	0.41	0.34	9.545	**0.002**
Rb	0.61	13.8	2.29	1.55	***1.32***	0.18	0.65	0.40	0.4	27.459	**$1.6e10^{-7}$**
Sb	0.10	2.21	0.36	0.28	***0.07***	0.01	0.10	0.04	0.03	28.139	**$1.7e10^{-7}$**
Se	0.16	0.78	0.40	0.34	***0.34***	0.02	12.10	3.71	1.91	9.889	**0.001**
Sr	315	1436	923	996	***110***	684	1756	1224	1210.	6.355	0.011
Th	<0.001	0.003	0.002	0.0015	***0.009***	<0.001	0.002	0.001	0.001	11.43	**$7.2e10^{-4}$**
Ti	<0.08	0.54	0.29	0.28	***0.90***	<0.08	0.35	0.16	0.15	16.674	**$4.4e10^{-5}$**
Tl	<0.002	0.012	0.010	0.008	***0.005***	<0.002	0.006	0.003	0.003	14.797	**$1.1e10^{-4}$**
U	0.37	1.96	1.06	1.08	***0.32***	0.06	8.54	2.33	2.22	5.795	0.016
V	0.12	0.97	0.43	0.31	***0.46***	<0.10	0.28	0.14	0.15	15.293	**$9.2e10^{-5}$**
Y	0.006	0.036	0.02	0.015	***0.064***	0.015	0.026	0.020	0.021	3.676	0.055
Zn	2.2	23.6	7.3	5.5	***2.7***	8.5	59.2	28.6	24.0	21.113	**$2.6e10^{-6}$**
Zr	0.007	0.151	0.050	0.022	***0.053***	0.086	0.141	0.120	0.122	13.786	**$2.1e10^{-4}$**

3.2 Trace elements

Focusing on trace element composition in surface and groundwaters, the significant differences outlined by the Kruskal-Wallis statistical test (**Table 2.2**) point to relatively higher concentrations in surface water for Ag, As, Be, Co, Cr, Cs, Cu, Ga, Mo, Rb, Sb, Sr, Th, Ti, Tl, V, whereas Fe, Mn, Pb, Se, Zn and Zr record higher median in groundwaters. Fe and Mn differences are consistent with Fe-Mn behavior strongly dependent on redox conditions: in oxidizing environments the two elements tend to be aggregated and form oxide-hydroxides minerals, while in reducing conditions, such as those that characterize deeper aquifers, they show a higher mobility (De Vivo et al. 2004).

Be, Co, Cr, Cs, Mo, Pb, Rb, V, Th, Ti, Tl, Zr and Zn concentrations in the environment are strongly controlled by clay minerals and Fe-Mn hydroxides: local adsorption-desorption processes may lead to their removal from or release into the solution (De Vivo et al. 2004), with the consequence of local depletions or enrichments in waters. Among all of them, Zn enrichments in groundwaters can reflect its mobilization after dissolution of Fe oxide minerals. Th differences between groundwaters and surface waters are mainly dependent on the fact that the element was detected in all groundwater samples in concentrations near to the instrument detection limit (0.001 μg/L); in surface waters Th values vary in a very low range (0.001-0.003 μg/L). Besides clay material, also carbonate rocks can control Pb distribution in the two types of waters; Pb difference in surface and groundwaters is not too high, though (median value of 0.26 μg/L in rivers and 0.34 μg/L in groundwaters, with a test *p*-value of 0.002).

Relatively higher concentrations of Ga detected in surface waters (with maximum concentration of 0.054 μg/L) can reflect water interactions and weathering of feldspathic sandstones or clay minerals of the *Marnoso-Arenacea Formation*, which can host this element (Dinelli et al. 1999).

Arsenic is a calcophile element found in the environment as accessory element in sulphide minerals and phosphatic sediments; it can be easily adsorbed on clay mineral surfaces, as well. The higher concentrations were detected in this study in correspondence of the river mouths of the Lamone and Fiumi Uniti rivers (for detailed concentrations see **Table S2.1** of the *Supplemetary Material*), where external inputs can be added to the terrigenous origin of the element in rivers. Anyway, concentrations were not too high (maximum value: 1.64 μg/L), and As median and mean are comparable to FOREGS median value (**Table 2.2**).

One element with a significant difference between the medians is Ag. Surface water has a median of 0.14 μg/L, in contrast with 0.003 μg/L for groundwaters and the median value (0.001 μg/L) reported by FOREGS. The maximum value (1.95 μg/L) is observed in sample R1 from the Lamone river (see **Table S2.1**), but it is interesting its distribution along the river course (**Figure 2.3**), since it is characterized by a constant concentration in its initial stretch, followed by a steadily increase from sample point R3 (located 70 km away from the river head) to the river mouth (R1 sample, 103 km from the river head). Silver abundances in

rocks and soils are generally low, being greater in clays, which could represent a natural source of the element, although various important anthropogenic sources may introduce the element in the environment. The most important ones could be represented by release associated to photographic materials and processing, mining and smelting activities, jewelry, silverware, solder, bearings, medical and dental applications, electrical contacts, circuit board manufacture, cloud seeding, catalysts, and sewage sludge (Evans and Barabash 2010). Silver is increasingly used in disposable products, often as nanoparticles, such as antimicrobial coatings for bandages, clothing, or water treatment devices, and these might represent an additional, unquantified, possibly widespread source of the element (Eckelman and Graedel 2007). Along its course, the river crosses progressively more densely populated and industrialized areas and receives the effluents from the WWTP of Faenza (located just before R3 sample point), which could represent a possible source of contamination.

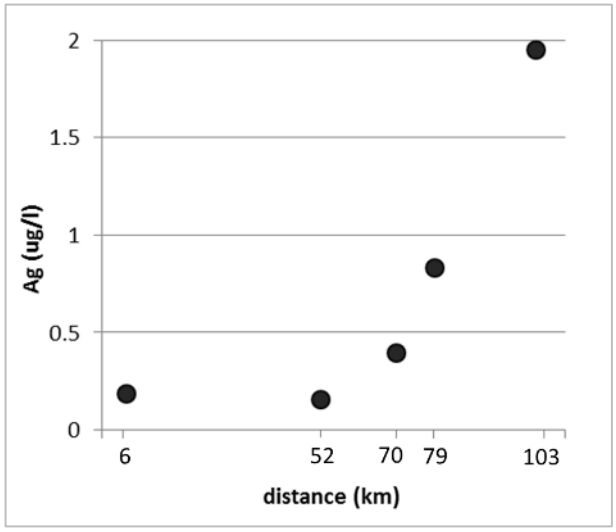


Figure 2.3 Ag spatial distribution along the Lamone river, from the river head (sample R5) to the river mouth (sample R1, left-to-right). On the x axis distances in km of sample points from the river head are reported: sample R5 (6 km), sample R4 (52 km), sample R3 (70 km), sample R2 (79 km) and sample R1 (103 km)

Petrini et al. (2014) carried out a study in rivers and channels of the Ravenna coastal plain in order to assess whether agricultural land use can affect water quality. Comparing trace elements (Sr, Ba, Li, Al, B, Mn, Fe, Ni, Cu, Zn) of Lamone and Fiumi Uniti samples of their study collected in winter time with R1 and R17 samples (which correspond in position to the samples analyzed by Petrini et al. 2014), it can be inferred that samples from this study have always maximum values less or equal to those found by Petrini et al. (2014). Only for Cu a 3-times enrichment was registered both for R1 and R17, although this enrichment is not too high (less than 2 μg/L).

3.3 REE distribution in surface and groundwaters

Particular attention was paid to the REE group. Total REE concentrations range from 33 to 301 ng/L in surface waters, with a mean value of 71±55 ng/L; REE in groundwaters show a mean value of 42±13 ng/L (range 28-87 ng/L). Summary statistics of individual and total REE content in surface and groundwaters is

reported in **Table 2.3.** The high REE content registered in surface waters (301 ng/L) is mainly dependent on La enrichment, which contributes to the overall REE content for the 75%, suggesting the presence of some local outliers, as it can be inferred by the mean value (27 ng/L) associated with a wide standard deviation (45 ng/L). Except from La, groundwater samples show on average a comparable REE content with that of surface waters.

Table 2.3 REE summary for surface and groundwaters. Concentrations are expressed in ng/L

	Surface waters *(n=24)*				**Groundwaters** *(n=17)*			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
La	4	226	27.2	44.5	4	19	6.8	3.3
Ce	3	18	6.8	3.3	4	13	7.4	2.0
Pr	1	2	1.2	0.4	1	2	1.1	0.3
Nd	3	11	5.4	1.6	4	7	5.2	0.9
Sm	1	6	3.5	1.9	1	9	3.7	2.1
Eu	1	5	1.9	1.1	1	22	3.2	4.9
Gd	2	46	11.5	12.8	2	11	3.7	2.1
Tb	1	1	1.0	0	1	1	1.0	0
Dy	2	5	2.8	0.9	2	3	2.2	0.4
Ho	1	1	1.0	0	1	1	1.0	0
Er	2	7	2.8	1.5	2	4	2.2	0.6
Tm	1	1	1.0	0	1	2	1.1	0.2
Yb	2	8	4.0	1.5	2	5	2.7	1.1
Lu	1	2	1.1	0.3	1	2	1.1	0.3
ΣREE	33	301	71.2	54.7	28	87	42.2	12.6

Figure 2.4 reports a boxplot showing the distribution values of all the lanthanides. Please note that R1 sample was removed from the boxplot since it was an outlier for La value (0.226 µg/L) and it caused a flattening of the other REE boxplots. As it can be easily noticed from the figure, La and Gd are, among surface water REEs, the most spread elements, with a wider range of values; in groundwaters, on the contrary, concentrations of all REEs are comparable.

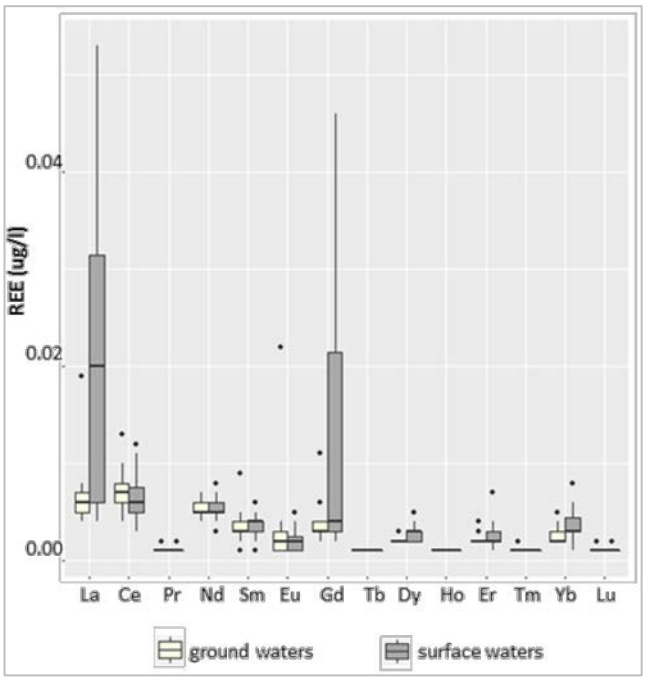


Figure 2.4 Boxplot showing REE concentrations

To better understand REE association in water, total REE content was compared with electrical conductivity, HCO_3, and Al and Fe, as well (**Figure 2.5**). Electrical conductivity can be considered as representative of the total dissolved ions present is solution. **Figure 2.5a** and **2.5b** show a different pattern of REE content in surface and groundwaters: while surface total REEs present a slight increase as electrical conductivity increases (R^2 determination coefficient of Lamone-CER, removing the two main outliers: 0.85, R^2 Fiumi Uniti: 0.45; R^2 Marecchia: 0.70; R^2 Conca: 0.24), groundwater REEs seem not to be related to electrical conductivity, as total REE values are constant at increasing electrical conductivity. This may suggest that weathering processes of rock aquifers regarding REEs happen to a longer time scale than for major ions. Same absence of relation can be observed for HCO_3 (**Figure 2.5d**), Fe (**Figure 2.5f**) and Al (**Figure 2.5h**), with R^2 values always below 0.15. On the other hand, surface waters show a slight positive relation with HCO_3, the most dominant component in the analysed waters (**Figure 2.5c**), even though the presence of two outliers from the Lamone river system interfere with the overall relation. Except these two outliers, whose REE content is likely to be related to other sources, REE pattern are partially dependent on carbonates dissolved in waters (R^2 Fiumi Uniti: 0.61; Lamone-CER, removing the two outliers: 0.62). Other elements that can affect REE distribution in waters are Al (R^2 Fiumi Uniti: 0.91, R^2 Lamone: 0.42; **Figure 2.5e**) and Fe (R^2 Fiumi Uniti: 0.38, R^2 Lamone: 0.90; **Figure 2.5g**). In alkaline conditions Al and Fe are present in their anionic form; the correlation between REEs and Al and Fe found in surface waters may thus suggest REE adsorption on Al-Fe-colloidal particles (Goldstein and Jacobsen 1988). This inference is confirmed by the work of Cidu et al. (2013), that demonstrated that at <0.4 µm water fraction REE dissolution in water is controlled by Al- and, to a lesser extent, Fe-colloids.

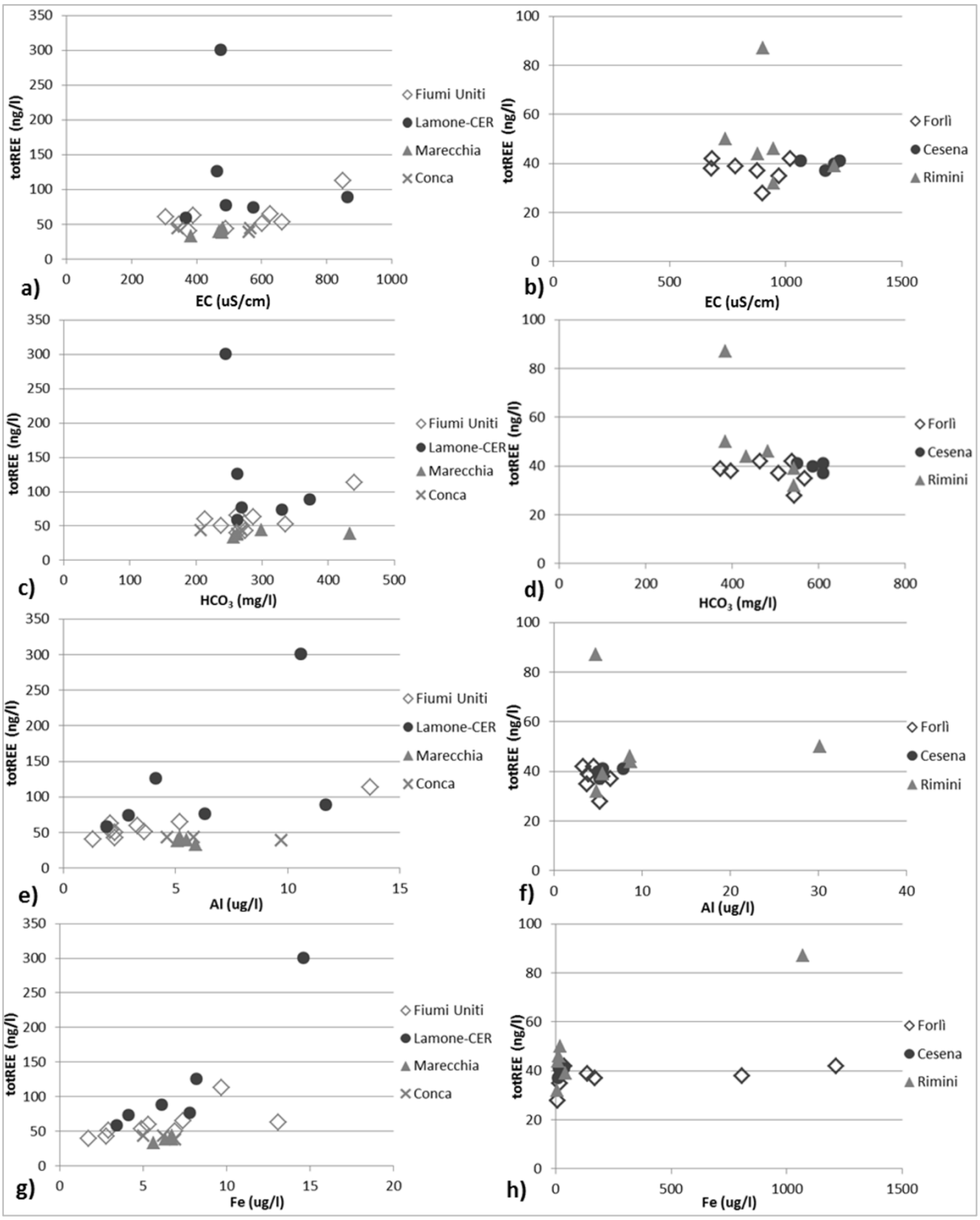


Figure 2.5 Total REE concentration in surface waters (columns on the left) and groundwaters (columns on the right) versus electrical conductivity (a, b), HCO_3 (c, d), Al (e, f) and Fe (g, h)

3.4 REE identification of anomalies

The normalized (to PAAS) REE patterns in surface and groundwaters allow the identification of possible anomalies (**Figure 2.6** for groundwaters samples, **Figure 2.7** for surface waters). In both water types the

REE pattern is slightly enriched in heavy REE (HREE, from Gd to Lu) compared with light REE (LREE, from La to Eu), with a ratio La_N/Lu_N always below 0.5. This pattern suggests that in the analysed water REEs are mostly present in the dissolved phase, since HREEs are generally dominant in the dissolved or colloidal phase, while LREEs are more abundant in the particulate material (Elderfield et al. 1989; Merschel et al. 2015). In fact, HREEs tend to form stronger complexes with ligands in solution than do LREEs, which are on the other hand preferentially adsorbed on particle surfaces of the suspended matter (Elderfield et al. 1989). Focusing on intermediate REEs (MREE, from Sm to Ho), it can be noticed that the pattern in both water types follows the enrichment towards HREE content, with exclusion of some anomalies of Eu or Gd which highlight local abnormal behaviors. All rivers (**Figure 2.7**) show a depletion of Ce, highly remarked in Lamone and Fiumi Uniti rivers (Ce^*_N values below 0.2). This anomaly is commonly found in river systems and is related to the redox-sensitivity of Ce: in oxidizing conditions, Ce^{3+} is rapidly oxidizied to Ce^{4+}, which precipitates, generating a decrease of concentration in water (Goldstein and Jacobsen 1988). All groundwater samples show a positive Eu anomaly, ranging from 1.6 to 10.7 (**Figure 2.6**), related to redox conditions, as well. In fact, under reducing conditions, such as those that characterize groundwater samples, Eu is reduced in its form Eu^{2+}, which is more mobile and soluble than Eu^{3+} (Goldstein and Jacobsen 1988). Marecchia and Conca rivers (**Figure 2.7c**, **2.7d**) show a slight enrichment in Eu in all samples with the exception of the samples close to the headwaters. This anomaly can be explained by the geological setting of the area. Eu^{2+} is similar in size and charge to Ca^{2+}; during rock forming, it is preferentially incorporated into plagioclase feldspars, substituting Ca^{2+} (Henderson 1984). Weathering interactions between waters and the *Umbro-Marchean Succession* (the geological formation enriched in plagioclase content that characterizes the southern portion of the area) can lead to a slight enrichment of Eu in rivers. In addition, the Eu enrichment found in the Marecchia river can testify local interactions between surface and groundwaters, with a major contribution of groundwaters, characterized by higher concentrations of the element. In fact, the highest Eu anomaly (Eu^*_N=3.9) was detected in sample R20, which correspond to the aquifer recharge area of the Marecchia conoid. Eu surface enrichment however is not so high: values of Eu detected in Marecchia and Conca rivers are always below 6 ng/l (median European Eu concentration in water reported by FOREGS database is 5 ng/L).

La enrichments are found in almost all points of Lamone and Fiumi Uniti rivers and in CER canal, as well (**Figure 2.7a**, **2.7b**), with La/La^* ratios between 1.4 and 9.7, the only exception being R1 sample (Lamone river) with a ratio of 26.1. The distribution of such La enrichments all along the two river bodies suggests that this effect could be related to the geological setting of the study area rather than to anthropogenic inputs. In sedimentary rocks La can be adsorbed onto clays minerals; under alkaline conditions it can precipitate as La-carbonate, as well (Balashov et al. 1964). Interactions between river water and stream rocks can then lead to the transfer of the element into solution and to a higher concentration in those areas characterized by lower flow rates such as the river heads, whereas higher flow rates come along with a

greater dilution of the element. This can explain the high La/La^* ratios obtained for samples collected in proximity of the river heads, far from potential contamination sources. A different remark needs to be done for sample R1. The high anomaly found only in this sampling point (La/La^*=26.1, with La value of 226 ng/L) is far different from all the other samples of the same river body, which have concentrations ranging between 26 and 53 ng/L, in accordance with the median La value in river waters reported by FOREGS (34 ng/L). This different pattern implies a La input that is not completely determined only by the geological conditions. The abovementioned relation between REEs and electrical conductivity (**Figure 2.5a**), as well as REEs boxplot (**Figure 2.4**), have already highlighted the anomalous behaviour of this sample compared to the other river samples. R1 sample is located in proximity of the Lamone river mouth (**Figure 2.1**), in an industrialized area where activities of petrochemistry and petroleum manufacturing are concentrated. As some authors have pointed out in their studies (Kulkarni et al. 2006; 2007), La is highly used for fluid catalytic cracking catalysts in petroleum manufacturing and refining, and it has been found to be enriched in dust particles coming from these plants. Later deposition of such La enriched ashes can be the explanation of the high La enrichment registered in R1 sample. However, it should be noted that anthropogenic input is not so high: the calculated La_{anthr} values range from 2 to 5 ng/L, in all samples in which an anomaly was detected; only for R1 sample La coming from anthropogenic sources reached 18 ng/L.The aforementioned work by Cidu et al. (2013) reported La values in the Ravenna coastal plain similar and comparable to those found in the Lamone site R1; no enrichment though was highlighted since all the other REEs had higher concentrations, as well, with a resulting smoother pattern. Further studies should thus be done in order to better understand the cause of La enrichment in the area.

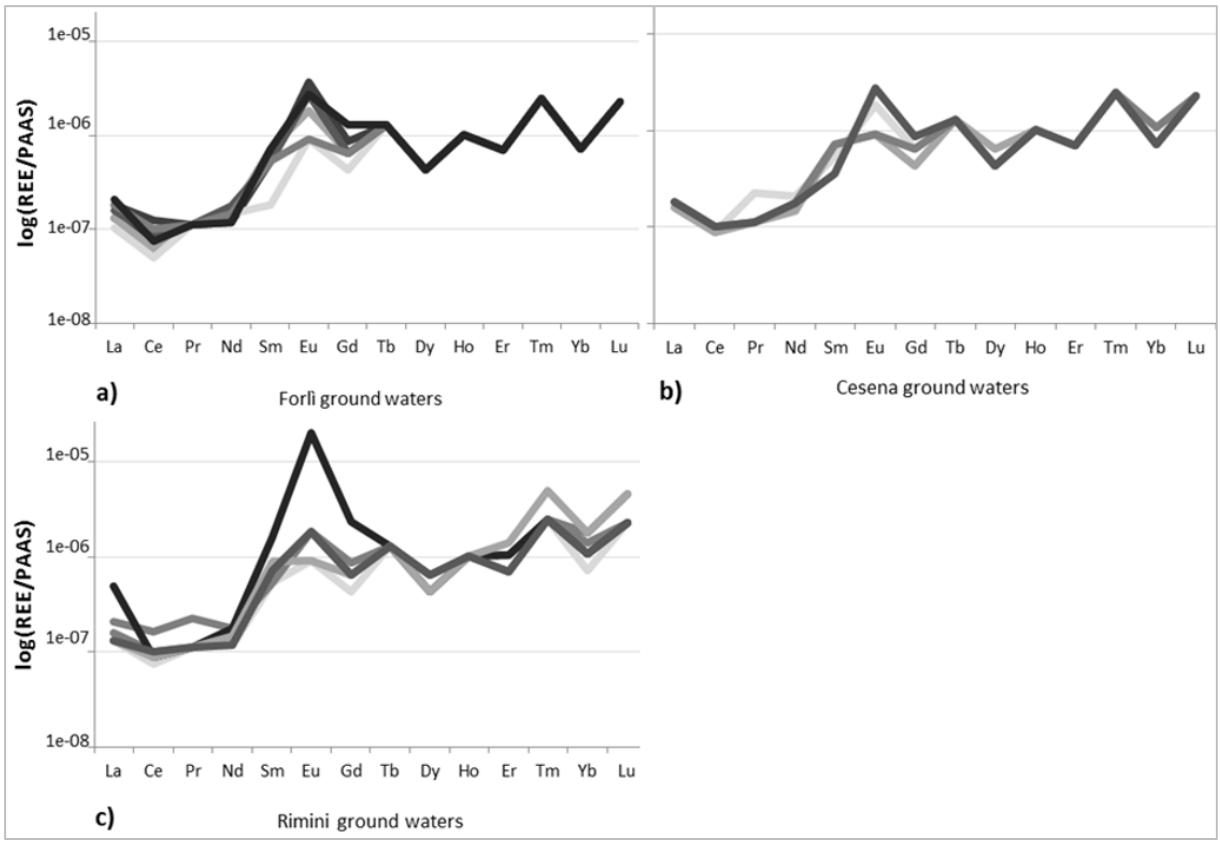


Figure 2.6 REE pattern in groundwater wells of Forlì (a), Cesena (b) and Rimini (c)

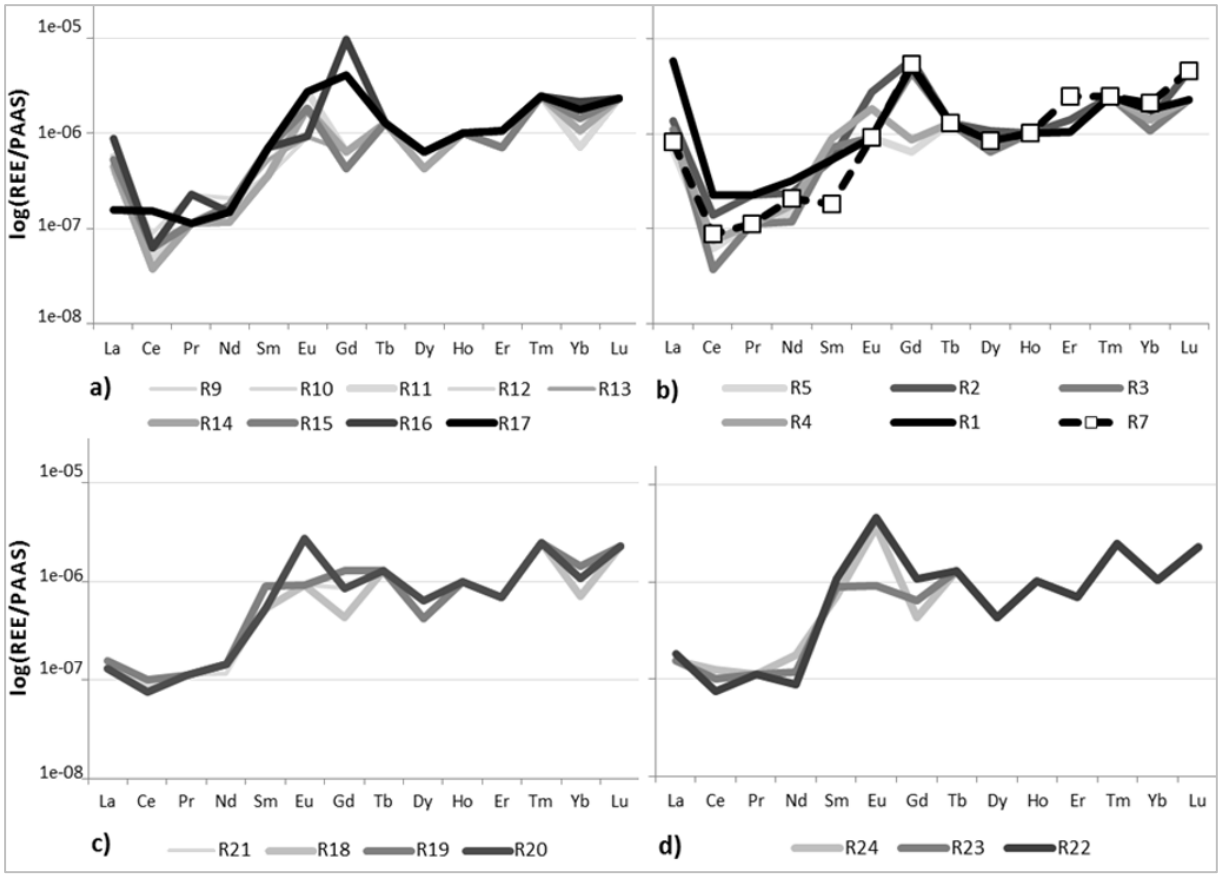


Figure 2.7 REE pattern in in surface waters: Fiumi Uniti system (a), Lamone river (continuous line) and CER canal (dotted line) (b); Marecchia river (c); Conca river (d)

In addition to La anomaly, the Lamone river (**Figure 2.7b**), together with the Fiumi Uniti system (**Figure 2.7a**) and CER canal (**Figure 2.7b**) are characterized by an evident anomaly in Gd, expecially in proximity of the river mouths (samples R1, R6, R7, R16). This anomaly is of one order of magnitude higher than the natural REE pattern, with ratios Gd/Gd^* ranging from 0.6 to 9.3, thus indicating a clearly anomalous source of Gd in these rivers. The Marecchia and Conca rivers situation is different (**Figure 2.7c, 2.7d**): ratios of Gd/Gd^* are between 0.4 and 1.1, with little or no anthropogenic input of Gd. Groundwaters (**Figure 2.6**) show low concentrations of Gd, and low Gd/Gd^* (0.6-1.2), indicating that no interaction or exchange processes between contaminated surface rivers and groundwaters have taken place. Positive Gd anomalies have also been found at the exit of the drinking water treatment plant in the northern side of Ravenna (sample R6, **Figure 2.8a**). This plant receives waters from Lamone and CER during summer; waters at the entrance of the DWTP are quite highly enriched in Gd (Gd/Gd^*=6.6), coherently with the two water bodies status. The sample at the exit of the plant has a lower Gd/Gd^* value (2.7), but it can not be considered completely free from this microcontaminant. On the contrary, the DWTP in the south of Ravenna (sample R8, **Figure 2.8b**) has proven to be more efficient, since Gd concentration at the exit of the plant is significantly lower compared to the concentration input (3 ng/L at the exit versus 28 ng/L at the entrance of the plant, with a Gd/Gd^* ratio of 0.6 at the exit of the plant and 5.6 at the entrance). This result confirms the efficiency of the advanced technologies for water treatment installed in the new DWTP R8 compared with the other DWTP R6.

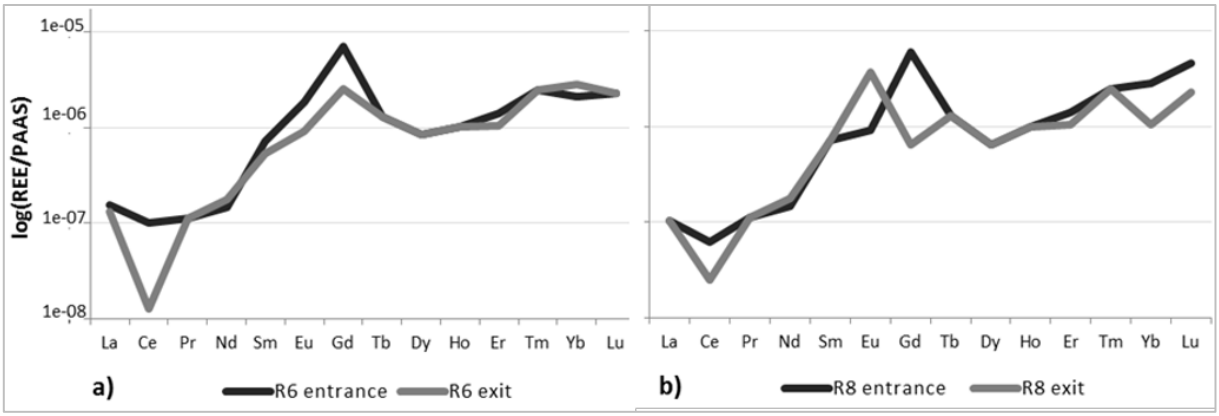


Figure 2.8 REEs in the drinking water treatment plants to the north (a) and to the south (b) of Ravenna

Spatial distribution of Gd anomalies is shown in **Figure 2.9**. Fom the map it is evident that all the higher anomalies are concentrated in the north-eastern part of the study area. It is a moderately high urbanized and industrialized area, where the sources of contamination can be different and multiple, the most important ones being the WWTPs of the cities of Faenza and Forlì that release their treated effluents directly in the river bodies (Faenza WWTP is located between points R3 and R4; Forlì WWTP is between points R13 and R16). Moreover, the presence of two highly technologically advanced hospital centres in the

two cities can be an additional source of contamination. The two rivers show comparable Gd levels of contamination: calculated Gd anthropogenic input ranges from 16 to 28 ng/L in the Lamone river, while in the Fiumi Uniti an anthropogenic concentration of 41 ng/L was detected in R16 sample and 14 ng/L in sample R17. However, a slight difference between the two water systems needs to be noted. In the Lamone river high anomalies of Gd are detected downstream of the city of Faenza until the river-mouth, suggesting that different sources of contamination in this portion of the river contribute to the high concentrations (such as hospitals, the Faenza WWTP and CER water input into Lamone). In the Fiumi Uniti system, instead, contamination is more restricted: the effect of dilution as a consequence of the confluence of Ronco and Montone rivers into the Fiumi Uniti plays an important role in decreasing Gd concentrations in the final stretch of the river.

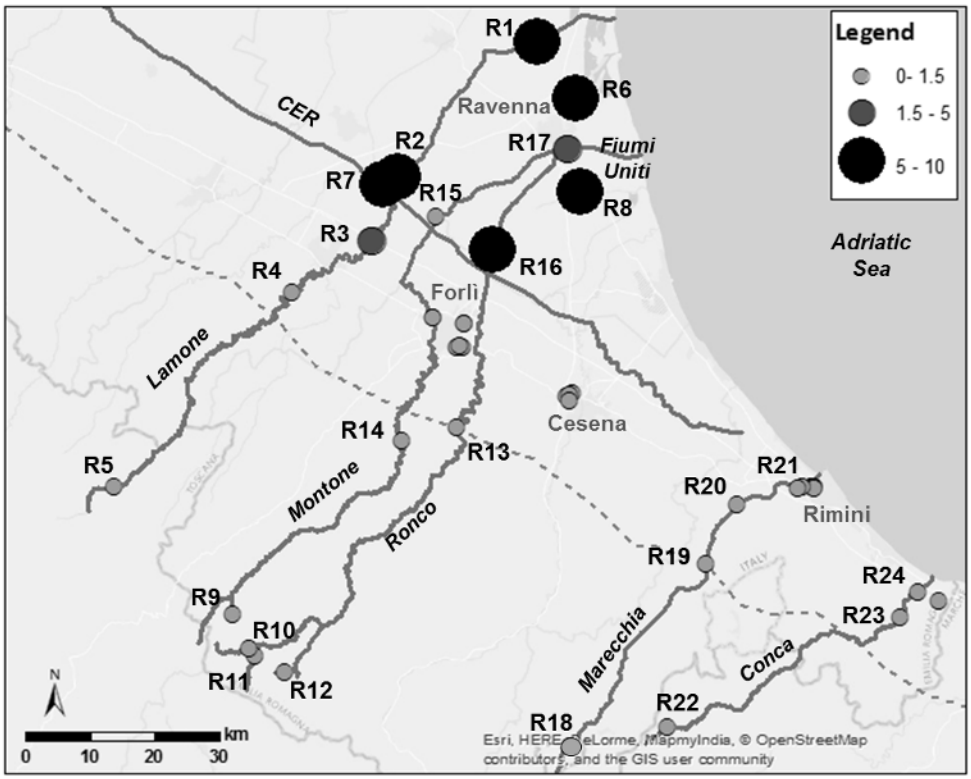


Figure 2.9 Spatial distribution of Gd anomalies in both ground and river waters

4 CONCLUSIONS

This work investigated water chemistry of surface and groundwaters based on an extensive set of elements; detailed discussion was also provided for REE content. Surface and groundwaters can be classified as Ca-HCO_3 type. The comparison between the two groups of waters revealed that surface water are enriched in elements generally associated with clay minerals (As, Be, Co, Cr, Cs, Cu, Mo, Ni, Rb, Th, Ti, V), whereas groundwaters record higher medians for Pb and Zn, calcophile elements that are related to carbonate materials. Fe and Mn enrichments in groundwaters reflect the presence of reducing conditions

for some of the analysed aquifers. Ag high values found in surface waters of the Lamone river can suggest anthropic inputs for this element.

Rare Earth Elements can be used in geochemical studies to investigate anomalies in their behavior related to anthropogenic sources. In particular, Gd is a micropollutant introduced in the aquatic environment by the incorrect disposal of WWTP effluents receiving hospital wastewaters. This element can thus be considered as a tracer of anthropogenic input in surface and groundwaters. In this study only rivers in the north-eastern part of the Romagna area show Gd anomalies, while streams in the south portion of the area seem not to be affected by this kind of contamination, as well as groundwaters of confined aquifers. La enrichments give evidence of further potential sources of contamination, even though more studies need to be done about this topic. Drinking water treatment plants receiving contaminated river waters are not always efficient to remove the microcontamination.

Supplementary Material

GEOCHEMICAL CHARACTERIZATION AND RARE EARTH ELEMENTS ANOMALIES IN SURFACE- AND GROUNDWATERS OF THE ROMAGNA AREA (Italy)

Table S2.1 Coordinates (*WGS84 – EPSG: 4326*), physical-chemical parameters, major ions and trace elements of the Lamone river, CER canal and DTWPs; Fiumi Uniti rivers; Marecchia and Conca rivers

		Lamone river (n=5)					*CER*	*DWTP (n=4)*			
Sample	***DL***	**R1**	**R2**	**R3**	**R4**	**R5**	**R7**	**R6 in**	**R6 out**	**R8 in**	**R8 out**
X coord		12.17148	11.97791	11.94272	11.83286	11.58618	11.95714	12.22568	12.22568	12.23112	12.23112
Y coord		44.50456	44.37099	44.30651	44.25477	44.06146	44.36230	44.44815	44.44815	44.35606	44.35606
T *(°C)*	*0.1*	25.6	25.3	24.6	24.1	23.2	26.2	29.6	30.1	28.9	27.8
pH	*0.1*	8.2	8.2	7.92	8.0	8.1	8.2	7.9	7.9	7.7	7.8
EC *(µS/cm)*	*0.1*	474	461	865	573	368	271	491	365	544	395
mg/L											
K	*0.1*	15.0	7.5	17.1	3.8	2.0	2.6	6.9	2.8	6.9	2.7
Na	*0.1*	27.8	28.3	66.8	34.8	13.4	10.7	29.6	18.1	36.8	18.9
Mg	*0.01*	18.3	18.1	32.2	27.9	18.9	9.57	19.2	14.4	18.9	14.7
Ca	*0.01*	60.1	58.1	86.0	68.0	53.0	40.2	60.4	47.8	64.4	51.6
Cl	*0.01*	41.9	39.3	85.0	42.2	8.0	14.9	39.0	27.7	46.4	39.4
SO_4	*0.01*	69.8	63.9	138.0	81.8	38.0	32.3	68.9	43.7	68.9	47.5
HCO_3	*0.1*	244	262	372	329	262	159	269	236	171	158
NO_3	*1*	6.02	6.31	12.21	2.84	<1	4.36	4.16	4.31	<1	<1
F	*0.003*	0.133	0.113	0.112	0.139	0.105	0.090	0.098	0.093	0.123	0.124
µg/l											
Ag	*0.002*	1.950	0.830	0.400	0.160	0.190	0.210	0.010	0.010	0.010	0.010
Al	*0.5*	10.60	4.10	11.70	2.90	1.90	2.40	6.30	13.40	17.20	40.90
As	*0.03*	1.29	1.41	1.24	0.97	0.16	1.64	1.24	0.44	1.21	0.99
B	*2*	70	68	140	122	49	30	61	66	48	30
Ba	*0.05*	38.9	40.7	54.4	52.7	62.8	31.3	46.7	49.4	40.5	74.8
Be	*0.005*	0.007	0.005	0.006	0.005	0.007	0.008	0.005	<0.005	<0.005	0.021
Cd	*0.003*	0.072	0.091	0.026	0.031	0.011	0.027	0.024	0.006	0.016	<0.001
Ce	*0.001*	0.018	0.011	0.003	0.006	0.005	0.008	0.005	0.001	0.007	0.002
Co	*0.01*	0.12	0.10	0.23	0.09	0.03	0.06	0.10	0.09	0.04	0.15
Cr	*0.10*	0.17	0.22	0.13	<0.10	0.12	0.25	0.12	0.12	0.13	<0.10
Cs	*0.002*	0.109	0.030	0.028	0.009	0.008	0.011	0.005	0.011	0.033	0.043
Cu	*0.10*	4.56	3.00	2.83	2.29	1.01	2.53	1.92	0.80	1.63	0.02
Er	*0.001*	0.003	0.004	0.002	0.003	0.001	0.007	0.004	0.003	0.002	0.003
Eu	*0.001*	0.001	0.003	0.001	0.002	0.001	0.001	0.002	0.001	0.001	0.004
Dy	*0.001*	0.004	0.005	0.003	0.004	0.002	0.004	0.004	0.004	0.003	0.003
Fe	*0.5*	14.6	8.2	6.1	4.1	3.4	3.3	7.8	0.7	5.6	2.0
Ga	*0.005*	0.032	0.024	0.054	0.014	0.012	0.019	0.018	0.014	0.017	0.061
Gd	*0.002*	0.024	0.028	0.021	0.004	0.003	0.025	0.033	0.012	0.028	0.003
Ge	*0.03*	0.04	0.02	0.03	0.02	0.05	0.03	0.04	0.02	0.03	0.42
Ho	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
La	*0.001*	0.226	0.053	0.042	0.035	0.026	0.031	0.006	0.005	0.004	0.004
Li	*0.2*	9.5	9.1	22.9	16.9	9.7	2.8	6.3	6.1	3.4	4.1
Lu	*0.001*	0.001	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.001
Mn	*0.1*	3.0	2.4	2.6	1.0	1.1	1.0	0.9	0.6	0.7	8.3
Mo	*0.02*	1.02	1.08	0.96	0.68	0.49	1.19	1.21	1.23	1.30	0.39
Nd	*0.001*	0.011	0.008	0.004	0.006	0.005	0.007	0.005	0.006	0.005	0.006
Ni	*0.02*	2.56	2.12	2.79	1.66	0.24	1.49	1.90	1.92	1.28	4.52
Pb	*0.01*	1.05	0.43	0.16	0.15	0.14	0.24	0.29	0.04	0.23	0.05
Pr	*0.001*	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Rb	*0.01*	4.24	4.29	13.80	1.44	0.83	1.64	3.87	4.23	2.48	2.64
Sb	*0.01*	0.42	0.38	0.24	0.15	0.10	0.27	0.31	0.48	0.38	0.38
Se	*0.02*	0.27	0.29	0.42	0.31	0.40	0.20	0.27	0.08	0.24	0.07
Sm	*0.001*	0.003	0.003	0.004	0.005	0.004	0.001	0.004	0.003	0.004	0.004
Sr	*1*	551	566	1012	996	812	315	511	558	381	449
Tb	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Te	*0.02*	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Th	*0.001*	0.001	0.002	0.002	0.001	0.001	0.003	0.002	0.001	0.001	<0.001
Ti	*0.08*	0.54	0.42	0.27	0.26	0.28	0.30	0.16	0.11	0.12	0.08
Tl	*0.002*	0.008	0.007	0.001	0.008	0.01	0.006	0.009	0.01	0.011	0.015
Tm	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
U	*0.001*	1.21	1.32	1.88	1.40	0.65	1.01	1.40	0.42	1.35	0.28
V	*0.10*	0.89	0.88	0.97	0.58	0.20	0.79	1.12	0.90	0.85	1.76
W	*0.05*	0.05	0.07	<0.05	<0.05	<0.05	0.08	0.05	<0.05	0.07	<0.05
Y	*0.001*	0.03	0.03	0.019	0.021	0.012	0.027	0.027	0.028	0.022	0.031
Yb	*0.001*	0.005	0.004	0.002	0.004	0.001	0.006	0.006	0.008	0.008	0.003
Zn	*0.2*	15.5	13.8	8.7	6.2	3.6	5.7	4.3	1.2	4.5	21.1
Zr	*0.001*	0.024	0.017	0.027	0.015	0.007	0.01	0.093	0.009	0.075	0.005

Table S2.1 (continued)

		Fiumi Uniti rivers (n=9)								
Sample	***DL***	**R9**	**R10**	**R11**	**R12**	**R13**	**R14**	**R15**	**R16**	**R17**
X coord		11.74926	11.77173	11.78148	11.82139	12.06147	11.98376	12.03138	12.11078	12.21547
Y coord		43.93492	43.90056	43.89241	43.87684	44.12163	44.10794	44.33009	44.2977	44.39761
T *(°C)*	*0.1*	16.3	17.5	17.6	20.7	23	24.2	23.2	22.3	28.8
pH	*0.1*	8.2	7.9	8.05	7.8	8.1	8.1	8.0	7.9	7.5
EC *(µS/cm)*	*0.1*	376	346	304	390	602	489	662	850	627
mg/l										
K	*0.1*	1.9	1.9	1.7	1.7	5.3	4.0	4.8	8.4	5.8
Na	*0.1*	8.7	8.4	7.5	10.6	38.4	20.7	37.4	68.1	45.0
Mg	*0.01*	19.0	14.8	11.1	15.1	29.3	29.2	35.3	36.1	32.3
Ca	*0.01*	56.0	58.6	52.5	68.4	58.0	58.0	77.3	89.2	61.7
Cl	*0.01*	6.1	4.7	6.4	4.5	43.3	14.7	39.2	87.2	51.8
SO_4	*0.01*	39.6	37.2	23.4	33.3	111.0	95.4	123.0	119.0	106.0
HCO_3	*0.1*	262	238	214	287	269	275	336	439	262
NO_3	*1*	<1	<1	1.40	<1	6.12	2.58	7.29	11.58	6.14
F	*0.003*	0.092	0.079	0.044	0.095	0.167	0.158	0.121	0.13	0.152
µg/l										
Ag	*0.002*	0.010	0.090	0.590	0.200	0.280	0.310	0.120	0.220	0.100
Al	*0.5*	5.2	13.7	3.6	2.1	3.3	2.3	1.3	2.3	2.1
As	*0.03*	1.04	0.94	0.51	0.21	0.28	0.22	0.13	0.27	0.70
B	*2*	179	202	183	42	26	36	36	113	179
Ba	*0.05*	63.10	74.60	52.70	52.90	51.20	58.70	60.20	49.80	56.60
Be	*0.005*	0.007	<0.005	0.005	0.006	0.006	0.005	0.005	<0.005	0.006
Cd	*0.003*	0.026	0.082	0.011	0.013	0.014	0.013	0.006	0.008	0.011
Ce	*0.001*	0.012	0.005	0.005	0.006	0.007	0.004	0.003	0.003	0.005
Co	*0.01*	0.24	0.37	0.08	0.02	0.05	0.05	0.02	0.05	0.09
Cr	*0.10*	0.11	0.19	0.14	0.10	0.18	0.11	0.12	0.10	0.10
Cs	*0.002*	0.005	0.01	0.005	0.008	0.005	0.007	0.005	0.007	0.004
Cu	*0.10*	3.41	2.33	2.59	0.91	1.26	1.00	0.70	1.32	1.65
Er	*0.001*	0.003	0.003	0.002	0.002	0.003	0.002	0.002	0.002	0.002
Eu	*0.001*	0.003	0.001	0.001	0.002	0.002	0.003	0.001	0.002	0.002
Dy	*0.001*	0.003	0.003	0.002	0.002	0.003	0.002	0.002	0.002	0.003
Fe	*0.5*	7.4	9.7	2.9	13.1	5.3	6.9	1.7	2.8	4.9
Ga	*0.005*	0.016	0.046	0.008	0.006	0.008	0.006	0.007	0.006	0.008
Gd	*0.002*	0.019	0.046	0.003	0.002	0.003	0.003	0.003	0.003	0.002
Ge	*0.03*	<0.03	0.04	0.03	<0.03	<0.03	<0.03	0.03	<0.03	0.03
Ho	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
La	*0.001*	0.006	0.034	0.021	0.032	0.022	0.022	0.015	0.017	0.020
Li	*0.2*	22.4	24.6	26.9	8.6	4.2	6.9	8.0	21.4	26.0
Lu	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Mn	*0.1*	1.2	5.3	0.5	0.5	1.8	4.2	0.3	0.4	0.4
Mo	*0.02*	1.42	1.74	0.94	0.32	0.37	0.44	0.48	0.63	0.81
Nd	*0.001*	0.005	0.005	0.006	0.005	0.007	0.005	0.004	0.004	0.006
Ni	*0.02*	3.27	4.47	1.90	0.47	0.37	0.40	0.15	0.89	2.10
Pb	*0.01*	0.47	0.27	0.10	0.14	0.16	0.12	0.07	0.08	0.08
Pr	*0.001*	0.001	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001
Rb	*0.01*	2.18	3.66	1.77	0.91	0.84	0.90	0.61	1.12	1.38
Sb	*0.01*	0.32	0.45	0.52	0.27	0.24	2.21	0.29	0.23	0.31
Se	*0.02*	0.57	0.64	0.50	0.38	0.16	0.25	0.58	0.57	0.70
Sm	*0.001*	0.004	0.004	0.003	0.004	0.002	0.002	0.002	0.002	0.004
Sr	*1*	1140	1436	1364	743	435	599	742	1312	1261
Tb	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Te	*0.02*	<0.02	0.02	0.02	<0.02	0.02	<0.02	<0.02	<0.02	<0.02
Th	*0.001*	0.002	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.002
Ti	*0.08*	0.27	0.27	0.31	0.31	0.30	0.19	0.25	0.24	0.20
Tl	*0.002*	0.008	0.004	0.003	0.004	0.005	0.008	0.005	0.004	0.007
Tm	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
U	*0.001*	1.68	1.62	1.51	0.52	0.39	0.53	0.63	1.38	1.96
V	*0.1*	0.80	0.71	0.32	0.12	0.22	0.14	0.14	0.24	0.37
W	*0.05*	<0.05	0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Y	*0.001*	0.020	0.036	0.013	0.015	0.023	0.017	0.006	0.011	0.021
Yb	*0.001*	0.005	0.006	0.003	0.003	0.005	0.002	0.003	0.003	0.004
Zn	*0.2*	10.5	23.6	4.0	4.0	5.2	4.2	2.2	3.1	3.0
Zr	*0.001*	0.151	0.039	0.017	0.007	0.009	0.009	0.007	0.011	0.020

Table S2.1 (continued)

		Marecchia river (*n*=4)				*Conca river* (*n*=3)		
Sample	***DL***	**R18**	**R19**	**R20**	**R21**	**R22**	**R23**	**R24**
X coord		12.21737	12.40590	12.45113	12.53548	12.3523	12.67763	12.7013
Y coord		43.80233	43.98533	44.04374	44.06094	43.82106	43.93176	43.95930
T (°C)	*0.1*	30.3	29.7	32.8	34	16.6	35.2	28.9
pH	*0.1*	7.6	7.9	7.9	7.9	8.0	7.9	7.9
EC *(µS/cm)*	*0.1*	381	479	469	481	342	566	561
mg/l								
K	*0.1*	2.7	4.1	4.6	4.8	1.4	5.8	6.2
Na	*0.1*	18.8	25.4	29.5	30.7	9.7	48.9	44.5
Mg	*0.01*	18.8	19.2	20.3	20.3	12.0	27.6	25.3
Ca	*0.01*	47.4	64.7	56.3	60.0	56.5	46.2	61.0
Cl	*0.01*	8.8	15.4	17.0	19.8	6.3	45.4	38.7
SO_4	*0.01*	43.0	93.9	96.3	101.0	16.9	115.0	109.0
HCO_3	*0.1*	256	262	433	299	269	208	262
NO_3	*1*	<1	1.59	<1	1.08	2.46	2.15	2.08
F	*0.003*	0.140	0.162	0.147	0.173	0.123	0.257	0.256
µg/l								
Ag	*0.002*	0.010	0.010	0.010	0.020	0.010	0.010	0.010
Al	*0.5*	5.9	5.2	5.5	5.1	4.6	5.8	9.7
As	*0.03*	0.25	0.35	0.45	0.18	0.11	0.71	0.72
B	*2*	140	242	249	213	79.5	295	207
Ba	*0.05*	77.0	80.1	88.0	77.0	233.0	73.0	73.3
Be	*0.005*	0.005	0.008	0.006	0.005	0.005	0.006	<0.005
Cd	*0.003*	0.019	0.016	0.021	0.014	0.011	0.024	0.026
Ce	*0.001*	0.006	0.008	0.006	0.007	0.006	0.010	0.008
Co	*0.01*	0.03	0.06	0.07	0.05	0.05	0.11	0.03
Cr	*0.1*	0.12	0.08	0.08	0.10	0.14	0.10	0.12
Cs	*0.002*	0.020	0.011	0.011	0.010	0.004	0.009	0.009
Cu	*0.1*	2.11	1.86	1.82	1.84	1.23	2.32	2.20
Er	*0.001*	0.002	0.002	0.002	0.002	0.004	0.002	0.002
Eu	*0.001*	0.001	0.001	0.003	0.001	0.005	0.004	0.001
Dy	*0.001*	0.002	0.002	0.003	0.003	0.002	0.002	0.002
Fe	*0.5*	5.6	6.7	6.7	6.3	5.0	6.2	6.9
Ga	*0.005*	0.007	0.010	0.010	0.007	0.006	0.012	0.007
Gd	*0.002*	0.002	0.006	0.004	0.004	0.005	0.002	0.003
Ge	*0.03*	<0.03	0.03	0.03	<0.03	0.04	0.03	0.04
Ho	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001
La	*0.001*	0.005	0.006	0.005	0.006	0.007	0.006	0.006
Li	*0.2*	16.6	27.5	27.5	24.7	9.3	38.1	27.1
Lu	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Mn	*0.1*	0.7	1.2	0.9	1.6	0.7	0.9	1.1
Mo	*0.02*	0.72	1.35	1.19	0.94	0.31	2.02	1.56
Nd	*0.001*	0.005	0.005	0.005	0.004	0.003	0.006	0.004
Ni	*0.02*	0.47	1.41	1.53	0.74	0.62	1.98	2.13
Pb	*0.01*	0.31	0.29	0.31	0.29	0.30	0.33	0.40
Pr	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Rb	*0.01*	1.32	1.87	1.88	1.52	0.70	1.96	1.58
Sb	*0.01*	0.18	0.32	0.34	0.17	0.15	0.29	0.17
Se	*0.02*	0.36	0.26	0.27	0.30	0.78	0.32	0.32
Sm	*0.001*	0.003	0.005	0.003	0.003	0.006	0.004	0.005
Sr	*1*	786	1021	1007	1012	1267	996	921
Tb	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Te	*0.02*	0.024	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Th	*0.001*	0.001	0.002	0.002	0.001	<0.001	0.002	0.001
Ti	*0.08*	0.32	0.34	0.26	0.34	0.38	0.27	<0.08
Tl	*0.002*	0.011	0.011	0.012	0.011	0.005	0.008	0.010
Tm	*0.001*	0.001	0.001	0.001	0.001	0.001	0.001	0.001
U	*0.001*	0.60	0.63	0.60	0.58	0.37	1.74	1.14
V	*0.1*	0.30	0.28	0.34	0.17	0.21	0.51	0.28
W	*0.05*	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Y	*0.001*	0.010	0.015	0.012	0.012	0.015	0.013	0.013
Yb	*0.001*	0.002	0.004	0.003	0.003	0.003	0.003	0.003
Zn	*0.2*	4.4	5.9	4.5	5.9	4.6	6.2	16.3
Zr	*0.001*	0.117	0.107	0.113	0.111	0.108	0.103	0.100

Table S2.2 Coordinates (*WGS84 – EPSG: 4326*), phisical-chemical parameters, major ions and trace elements of ground waters. Detection limits are the same as the ones reported for surface waters (see Table S2.1)

	***Forlì** (n=7)*								***Rimini** (n=6)*				***Cesena** (n=4)*				
Sample	**GW28**	**GW29**	**GW30**	**GW31**	**GW32**	**GW33**	**GW34**	**GW35**	**GW36**	**GW37**	**GW38**	**GW39**	**GW40**	**GW41**	**GW42**	**GW43**	**GW44**
X coord	12.0606	12.0700	12.0644	12.0283	12.0283	12.0707	12.0706	12.5567	12.5535	12.5573	12.5572	12.5398	12.7298	12.2222	12.2182	12.2139	12.2179
Y coord	44.2010	44.2002	44.2016	44.2302	44.2301	44.2243	44.2243	44.0603	44.0624	44.0616	44.0617	44.0624	43.9483	44.1550	44.1538	44.1520	44.1472
T *(°C)*	16.8	18.5	20.9	17.5	16.8	15.9	19.2	18.2	16.3	15.9	16.5	17.6	18.5	17.6	18.8	17.5	18.3
pH	7.6	7.6	7.4	7.4	7.5	7.7	7.8	6.7	7.5	7.2	7.2	7.6	7.5	7.3	7.1	6.9	6.7
EC *(µS/cm)*	874	970	899	1017	781	679	683	948	947	739	901	878	1210	1209	1169	1233	1062
mg/l																	
K	3.8	4.6	5.3	5.0	3.8	3.4	3.4	2.8	2.8	7.8	2.4	3.5	4.8	3.6	3.1	2.5	2.4
Na	34.3	59.3	32.5	42.8	46.9	30.3	38.0	55.8	49.4	37.6	56.3	44.1	93.4	54.0	52.7	49.0	69.4
Mg	41.9	47.3	46.1	46.0	35.7	34.3	34.9	27.7	26.3	24.4	29.7	25.7	48.8	56.3	55.4	54.4	45.9
Ca	136.1	139.0	160.3	158.0	104.0	95.3	99.2	162.0	157.0	118.0	115.0	156.1	156.1	197.8	195.2	192.3	143.5
Cl	57.2	113.0	49.9	71.7	107.0	45.8	50.7	63.4	64.3	50.0	150.0	60.4	150.0	72.7	80.0	96.2	99.6
SO_4	97.1	73.1	111.0	130.0	64.6	62.8	36.9	123.4	101.5	105.6	28.9	101.0	158.2	267.0	223.0	179.1	76.2
HCO_3	507	567	543	537	372	397	464	543	482	384	384	433	543	586	610	610	549
NO_3	38.5	13.0	57.0	48.0	<1	<1	<1	34.3	65.4	21.3	7.0	69.9	39.1	13.4	45.0	50.8	64.7
F	0.056	0.054	0.065	0.100	0.082	0.064	0.160	0.102	0.085	0.086	0.139	0.185	0.169	0.108	0.115	0.156	0.249
µg/l																	
Ag	0.003	0.003	0.004	0.003	0.003	0.005	0.003	0.003	0.003	0.114	0.002	0.003	0.003	0.005	0.003	0.003	0.003
Al	6.4	3.7	5.2	4.5	3.9	5.4	3.3	4.8	8.6	30.2	4.7	8.7	5.5	5.0	5.2	5.5	7.8
As	0.26	0.14	0.11	0.13	0.13	0.82	0.37	0.08	0.07	0.07	0.10	0.07	0.08	0.12	0.10	0.12	0.13
B	90.1	97.4	79.1	112.0	85.6	68.4	83.3	114.0	129.0	163.0	162.0	136.0	228.0	177.0	145.0	138.0	122.0
Ba	116.0	112.0	74.4	60.3	122.0	138.0	220.0	54.7	47.8	55.9	1075.0	51.0	43.4	35.1	51.2	60.3	111.0
Be	<0.005	0.007	<0.005	<0.005	<0.005	<0.005	<0.005	0.005	<0.005	<0.005	<0.005	0.005	0.005	<0.005	<0.005	<0.005	<0.005
Cd	0.021	0.013	0.051	0.028	0.023	0.020	0.017	0.026	0.025	0.057	0.018	0.019	0.024	0.017	0.022	0.029	0.020
Ce	0.006	0.005	0.004	0.010	0.007	0.008	0.006	0.006	0.008	0.013	0.007	0.007	0.008	0.007	0.007	0.008	0.008
Co	0.09	0.01	0.03	0.12	0.23	0.05	0.01	<0.01	0.05	0.03	0.04	0.04	0.10	0.02	0.02	0.04	0.04
Cr	0.12	<0.1	0.18	0.04	<0.1	<0.1	<0.1	<0.1	<0.1	0.15	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cs	0.003	0.002	0.002	0.003	0.005	0.004	0.005	0.004	0.003	0.004	0.002	<0.002	0.004	0.002	0.010	0.009	0.007
Cu	1.15	1.55	3.45	1.52	0.77	0.58	0.20	1.36	1.73	5.40	0.24	0.55	1.62	0.67	0.91	1.68	2.50
Er	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.003	0.004	0.002	0.002	0.002	0.002	0.002
Eu	0.004	0.002	0.001	0.003	0.003	0.001	0.003	0.001	0.002	0.002	0.022	0.001	0.002	0.002	0.001	0.001	0.003
Dy	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.003	0.002	0.003	0.002	0.002
Fe	166.0	17.1	6.4	36.2	136.0	805.0	1213.0	4.6	12.0	20.2	1069.0	8.6	40.5	18.2	8.3	33.9	13.2
Ga	0.007	0.006	0.006	0.008	0.023	0.015	0.015	<0.005	0.005	0.006	0.015	0.006	<0.005	<0.005	<0.005	<0.005	0.005
Gd	0.004	0.003	0.002	0.003	0.003	0.003	0.006	0.002	0.004	0.003	0.011	0.003	0.003	0.003	0.002	0.003	0.004
Ge	<0.03	0.03	<0.03	0.03	<0.03	0.06	0.05	0.03	0.03	<0.03	0.08	<0.03	0.04	0.04	0.04	0.03	0.03
Ho	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
La	0.005	0.005	0.004	0.007	0.006	0.007	0.008	0.005	0.006	0.008	0.019	0.005	0.005	0.006	0.006	0.007	0.007
Li	14.1	15.9	13.2	15.3	11.2	12.1	10.6	6.8	7.4	12.3	8.4	9.4	24.9	20.8	19.3	17.1	17.3
Lu	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001
Mn	46.6	2.6	1.5	107.0	392.0	123.0	127.0	0.9	1.4	2.4	263.0	1.0	2.7	7.1	0.9	1.2	31.7
Mo	0.31	0.41	0.31	0.50	0.30	0.75	0.64	0.19	0.23	0.34	0.92	0.28	0.50	0.37	0.29	0.37	0.58
Nd	0.004	0.005	0.005	0.005	0.006	0.005	0.004	0.004	0.005	0.006	0.006	0.005	0.004	0.007	0.005	0.006	0.006
Ni	18.60	2.11	0.36	2.00	1.28	2.99	1.78	0.72	2.02	1.71	0.43	0.95	1.63	1.28	1.11	1.90	1.02
Pb	0.46	0.32	0.34	0.36	0.30	0.38	0.27	0.34	0.35	0.89	0.28	0.32	0.29	0.34	0.45	0.75	0.54
Pr	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.001
Rb	0.40	0.34	0.56	0.18	0.65	0.28	0.34	0.28	0.23	0.47	0.22	0.25	0.63	0.51	0.60	0.45	0.44
Sb	0.03	0.03	0.04	0.07	0.04	0.02	0.01	0.03	0.03	0.10	0.02	0.03	0.04	0.03	0.04	0.04	0.03
Se	2.97	1.91	4.86	9.68	0.03	0.22	0.02	1.70	1.76	1.04	0.32	1.13	3.67	12.10	9.46	7.82	4.42
Sm	0.003	0.004	0.001	0.003	0.003	0.003	0.004	0.003	0.004	0.003	0.009	0.005	0.004	0.003	0.004	0.004	0.002
Sr	1415	1617	1210	1013	1195	1460	992	684	697	1025	1290	878	966	1756	1646	1521	1444
Tb	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Te	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.02
Th	0.001	<0.001	0.001	<0.001	0.001	0.001	0.001	0.001	0.001	0.001	<0.001	0.001	0.002	0.001	0.001	<0.001	0.001
Ti	0.12	0.19	0.35	0.17	0.11	0.23	0.11	0.09	0.18	0.24	<0.08	0.14	0.15	0.15	0.12	0.25	0.12
Tl	0.002	0.006	0.005	0.003	0.002	0.006	0.004	0.001	0.002	0.003	0.003	0.002	0.002	0.005	0.003	<0.001	0.005
Tm	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
U	2.22	2.20	2.48	3.06	8.54	1.58	0.055	0.90	0.99	0.59	0.29	0.86	2.94	3.38	3.08	3.27	3.19
V	0.25	0.26	0.28	0.15	<0.1	<0.1	<0.1	0.10	0.10	<0.1	<0.1	0.11	0.15	0.21	0.18	0.16	0.21
W	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Y	0.018	0.020	0.018	0.024	0.021	0.019	0.015	0.015	0.021	0.019	0.026	0.022	0.016	0.024	0.022	0.026	0.021
Yb	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.004	0.003	0.005	0.003	0.002	0.002	0.003	0.002
Zn	45.4	12.1	35.8	20.9	19.5	39.1	24.0	11.6	28.6	35.8	13.9	8.5	59.2	19.0	46.1	46.7	19.4
Zr	0.090	0.116	0.122	0.109	0.087	0.086	0.087	0.121	0.141	0.139	0.087	0.136	0.139	0.132	0.135	0.138	0.130

Chapter 3

OCCURRENCE AND DISTRIBUTION OF SIX SELECTED ENDOCRINE DISRUPTING COMPOUNDS IN SURFACE- AND GROUNDWATERS OF THE ROMAGNA AREA (North Italy)

1 INTRODUCTION

Endocrine Disrupting Compounds (EDCs) are a wide group of chemicals that can alter functions of the endocrine system, consequently causing adverse effects in an intact organism, its progeny or (sub)populations (EC 1996). These contaminants of emerging concern can be both natural, produced by humans, animals or plants, or synthetic, being present in human-made compounds such as personal care products, plastics, pharmaceuticals, industrial compounds and their by-products. Among EDCs, estrogens, phenolic compounds and perfluorinated compounds have raised increasing concern among scientists due to their widespread use and detection in rivers all around the world (Lapworth et al. 2012; Meffe and Bustamante 2014; Sunantha and Vasudevan 2016). Estrogens are both natural (estrone, 17-ß estradiol, estriol) and synthetic (17α-ethinylestradiol) hormones produced by humans and animals or administered to them to treat estrogenic dysfunctions or improve productivity. Perfluorinated compounds (PFCs) have been used for decades in several industrial and household applications for their high thermal and oxidative resistance, and they are commonly found in everyday products, such as cleaning products, non-stick cookware, food packaging, carpets and textile manufactures, fire-fighting foams, semiconductors (Knepper and Lange 2012). Among phenolic substances, the most studied compound is bisphenol A (BPA), a monomer used for the production of polycarbonate and epoxy resins, and present in food and beverage cans, powder paints, as additive in thermal paper, plastics and for the encapsulation of electrical parts (Geens et al. 2012). The aquatic environment is the primary most affected compartment, since EDCs are mainly released in the environment through municipal and industrial wastewater treatment plants (WWTPs). As a consequence of their low degradation, they are detected in the receiving river bodies, groundwaters, sediments and even biota (Zhang et al. 2016). The potential harmful effects that these substances can have upon organisms have been only partially recognized by public authorities. EU WFD (2013) identified PFOS as one of the 45 priority substances to be monitored in water bodies, along with a watch list of substances, including EE2 and E2, in order to gather information data and determine appropriate measures to manage the risk posed by those substances. As regards BPA, no regulations have still been provided by public authorities, though the US FDA in 2013 banned its use for the production of

baby bottles and sippy cups (FDA 2013) and the EU EFSA (EFSA 2015a) posed a recommended Total Dietary Intake (TDI) of BPA of 4/μg/kg body weight/day.

The aim of this study was to evaluate the occurrence of perfluorinated compounds (perfluorooctanoic acid – PFOA; perfluorooctane sulfonate - PFOS), estrogens (estrone - E1; ß-estradiol – E2; 17α-ethinylestradiol – EE2) and phenolic compounds (bisphenol A - BPA) in surface- and groundwaters of the Romagna area. As far as the authors know, this is the first report on the area and it will be useful to gather information on how far contamination by EDCs is spread in Italian river bodies, to fulfill EU Water Framework Directive purposes. In this sense it is a preliminary study on EDCs occurrence and behavior in the aqueous environment, which is essential for the future management of the risk related to EDCs.

2 MATERIALS AND METHODS

2.1 Study area

The study area is located in the north-eastern part of Emilia-Romagna region (Italy), south of the Po River delta (**Figure 3.1**). The area is a quite high urbanized area counting a total of 1,281,243 inhabitants with a population density of 200.8 pop/km^2 that increases of 54.6% for tourism along the coast during the summer season (Emilia-Romagna Region 2014). Two different sections of the study area can be identified: a south-western part, dominated by Apennines Mountains, and a north-eastern one, characterized by the Apenninic alluvial plain where most of the population and human activities are concentrated.

The main river bodies of the Romagna area are Lamone and Fiumi Uniti, on the northern side, and Marecchia and Conca rivers, on the southern portion. All rivers have a SW-NE flow direction, from the Apennines Mountains to the Adriatic Sea. Their flow rate is highly variable throughout the year, being mostly dependent on the rainfall rates; water abstractions for irrigation purposes are a further, remarkable pressure factor for river water quantity, leading to water shortage during summer periods. Embankment of the northern rivers (Lamone and Fiumi Uniti) is present from the closure of their mountain basin up to their mouth, so that in the plain section the rivers do not receive any water input, except from some artificial irrigation channels. In particular, CER channel represents the most important artificial water input into the Lamone River, with 224 Mm^3/year of water introduced in the river to prevent its summer drought (Laghi 2010).

Regarding land use (**Figure 3.1**), the Apennine section is dominated by a semi-natural environment made of beech, chestnut and oak woods with minor farming and orchard. The plain section is mainly characterized by farming activities, with the presence of vineyards, orchards, olive groves and arable crops. The major cities located in the area are Faenza, at the closure of the mountain basin of the Lamone River; Ravenna,

between Lamone and Fiumi Uniti river mouths; Forlì, located between Ronco and Montone rivers; and Rimini, on the south in correspondence of the Marecchia river mouth. The main industrial activities which may be related to the production of the EDCs selected for this study are industries of plastic production (43 plants in Forlì, 18 in Ravenna and 15 in Rimini areas), food industry (290 plants in Ravenna, 214 in Rimini, 177 in Forlì, 144 in Cesena and 90 in Faenza) and industries for electrical devices production (41 plants in Ravenna, Cesena and Rimini, 29 in Forlì and 22 in Faenza) (ISTAT 2014). Major stock-breeding is concentrated in the Forlì province, with livestock (cattle, pig, sheep and poultry farms) going from 122,371 to 3,366,090 units that require a huge amount of water and whose organic sewages can potentially affect river water quality. The presence of wastewater treatment plants (WWTPs) is a further pressure of contamination by EDCs. Faenza WWTP has been designed to treat wastewaters coming from 100,000 Population Equivalents (PE), processing 6.5 millions of sewages and releasing around 17,800 m^3/day of treated water directly in the Lamone River. Forlì WWTP treats effluents of 250,000 PE, for a total of 18,485 Mm^3/year of input sewages that are then released into Ronco River. Rimini WWTP, which discharges wastewater effluents in the Marecchia River mouth, can receive sewages coming from 250,000 PE, with an annual inflow of 29,642 Mm^3 (HERA 2015).

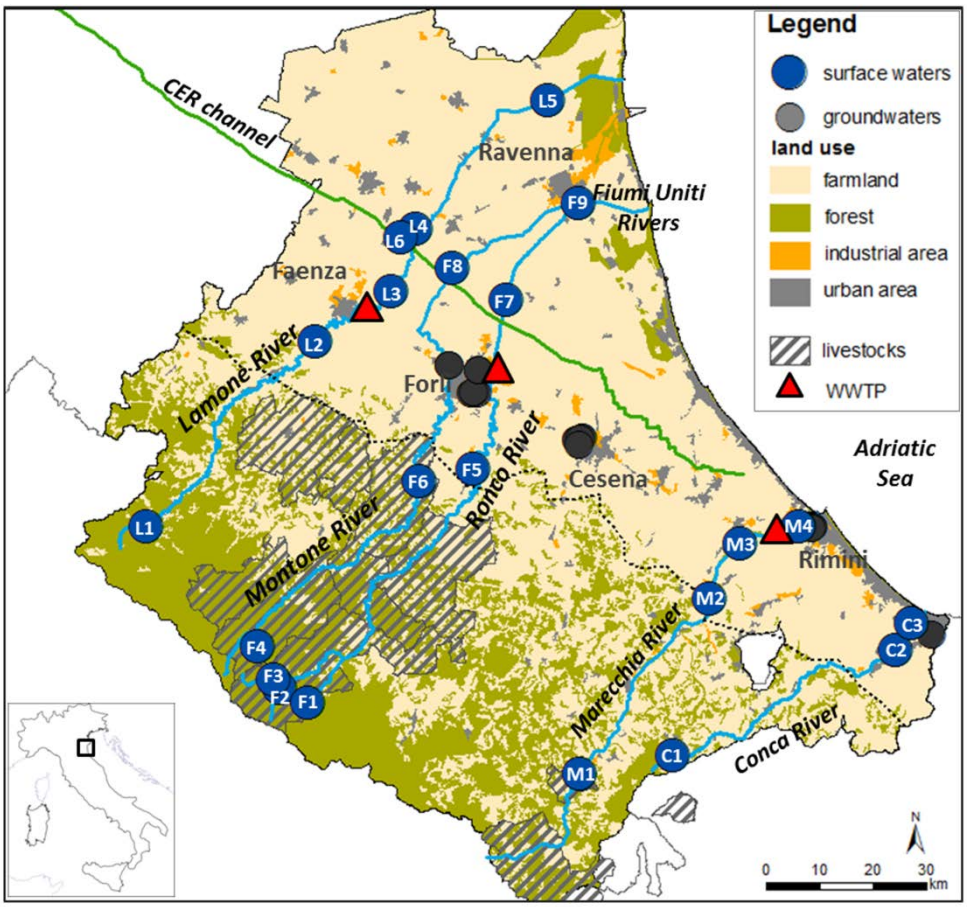


Figure 3.1 Summary map representing surface and groundwater sampling points, land use and WWTP distribution in the study area. The diagonal black dotted line represents the border between the mountain section and the alluvial plain of the study area

2.2 Chemicals and reagents

All native standards of ß-estradiol (E2), estrone (E1), 17α-ethinylestradiol (EE2), bisphenol A (BPA), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) were purchased by Sigma Aldrich. Isotope- labeled compounds used as internal standards were purchased by CDN Isotopes (E2-d_2 and BPA-d_6) and Wellington Laboratories Inc. ($^{13}C_4$-PFOA). Solvent reagents from Sigma Aldrich (water, methanol, ammonium acetate and ammonium hydroxide) were HPLC- analytical grade.

2.3 Sampling, sample storage and treatment

Sampling campaign was carried out in July 2015, which corresponded to the dry season. Summer sampling period was chosen with the purpose of analysing the worst scenario regarding EDC contamination in the study area. It is well known, in fact, that contamination in the aquatic compartment is highly influenced by weather conditions; generally, contaminants are diluted during the wet season as a consequence of the increase in rainfall and river flow rates, while they get more concentrated in summer periods, in correspondence to the absence of rainfall and to high evaporation rates. In this context, analysing EDCs in the summer period allowed to describe the most anomalous situation regarding EDCs occurrence in water.

Rivers crossing the major cities of the study area (Lamone; Ronco and Montone, which form the Fiumi Uniti River; Marecchia; Conca) were selected to investigate the anthropogenic impact on water quality. In addition, a sample from the artificial CER channel was studied (sample point L6, **Figure 3.1**), since part of its flow is diverted into the Lamone River to manage its water shortage in the summer season. Selection of sampling points of surface waters was made in order to have a homogeneous distribution of samples from the headwater to the river mouth, taking into account also possible anthropogenic factors that could be relevant for the occurrence of EDCs in rivers. Detailed information about each sampling station can be found in **Table S3.1** of the *Supporting Material*. Groundwaters were sampled selecting those wells of confined aquifers used to produce drinking waters, with the purpose of examining possible threats to human health. A total of 39 samples were collected: 22 from surface waters and 17 from groundwater wells. For each sampling point, 500 mL of water were collected in PE bottles previously rinsed with methanol to avoid EDCs adhesion on bottle surfaces. Samples were stored in the laboratory at 4 °C in darkness until analysis. All samples were processed within 36 h from the sampling to prevent degradation of analytes, following the method described by the Italian Institute of Health (Achene et al. 2011a). Briefly, 500 mL of water were spiked with a mixture of labeled- internal standards at 30 ng/L and filtered firstly with glass microfiber filters (1.60 µm pore size, 47 mm diameter; Whatman, Kent, UK) and then with cellulose acetate filters (0.45 µm pore size, 47 mm diameter; Whatman, Kent, UK). Solid phase extraction was subsequently performed through Oasis HLB cartridges (6 cc, 200 mg; WatersCorp, Milford, MA) previously conditioned with 6 mL of methanol and 6 mL of water. Samples were passed through the

cartridge under vacuum, at a flow rate of 10 mL/min. Cartridges were then vacuum-dried for 15 minutes and eluted with 6 mL of methanol, evaporated under a N_2 gentle stream up to a volume of 500 µL and split in two vials of 250 µL each. The first set of vials was evaporated until dryness and reconstituted in 125 µL of a mixture of water:methanol (90:10) for PFCs detection. The remaining vials were evaporated until dryness and reconstituted in 125 µL of (50:50) water:methanol for estrogens and BPA analysis. Procedural blanks were prepared in parallel with 500 mL of deionized water to detect any kind of contamination occurring during sample treatment. All samples were analysed in triplicates.

2.4 PFCs chromatographic conditions and mass spectrometry detection

PFOA and PFOS were analysed by an UPLC-MS/MS system, equipped with an electrospray ionization source (Waters Corporation). Separation of compounds was achieved through an Acquity UPLC BEH C18 column (Waters Corporation); the flow rate was 0.4 mL/min and the volume injection 10 µL. Analyses were done in negative ion mode using 20 mM NH_4Ac methanol (A) and 20 mM NH_4Ac water (B) as mobile phases. Elution gradient started with 20% of A and gradually increased to 80% in 3.50 min and to 90% in 2 min, followed by 1.50 min isocratic elution and a 2 min linear gradient back to initial conditions, kept for 2 min to equilibrate the column before a new injection. The optimized mass spectrometry parameters were as follows: capillary 2.80 kV; source offset 50 V; desolvation temperature 600 °C; desolvation gas flow 1000 L/Hr; cone gas 150 L/Hr; nebulizer gas 7.0 bar; collision gas flow 0.15 ml/min. Analyses were performed in Multiple Reaction Monitoring (MRM). **Table S3.2** summarizes the mass transitions selected for each compound and further MS parameters details.

2.5 BPA and estrogens chromatographic conditions and mass spectrometry detection

Estrogens and BPA were analysed by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS, Thermo Fisher Scientific, San Jose, CA) equipped with an electrospray ionization source operating in negative conditions. Mobile phases consisted in 0.02% NH_4OH methanol (A) and 0.02% NH_4OH water (B) and compounds were separated using an Acquity UPLC BEH C18 column (Waters Corporation). The elution gradient started at 50% of A and rapidly increased to 70% A (1 min) and then to 100% A in 7.50 min and kept at isocratic conditions for 1 min; after the elution of all compounds, turned back to initial conditions in 1 min and stayed at 50% A for 3 min to let the column equilibrate. A 100% methanol sample was injected every three samples. MS parameters were as follows: 40 (arb) sheat gas; 15 (arb) aux gas; 1 (arb) sweep gas; 350 °C ion transfer tube temperature; 300 °C vaporizer temperature. In **Table S3.3** the selected mass transitions, as well as MS detection parameters, are reported.

2.6 QA/QC

Each water sample was analysed in triplicate. Procedural blanks were analysed as well, and blank concentrations were subtracted, when present. To rule out any system contamination or compounds retention in the column, blanks of pure water:methanol (50:50) were run every three sample injections.

Six-point calibration curves (0-100 ng/mL) for the two analytical groups of EDCs were prepared in a mixture of water:methanol at the same initial conditions of samples. A good linearity was achieved for all compounds, with a coefficient of determination (R2) always greater than 0.97. Method Limit of Detection (MDL) and Method Limit of Quantification (MQL) were calculated as the concentration that yielded a signal-to-noise ratio of 3 (MDL) and 10 (MQL). For each compound, recovery rates were calculated by spiking blank samples (n=3) with 20 ng/L of a mixture of all EDCs. Information about MDL, MQL and Recovery are available in **Table 3.1**. Quantification of EDC analytes was performed by the internal standard method in order to correct for recovery and matrix effect, by adding a mixture of labelled standards to water samples before the filtration step.

Intra-day 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, %). Values concerning precision are also reported in **Table 3.1**.

Table 3.1 List of the investigated EDCs, mass transitions, MDL and MQL

Compounds	Mass transitions *(m/z)*	MDL *(ng/L)*	MQL *(ng/L)*	Compounds	Mass transitions *(m/z)*	MDL *(ng/L)*	MQL *(ng/L)*
PFOA	413>369 413>369	0.06	0.21	**E1**	269.1>145 269.1>143	0.16	0.52
PFOS	499>99 499>80	0.08	0.25	**E2**	271.1>183 271.1>145	0.45	1.50
BPA	227>212 227>133	0.61	2.02	**EE2**	295.1>145 295.1>159	0.56	1.88

2.7 Statistical analyses

Values below the MQL were substituted with half the quantification limit. All statistical analyses (mean, median, standard deviation, correlation analysis and cluster analysis) were performed with R software. Prior to the execution of multivariate analysis, variables were standardized. Pearson correlation coefficient among variables was calculated, setting the statistical significance level at α=0.05. Hierarchical cluster analysis was performed using correlation distance among EDC variables and inorganic water elements obtained from an already published work conducted on the same sampling campaign (Pignotti et al. 2017a), in order to investigate the degree of relation among them. All observations of surface waters

(n=22) were used for statistical analyses. Distribution maps were realized using ArcGIS 10.1 software; concentration values in the maps were grouped into 5 classes, according to percentiles: I class (0-20th percentile); II class (20th – 40th percentile); III class (40th – 60th percentile); IV class (60th – 80th percentile); V class (80th -100th percentile).

3 RESULTS

3.1 PFCs occurrence in the Romagna area

Table 3.2 reports concentrations of the selected endocrine disruptors in the analysed surface- and groundwaters. PFCs were lower than the MQL in all groundwater samples and in Conca River.

Table 3.2 EDCs minimum and maximum concentrations (ng/L), mean (ng/L), standard deviation (SD, ng/L) and frequency (%) in surface- and groundwaters of the Romagna area

	Lamone – CER system *(n=6)*					***Fiumi Uniti Rivers*** *(n=9)*					***Marecchia River*** *(n=4)*					***Conca River*** *(n=3)*		
	Min	**Max**	**Mean**	**SD**	**%**	**Min**	**Max**	**Mean**	**SD**	**%**	**Min**	**Max**	**Mean**	**SD**	**%**	**Min**	**Max**	**%**
PFOA	<MQL	9.1	4.3	3.4	83	<MQL	17.7	3.4	6.1	33	<MQL	1.5	0.7	0.7	75	<MQL	<MQL	-
PFOS	<MQL	1.6	0.7	0.6	67	<MQL	2.9	0.6	0.9	22	<MQL	5.5	1.7	2.5	50	<MQL	<MQL	-
BPA	2.8	117.7	36.7	42.1	100	<MQL	93.1	30.7	32.1	89	44.7	171.3	97.5	53.9	100	<MQL	<MQL	-
E1	<MQL	26.0	8.6	11.0	50	<MQL	28.0	8.9	12.0	44	<MQL	3.6	1.7	1.7	50	<MQL	<MQL	-
E2	<MQL	10.14	-	-	17	<MQL	39.7	8.5	14.7	44	<MQL	<MQL	-	-	-	<MQL	<MQL	-
EE2	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-

	Forlì groundwaters *(n=7)*					***Cesena groundwaters*** *(n=4)*					***Rimini groundwaters*** *(n=6)*				
	Min	**Max**	**Mean**	**SD**	**%**	**Min**	**Max**	**Mean**	**SD**	**%**	**Min**	**Max**	**Mean**	**SD**	**%**
PFOA	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-
PFOS	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-
BPA	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-
E1	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-
E2	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-
EE2	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-	<MQL	<MQL	-	-	-

PFOA and PFOS concentration levels were comparable in Lamone, Fiumi Uniti and Marecchia rivers. PFOA showed higher concentrations than PFOS in both Lamone and Fiumi Uniti rivers, with mean values of 4.3±3.4 ng/L and 3.4±6.1 ng/L, respectively. In Marecchia River, on the contrary, PFOS was detected at slightly higher concentrations (maximum value of 5.5 ng/L, in contrast with 1.5 ng/L for PFOA).

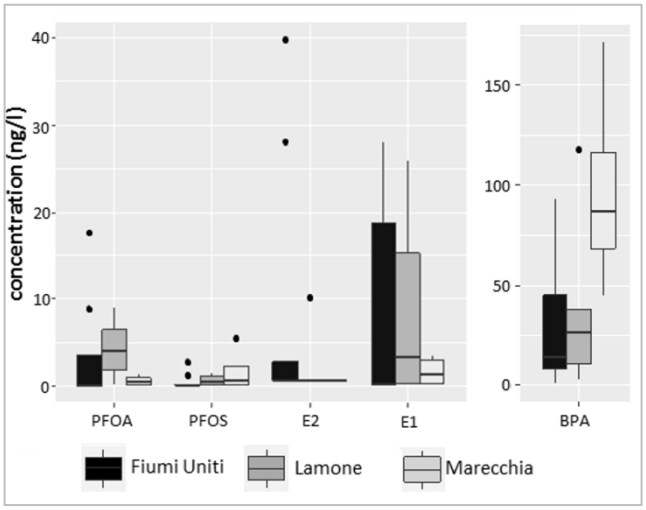


Figure 3.2 Boxplots representing the distribution of EDC concentrations (ng/L) detected in surface waters. Neither Conca River nor groundwaters were included because all EDCs were below the MQL

Figure 3.2 reports distribution of EDC concentration values in surface waters. As regards PFCs, it can be noticed that PFOA was more common than PFOS in the northern rivers (Lamone and Fiumi Uniti), with a slight higher variability in comparison with PFOS, which in turn showed a narrower range of concentrations. Lamone River displayed a more homogeneous distribution of PFOA values, with a median centering the range of concentrations, while Fiumi Uniti Rivers showed a right-skewed population, with two outliers at the highest concentrations. PFOS was detected in few samples of the Fiumi Uniti Rivers (22% of detection), while in the Lamone River concentrations were in a restricted range (<MQL-1.7 ng/L), resulting in the flattened boxplots of **Figure 3.2**. The Marecchia River, on the other hand, showed a slight higher variability in PFOS concentrations (range <MQL-5.5 ng/L) and lower values of PFOA (mean concentration of 0.6 ± 0.7 ng/L). Overall, concentrations of PFCs found in waters of the Romagna area were not so high, since the maximum value recorded for PFOA was 17.7 ng/L and 5.5 ng/L for PFOS. **Table 3.3** summarizes PFC concentrations reported by other studies on surface waters. PFC occurrence in the Romagna area was comparable to German rivers (Ahrens et al. 2009a; 2009b; Möller et al. 2010), Brazilian rivers (Quinete et al. 2009), Indian rivers (Yeung et al. 2009), Spanish Catalan rivers (Sanchez-Avila et al. 2010) and China rivers and coastal waters (Yang et al. 2011). On the other hand, concentrations were lower than the range of values recorded in Ebro and Guadalquivir river basins (Lorenzo et al. 2016), in French rivers (Munoz et al. 2015), Taihu river, in China (Yang et al. 2011) or in Hudson River (Sinclair et al. 2006). The results reported by Valsecchi et al. (2015) and Castiglioni et al. (2015) in the northern part of Italy (Po River and its tributaries) were up to 10 times higher than those found in this work, reaching values of 122.4 ng/L and 303 ng/L for PFOA. This suggests that, even if the Po River basin and Romagna rivers are quite close, the two areas are characterized by different sources of these contaminants of emerging concern. That aside, PFOA and PFOS concentrations were below the threshold concentrations recently proposed by European and Italian legislations as the maximum acceptable concentration in inland waters of 36 µg/L for PFOS (EU 2013) and 0.1 µg/L for PFOA (Legislative Decree No. 172/2015).

3.2 Estrogens and BPA occurrence in the Romagna area

Both estrogens and BPA were below the MQL for all the groundwater samples. Conca River was found not to be affected by contamination of these classes of compounds, as well. Estrogens (E2, E1) were detected at concentrations above the MQL in Lamone-CER channel and Fiumi Uniti Rivers, while only E1 was recorded in the Marecchia River. The synthetic hormone 17α-ethinylestradiol was never detected in any of the analysed samples (**Table 3.2**). Among the estrogen class, E1 was the most common, being detected in 50% of samples of the Lamone River, 44% of Fiumi Uniti Rivers and 50% of samples in the Marecchia River, as shown in **Figure 3.2**. In detail, Fiumi Uniti resulted to be the most affected river as regards estrogens occurrence, displaying the highest values and the highest frequency of detection for both E1 and E2. Moreover, E2 in the Fiumi Uniti Rivers was recorded with a very high variability, as the mean value and its wide standard deviation suggest (8.5±14.7 ng/L), as well as the boxplot in **Figure 3.2**, which shows a narrow interquartile distance (<MQL-2.9 ng/L) and two higher outliers very distant from the population data (39.7 and 27.9 ng/L). Concentrations of E2 in Lamone were occasional and below detection limit in Marecchia and Conca rivers (**Figure 3.2**). E1 displayed a higher detection and variability in Fiumi Uniti and Lamone rivers, with no outliers present, revealing a more homogenous behavior of the population data. Marecchia River recorded the presence of E1 in the 50% of the samples, but in a more restricted range if compared with the other two rivers (<MQL-3.6 ng/L).

Regarding BPA, concentration levels in the three rivers were of the same order of magnitude (mean values of 36.7 ± 42.1 ng/L in Lamone River, 30.7±32.1 ng/L in Fiumi Uniti Rivers and 97.5±53.9 ng/L in Marecchia River). Though, it should be noticed that Marecchia River was the most affected river by BPA contamination, since the compound was detected in all sampling points and with the highest mean and maximum value. This different behavior is highlighted by the boxplots in **Figure 3.2**, which show a similar distribution pattern for the Lamone and Fiumi Uniti population data, with the upper quartile below 50 ng/L. Contrariwise, the population of the Marecchia River is characterized by higher values, with the lowest quartile at 67.9 ng/L and the upper quartile at 116.5 ng/L.

Table 3.3 PFOA and PFOS concentrations around the world. Concentration and mean values are expressed as ng/L

Location	Water typology	Compounds	Concentration range (mean)	References
Italy				
Arno	river water	PFOA	16 - 40 (28)	Valsecchi et al. 2015
		PFOS	0 - 6 (3)	
Tevere	river water	PFOA	<0.5	
		PFOS	<2.5	
Po	river water	PFOA	17 - 93 (46)	
		PFOS	<2.5 - 10 (5)	
Po tributaries		PFOA	3 - 303 (42)	Castiglioni et al. 2015
		PFOS	5 - 43 (14)	
Spain				
Ebro	river water	PFOA	2.0 - 125.0 (7.3)	Lorenzo et al. 2016
		PFOS	0.1 - 27.0 (2.2)	
Guadalquivir	river water	PFOA	4.1 – 188.6 (11.6)	
		PFOS	0.01 – 42.6 (1.8)	
Catalonian rivers	river water	PFOA	0.79 – 9.63	Sanchez-Avila et al. 2010
		PFOS	1.09 – 9.56	
Germany				
Elbe River	river water	PFOA	2.8 – 9.6 (6.36)	Ahrens et al. 2009a
		PFOS	0.5 – 2.9 (1.62)	
German Bight	coastal waters	PFOA	2.67 – 7.83 (4.05)	Ahrens et al. 2009b
		PFOS	0.69 – 3.95 (1.39)	
Rhine River	river water	PFOA	0.61 – 4.07 (2.38)	Möller et al. 2010
		PFOS	1.41 – 7.34 (3.81)	
China				
Liao river	river water	PFOA	<0.7 – 27.9 (10.9)	Yang et al. 2011
		PFOS	<0.7 – 6.6 (0.33)	
Taihu river	river water	PFOA	10.6 – 36.7 (21.7)	
		PFOS	3.6 – 394 (26.5)	
India				
India rivers	river water	PFOA	<1 – 23.1	Yeung et al. 2009
		PFOS	<0.083 – 3.91	
Brazil				
Paraiba do Sul River	river water	PFOA	<0.09 – 1.22 (0.65)	Quinete et al. 2009
		PFOS	<0.1 – 1.32 (0.50)	
U.S.A.				
Hudson river	river water	PFOA	22 – 173 (35)	Sinclair et al. 2006
		PFOS	1.5 – 3.4 (1.7)	
Niagara river	river water	PFOA	18 – 22 (19)	
		PFOS	3.3 – 6.7 (5.5)	

Concentrations of estrogens found in this study were similar to those reported by Zhang et al. (2014) for estrone and by Chen et al. (2007) for ß-estradiol, and comparable to rivers on the southwest of Germany, as well (Körner et al. 2001); they were lower than concentrations registered in the Venice lagoon (Italy), which highlighted very anomalous situations (Pojana et al. 2007), as shown in **Table 3.4**.

BPA range of values detected in this study were comparable with those ones reported on already published studies (Table 3.4), such as the Iberian rivers (maximum value: 281 ng/L; Gorga et al. 2015), Tagus River (maximum: 190 ng/L; Rocha et al. 2015) or Songhua River, in China (Zhang et al. 2014). Nonetheless, they

were slightly higher than concentrations found in Krähenbach River, Germany (Körner et al. 2001) and in coastal environments of South Florida (Singh et al. 2010).

Table 3.4 Estrogens and BPA concentrations around the world. Concentration and mean value are axpressed as ng/L

Location	Water typology	Compound	Concentration range (mean)	References
Italy				
Italian WWTPs effluents	river waters	E1	<LOD - 48	Castiglioni et al. 2005
		E2	<5.2	
		EE2	<4.6	
Venice lagoon	coastal waters	E1	<1.2 – 10 (1.81)	Pojana et al. 2007
		E2	<1.0 – 175 (14.23)	
		EE2	<0.8 – 34 (8.20)	
		BPA	<1.0 – 145 (13.58)	
Spain				
Iberian rivers	river waters	E1	<0.17 – 7.3	Gorga et al. 2015
		E2	<0.12 – 7.8	
		EE2	<0.47 – 2.2	
		BPA	<0.39 - 281	
Portugal				
Tagus River	river waters	E1	2.4 – 4.0 (3.08)	Rocha et al. 2015
		E2	4.9 – 10.1 (8.43)	
		EE2	4.6 – 9.14 (5.42)	
		BPA	15.1 – 190 (69.98)	
Germany				
Körsch river	river waters	E1	<2.5 – 49 (7.6)	Körner et al. 2001
		E2	<0.66 - 1.8 (0.78)	
		BPA	<LOD - 272 (72)	
Krähenbach river	river waters	E1	<2.5 - 22 (1.7)	
		E2	<0.66	
		BPA	<LOD - 59 (21)	
China				
Dan-Shui River	river waters	E1	22.4 – 66.2 (34.7)	Chen et al. 2007
		E2	1.40 – 33.9 (14.4)	
		EE2	7.53 – 27.4 (15.3)	
Ningbo City rivers	river waters	E1	<0.2 – 8.65	Wang et al. 2015
		E2	<1.4	
		EE2	<1.2 – 38.1	
		BPA	13.57 – 3336.7	
Songhua river	river waters	E1	0.84 – 17.8 (4.20)	Zhang et al. 2014
		E2	<LOD – 1.16 (0.11)	
		EE2	<LOD	
		BPA	8.24 – 263 (52.0)	
South Korea				
Han River	river waters	E1	0.2 – 4.2 (1.6)	Yoon et al. 2010
		E2	<0.5	
		EE2	<1.0	
		BPA	6.9 – 59 (27)	
U.S.A. (South Florida)				
Miami River	coastal waters	E1	0.90 - 2.9	Singh et al. 2010
		E2	<LOD	
		BPA	4.4 - 190	
Key Largo Harbor	coastal waters	E1	0.66 - 5.2	
		E2	<LOD - 1.8	
		BPA	4.8 - 32	
Looe Key	coastal waters	E1	<LOD - 0.88	
		E2	<LOD	
		BPA	<LOD	

4 DISCUSSION

4.1 EDCs occurrence in surface- and groundwaters

None of the selected EDCs were detected at concentrations above the MQL in the groundwater samples. These samples come from confined aquifers belonging to the Quaternary succession (Upper Pleistocene) and are used to provide drinking water to the areas of Forlì, Cesena and Rimini. These confined aquifers are recharged indirectly by rainfalls and river waters in the alluvial fan located at the end of the mountain sections. Recharge rates are very slow, with an average of 1.18 m^3/s/year for aquifers belonging to the Ronco-Montone alluvial fan (Emilia-Romagna Region, 2009) and 0.7-1.3 m^3/s/year for the aquifers of the Marecchia alluvial fan (Emilia-Romagna Region, 2006). The absence of any of the EDCs in groundwaters implies an effect of dilution by rainfalls that leads to undetectable concentrations of contaminants in the aquifers. Spatial distance of the selected wells from the recharge areas, more vulnerable to contamination, is an additional factor that influences EDCs absence in aquifers. In addition, the natural attenuation processes occurring during water infiltration through the aquifer are another cause for the removal of these microcontaminants. Ma et al. (2015) conducted a lab- scale experiment simulating aquifer recharge to assess EDCs transport through the aquifer medium. Their results demonstrated an effective attenuation of E2, EE2 and BPA, mainly due to the physical adsorption on the aquifer medium and degradation by bacteria and other microorganisms present in the vadose zone. Sorption and biodegradation processes can thus play a great role in the removal of EDCs in groundwater, as suggested also by Ying et al. (2004).

Concerning surface waters, the northern rivers Lamone and Fiumi Uniti were more affected by EDCs contamination than the southern Marecchia and Conca rivers. Among the analysed EDCs, perfluorinated compounds showed the lowest concentrations compared to the other EDCs. The higher abundance of PFOA in contrast to PFOS found in this work reflects a general behavior commonly found in the majority of study cases (see **Table 3.3**) and is likely explained by the lower solubility of PFOS in the aqueous environment (Lein et al. 2008). Levels of BPA were comparable in the Lamone and Fiumi Uniti rivers, but slightly lower if compared to the Marecchia River. Estrogens were the contaminants that presented the highest variability, being E1 more abundant and more common than E2, excluding two maxima E2 values of 39.7 and 27.9 ng/l, which represent *hot spots* of contamination. Estrogens abundance followed the sequence E1>E2>EE2, which is consistent with previous works stating E1 as the most abundant estrogenic hormone in the environment as a consequence of the partial degradation of E2 and EE2 into E1 (Baronti et al. 2000; Andersen et al. 2003).

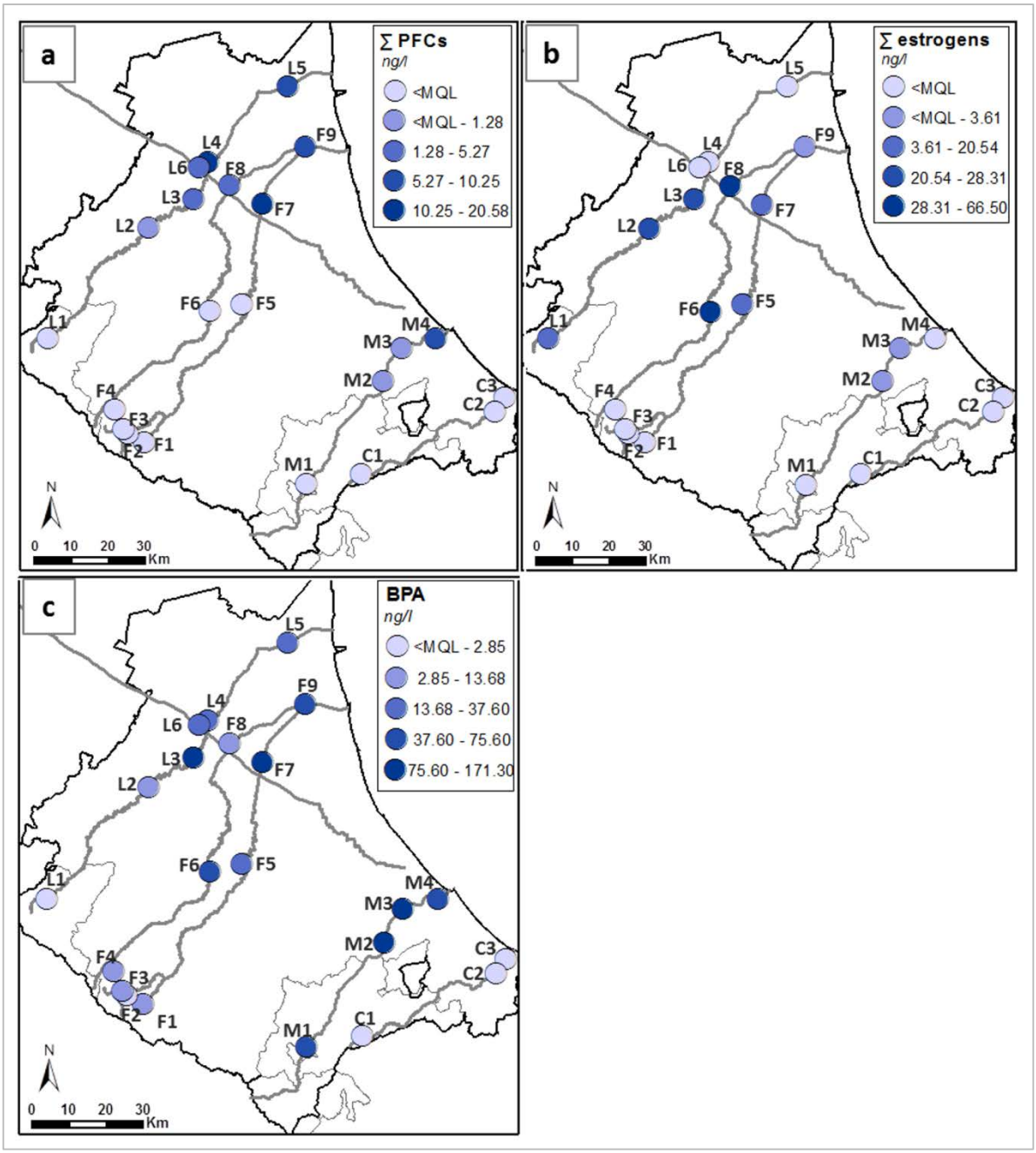


Figure 3.3 Spatial distribution of ΣPFCs (as sum of PFOA and PFOS; a), Σestrogens (sum of E1, E2 and EE2; b) and BPA (c) in the study area. Class values are grouped on the basis of percentiles (I class: 0-20th, II class: 20th-40th, III class: 40th-60th; IV class: 60th-80th; V class: 80th-100th)

Figure 3.3 shows spatial distribution of PFCs (as the sum of PFOA and PFOS concentrations), estrogens (as sum of E1, E2 and EE2 concentrations) and BPA in the study area. PFCs displayed a linear- gradient distribution pattern of concentrations gradually increasing as the rivers flow towards the sea, reflecting human presence and anthropogenic activities influence in the occurrence of these compounds. The absence of contaminants at the river heads identifies these samples as free from anthropogenic contamination. The influence of WWTPs in the release of PFCs into the aquatic compartment needs to be remarked: the WWTP of Faenza located between sample points L2 and L3, the WWTP of Forlì between F5

and F7 sample points, and the WWTP of Rimini, just before M4. Downstream of the main WWTPs, in fact, the highest concentrations of PFCs, above 10 ng/L (80^{th} percentile), are registered. Pignotti et al. (2017a) in a previous study conducted in the same area detected evident Gd anomalies in the northern rivers. As Gd anomaly can be used as tracer of wastewater contamination in freshwater systems (Möller et al. 2000; Williams et al. 2013; Barber et al. 2015), this value was compared to PFCs in order to check for possible relation among the variables. The two variables resulted to be significantly correlated (Pearson correlation coefficient: 0.88; *p*-value: $2.03*10^{-7}$; α=0.05), suggesting that the presence of these two different types of contaminants is associated to a common source of introduction in the environment, represented by effluents from wastewater treatment plants. Furthermore, the increase of concentrations at the final stretch of the water bodies may reflect the influence of the several industries located in Ravenna and Rimini districts. Lin et al. (2009a) pointed out in their study a strong influence of semiconductor, electronics and optoelectronics industries on the distribution of PFCs in the receiving river waters; the presence of industries of electrical and electronic devices in the Ravenna area (42 local units; ISTAT 2014), as well as those in Rimini district (43 local units; ISTAT 2014), can thus be responsible for the higher concentrations found at the river mouths, especially for the increase of PFOS.

Concerning estrogens, the highest concentrations of E1 and E2 were registered in the northern rivers, Lamone and Fiumi Uniti, while Marecchia River displayed only some positive measurements, below 3.61 ng/L (40^{th} percentile) and no estrogenic compound was detected in Conca River. The high concentrations of E1 and E2 recorded in the northern rivers are likely related to the sewages coming from the animal farms present in the catchment of those rivers, as depicted in **Figure 3.1**. Different studies identified livestock as one of the main sources of estrogens in the aquatic system (Gadd et al. 2010; Aris et al. 2014). A study conducted in the UK farming lands revealed that animal farming activities are responsible for the production and release of 789 kg E2 equiv/year, much higher than 219 kg E2 equiv/year produced by humans (Johnson et al. 2006). Another major additional source of contamination can be represented by surface runoff from agricultural lands where animal manure is applied (Combalbert and Hernandez-Raquet 2010). Considering that most of the land use in the study area is represented by farming activities, the use of fertilizers cannot be excluded. Lower concentrations found at the final stretch of Lamone and Fiumi Uniti rivers, in contrast to their pattern in the upper portion of the rivers, are likely to be dependent on the embankment of the river bodies in the plain section that avoids any direct water input from the adjacent farming lands into the rivers. Leakages from septic tanks, as well as illicit sewage discharges into the rivers may not be negligible and can further affect water quality, especially in “pristine” environments such as the headwaters.

Contamination by BPA was found to be spread along the whole study area, except from the southern Conca River. BPA spatial distribution was only partially related to the presence of WWTPs, as in the case of Ronco

River, where high concentrations greater than 80th percentile of the population data were detected in point F7, near to Forlì WWTP, and in the sample point L3 in the Lamone River, which receives Faenza WWTP effluents. However, no linear gradient was found, in contrast to that one of perfluorinated compounds, or a correlation with Gd anomaly, meaning that other different sources influenced its occurrence in the water compartment. BPA has been widely used for the production of epoxy resins and polycarbonate plastics for coating tanks and pipes; it is also present in powder paints, food and beverage cans, optical lenses, protective window glazing, thermal paper and in medical and healthcare applications (Geens et al. 2012). All the various industries located in Rimini and Ravenna districts (food packaging, plastics manufacturing and printing industries) can be potential sources of this compound in the environment. Leachates from the municipal landfills, as well as inappropriate disposal of wastes, can be additional inputs (Morin et al. 2015). Marecchia River showed the highest concentration values (range 44.7-171.3 ng/L) and a very different pattern from the one displayed by the two northern rivers, as already stated from the boxplots (**Figure 3.2**). Such a difference in concentrations distribution is to be related not only to the various anthropic activities located in proximity of the rivers, but also to the different morphology of the three river bodies. In fact, Marecchia River is characterized by lower flow-rates during the summer season, due to the high temperatures and high permeability of the river bed, as well as water abstractions for irrigation purposes that may lead to an enhanced concentration of the compound in solution. Moreover, the absence of embankments along the Marecchia river body, in contrast with the northern rivers, makes the river more vulnerable to anthropic contamination coming from the surrounding human activities.

4.2 EDCs correlation with inorganic water chemistry

As a further, complementary step, we compared the results of EDCs to those of inorganic water chemistry, on the same samples, discussed in Pignotti et al. (2017a). Multivariate analysis was applied and the dendrogram resulting from the hierarchical cluster analysis based on the correlation matrix is reported in **Figure 3.4**.

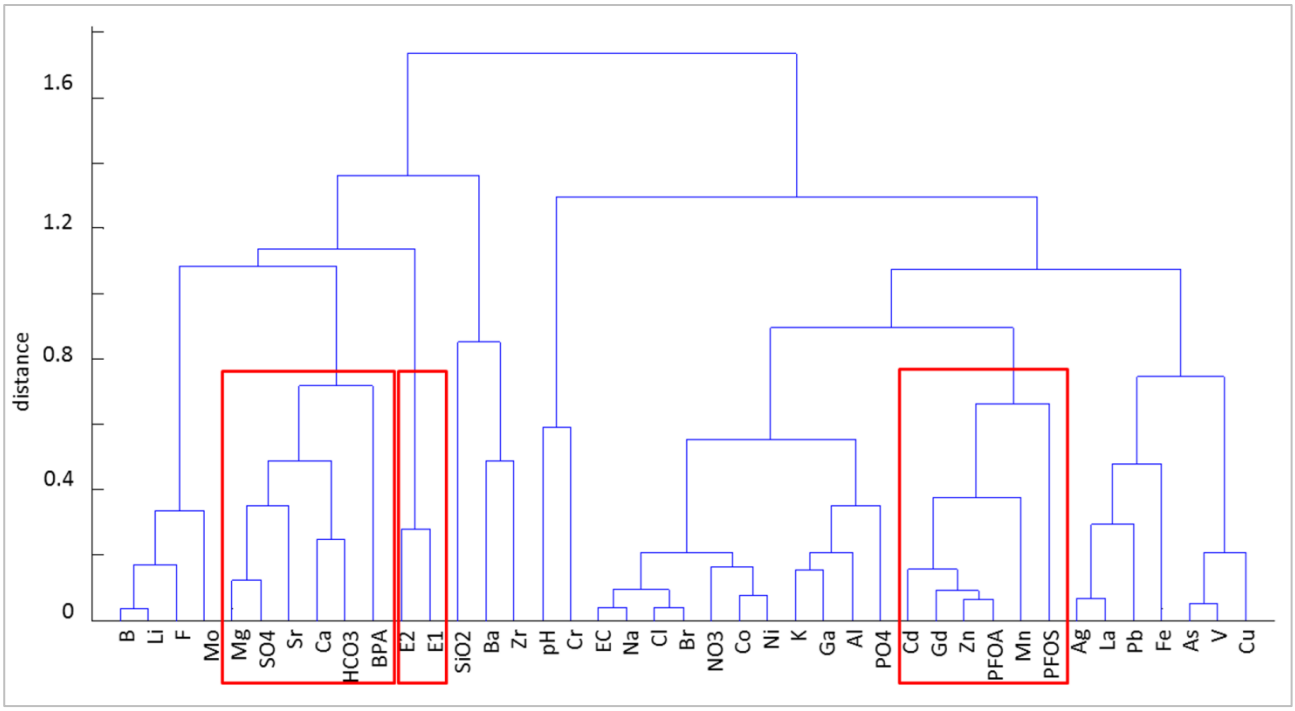


Figure 3.4 Hierarchical cluster analysis showing correlation between EDCs and water chemistry

BPA resulted to be related to HCO_3, Ca, Sr, SO_4 and Mg, elements mainly derived from water/rock interactions considering the geochemical features of the area (Lancianese and Dinelli 2015; 2016). Dolomite, calcite and gypsum represent the likely sources of these elements, which have basically a conservative attitude in surface environments. The association of BPA with these elements hence suggests a conservative behavior of the microcontaminant, as well, since it is related to the hydrogeochemical characteristics of the Romagna surface waters. Estrogens constituted an own group, confirming the relation between the two compounds, as they have similar physical and chemical properties and the same sources of introduction in the environment. Only a weak relation with the group formed by BPA and elements typical of carbonatic environments was highlighted by the dendrogram, and it may be related to the different spatial distribution pattern of the two classes of microcontaminants. In fact, estrogens showed an aggregate distribution of concentrations, being mostly detected in the upper portion of Lamone and Fiumi Uniti rivers, where the main stock-breeding activities are located. Therefore, their occurrence in river water does not follow primarily carbonatic elements distribution, but is mainly dependent on local inputs. The absence of correlation between estrogens and N, P or K, indicators of organic input in the water compartment (Hooda et al. 2000), on the other hand, suggests a total removal of organic loads from the treated effluents of farming activities; the same treatment processes, though, are not efficient in the removal of estrogens. Conversely, BPA occurrence in the Romagna rivers was not related to local inputs ascribed to a particular portion of the study area, but was rather related to a combination of sources of contamination (e.g. WWTP effluents, dusts coming from industrial activities and waste incinerators, leakages from coatings of tanks and pipes). The various BPA anthropogenic sources lead the contaminant to

be present in the aquatic compartment in a more homogeneous pattern, similar to the one of carbonatic elements. Even though BPA was detected in all rivers at trace concentrations (ppt level), its widespread distribution in the study area might be of concern, especially in the case of Marecchia River, given its quite high resistance to degradation (half-life of BPA in water: 66h-160 days; Im and Löffler 2016). Moreover, it should be noted that the lower similarities between estrogens and carbonatic elements may also be due to the lower frequency of detection obtained for estrogens (24% for E2 and 43% for E1) in contrast to BPA (76%); the high number of <MQL values registered for estrogens can influence the final correlation results.

Concerning perfluorinated compounds, PFOA and, to a lesser extent, PFOS were related to Zn, Cd and Mn. These trace elements can be present in the aqueous environment as a consequence of the weathering of Fe and Mn oxides and hydroxides, clay minerals and organic matter. Anthropogenic sources of these metals, however, are not negligible and mainly include industrial activities, such as waste combustion, traffic emissions, steel processing, fertilizers, anticorrosion coatings and painting products (Araújo et al. 2017). Another important input of heavy metals is represented by the discharge of WWTP effluents into surface waters (Maranho et al. 2015). Even if concentrations of the three metals never reached very high values (Pignotti et al. 2017a) and did not exceed the Italian water quality standards (Legislative Decree No. 152/2006), a slight increase of their concentrations in the selected water bodies related to human activities cannot be excluded. Furthermore, results of the hierarchical clustering showed a close relation between Gd and PFCs, especially as regards PFOA, confirming the strong influence of WWTP discharges on river water quality, as already stated in this work.

4.3 Comparison of EDCs found in the Romagna area with toxicity data

Estrogens are the most harmful among all the EDCs, since they can exert adverse effects on reproduction and can highly influence the endocrine system functionalities even at very low concentrations (1-2 ng/L). Luzio et al. (2016) in their study pointed out an induced gonad maturation in both males and females of zebrafish, and delay in male gonad development, in individuals exposed to a concentration of 4 ng E2/L. Raimondo et al. (2009) conducted a research study on the effects induced by E2 in two generations of the sheephead minnow (*Cyprinodon variegatus*). The results showed a reduced reproduction at 80 and 200 ng/L in both generations and a decreasing survival of the embryo and larval stages at 200 ng/L in the second generation only, thus revealing the compounding effects of long-term exposure to E2. Changing of sex ratio to all female individuals was also observed in fathead minnow *(Pimephales promelas)* when exposed to 60 ng E2/L (Nazari and Suja 2016). Caldwell et al. (2012) reported a Predicted No-Effect Concentration (PNEC) of 6, 2 and 0.1 ng/L of E1, E2 and EE2, respectively. Concentrations of estrogens found in this study partially exceed these PNEC values, especially in the upstream section of Lamone and Fiumi Uniti, near the municipalities of Faenza and Forlì (points L2 and L3; points F6 and F8 respectively). As already discussed, concentrations in these areas can be both dependent on animal breeding and on the

presence of WWTPs. The high concentrations detected in the northern area (∑estrogens of 20.6 to 66.5 ng/L) can be of concern because they might cause a risk for the aquatic population.

On the other hand, the southern Marecchia river body showed very high concentrations of BPA, in contrast to the lower values detected in the northern section of the study area. BPA is acutely toxic for aquatic species at 1.34-17.9 mg/L, and chronically lethal at 0.50-0.78 mg/L, being more toxic to aquatic invertebrates than fish (Mathieu-Denoncourt et al. 2016). In comparison, concentrations found in this work were far lower and can thus be considered safe for aquatic biota. However, Canesi and Fabbri (2015) reported endocrine disrupting effects at much lower doses (1 µg/L), with reduction of sperm density and mobility in brown trout (*Salmo trutta*) and general alterations in gonad functionality in several fish species. Moreover, in-field studies conducted by Viganò et al. (2007) in a heavily polluted section of Po River (North Italy) evidenced intersexuality in immature barbels (*Barbus* sp) at environmentally BPA concentrations of 300 ng/L. Therefore, a risk for the aquatic compartment in the Marecchia River should not be excluded, and further research on this topic should be addressed.

Regarding PFCs, PFOS toxicity is known to be generally higher than for PFOA, having this compound also a greater bioaccumulation potential. Li (2009) in his study highlighted significant reproductive changes in *Moina Macrocopa* at 0.31 mg/L of PFOS, and LC_{50} median values of 17.95 mg PFOS/L and 199.51 mg PFOA/L. Giesy et al. (2010) stated a no-effect concentration level (NOEC) of 0.3 mg/L for PFOS in the fathead minnow (*Pimephales promelas*), with an LC_{50} at 7.2 mg/L, while in the northern leopard frog (*Rana pipiens*) an LC_{50} of 6.2 mg/L was obtained. Considering literature toxicity data, current PFOS and PFOA levels registered in the Romagna area can be considered out of concern, since they are far lower than these toxicity reports.

5 CONCLUSIONS

This study focused on the occurrence of six EDCs belonging to three different classes of compounds (PFOA and PFOS as perfluorinated compounds; E1, E2, EE2 as estrogens; BPA for phenolic compound class) in surface- and groundwaters of the Romagna area. Regarding surface waters, perfluorinated compounds were the least abundant compounds. Their occurrence in the aqueous environment was mainly due to WWTP effluents of the main cities, proving their inefficiency in the complete removal of microcontaminants; additional industrial sources, such as the presence of electronic devices production, may be a further source of contamination. Estrogens were detected at higher concentrations and variability, and were mainly dependent on livestock located in the northern part of the study area, in the surroundings of Forlì province. BPA showed to be spread in all the study area at a wider range of concentrations than the other EDCs. Due to its widespread use in human activities, BPA was not related to any specific anthropogenic source, but rather a mixture of both domestic and industrial sources can be

responsible for its occurrence in the aquatic environment. Overall, the northern part of the study area, dominated by Lamone and Fiumi Uniti river basins, resulted to be more affected by poor water quality as regards EDCs; conversely, the southern part showed only a remarkable contamination by BPA in the Marecchia River, whereas no compound was found at concentrations above the MQL in the Conca River. All confined groundwaters sampled showed not to be affected by EDCs contamination, as well. A comparison of EDC concentrations in the Romagna area with toxicity data taken from literature suggests that more studies should be addressed on the real harmful effects of BPA and estrogen concentrations on the aquatic organisms of the study area.

Supplementary material

OCCURRENCE AND DISTRIBUTION OF SIX SELECTED ENDOCRINE DISRUPTING COMPOUNDS IN SURFACE- AND GROUNDWATERS OF THE ROMAGNA AREA (North Italy)

Table S3.1 Description of surface water sampling points

Sampling station	River	Description
L1	Lamone	headwater
L2	Lamone	upstream of Faenza
L3	Lamone	downstream of Faenza WWTP
L4	Lamone	downstream of CER channel input into Lamone
L5	Lamone	proximity of Ravenna; river mouth
L6	CER channel	CER water before its divergence into Lamone
F1	Fiumi Uniti (Ronco)	headwater
F2	Fiumi Uniti (Ronco)	headwater
F3	Fiumi Uniti (Ronco)	headwater
F4	Fiumi Uniti (Montone)	headwater
F5	Fiumi Uniti (Ronco)	upstream of Forlì; mountain basin closure
F7	Fiumi Uniti (Ronco)	downstream of Forlì
F6	Fiumi Uniti (Montone)	upstream of Forlì; mountain basin closure
F8	Fiumi Uniti (Montone)	downstream of Forlì
F9	Fiumi Uniti	proximity of Ravenna; confluence of Ronco and Montone rivers; river mouth
M1	Marecchia	headwater
M2	Marecchia	mountain basin closure
M3	Marecchia	aquifer recharge area
M4	Marecchia	proximity of Rimini; river mouth
C1	Conca	headwater
C2	Conca	upstream of Conca dam
C3	Conca	downstream of Conca dam; river mouth

Table S3.2 MS detection parameters for PFC detection: precursor and product ions, cone voltage and collision energy

Compound	**Precursor ion** (m/z)	**Product ion** (m/z)	**Cone voltage** (V)	**Collision energy** (V)
PFOA 1	413	369	17	10
PFOA 2	413	169	17	12
PFOS 1	499	99	45	40
PFOS 2	499	80	45	38
PFOA-C_{13}	417	372	45	40

Table S3.3 MS detection parameters for estrogens and BPA detection: precursor and product ions, RF lens and collision energy

Compound	**Precursor ion** (m/z)	**Product ion** (m/z)	**RF Lens** (V)	**Collision energy** (V)
E1 1	269.1	145	235	53
E1 2	269.1	143	235	40
E2 1	271.1	183	235	41
E2 2	271.1	145	235	38
EE2 1	295.1	145	88	45
EE2 2	295.1	159	88	40
E2-d_3	273	185	235	41
BPA 1	227	133	66	29
BPA 2	227	211	66	21
BPA-d_6	233	215	66	21

Chapter 4

DISTRIBUTION AND PARTITION OF ENDOCRINE DISRUPTING COMPOUNDS IN WATER AND SEDIMENT: CASE STUDY OF THE ROMAGNA AREA (North Italy)

1 INTRODUCTION

River waters are the primary environmental compartment to be affected by a variety of harmful chemicals, mainly released from civil and industrial wastewaters, livestock sewages, and farmland run-offs. Among these chemicals, Endocrine Disrupting Compounds (EDCs) are of growing concern because of the potential harmful effects they can pose on organisms (Tijani et al. 2016; Li et al. 2017). They encompass a wide variety of both natural and man-made compounds that are present in every-day products, such as detergents, plastics, cookware, and water-repellent materials. Among these chemicals, estrogens, phenolic compounds and perfluorinated compounds are of great interest because of their high estrogenicity potential (estrogenic hormones) and their widespread application (perfluorinated and phenolic compounds) (Ahrens 2011; Geens et al. 2012). Several studies dealt with the occurrence of EDCs in the aquatic compartment, focusing on surface waters (Valsecchi et al. 2015), groundwaters (Jurado et al. 2012), drinking waters (Kleywegt et al. 2011) or wastewater treatment plant effluents (Muz et al. 2012). Nevertheless, still little is known about their fate and transport once they are released into the environment. Sediments are known to be one major sink for these chemicals due to their high hydrophobicity (Gong et al. 2012), as suggested by their high $\log K_{ow}$ values, between 3.43 and 5.76. Hydrophobic chemicals easily bind to sediments and to the suspended particulate material that is deposited along the waterways and can then be mobilized if changes in river flow conditions occur, enhancing their bioavailability and entrance into the food chain (Zoppini et al. 2014). Moreover, they can cause adverse effects on benthic- dwelling organisms. So far, different batch experiments have been focused on the partitioning behavior of emerging contaminants in sediments (Ahrens et al. 2011; Liao et al. 2014; Chen et al. 2016), but only few studies have been carried out on real environmental matrices.

This work was aimed to assess the occurrence and distribution of estrogens, phenolic compounds, and perfluorinated compounds in surface waters and sediments of the Romagna rivers and a coastal lagoon (NE Italy) in order to elucidate their behavior and fate once they enter the aquatic environment. The study was integrated with a characterization of the bulk chemical composition of sediments as well as with basic hydrogeochemistry.

2 MATERIALS AND METHODS

2.1 Study area and sampling

The study was focused on the main river bodies of the Romagna area, in the north-eastern part of Italy. This is a quite high urbanized area with a population density of 200.8 inhabitants/km^2 mostly concentrated around the main cities of Ravenna, Faenza, Forlì, Cesena and Rimini (Emilia-Romagna Region 2014). Major human activities in the area are farming activities (vineyards, orchards, arable crops), and stock-breeding. These activities require a huge water demand and are one major cause of water scarcity during the summer period. Furthermore, there are various industries located in the main cities of the study area that can be related to a potential EDCs contamination. River quality can also be affected by the discharge of sewages coming from the wastewater treatment plants (WWTP) of the cities of Faenza, Forlì and Rimini that release their treated effluents directly into Lamone, Ronco and Marecchia rivers. Detailed information about the main activities located in the Romagna area is reported in **Table S4.1** of the Supplementary Material.

The rivers selected for the study were Lamone, Fiumi Uniti (formed by the union of Ronco and Montone rivers), Bevano, Savio and Marecchia (**Figure 4.1**). One additional sample was taken from the "*Canale Emiliano-Romagnolo*" (CER) channel, which is an artificial channel that crosses all the study area in a NW-SE direction and delivers Po river water in the Romagna farming area in order to supply for water deficiency during the summer season. All rivers have a SW-NE flow direction, from the Apennine Mountains to the Adriatic Sea. Lamone, Fiumi Uniti and Savio are characterized by artificial embankments in their plain section to prevent seasonal floods. Bevano is a short river that differs from the others because it is characterized by a strong torrential behavior and is mainly fed by inputs coming from the surrounding channels, in contrast to the other rivers which are mainly fed by rainfall inputs. In addition to river waters, water quality of Pialassa Baiona was also investigated. Pialassa Baiona is a wetland area on the south of the Lamone River connected both to inland waters and sea waters. It is a highly naturalistic environment, whose quality is though affected by the surrounding anthropic activities, especially the petrochemical plant of the city of Ravenna. To better evaluate Pialassa Baiona water quality, two samples were also taken from the artificial freshwater channels (P1 and P2 sample points in **Figure 4.1**) that are in close connection with the coastal lagoon.

The main geological units that characterize the study area are summarized in **Figure 4.1**. Briefly, the geological setting is primarily composed by the *Marnoso-Arenacea Formation,* characterized by the alternation of sandstones, marls and clays, and the *Gessoso-Solfifera Formation*, made of gypsum evaporitic rocks. The plain section is characterized by the alluvial deposits of Apennines rivers.

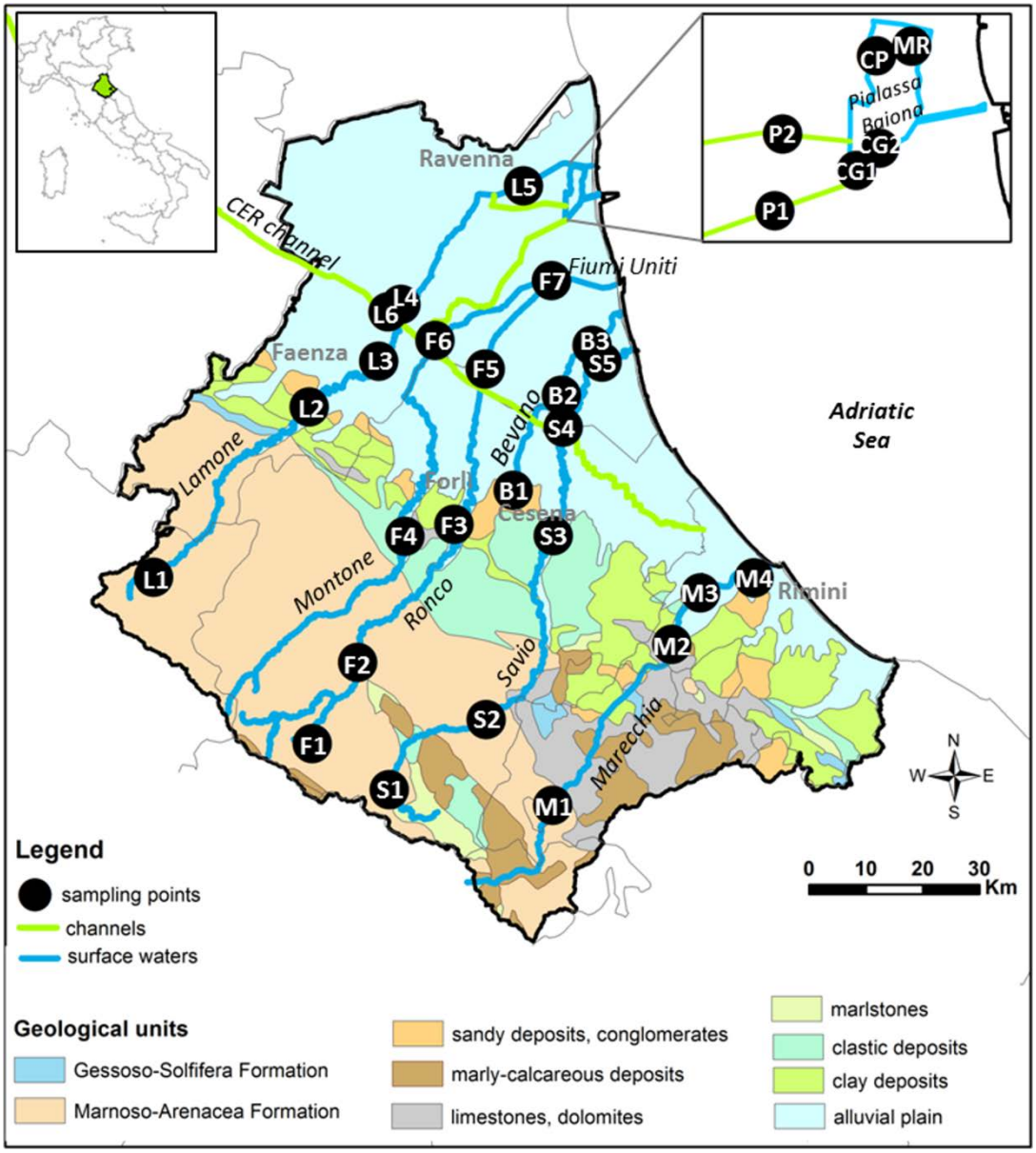


Figure 4.1 Map of the study area and sampling points

Sampling campaign was carried out in July 2016, during the dry season. Grab samples (n=27) were collected from the active bed of the rivers and from the transitional environment of Pialassa Baiona (n=3). At each sampling site, 500 mL were taken for hydrogeochemical analyses, and 500 mL were collected in PE bottles for EDCs determination. Sediment samples were collected with a Van Veen grab sampler and wrapped in aluminum foil. When it was possible, as in the case of headwaters, a stainless steel hand trowel was used for sediment collection. All samples were stored at 4 °C in darkness and extracted within 72 h to prevent the compounds degradation.

2.2 Sample treatment and analysis

2.2.1 Geochemical analyses

Waters were filtered immediately in field with 0.45 µm mixed cellulose ester filters; for each sample, 100 mL were used for major anions determination, and 250 mL were subsequently acidified with ultrapure HNO_3 (pH=2) for cations determination. Major anions were analysed by a Metrohm 883 Basic IC Plus ion chromatogram, while cations were analysed by a Perkin Elmer AAnalyst-100 flame atomic absorption spectrometer.

Sediments were sieved (180 µm particle size) to divide the coarse and the fine fractions. For some samples it was not possible to divide the two fractions, since the coarse fraction was very low; in other few station points it was not possible to take sediments from bed rivers. **Table S4.2** reports additional information of available sediments at each sampling station. Sediment samples were ground with an agate mortar. 3 g of sediments were used to prepare pressed powder pellets to be analysed using a Panalytical Axios 4000 X-Ray fluorescence spectrometer equipped with a Rh tube. Loss On Ignition (LOI) was calculated with 1 g of sediment sample after heating for 12 h at 950 °C. To determine Total Organic Carbon (TOC) content, 40 µL of diluted HCl were added to 8-10 mg of powder sediment samples to remove carbonates and dried at 60 °C for 1 hour. The same procedure was repeated several times until all the inorganic carbon was removed. TOC determination was performed using a CHNS Element Analyser Flash 2000 (Thermo Scientific; Waltham, MA).

2.2.2 EDCs analyses

2.2.2.1 Reagents and standards

All solvent reagents (water, methanol, acetonitrile, ammonium acetate, ammonium hydroxide) were LC-MS analytical grade and were purchased from Sigma-Aldrich. Native standards of the compounds ß-estradiol (E2), estrone (E1), 17α-ethinylestradiol (EE2), bisphenol A (BPA), 4-nonylphenol (NP), octylphenol (OP), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) were purchased from Sigma Aldrich. Isotope- labelled compounds used as internal standards were purchased from CDN Isotopes (ß-estradiol-d_2, E2-d_2; bisphenol A-d_6, BPA-d_6) and Wellington Laboratories Inc. ($^{13}C_4$-perfluorooctanoic acid, $^{13}C_4$-PFOA). Stock solutions of all target compounds were prepared at concentration of 1 g/L in methanol and stored at -20 °C. Diluted working solutions were prepared at the time of analyses.

2.2.2.2 Extraction procedure and analysis

For the extraction of the selected EDCs in water samples, the method described in Pignotti et al. (2017) was followed. Briefly, 500 mL of water were spiked with a mixture of internal standards at a concentration of 30

ng/L and filtered firstly with glass microfiber filters (1.60 µm pore size, 47 mm diameter; Whatman, Kent, UK) and then with cellulose acetate filter (0.45 µm pore size, 47 mm diameter; Whatman, Kent, UK). Sample extraction was performed by solid phase extraction (SPE), using OASIS HLB cartridges (6 cc, 200 mg; WatersCorp, Milford, MA, USA) previously conditioned with 6 mL methanol and 6 mL ultrapure water. After all the filtrate sample passed through the cartridge at a flow rate of 12 mL/min, cartridges were dried for 30 min and then eluted with 6 mL of methanol. The eluate was subsequently evaporated under a N_2 gentle stream near dryness, and split into two vials, each one reconstituted in 250 µL of a mixture of water/methanol (50:50) for estrogens and phenolic compounds determination, and 250 µL water/methanol (90:10) for perfluorinated compounds analysis. Samples were analysed in triplicates.

Concerning sediment samples, 1 g dry weight (dw) of sediment was spiked with 50 µL of a mixture of internal standards (250 µg/L) and left 30 min to reach equilibrium. After that, 10 mL of methanol were added and EDCs were extracted by ultrasonic assisted extraction (UAE) for 1 hour. Samples were then centrifuged for 20 min at 4000 rpm at room temperature. The extraction and clean-up procedure was performed by solid phase extraction (SPE) adding 90 mL of ultrapure water to the extracted sample, and following the same procedure described for waters. Procedural blanks were prepared in parallel with 10 mL of methanol and 90 mL of ultrapure water, following all the above-mentioned extraction steps.

EDCs determination was performed by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) using an Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) coupled to a Quattro Premier XE mass spectrometer (Waters Corp). Additional information about the chromatographic conditions adopted is reported in **Table S4.3** and **Table S4.4**.

2.2.2.3 Quality assurance and quality control

Procedural blanks for both waters and sediments were prepared and analysed together with field samples in order to check for possible contamination during the whole analytical process. Blank concentrations were subtracted to the final concentration result, if present. All samples were analysed in triplicates.

Six-point calibration curves (0-100 ng/mL) were prepared for each set of analyses, and a good linearity was achieved for all compounds (R^2>0.99). Limit of Detection (LOD) and Limit od Quantification (LOQ) were calculated as the concentration that yielded a signal-to-noise ratio of 3 and 10, respectively. For both water and sediment analysis, recoveries were calculated by spiking a representative sample matrix with a mixture of 20 ng/L (20 ng/g for sediment) of native compounds. **Table S4.5** summarizes LOD, LOQ and Recovery values for each of the analysed compounds.

2.3 Data analysis

Concentrations in samples which were below mLOQ were substituted with half the mLOQ. Statistical analyses were performed with R software. Since all EDC variables were not-normally distributed, the Spearman rank correlation test was used to test their similarities, setting the significance level at α=0.05.

For EDCs detected both in waters and sediments, the field-based partition coefficient (K_d, mL/g) between sediment and water was calculated according to the equation (OECD 2001):

$$K_d = C_s / C_w$$

where K_d was the partition coefficient between sediment and waters (mL/g), C_s was the concentration of the compound detected in sediment (ng/g dw) and C_w the concentration detected in water (ng/L). A K_d value was calculated at each sampling site.

The field-based K_{oc} value and its corresponding logarithmic $\log K_{oc}$ were calculated, as well, normalizing the K_d to the organic carbon fraction registered in sediment, according to the equation (OECD 2001):

$$K_{OC} = K_d \cdot 100 / f_{OC}$$

Where K_{OC} is the organic carbon-normalized partition coefficient and f_{OC} is the percentage of organic carbon (%) detected at each sampling point.

In order to evaluate the potential estrogenic effects of EDCs in the study area, concentrations of each compound were converted to Estradiol Equivalent concentrations (ng/L), according to the following formula (Morales et al. 2013):

$$EEQ_{tot} = \sum (C_i \cdot EEF_i)$$

Where *i* stands for each of the EDCs analyzed in the study, *C* represents the compound concentration and *EEF* is the *Estrogenic Equivalent Factor* relative to E2 and is a useful tool to represent the ability of each of the EDCs to cause estrogenic effects, compared to the estrogenic effects naturally induced by estradiol. For the calculation of EEQs, EEFs suggested by Morales et al. (2013) were adopted and reported in **Table S4.6**. Since no data regarding EEFs for perfluorinated compounds are available in the literature, this class of compounds was excluded from the elaboration.

3 RESULTS AND DISCUSSION

3.1 Water and sediment characterization

River waters of the Romagna area can be classified as Ca-HCO_3 type, since they flow in a geological area with abundant limestones and marls (Lancianese and Dinelli 2015), and this is reflected in the high content of HCO_3^-, SO_4^{2-}, and Ca^{2+}, as reported in **Table S4.7**. The highest concentrations of all the water major components, along with the highest conductivity value of 1506 µS/cm, were recorded in sample L3 from Lamone River. This sample was taken after the release of WWTP effluents of the city of Faenza into the Lamone River, and reflects the high influence of the WWTP on water quality of this section of the river. Samples taken from Pialassa Baiona are typical of lagoon environments influenced by seawater, as can be seen from the high values of water major components (see **Table S4.7**).

Data regarding major oxides and trace elements in sediments are presented in **Table S4.8**, while additional graphical boxplots are reported in **Figure S4.1** (major oxides) and **S4.2** (trace elements). River sediments showed a higher Al_2O_3 content (clays) in the fine fraction than in the coarse one, which in turn was more enriched in SiO_2 (silicates), whereas in the lagoon sediments the two oxides were quite comparable, suggesting presence of clay minerals and feldspars. CaO, indicative of carbonatic material, was higher in the coarse fraction of river sediments, and lower in the lagoon environment. Trace elements in both river and lagoon samples were higher in the fine fraction, suggesting association with clay material and hydroxides. Cr, Cu, Pb and Zn showed much higher values in the fine fraction of Pialassa Baiona lagoon samples, in contrast with river sediments, likely related to anthropogenic inputs of the surrounding industrial activities and agricultural areas (Matteucci et al. 2005; Migani et al. 2015), especially in the southern portion of the lagoon (sample CG), which is in close connection with the Ravenna industrial harbor.

TOC and S content suggest a higher amount of organic matter in the fine fraction of sediments, especially in those collected in the lagoon environment of Pialassa Baiona.

3.2 EDCs in surface waters

Spatial distribution of EDCs in surface waters is shown in **Figure 4.2**, while summary statistics calculated for each river is reported in **Table 4.1**. For a better understanding of EDCs concentration distribution at each sampling point, please refer to the bar plots reported in **Figure S4.3**. Estrogens were the least detected class of contaminants, since estrone was the only steroid hormone detected in water samples, with a frequency of 16%, whereas E2 and EE2 were below the quantification limit in all samples. Focusing on the different river waters, E1 occurrence was rather sporadic, being present only in one sample of Fiumi Uniti, Savio and Bevano rivers, and in two of the three sample points taken from Pialassa Baiona. Concentration

values were very low, as well, with a maximum registered in the Bevano river (6.9 ng/L), followed by Pialassa Baiona, which recorded 4.3 ng/L in the northern part and 3.3 ng/L in the southern one.

Table 4.1 Summary statistics of EDCs detected in water samples

	Min[1]	Max[1]	Median[1]	Mean[1]	SD[1]	Frequency[2]
Total						
E1	<mLOQ	6.9	<mLOQ	0.82	1.5	17
E2	<mLOQ	<mLOQ	-	-	-	-
EE2	<mLOQ	<mLOQ	-	-	-	-
BPA	<mLOQ	244	22	58	72	90
NP	<mLOQ	135	41	50	44	87
OP	<mLOQ	<mLOQ	-	-	-	-
PFOA	<mLOQ	47	11	13	11	93
PFOS	<mLOQ	13	0.24	1.7	3.1	50
Lamone (n=6)						
E1	<mLOQ	<mLOQ	-	-	-	-
BPA	<mLOQ	99	16	27	36	83
NP	7.2	123	39	44	42	100
PFOA	<mLOQ	43	13	16	15	83
PFOS	<mLOQ	13	0.52	2.6	4.9	67
Fiumi Uniti (n=7)						
E1	<mLOQ	0.84	<mLOQ	<mLOQ	<mLOQ	14
BPA	9.6	96	19	35	31	100
NP	36.8	134	94	89	39	100
PFOA	0.51	18	3.6	8.7	8.2	100
PFOS	<mLOQ	0.92	<mLOQ	0.25	0.37	29
Bevano (n=3)						
E1	<mLOQ	6.9	<mLOQ	2.5	3.8	25
BPA	<mLOQ	139	46	62	71	75
NP	<mLOQ	61	41	34	31	75
PFOA	9.5	47	11	23	21	75
PFOS	<mLOQ	11	1.3	4.2	6.2	75
Savio (n=5)						
E1	<mLOQ	1.5	<mLOQ	0.54	0.52	20
BPA	5.9	226	23	73	92	100
NP	<mLOQ	119	79	66	52	80
PFOA	0.6	18	11	9.6	6.4	100
PFOS	<mLOQ	5.8	<mLOQ	1.2	2.6	20
Marecchia (n=4)						
E1	<mLOQ	<mLOQ	-	-	-	-
BPA	<mLOQ	244	195	158	110	75
NP	<mLOQ	72	9.7	23	33	75
PFOA	<mLOQ	15	7.5	7.6	6.9	75
PFOS	<mLOQ	3.0	2.0	1.8	1.3	75
Channels (n=2)						
E1	<mLOQ	<mLOQ	-	-	-	-
BPA	38	69	53	53	22	100
NP	9.1	29	19	19	14	100
PFOA	24	27	25	25	2	100
PFOS	12	4.1	3.1	3.0	1.4	100
Pialassa Baiona (n=3)						
E1	<mLOQ	4.3	3.3	2.6	2.1	67
BPA	3.9	17	14	12	7	100
NP	<mLOQ	47	8.0	19	25	67
PFOA	5.9	18	8.8	11	6	100
PFOS	<mLOQ	2.6	<mLOQ	0.9	1.5	33

[1] Concentrations are expressed as ng/L. SD: standard deviation; [2] Frequency of detection is expressed as %

Concerning perfluorinated compounds, PFOA showed a wider distribution than PFOS, being registered in 93% of the analysed samples, whereas PFOS was detected in 50% of samples. Lamone and Bevano were the most affected rivers concerning PFOA occurrence in waters, with the highest concentrations detected in point L3 (43 ng/L) and point B2 (47 ng/L). PFOA was also detected in all samples of Marecchia, Savio, Fiumi Uniti Rivers and in Pialassa Baiona, even if at slight lower concentrations. Lamone and Bevano were the main rivers also affected by PFOS contamination (67% of detections in Lamone and 75% in Bevano), along with Marecchia River (67%).

Phenolic compounds were the most abundant group among the analysed EDCs. BPA was registered in 90% of samples, followed by NP, recorded in 87% of water samples, whereas OP was never detected at concentrations above LOQ. BPA and NP showed also a wider range of detections if compared to the other EDCs (BPA: <LOQ-244 ng/L; NP: <LOQ-135 ng/L). The highest BPA concentrations were registered in Marecchia River, followed by Bevano River. On the other hand, Fiumi Uniti showed to be the most affected river body regarding NP occurrence. It should be remarked that, even though NP maxima concentrations in the other rivers of the Romagna area were comparable to sample F6 (135 ng/L) of Fiumi Uniti River, median values in Romagna rivers were much lower than the median registered in Fiumi Uniti, suggesting local sources of contamination by alkylphenols in contrast to a more widespread contamination present in Fiumi Uniti.

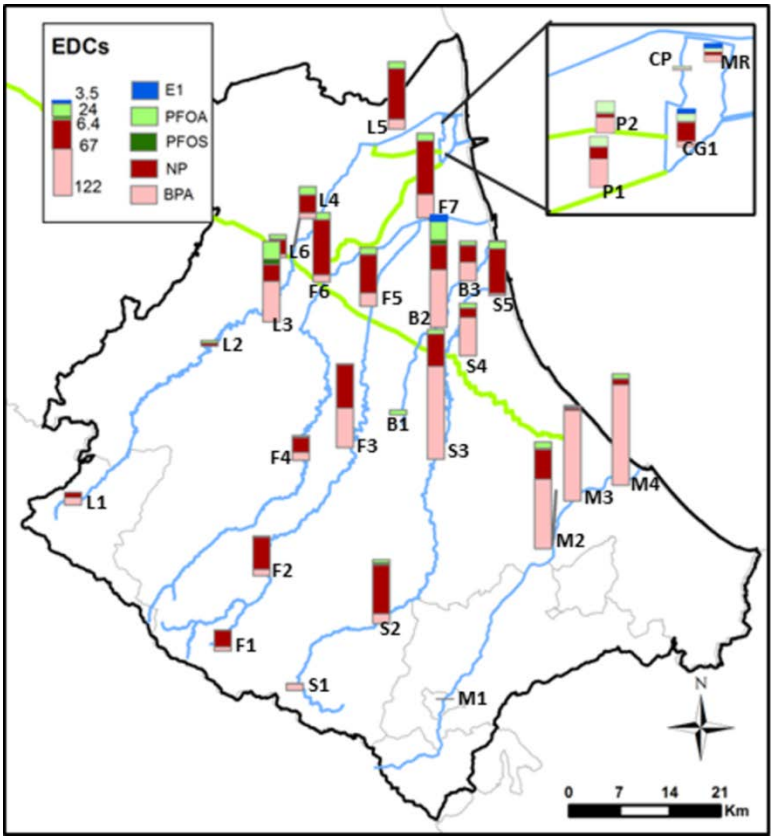


Figure 4.2 Distribution of the detected EDCs in water samples of the study area. Values reported in the legend are expressed as ng/L and correspond to half of the maximum value for each compound

Overall, all the rivers evidenced increasing concentrations in their plain section compared to the mountain section. This is quite consistent with the influence of human activities, especially WWTP release into river waters and industries presence in the surroundings of the main cities crossed by the Romagna rivers. In detail, point L3 of the Lamone River registered very high values of both perfluorinated and phenolic compounds and also water geochemistry was anomalous at this sample point. This sample was taken just after the discharge of WWTP effluents of the city of Faenza into the Lamone River; the very high values recorded at this point for both inorganic components and organic chemicals suggest a non-optimal functioning of the water treatments adopted at the Faenza WWTP, an effect eventually increased by the sampling season in a low discharge condition. The high anomalous BPA concentrations detected in the Marecchia River could reflect the influence of various painting industries and body car shops in which epoxy resins are used as primers to promote the adhesion of paints on metal surfaces (American Chemistry Council, 2017). The absence of artificial embankments lead the river to be in close connection with the surrounding environment; the very low flow rates that normally characterize this river body during summer periods enhance its concentrations in water.

A previous study on the occurrence of EDCs in surface waters was conducted in summer 2015 (Pignotti et al. 2017b) on the same area in Lamone, Fiumi Uniti and Marecchia rivers, and values were compared with the ones obtained in this study. A rough comparison among the distributions is presented in **Figure 4.3**.

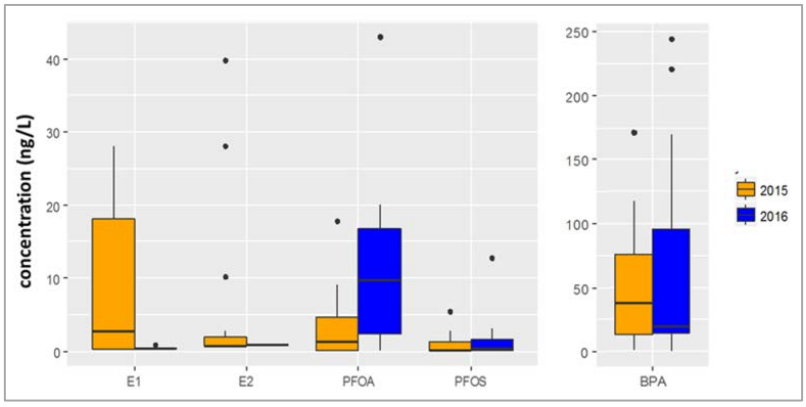


Figure 4.3 Comparison of EDC concentrations in waters during summer 2015 (Pignotti et al. 2017b) and 2016 (this study). For details about sampling and EDCs analysis in 2015, please refer to the work of Pignotti et al. (2017b)

BPA did not show a global variation in its distribution in Lamone and Fiumi Uniti rivers, while greater values were detected in Marecchia River in 2016, with an increase of values moving from the head of the river to the mouth, suggesting a continuous introduction of this compound in the aquatic compartment. Regarding perfluorinated compounds, PFOS showed no substantial variation in the two years, whereas PFOA was detected at higher concentrations in this study, as a result of an increased introduction of this compound or its precursors. However, its spatial distribution in rivers was comparable in the two years, with the highest

concentrations detected downstream of the WWTPs of Faenza and Forlì, as can be noticed by comparing the two works. A great difference was registered regarding estrogens, since they were almost undetected in this study, in contrast to their behavior in the previous year, in which local high anomalies (up to 66.5 ng/L of estrogen total amount) were detected and were mostly concentrated in the northern mountain section of the study area. This difference in their occurrence over time is probably due to some local and sporadic sources of contamination rather than to a continuous input of these chemicals into the river bodies. However, even though the two sampling campaigns were conducted in the same season, degradation of compounds occurring as a consequence of the high temperatures typical of the summer period cannot be excluded (Havens et al. 2010).

Regarding Pialassa Baiona, the highest EDCs concentrations were detected at sample point CG1, in the southern portion of the lagoon; EDCs in the northern section (sample point MR) were also quite comparable, while the central section of the lagoon (sample point CP) recorded the lowest values for all compounds. EDCs distribution in waters of Pialassa Baiona likely reflects water inputs into the lagoon and water currents of the lagoon itself. In fact, Pialassa Baiona is connected in its southern portion to inland waters which showed appreciable contamination by EDCs. Furthermore, the lagoon is in connection with the Adriatic Sea in its northern portion. Seawater has been found to be heavily affected by human presence, especially in coastal environments characterized by various human activities and high population density (Cocci et al. 2017; Miccoli et al. 2017). Detection of EDCs at the connection point between the Adriatic Sea and Pialassa Baiona is thus not surprising, and can be an evidence of a coastal environment affected by EDCs contamination, as well. Notwithstanding these EDCs introduction points into the lagoon, the central body of Pialassa Baiona showed lower concentrations, as an effect of the dilution and mixing of waters that enter the lagoon. The lower solubility of EDCs in seawater further influenced their lower detection in the central part of the lagoon.

3.3 EDCs in sediments

Distribution of EDCs in sediment is displayed in **Figure 4.4**, while **Table 4.2** reports summary statistics calculated for riverbed sediments. Moreover, bar plots of EDCs concentrations at each sampling site are reported in **Figure S4.4**. Estrogens occurrence in sediments was extremely sporadic: E2 was detected only in one sample (F5); E1 in the southern portion of Pialassa Baiona and in one channel delivering waters into the lagoon, while all other samples registered concentrations below the LOQ. This is consistent with water results, proving that the study area was not affected by estrogens release into the environment.

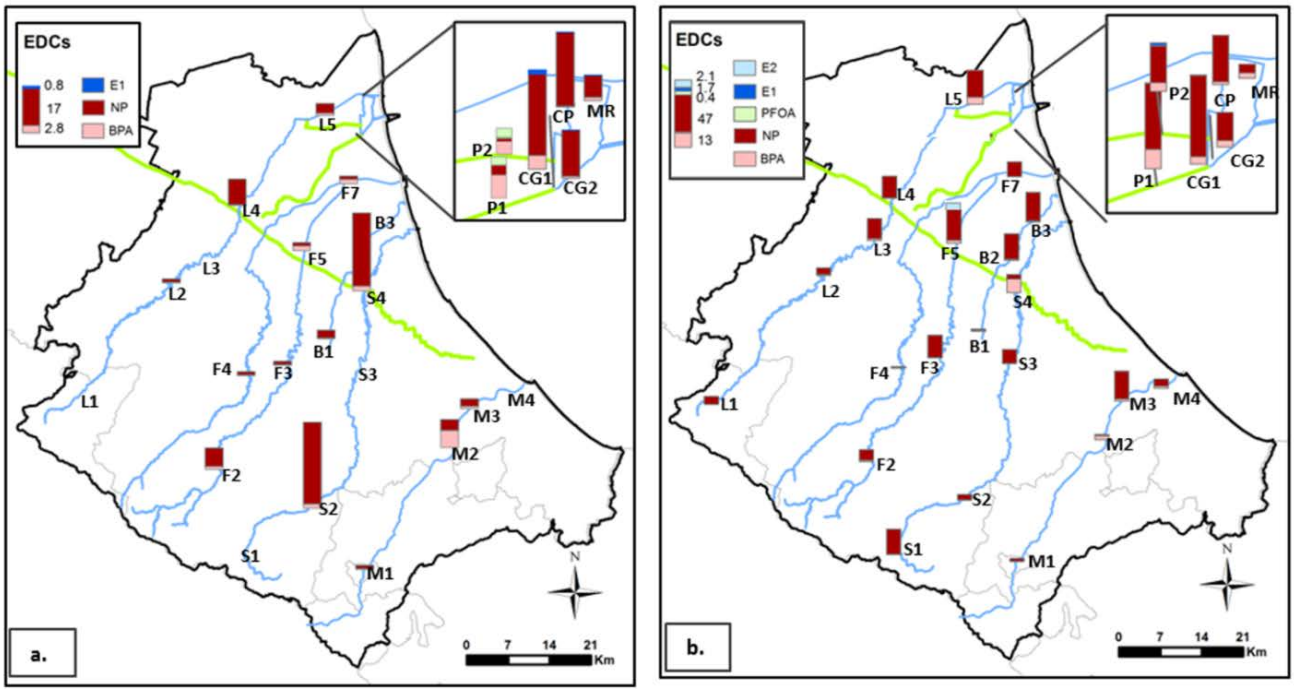


Figure 4.4 Distribution of EDCs in the coarse fraction (a) and in the fine fraction (b) of sediment samples. Values reported in the legend are expressed as ng/g dw and correspond to half of the maximum value for each compound

Perfluorinated compounds showed very low frequencies of detection, as well. In detail, PFOS was not detected at concentrations above the LOQ in any of the samples, whereas PFOA was registered in some samples of Lamone, Pialassa Baiona and its channels, at very low concentrations (<1 ng/g dw).

The most abundant compounds were the phenolic compounds BPA and NP (61 and 67% of detections for BPA; 67 and 85% of detections for NP in the coarse and fine fraction, respectively). Contrarily, OP was not recorded in any of the samples, in agreement with its absence in the water compartment.

Overall, the coarse fraction of sediments showed lower values if compared to the fine one. Concerning the coarse fraction, very low range of values were detected in the Fiumi Uniti River (<LOQ-1.9 ng/g dw for BPA; <LOQ-7.6 ng/g dw for NP). The highest values were registered for NP in sediments of the Savio River. Pialassa Baiona also showed to be quite affected by NP contamination in sediments, with values ranging from 8.6 ng/g dw (sample MR) to 32 ng/g dw in its southern portion (sample CG1), near to the Ravenna harbor. On the other hand, BPA concentrations were comparable in all coarse sediments.

In the fine fraction, BPA was detected at the highest concentrations in Savio River and in the two channels delivering waters into Pialassa Baiona, but NP showed much higher values, up to 97 ng/g dw, also concentrated in Pialassa Baiona and the two connected channels. Moreover, contrarily to the coarse fraction, sediments from the Fiumi Uniti River showed detectable concentrations of NP, from <LOQ to 37 ng/g dw, in a range comparable to the other inland rivers.

Table 4.2 Summary statistics of EDCs detected in sediment samples

	Coarse fraction							*Fine fraction*					
	Min[1]	**Max[1]**	**Median[1]**	**Mean[1]**	**SD[1]**	**%**		**Min[1]**	**Max[1]**	**Median[1]**	**Mean[1]**	**SD[1]**	**%**
Total (n=18)							*Total (n=27)*						
E1	<mLOQ	<mLOQ	-	-	-	-	E1	<mLOQ	3.5	<mLOQ	<mLOQ	<mLOQ	7
E2	<mLOQ	<mLOQ	-	-	-	-	E2	<mLOQ	4.2	<mLOQ	<mLOQ	<mLOQ	4
EE2	<mLOQ	<mLOQ	-	-	-	-	EE2	<mLOQ	<mLOQ	-	-	-	-
BPA	<mLOQ	6.9	1.0	1.6	1.8	61	BPA	<mLOQ	2.0	1.9	3.9	5.4	67
NP	<mLOQ	34	4.2	11	12	67	NP	<mLOQ	97	17	25	24	85
OP	<mLOQ	<mLOQ	-	-	-	-	OP	<mLOQ	<mLOQ	-	-	-	-
PFOA	<mLOQ	<mLOQ	-	-	-	-	PFOA	<mLOQ	0.88	<mLOQ	<mLOQ	<mLOQ	11
PFOS	<mLOQ	<mLOQ	-	-	-	-	PFOS	<mLOQ	<mLOQ	-	-	-	-
Lamone (n=3)							*Lamone (n=5)*						
BPA	<mLOQ	1.0	<mLOQ	0.59	0.39	33	BPA	<mLOQ	3.3	1.4	1.5	1.2	50
NP	<mLOQ	11	3.8	5.2	4.7	67	NP	7.8	26	12	16	9	100
PFOA	<mLOQ	<mLOQ	-	-	-	-	PFOA	<mLOQ	0.33	<mLOQ	<mLOQ	<mLOQ	20
Fiumi Uniti (n=3)							*Fiumi Uniti (n=5)*						
E2	<mLOQ						E2	<mLOQ	4.2	<mLOQ	<mLOQ	<mLOQ	20
BPA	<mLOQ	1.9	1.3	1.2	0.8	60	BPA	<mLOQ	3.4	<mLOQ	1.3	1.4	40
NP	<mLOQ	7.6	<mLOQ	2.6	2.8	20	NP	<mLOQ	37	17	19	14	80
Bevano (n=1)							*Bevano (n=3)*						
BPA	<mLOQ	<mLOQ	-	-	-	-	BPA	0.97	1.4	1.3	1.2	0.2	67
NP	3.2	3.2	-	-	-	-	NP	<mLOQ	34	31	22	18	67
Savio (n=2)							*Savio (n=4)*						
BPA	1.8	2.0	1.9	1.9	0.2	100	BPA	<mLOQ	16	<mLOQ	4.5	7.9	50
NP	31	34	32	32	2.5	100	NP	5.3	30	11	14	12	75
Marecchia (n=3)							*Marecchia (n=4)*						
BPA	<mLOQ	6.9	1.1	2.8	3.6	67	BPA	<mLOQ	4.7	2.6	2.6	1.8	75
NP	<mLOQ	4.5	3.0	2.9	1.6	67	NP	<mLOQ	33	5.9	12	15	75
Channels (n=0)							*Channels (n=2)*						
E1	-	-	-	-	-	-	E1	<mLOQ	3.5	<mLOQ	<mLOQ	<mLOQ	50
BPA	-	-	-	-	-	-	BPA	10	23	17	17	9	100
NP	-	-	-	-	-	-	NP	44	79	62	62	25	100
PFOA	-	-	-	-	-	-	PFOA	<mLOQ	0.84	<mLOQ	<mLOQ	<mLOQ	50
Pialassa Baiona (n=4)							*Pialassa Baiona (n=4)*						
E1	<mLOQ	<mLOQ	-	-	-	-	E1	<mLOQ	1.6	<mLOQ	<mLOQ	<mLOQ	25
BPA	<mLOQ	5.5	1.1	2.0	2.3	75	BPA	3.2	8.9	6.9	6.5	2.4	100
NP	8.6	32	24	22	11	100	NP	11	97	45	49	37	100
PFOA	<mLOQ	<mLOQ	-	-	-	-	PFOA	<mLOQ	0.88	<mLOQ	<mLOQ	<mLOQ	25

[1] Concentrations are expressed as ng/L. SD: standard deviation

A few studies have been carried out all over the world to determine EDCs occurrence in sediments, and the obtained range of concentrations were quite variable from site to site, according to the different types of environments and anthropic pressures. In Italy, a study conducted in the Venice lagoon, located approximately at 200 km north of the Romagna area, recorded quite comparable concentrations of NP, which ranged from 47 to 192 ng/g dw, and slight higher concentrations of BPA, going from <2.0 up to 118 ng/g dw in some stations (Pojana et al. 2007). Higher values than those reported in this study were found by Peng et al. (2017) in Chinese river sediments, with concentrations of 2.54-269 (mean of 121) ng/g dw of BPA and 10.9-14,400 (mean of 4,440) ng/g dw for NP. Klosterhaus et al. (2013) reported comparable concentrations of NP (range of 21.5-86.3 ng/g dw; median of 34.7 ng/g dw) in San Francisco Bay, and Liao

et al. (2012) <0.25-106 (median of 1.49) ng/g dw of BPA in US rivers and 1.88-23.0 (median 8.30) ng/g dw of BPA in Japan. A high contamination by NP was detected in the Iberian peninsula, with concentrations ranging from <1.6 to 1,693 (median of 62) ng/g dw in Ebro River, 19-470 (median 50) ng/g dw in Llobregat River, <1.6-175 (median 50) ng/g dw in Jucar River and 61-190 (median 43) ng/g dw in Guadalquivir River (Gorga et al., 2015).

Both BPA and NP concentrations in the coarse and fine fractions showed the highest values in the Pialassa Baiona, probably due to the close vicinity of this wetland area to the Ravenna harbor, characterized by petrochemical and metallurgical manufactories, as well as platform yards activities and a huge cargo vessel traffic. Moreover, the lagoon is in close connection with inland waters of Lamone River and the surrounding channels which were proven by this study to be affected by phenolic contamination in waters. Hence, inputs of phenolic compounds could come both from inland waters and from the surrounding activities located in the coastal area of Ravenna; the higher salinity of Pialassa Baiona waters in comparison to the inland freshwater system could enhance phenolic deposition in sediments.

In river fine sediments the highest phenolic concentrations were registered in sample F5 of the Fiumi Uniti River, which is highly influenced by the discharge of Forlì WWTP effluents that can thus be considered one major source of BPA and NP. In line with waters results, Bevano sediments were affected by phenolic contamination, especially in points B2 and B3, with concentrations greater than the 70^{th} percentile (29 ng/g dw) of the total phenolic population data. Sediment data of Lamone, Marecchia and Savio systems were quite comparable and revealed a distribution of contaminants gradually increasing at the final stretch of the river bodies, in accordance with phenolic distribution in water. The only exception to this behavior was sample S1, which recorded high concentrations of NP (30 ng/g dw), though its concentration in water was <LOQ. This can be due, once again, to the low flow rates that characterize the uppermost section of the river that lead to strong interactions with waters and sediments, with a subsequent enrichment and transfer of the contaminant in the solid phase.

3.4 EDCs interactions between water and sediment

Highly significant correlations in water samples were found for PFOA and PFOS (p-value: $5 \cdot 10^{-4}$). The close relation between the two compounds states that their occurrence in the aquatic compartment was dependent on the same sources of contamination, primarily depending on WWTP effluents release in river waters (Pistocchi and Loos 2009; Dauchy et al. 2017).

Even if at a lower order of magnitude, statistically significant correlations were also obtained between BPA and PFOS (p-value: $4.6 \cdot 10^{-3}$) and between NP and PFOA (p-value: $3.1 \cdot 10^{-2}$), suggesting that BPA and NP in some extent share similar sources of contamination with perfluorinated compounds. In detail, BPA and

PFOS correlation is likely explained by the ubiquitous presence of these two chemicals in the Marecchia river as a consequence of the presence of several industries of painting manufactories, potteries and mechanic workshops. On the other hand, PFOA was mainly present on the northern part of the study area, especially in correspondence of Ravenna and Forlì districts, clearly reflecting the influence of WWTPs located in the area, as pinpointed also by Pignotti et al. (2017b).

Correlation in sediments was investigated only for the phenolic group of contaminants, since the sporadic presence of perfluorinated compounds and estrogens in sediments could not allow to infer statistical results. Both BPA and NP showed to be highly related with TOC (p-value of $1 \cdot 10^{-5}$ and $8 \cdot 10^{-3}$, respectively). This is consistent with other previous studies stating the great influence of organic matter in the distribution of contaminants in soils and sediments (Jin and Zhu 2016; Pintado-Herrera 2017). Moreover, a weak relation was also observed for BPA and NP concentrations in sediments (p-value=0.03), suggesting a similar introduction in the environment and similar behaviors. Concerning a grain-size effect, concentrations recorded in the coarse fraction were more related to BPA and NP concentrations detected in water, whereas phenolic concentrations in the fine fraction were highly related with TOC content. This finding suggests that the coarse fraction of sediments is more involved in exchanges with the aquatic compartment, whereas the partition of phenolic compounds in the fine fraction is controlled by organic matter.

The partition coefficients between water and sediment K_d (mL/g) and the organic carbon- normalized coefficient $logK_{oc}$ values were calculated, both for BPA and NP. Boxplots representing the distribution of $logK_{oc}$ values are reported in **Figure 4.5**. Samples where both concentrations in water and sediment were <LOQ were not considered. $LogK_{oc}$ values ranged between 2.5 and 5.6 for BPA, and between 3.5 and 6.8 for NP in the fine fraction of sediments, while in the coarse fraction were slightly lower (3.2-4.4 for BPA; 3.2-6.8 for NP). In the case of NP, it is noteworthy that even if the range of $logK_{oc}$ values were quite comparable in the coarse and fine fractions, median values were slightly higher in the fine fraction (value of 4.7) than in the coarse one (value of 4.1), as also shown in **Figure 4.5**. Higher $logK_{oc}$ values of NP compared to BPA were registered in both sediment fractions, and are to be ascribed to the higher hydrophobicity of NP.

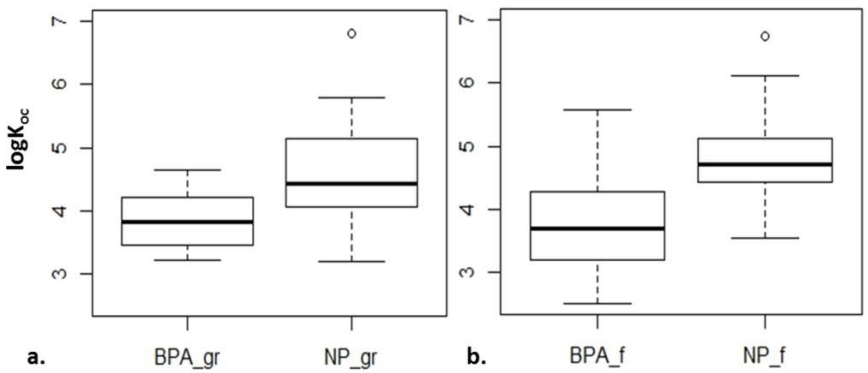


Figure 4.5 Distribution of $logK_{oc}$ values of BPA and NP in the coarse fraction (a) and fine fraction (b) of sediments

Overall, the greatest partitioning values for BPA and NP were registered in the samples taken from Pialassa Baiona, which is a brackish lagoon connected both with inland channels and with the marine environment. This behavior depends on the lower flow rates that characterize the lagoon and that enhance long-time sediment/water interactions, in contrast to the higher river flow rates. Furthermore, the difference in salinity might play an important role, enhancing precipitation of organic compounds in the solid phase as a consequence of the decrease of their solubility as the content of salts in water increases (Yang et al. 2016). $LogK_{oc}$ values in freshriver bodies were lower, since phenolic values in waters were much higher than those detected in sediments. This can be explained by a higher solubility of organic chemicals in the freshwater system and by higher river flow rates that limit long temporal interactions between waters and sediments.

K_d and K_{oc} could not be calculated for estrogens and perfluorinated compounds. Estrogens showed a very low frequency of detection in water (16% for E1), and an even more sporadic distribution in sediments (4% and 2% of frequency for E1 and E2). The very low presence of estrogens in waters is thus the explanation of their almost absence in sediments. Perfluorinated compounds, on the contrary, showed to be widely distributed in water samples (93% of frequency of distribution for PFOA; 50% of presence for PFOS), whereas in the sediment compartment they were almost undetected (7% of detections for PFOA, all concentrations <LOQ for PFOS). Partitioning of organic contaminants between water and sediment is influenced by many factors, such as physical and chemical properties of chemicals, composition of sediment samples, hydrological conditions of the water body and biological processes (Yang et al. 2011; Arditsoglou and Voutsa 2012). The almost absence of a partitioning process of perfluorinated compounds in sediments of this study can be explained by the lower hydrophobicity of these compounds with respect to the phenolic class, and by the high river flow rates that limit the achievement of an equilibrium between the sediment and water phases. Moreover, the lower values of pKa of PFOA (2.5) and PFOS (3.3) (EFSA 2008) strongly control their environmental behavior, leading to the prevailing of their anionic form in the aquatic environment, which is slightly alkaline. On the contrary, the high values of pKa for BPA and NP (9.46 and 10, respectively), indicate that these compounds are present in water in their neutral form, and hydrophobic interactions with sediments are more likely to occur.

3.5 Association between EDCs in sediments and sediment components

To further understand phenolic distribution in sediments, BPA and NP concentrations were compared with both major oxides and main trace elements. Scatterplots showing comparisons are reported in **Figure 4.6.**

No great correspondence was displayed between phenolic compounds and SiO_2, Al_2O_3 or CaO; only fine-grained samples of the Pialassa Baiona and some of the fine river sediments showed a slight positive relation with Al_2O_3, representative of aluminosilicates and clay minerals.

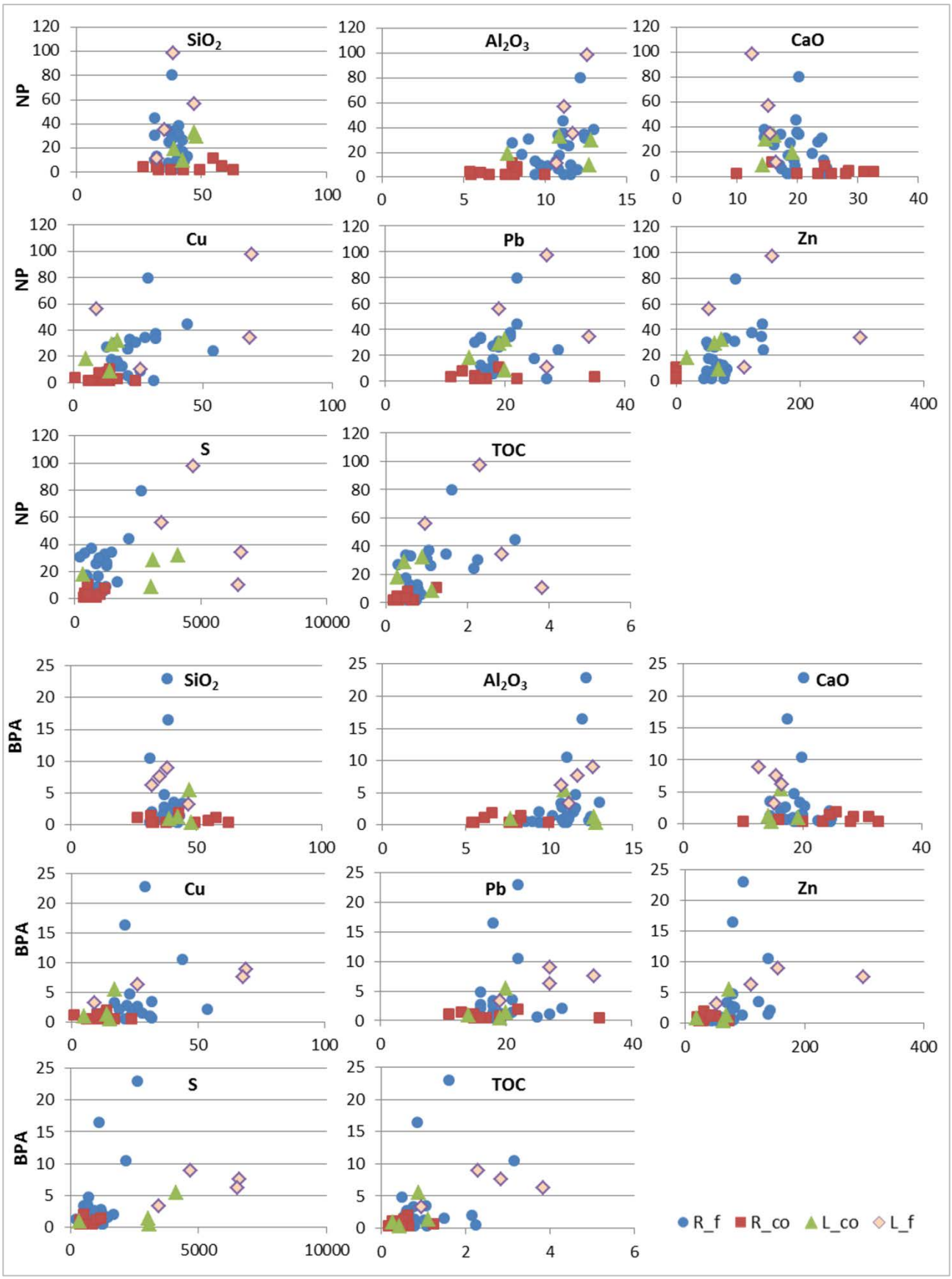


Figure 4.6 Relation between NP and BPA concentrations (ng/g dw) with major oxides SiO_2, Al_2O_3, CaO (wt%), trace metals Cu, Pb, Zn (mg/kg), S (mg/kg) and TOC (%). In each graph observations are grouped as river fine sediments (R_f), river coarse sediments (R_co), lagoonal coarse sediments (L_co) and lagoonal fine sediments (L_f)

Some similarities were observed between Cu, Pb and Zn in fine-grained river samples and the two phenolic compounds, BPA and NP. In particular, NP concentrations in river samples followed quite well Cu, Pb and Zn behavior in sediments, increasing as heavy metal concentrations increased. This is quite consistent with Cu, Pb and Zn distribution in sediments, since they can occurr as accessory elements in clay minerals or oxides and hydroxides, which are the main components of the fine-grained fraction. Such distribution pattern was barely present in Pialassa Baiona sediments, and this could be related to the different and local sources of introduction of the two groups of pollutants into the lagoon area; the differences in size of the two population data can also influence the global distribution pattern. BPA showed similar relations with the three heavy metals, as well, both in river and lagoon fine sediments. Though, it should be noticed that in all graphs three outliers (sample points P1, P2 and S4) masked the general behavior of values in sediments, stating the presence of particular sources of BPA. Association between the phenolic EDCs and TOC and S confirmed the affinity of compounds with organic matter, which exerts an important role in controlling their spatial distribution. In fact NP, and also BPA to a lesser extent, showed increasing concentrations at increasing of both TOC and S content, particularly in river fine sediments.

Notwithstanding the different patterns of concentrations displayed by the single graphs, it is worth to be noted that BPA and NP concentrations in the coarse fraction of sediments were always quite uncorrelated with any of the major oxides or trace elements analysed, and were characterized by the lowest concentration values. Therefore, concentrations in coarse-grained sediments can be considered as “background level” and can be used for comparison in order to identify possible anomalous situations that clearly differ from the general “background” behavior of concentrations. This can be useful, considering the wide distribution of phenolic compounds in the environment.

3.6 Environmental Quality Standards (EQS) evaluation

The growing concern of the potential threats to human health and the ecosystem from exposure to EDCs led European Union to set maximum allowable concentrations (MAC-EQS) and annual- average concentrations (AAC-EQS) not to be exceeded in surface waters for some of the EDCs analysed in this study (EU 2013). The Italian Legislative Decree N. 172 (2015), implementing the European legislation, set additional chemical substances to be monitored, such as PFOA. These thresholds are summarized in **Table 4.3**.

In this study OP was never found at concentrations above the LOQ in water samples, proving that the study area is not affected by contamination of this chemical. On the other hand, NP was detected at maxima concentrations slightly lower than 0.15 µg/L in some situations; nevertheless, no water sample exceeded the EU threshold of 0.3 µg/L. Regarding perfluorinated compounds, almost all sample stations where PFOS was detected at concentrations higher than its LOQ exceeded the threshold of 0.65 ng/L as annual average

concentration in river waters, but were far lower than the maximum allowable concentration of 36 μg/L set by the Directive. Sample CG1 of the Pialassa Baiona, belonging to a transitional environment, was found at concentrations higher than 0.13 ng/L, as well, but lower than the maximum of 7.2 μg/L. On the contrary, river waters did not exceed the threshold of 0.1 μg/L for PFOA proposed by the Italian law in any of the samples. Waters taken from Pialassa Baiona (transitional waters), as well, were lower than the threshold of 20 ng/L, even though sample CG1 was very close to the Italian threshold, registering a value of 18 ng/L, and hence indicating a situation of possible concern. No thresholds for estrogens have been set yet, though these compounds are included in the "watch list" of substances to be monitored in order to gather information about their occurrence in the environment (EU 2013). In this study only estrone was present at detectable concentrations, but its spatial occurrence was rather sporadic, regarding only Pialassa Baiona and some samples of Bevano, Savio and Fiumi Uniti basins. Concentrations were not too high, though (<7 ng/L). Concerning BPA, no restrictions have still been adopted, because of insufficient data on its risk assessment (US EPA 2017).

Table 4.3 Environmental Quality Standards reported by the Directive 2013/39/EU and Italian Legislative Decree No. 172/2015 for surface waters

	AA-EQS[1]	AA-EQS[2]	MAC-EQS[1]	MAC-EQS[2]	References
NP	0.3	0.3	2.0	2.0	EU 2013
OP	0.1	0.01	-	-	EU 2013
PFOS	$6.5 \cdot 10^{-4}$	$1.3 \cdot 10^{-4}$	36	7.2	EU 2013
PFOA	0.1	0.02	-	-	Legislative Decree No. 172/2015

[1] Annual Average (AA) and Maximum Allowable Concentrations (MAC) applicable to inland waters. Values are expressed as μg/L
[2] Annual Average (AA) and Maximum Allowable Concentrations (MAC) applicable to surface waters other than inland waters. Values are expressed as μg/L

Concerning sediments, no regulation is available, so far. Even if based on a limited set of data, EU assessed a theoretical predicted no-effect concentration in sediments ($PNEC_{sed}$), that is the maximum concentration at which no adverse effects on organisms are detected, of 39 ng/g dw for NP (EC 2002) and 63 ng/g dw for BPA (EU 2010). Comparing those values to BPA and NP total concentrations detected in sediments in this study, no critical situations were found for BPA, since its maximum concentration was 23 ng/g dw (as total content). On the contrary, concentrations of NP were very close or even exceeded the theoretical $PNEC_{sed}$, reaching 129 ng/g dw in sediments, with an average value of 31±29 ng/g dw. It should be remarked, however, that the theoretical $PNEC_{sed}$ was derived from the PNEC value calculated on aquatic organisms; therefore, it is advisable to revise these values by performing toxicity test on sediment organisms, as well (EC 2002).

To better analyse EDCs contamination in the study area, concentrations of both water and sediment samples for each compound were converted in terms of EEQs. For the precautionary principle, to calculate the total concentration of EEQ in each sample, concentrations of compounds <LOQ were substituted with

half the LOQ. EEQs in surface waters of the study area showed little variation, with values comprised between 1.5 and 2.3 EEQ ng/L, meaning that concentration of EDCs found in the study area can be comparable to the exclusive detection of 1.5-2.3 ng/L of E2 in the aquatic compartment. Sediment samples displayed a slightly higher variability, with values ranging from 3 to 6 EEQ ng/g dw. A few studies have reported the evidence of estrogenic effects at levels >1 ng/L of E2 in the aquatic environment (Esteban et al., 2014; Tiwari et al., 2016). In this study all rivers registered EEQ levels higher than 1 ng/L; it should be noted, however, that these values are highly dependent on the concentrations of estrogens, since they have the highest EEF values. Estrogens in this study were <LOQ in the majority of the samples and this should be kept into consideration when interpreting the results. When calculating EEQs considering only EDCs concentrations that were > LOQ, the obtained values were much lower (range 0.002 - 0.83 ng/L for waters; 0.0005 - 0.48 ng/g dw in sediments). Therefore, based on these results, EDCs in the study area are not likely to exert strong endocrine disruptions on aquatic or benthic organisms. It is to bear in mind, however, that PFOA and PFOS were not considered in the total estrogenic evaluation of EDCs; further studies are hence needed to deeply understand their estrogenic effects with respect to estradiol.

4 CONCLUSIONS

The study was aimed at assessing EDCs distribution in water and sediment of the Romagna area, integrating it with a general geochemical characterization of both compartments. Concerning the aquatic compartment, river bodies showed to be affected by the occurrence of perfluorinated and phenolic compounds, whereas estrogens were detected only at local stations. Overall, the highest EDCs concentrations were detected in the plain section of the rivers, reflecting the great impact of human presence on water quality. EDCs were also recorded in waters of the wetland area of Pialassa Baiona, which receives waters both from the inland area and from the Adriatic Sea, suggesting a contamination not restricted only to the freshwater system, but broadened also to the coastal environment. Concerning sediments, the only EDCs widely detected were NP and BPA, whereas estrogens and perfluorinated compounds showed only some local occurrence. The different behavior of phenolic compounds with respect to perfluorinated substances was mainly dependent on their difference in hydrophobicity. Salinity and grain size were found to be additional elements controlling phenolic partitioning between water and sediment. Globally, surface waters were not affected by a high contamination by EDCs, since concentrations were lower than the environmental quality standards set by the EU Directive for most of the selected compounds. Overall, no great estrogenicity effects are to be expected in the aquatic biota. A continuous monitoring is though needed in order to assess a long-term potential hazard in the study area.

Supplementary Material

DISTRIBUTION AND PARTITION OF ENDOCRINE DISRUPTING COMPOUNDS IN WATER AND SEDIMENT: CASE STUDY OF THE ROMAGNA AREA (North Italy)

Table S4.1 Principal human activities located in the main cities of the study area

Town/municipality	Main human activities	Details
Ravenna	WWTP	240,000 Population Equivalents
		15,944 Mm^3/year of input sewages
		Effluents discharged into P1 channel
	Plastic and paintings production	19 plants
	Food industry	290 plants
	Textile	30 plants
	Electrical devices production	41 plants
Faenza	WWTP	100,000 Population Equivalents
		17,800 Mm^3/year of input sewages
		Effluents discharged into Lamone River
	Plastic and paintings production	6 plants
	Food industry	90 plants
	Textile	40 plants
	Electrical devices production	22 plants
Forlì-Cesena	WWTP	250,000 Population Equivalents
		18,485 Mm^3/year of input sewages
		Effluents discharged into Ronco River
	Plastic and paintings production	47 plants
	Food industry	321 plants
	Textile	91 plants
	Electrical devices production	70 plants
Rimini	WWTP	250,000 Population Equivalents
		29,642 Mm^3/year of input sewages
		Effluents discharged into Marecchia River
	Plastic and paintings production	17 plants
	Food industry	214 food industry
	Textile	28 plants
	Electrical devices production	41 plants

Table S4.2 Availability of water and sediment samples

River	Sample point	Water	Sediment	
			Coarse fraction	*Fine fraction*
Lamone	L5	+	+	+
	L4	+	+	+
	L3	+	-	+
	L2	+	+	+
	L1	+	-	+
CER channel	L7	+	-	-
Fiumi Uniti	F7	+	+	+
	F5	+	+	+
	F6	+	-	-
	F4	+	+	+
	F3	+	+	+
	F2	+	+	+
	F1	+	-	-
Bevano	B3	+	-	+
	B2	+	-	+
	B1	+	+	+
Savio	S5	+	-	-
	S4	+	+	+
	S3	+	-	+
	S2	+	+	+
	S1	+	-	+
Marecchia	M4	+	-	+
	M3	+	+	+
	M2	+	+	+
	M1	+	+	+
channels	P2	+	-	+
	P1	+	-	+
Pialassa Baiona	CG1	+	+	+
	CG2	-	+	+
	CP	+	+	+
	MR	+	+	+

+ available
- not available

Table S4.3 Chromatographic conditions and MS parameters for perfluorinated compounds

LC chromatographic conditions				
Time (min:sec)	**A** (%)	**B** (%)	**Flow** (ml/min)	
00:00	90	10	0.2	
06:00	90	10	0.2	
13:00	1	99	0.2	**Pump A**: water + 1 mM ammonium acetate
18:00	1	99	0.2	**Pump B**: acetonitrile/water (95/5) + 1 mM ammonium acetate
20:00	90	10	0.2	
27:00	90	10	0.2	

MS detection parameters			
Capillary (V):	2.7	Source Temperature (°C)	130
		Desolvation Temperature (°C)	350
Extractor (V)	3	Desolvation gas flow (l/h)	850
		Cone gas flow (l/h)	85

MS transitions (m/z)				
	PFOA	413>369	**PFOS**	499>80
		413>169		499>99

Table S4.4 Chromatographic conditions and MS parameters for estrogens and phenolic compounds

LC chromatographic conditions				
Time (min:sec)	**A** (%)	**B** (%)	**Flow** (ml/min)	
00:00	90	10	0.2	
06:00	90	10	0.2	
08:00	20	80	0.2	
14:00	20	80	0.2	**Pump A**: water + 0.1% ammonium hydroxide
15:00	1	99	0.2	**Pump B**: acetonitrile+ 0.1% ammonium hydroxide
21:00	1	99	0.2	
23:00	90	10	0.2	
35:00	90	10	0.2	

MS detection parameters			
Capillary (V):	2.9	Source Temperature (°C)	130
		Desolvation Temperature (°C)	400
Extractor (V)	5	Desolvation gas flow (l/h)	800
		Cone gas flow (l/h)	80

MS transitions (m/z)				
	E1	269.1>145	**BPA**	227>212
		269.1>143		227>133
	E2	271.1>183	**NP**	219.1>133
		271.1>145		219.1>147
	EE2	295.1>145	**OP**	205.2>106
		295.1>159		

Table S4.5 Limit Of Detection (LOD), Limit of Quantification (LOQ) and recoveries for the selected EDCs

	Waters			Sediments		
	mLOD (ng/L)	**mLOQ** (ng/L)	**Recovery** (%)	**mLOD** (ng/g dw)	**mLOQ** (ng/g dw)	**Recovery** (%)
estrogens						
E1	0.19	0.62	89±2	0.48	1.60	74±7
E2	0.71	1.35	83±5	0.75	2.49	71±8
EE2	0.80	1.66	77±9	0.83	2.78	69±11
Phenolic compounds						
BPA	0.30	0.99	88±2	0.21	0.71	81±5
NP	0.62	2.05	84±4	0.78	2.60	80±6
OP	0.20	0.66	88±3	0.23	0.77	79±6
Perfluorinated compounds						
PFOA	0.02	0.07	96±2	0.09	0.30	79±12
PFOS	0.02	0.08	94±4	0.27	0.90	77±10

Table S4.6 Estrogenic Equivalent Factors (EEF) for each of the analyzed EDCs, as reported in Morales et al. (2013)

Compound	**EEF**[1]
E1	0.11
E2	1
EE2	1.25
BPA	$3.9 \cdot 10^{-4}$
NP	$2.7 \cdot 10^{-4}$
OP	$2.1 \cdot 10^{-4}$

[1] EEFs are defined as the quotient of half maximal effective concentrations of estradiol and other EDC ($EC50_{E2}/EC50_{EDC}$) and is set to 1 for estradiol (Morales et al., 2013)

Table S4.7 Summary statistics of major components of river and lagoonal water samples

	River					*Lagoon*				
Element	**Min**	**Max**	**Median**	**Mean**	**SD**	**Min**	**Max**	**Median**	**Mean**	**SD**
T (°C)	15.8	33.0	26.1	24.7	4.9	29.3	29.8	29.6	29.6	0.3
pH	7.3	8.2	7.6	7.7	0.2	7.9	8.1	8.0	8.0	0.1
EC (mS/cm)	0.282	1.506	0.549	0.564	0.253	33.5	45.3	45.1	41.3	6.7
mg/L										
Ca	32	116	59	63	19	310	409	396	371	54
Mg	8.4	38	21	22	9	960	1286	1262	1169	182
K	1.6	100	5.7	9.2	18.4	284	399	380	354	61
Na	6.5	124	34	37	29	7163	8408	8340	7970	700
Cl	7.9	200	29	44	43	13133	19465	18971	17190	3522
F	0.002	0.388	0.143	0.168	0.066	<0.002	4.6	<0.002	1.5	2.7
HCO_3	140	598	275	294	106	140	140	140	140	-
NO_2	<0.02	0.83	<0.02	0.08	0.16	<0.02	<0.02	-	-	-
NO_3	0.001	18	3.2	4.4	3.9	<0.001	<0.001	-	-	-
PO_4	<0.002	2.6	<0.002	0.13	0.51	<0.002	<0.002	-	-	-
SO_4	26	175	82	85	45	1717	2555	2421	2231	450

Table S8 Minimum, maximum and median concentrations of major oxides, TOC, N and trace elements content in river and lagoon coarse and fine fractions of sediments

	River						***Lagoon***					
	Coarse fraction *(n=14)*			Fine fraction *(n=23)*			Coarse fraction *(n=4)*			Fine fraction *(n=4)*		
Element	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**
%												
SiO_2	26.8	62.9	40.5	31.5	44.6	38.5	39.1	48	44.8	32.4	47.0	37.1
TiO_2	0.2	0.5	0.3	0.4	0.6	0.5	0.3	0.6	0.5	0.5	0.6	0.5
Al_2O_3	5.4	10	7.8	8	13	11.1	7.7	12.8	11.8	10.7	12.6	11.5
Fe_2O_3	2.1	5.1	3.3	3.7	5.9	4.9	3.3	5	4.1	3.7	5.8	5.2
MnO	0	0.3	0.2	0.1	0.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1
MgO	1.6	3.2	2.3	2.5	3.7	3.2	2.6	3.7	3.6	3.4	3.5	3.4
CaO	10	32.8	25.2	14.6	24.9	19.7	14.3	19.3	15.6	12.6	16.5	15.5
Na_2O	0.1	1.7	0.8	0.1	1	0.5	1	1.6	1.3	1.0	1.6	1.3
K_2O	1	2.4	1.65	1.4	2.5	2	2.1	2.6	2.5	2.2	2.6	2.4
P_2O_5	0.1	0.2	0.1	0.1	0.5	0.2	0.1	0.2	0.1	0.1	0.2	0.2
LOI	9.7	26.2	21.1	14.4	25	18.5	12.7	17.5	13.8	12.4	17.3	14.9
%												
TOC	0.2	1.3	0.5	0.3	3.2	0.8	0.3	2.3	0.8	0.9	3.8	1.9
N	0.01	0.1	0.06	0.1	0.4	0.1	0.04	0.33	0.08	0.1	0.3	0.1
mg/Kg												
As	2	7.8	3.2	3	8	5	3	7	5	5	9	6
Ba	88	282	208	176	346	260	218	279	262	227	287	259
Ce	23	42	31	34	66	50	31	61	55	38	58	49
Cl	22	65	34	7	701	26	152	2741	2048	1474	7288	2362
Co	4	11	5	7	13	10	4	12	8	6	12	8
Cr	42	132	63	97	166	126	74	152	121	104	281	134
Cs	0.6	4	3	1	4	2	2	3	3	2	4	2
Cu	1	24	12	11	54	21	5	69	15	9	68	21
Ga	6	13	7.6	9	17	12	6	16	12	8	14	12
La	10	33	18	11	31	20	16	29	26	19	27	21
Nb	7	14	8	11	15	13	6	14	11	8	13	11
Ni	28	55	36	48	88	65	44	92	66	52	93	66
Pb	11	35	16	15	29	18	14	27	19	19	34	23
Rb	32	81	56	58	119	78	51	116	90	70	101	86
S	360	1200	515	230	2640	950	340	4700	3060	3460	6590	5305
Sc	22	71	47	30	49	39	29	43	32	32	38	34
Sn	1	10.2	2	1	6	2	3	6	4	3	4	3
Sr	255	651	399	287	588	395	267	327	295	299	562	375
Th	1	11	7.8	9	20	14	2	19	13	8	16	14
U	1	2	1	1	3	2	1	3	2	1	4	2
V	42	205	67	116	191	162	94	172	139	107	193	143
Y	12	21	16	20	27	23	11	25	21	14	22	19
Zn	20	74	31	45	142	77	18	156	66	53	298	92
Zr	52	84	64	83	158	117	50	107	90	70	98	83

n refers to the number of sampling points considered for each group of sediments

Figure S4.1 Boxplots of major oxides analysed in this study, expressed as weight percentage (wt%). For each variable, data are subdivided as follows: river fine fraction (R_f); river coarse fraction(R_co); lagoonal fine fraction (L_f); lagoonal coarse fraction (L_co)

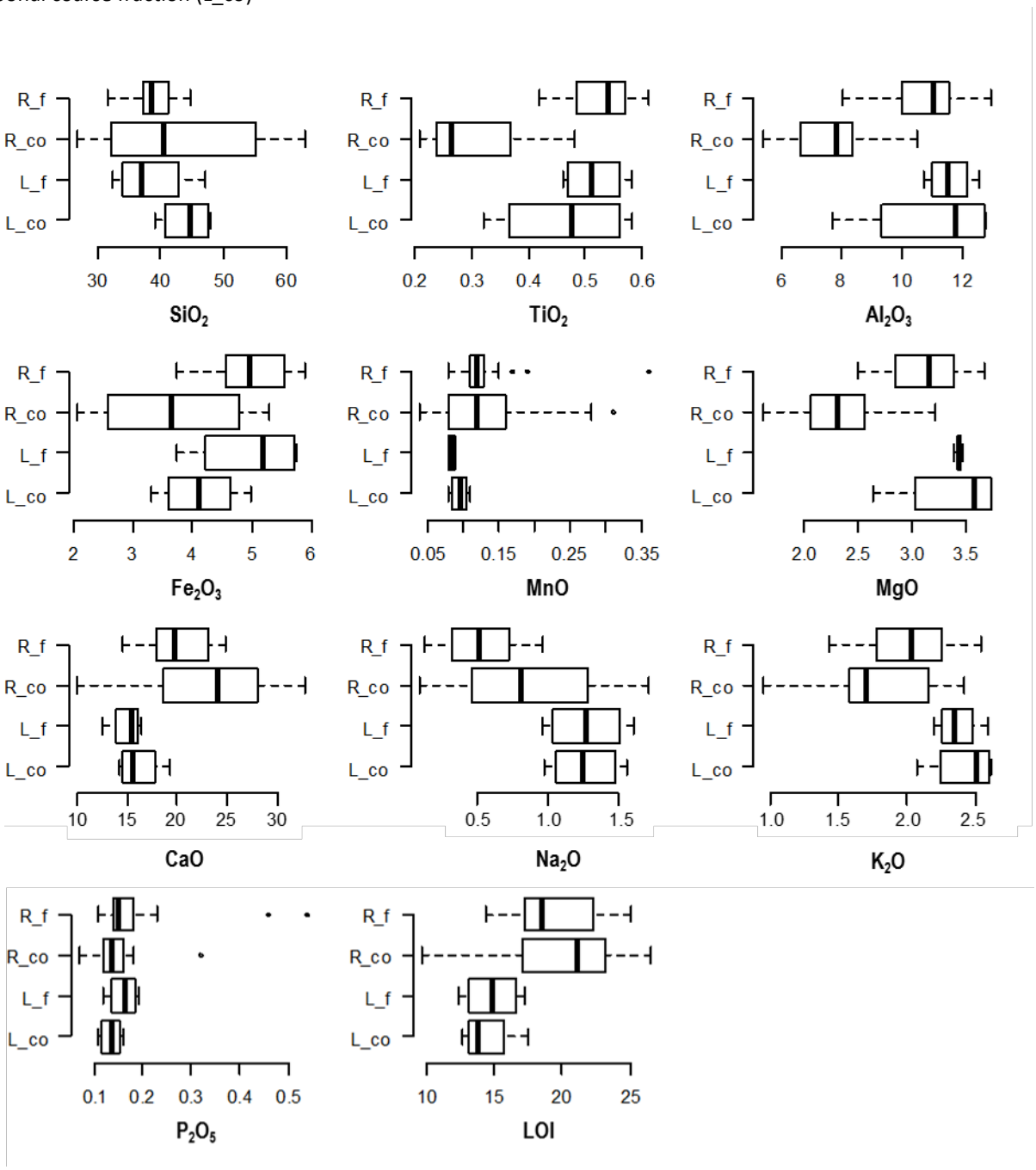


Figure S4.2 Boxplots of trace elements analysed in this study. All values are expressed in mg/kg. For each variable, data are subdivided as follows: river fine fraction (R_f); river coarse fraction(R_co); lagoonal fine fraction (L_f); lagoonal coarse fraction (L_co)

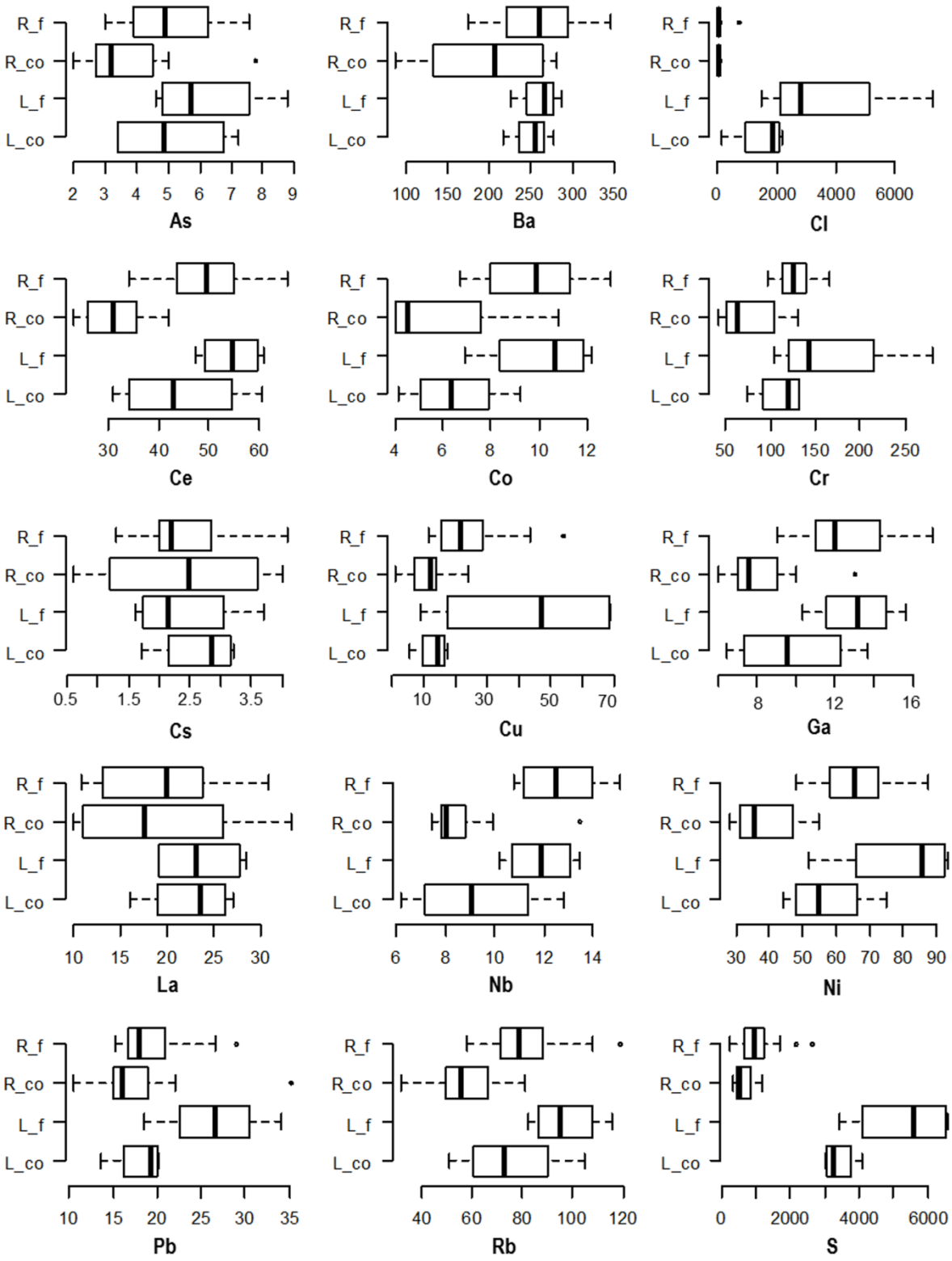


Figure S4.2 (continued)

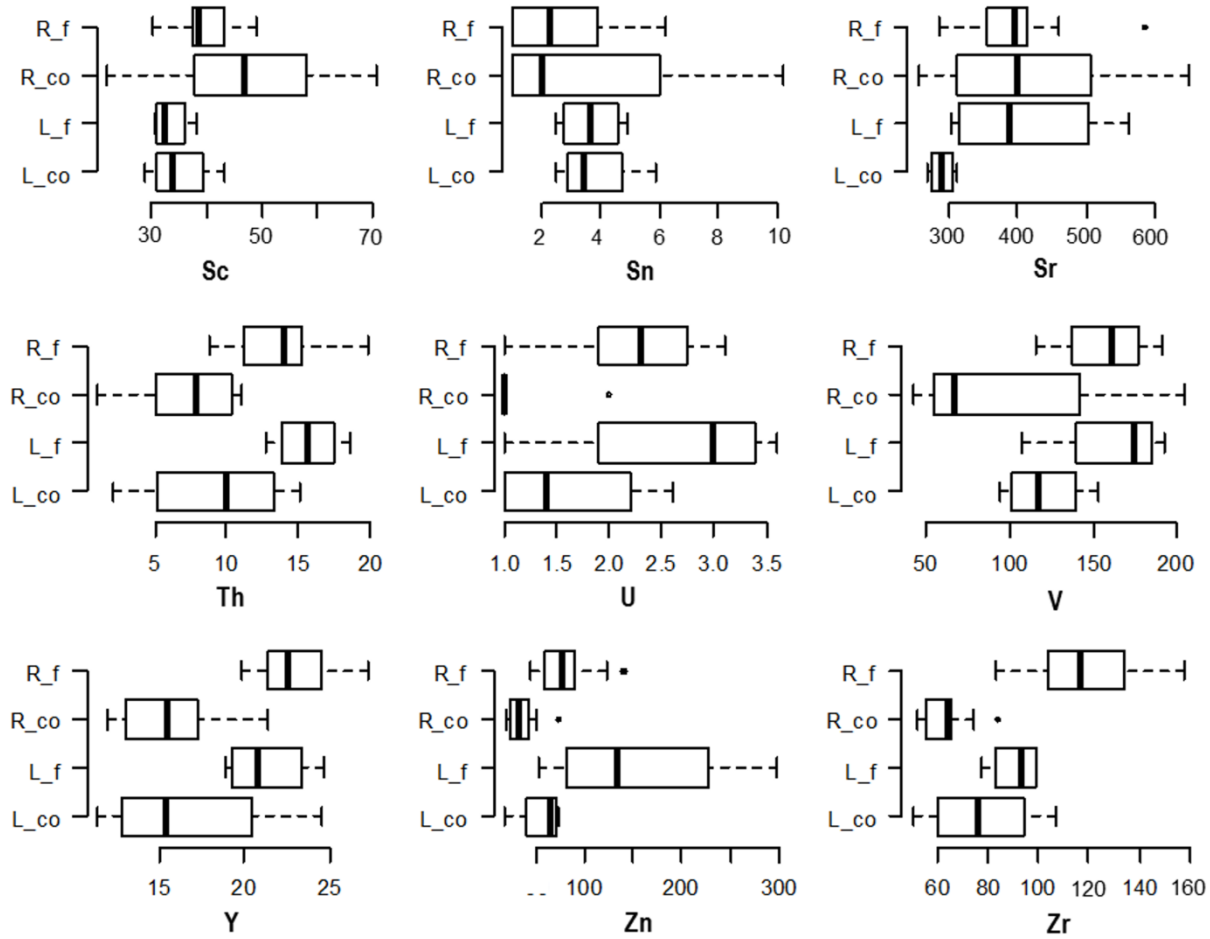


Figure S4.3 EDCs distribution in water samples

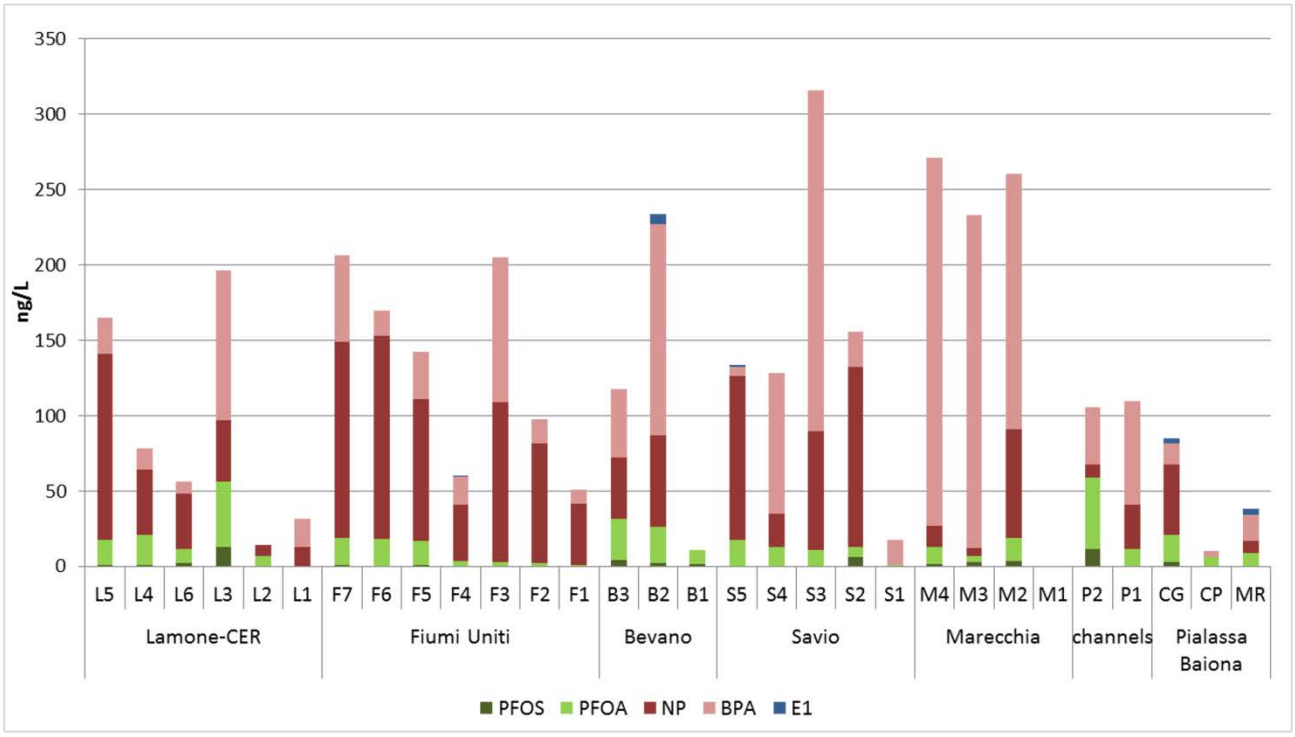
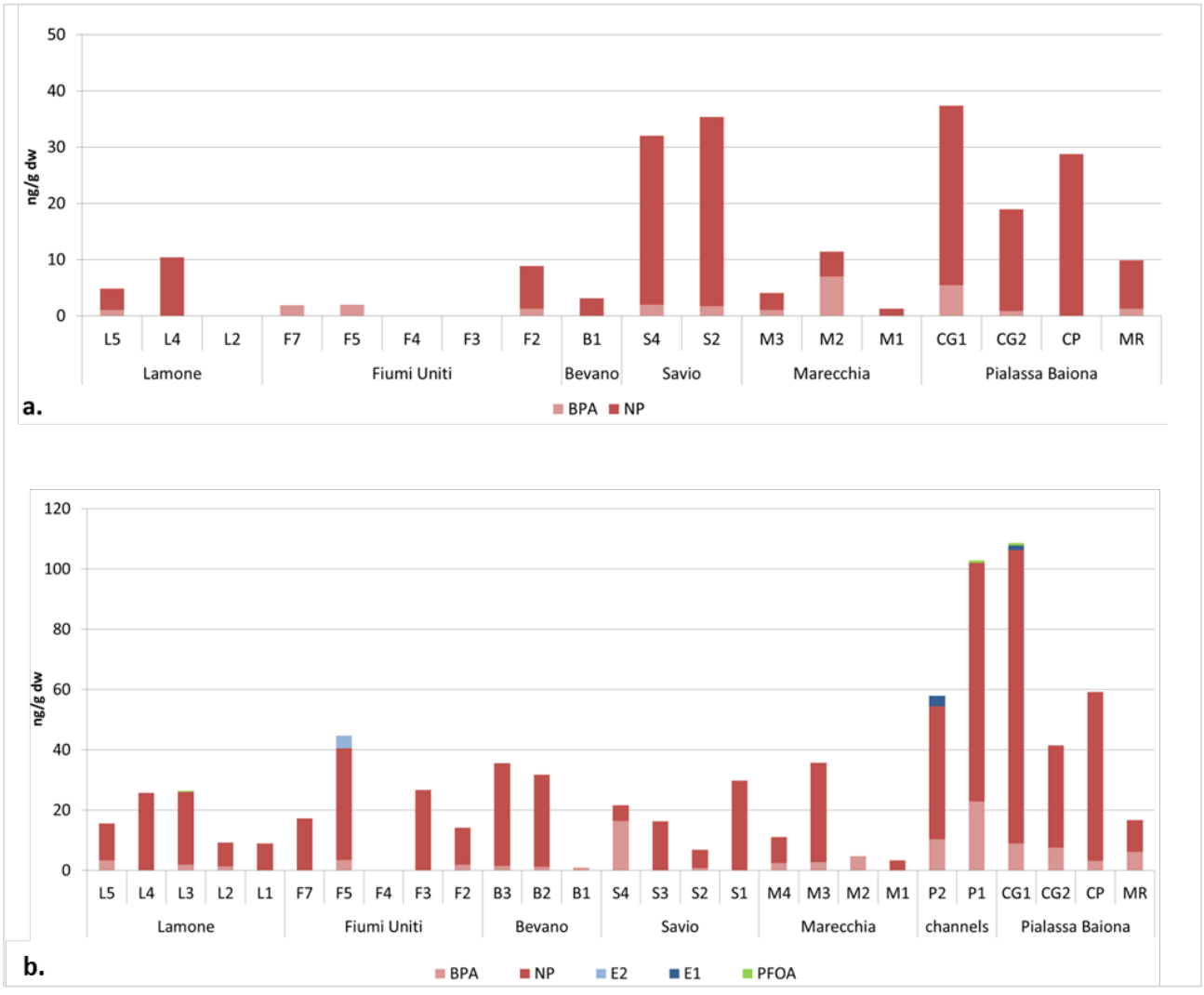


Figure S4.4 EDCs distribution in the coarse fraction (a) and in the fine fraction (b) of sediment samples



Chapter 5

SEASONAL VARIATIONS IN THE OCCURRENCE OF PERFLUORINATED COMPOUNDS IN WATER, SEDIMENT AND FISH SAMPLES FROM EBRO DELTA (Catalonia, Spain)

1 INTRODUCTION

Perfluorinated compounds (PFCs) are a wide group of synthetic substances with multiple industrial and domestic applications, such as stain repellents coatings for textiles and fire-fighting foams among many others (Arvaniti and Stasinakis 2015; Zareitalabad et al. 2013). Because of the strong carbon-fluorine bond, these compounds are characterized by high thermal, chemical and biological stability. However, due to this high stability, they have been found to be persistent in the environment, with compounds such as the perfluorooctanesulfonate (PFOS) having a half-life of more than two months in waters and over six months in soils and sediments (Renzi et al. 2013). Moreover, PFCs show a tendency to bioaccumulate and biomagnify through the food chain (Ahrens et al. 2011; Naile et al. 2010), potentially causing adverse effects on organisms, such as hepatotoxicity reduction of the immune function among others (Lau et al. 2007; Zhang et al. 2013). Therefore, due to their persistence, accumulation in living organisms, the toxicity of some compounds and their wide distribution in the environment, the occurrence of PFCs is a cause for concern, and nowadays they are considered as emerging organic contaminants. For these reasons, the European Commission has set PFOS and its derivatives in the list of priority hazardous substances and has identified water and fish threshold concentrations for environmental quality assessment under the Water Framework Directive (EU 2013). However, there is still a lack of legislation concerning most of these compounds in drinking water and food. Moreover, the Directive 2013/39/EU (EU 2013) laid down environmental quality standards (EQS) for priority substances in water and biota. The EQSs set for PFOS are 0.65 ng/L in inland surface waters (annual average concentration), 36 μg/L as maximum allowable concentration, and 9.1 μg/kg in biota. The US Environmental Protection Agency (US EPA 2016b) has proposed a provisional threshold (between 0.01 and 0.09 μg/L) for drinking water with respect to only 7 compounds, including PFOS and perfluorooctanoic acid (PFOA).

Manufacturing facilities are considered to be one of the main sources of contamination by PFCs (Prevedouros et al. 2006; Pistocchi and Loos 2009), along with wastewater treatment plants (WWTPs), which have been found to be inefficient in the removal of these compounds from wastewater influents (Ahrens et al. 2009a; Boulanger et al. 2005; Schultz et al. 2006). Once released into the aquatic

environment, they can easily be transferred into different environmental compartments, reaching groundwater (Houtz et al. 2013), soils (Houtz et al. 2013), sediments (Gao et al. 2015) and biota (Campo et al. 2016). Furthermore, these compounds have been found in remote environments, such as the Antarctica region (Llorca et al. 2012a). Once in the aquatic environment, PFCs are accumulated and biomagnified through the aquatic food chain whereby they reach human food (Pérez et al. 2014) and drinking water (Llorca et al. 2012b; Schwanz et al. 2016). The partitioning mechanism and their fate in the environment, though, are still not well-known (Ahrens 2011). In addition, most studies have been mainly focused on more persistent and accumulative compounds such as PFOS and PFOA, while less information has been reported regarding the use of short-chain PFCs in the substitution of the 8-carbon chain compounds.

Different studies have already investigated the occurrence of PFCs in the aquatic environment, mainly focusing on their distribution in freshwater, particularly rivers (Ahrens 2011; Loos et al. 2013b; Munoz et al. 2015; Valsecchi et al. 2015; Lorenzo et al. 2016). But, up until now, scarce information is available about their seasonal fluctuation in coastal and highly productive areas, such as estuarine habitats. Those are fragile ecosystems that can be highly affected by human activities since they receive urban sewages and other by-products of human activities (Jiang et al. 2014).

Within this context, the main aim of this study was to assess the occurrence and environmental fate of 13 PFCs in the Ebro Delta (NE of Spain), as well as the surrounding coast: 8 perfluorocarboxylic acids, 4 perfluorosulfonates and 1 sulfonamide in a total number of 213 samples (87 waters, 71 sediments and 55 fishes). These compounds were analysed in the water, sediment and fish samples during three different seasons.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Perfluorinated compounds standards were provided by Wellington Laboratories Inc. (Canada) and were composed of: (i) a mixture of PFCs (PFAC-MXB, 2 µg/ml in methanol, purity > 98%) containing perfluoropentanoic (PFPeA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), perfluoroundecanoic (PFUdA) and perfluorododecanoic (PFDoA) acids, and perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), perfluorooctanesulfonate (PFOS), perfluorodecane sulfonate (PFDS); and (ii) the perfluoroctanesulfonamide (PFOSA). Surrogate internal standards used for quantification normalization were supplied by Wellington Laboratories Inc. (Canada), and included: (i) a mixture of labelled PFCs (MPFAC-MXA, 2 µg/ml in methanol, purity > 98%), composed of $^{18}O_2$-perfluorohexanesulfonate (MPFHxS-$^{18}O_2$), $^{13}C_2$-perfluorohexanoic acid (MPFHxA $^{13}C_2$), $^{13}C_4$-perfluorooctanesulfonate (MPFOS-$^{13}C_4$), $^{13}C_4$-

perfluorooctanoic acid (MPFOA-$^{13}C_4$), $^{13}C_5$-perfluorononanoic acid (MPFNA-$^{13}C_5$), $^{13}C_2$-perfluorodecanoic acid (MPFDA-$^{13}C_2$), $^{13}C_2$-perfluorododecanoic acid (MPFDoA-$^{13}C_2$); and (ii) $^{13}C_8$-perfluorooctanesulfonamide (M8FOSA, >99%).

All solvents and reagents were of analytical grade. Water and methanol (CHROMASOLV® Plus), ammonium acetate (MW: 77.08, purity > 98%), and ammonium hydroxide (MW: 35.05, purity > 98%) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Area of study

The Ebro Delta is the third largest delta in the Mediterranean Sea. It is a wetland area of 320 km^2, highly relevant for conservation, which is included in the Ramsar Convention list. This estuarine habitat is characterized by a high biological productivity, thanks to the nutrients that are provided by the Ebro River (Lloret et al. 2004). The climate in the middle and lowland reaches of the River Ebro is typically Mediterranean, with rainfall concentrated in autumn and spring (200–300 mm) and intense summer drought (<50 mm). Flow regime is pluvio-nival because of the left-margin tributaries from the Pyrenees. The average annual temperature is between 10–15 °C. The lowest temperatures occur in winter (down to -5 °C) and the highest in summer (>40 °C). Substratum in the area is mainly calcareous, with Cenozoic limestones, gypsum and alluvial sediments. Aquatic vegetation consists of macrophytes such as water crowfoot *Ranunculus* spp. and *Scirpus* spp. The land use is mainly for agriculture and cattle rearing; approximately 13% of its total surface is composed of natural lagoons, bays and marshes, whereas the major part (77%) is dedicated mainly to agricultural activity such as rice and orchards. For this reason, since the 1960s, different dams and irrigation channels have been built in order to control Ebro River water and sediment inputs and to fulfill the surrounding water demand (Cardoch et al. 2002).

Amposta, Deltebre, Sant Jaume d'Enveja and Sant Carles de la Ràpita are the main towns that are located in this area, and they can potentially affect the environmental quality with the discharge of their treated sewages into the Ebro River. Chemical industries and a nuclear power plant on the northern side of the area (province of Tarragona) may be additional sources of contamination.

2.3 Sampling

Three sampling campaigns were carried out during October-November 2015 (autumn), February-April 2016 (winter), and June-July 2016 (spring-summer). A total number of 213 samples including 87 water, 71 sediment and 55 fish samples were collected. During the first campaign (i.e. autumn), only water and sediment were sampled, while fish samples were collected in the second (i.e. winter) and third (i.e. spring-summer) sampling campaigns, in addition to water and sediment. Detailed information about the locations of sampling sites and the samples are listed in **Table S5.1** from the *Supplementary Material*. In summary,

water samples were collected from the Ebro River at 7 irrigation channels, from the emissary of the wastewater treatments plant (WWTP) that is located in Sant Carles de la Ràpita, at the influents and the effluents of 2 WWTPs (Sant Carles de la Rapita and Amposta); estuarine water samples were collected in different lagoons (Illa de Buda, L'Encanyissada, La Tancada and Canal Vell) and seawater samples from the Fangar and Alfacs bays and at the open sea adjacent to these bays. Fish samples were collected both from seawater (during winter season) and from freshwater (during spring-summer period). Detailed information about fish communities is shown in **Table 5. 1**.

Table 5.1 Fish communities

Habitat	Scientific name	Common name	Taxonomic family	Origin
sea	*Torpedo torpedo*	common torpedo	Torpedinidae	native
sea	*Trachurus mediterraneus*	jack mackerel	Carangidae	native
sea	*Boops boops*	bogue	Sparidae	native
sea	*Diplodus annularis*	annular sea bream	Sparidae	native
sea	*Sarpa salpa*	salema	Sparidae	native
delta	*Anguilla anguilla*	European eel	Anguillidae	native
delta	*Micropterus salmoides*	largemouth bass	Centrarchidae	non-native
delta	*Mugil cephalus*	flathead grey mullet	Mugilidae	native
delta/river	*Squalius laietanus*	Ebro chub	Cyprinidae	native
delta/river	*Cyprinus carpio*	common carp	Cyprinidae	non-native
river	*Alburnus alburnus*	bleak	Cyprinidae	non-native
river	*Rutilus rutilus*	roach	Cyprinidae	non-native
river	*Scardinius erythrophtalmus*	rudd	Cyprinidae	non-native
river	*Silurus glanis*	European catfish	Siluridae	non-native
river	*Liza* sp.	mullet sp.	Mugilidae	native

Regarding seawater fish, a total of 15 specimens of different species were sampled from two sites of the Mediterranean Sea (Fangar bay, n=4; Alfacs Bay, n=5) and from one Ebro Delta site (Illa de Buda lagoon, *n* = 6). In particular, fish species were *Mugil cephalus*, *Squalius laietanus*, *Cyprinus carpio*, *Anguilla anguilla*, *Torpedo torpedo*, *Sarpa salpa*, *Trachurus mediterraneus*, *Boops boops*, *Diplodus annularis* and *Micropterus salmoides.* Fish were sampled by local fishers using nets. Individual fish samples were measured for fork/total length (FL/TL, ±1 mm), weighed (wet body weight, ±0.1 g), labelled, stored in ice and frozen (-20 °C) on the same date of collection.

Riverine fishes (n=40) sampled during the spring-summer period were collected from two sites of the Ebro River upstream of the Delta: Xerta (n=21) and Tortosa (n=19). In particular, fish species were *Alburnus alburnus*, *Cyprinus carpio*, *Liza* sp., *Rutilus rutilus*, *Scardinius erythrophtalmus*, *Silurus glanis*, and *Squalius laietanus*. To encompass the existing environmental variability, fish were collected from all meso-habitats present in the river (e.g. runs, riffles and pools), from the left and right margins along each sampling site (500 m river length). This allowed collecting a representative sample of fishes. Fishes were sampled by electrofishing from a boat (4.5-m aluminium hull) by using a 2000 W DC generator at 1000 V and 16 A (Model: 5.0-GPP Smith-Root Inc., Vancouver, WA, USA), along with dip nets (2.5 m long pole, 50 cm

diameter net, 10 mm mesh size). Two anodes were suspended from booms and mounted on the bow of the boat, and a cathode was mounted along each side of the hull. A single pass was made following a zigzagging and upstream direction without using block nets in every sampling site. After each survey was concluded, fish were identified until the species level and counted. Then, a fish sub-sample was immediately immersed in an overdose solution of anaesthetic (MS-222) for 15 min. Euthanized fish were measured for fork/total length (FL/TL, ±1 mm), weighed (wet body weight, ±0.1 g), labelled, stored in ice and frozen (–20 ºC) on the same date of collection (<2 h since euthanasia) until laboratory processing. The remaining individuals of non-native species were euthanized according to the same procedure described above, while those of native fish species were kept in a tank with supplied oxygen (two battery operated aerators with portable pump) until fully recovered before being released. All field procedures complied with animal use and care regulations of Europe and Spain (specific licences were granted for Scientific Field Research in the River Ebro). Fish were collected by trained personnel (i.e. the holder of the licence, D. Almeida). Thus, no adverse effects were caused on the wildlife in the study habitats and all native fish fully recovered from the anaesthetic.

2.4 PFCs analysis

2.4.1 Analysis of water samples

Extraction and clean-up were carried out by using the method described by Llorca et al. (2012b). Briefly, 500 mL of seawater, 250 mL of river water and wastewater effluents and 150 mL of wastewater influents were spiked with 10 µL of a mixture of surrogate internal standards at 100 ng/L. After 15 min, a time period that is necessary in order to reach the equilibrium, the samples were filtered and extracted by solid phase extraction (SPE) with Oasis WAX cartridges (30 cc, 60 mg, 30 µm; Waters Corporation, MA, USA) that were previously conditioned with methanol and water. Cartridge elution was carried out with 4 mL of 10% NH_4OH in methanol. The extracts were evaporated under a gentle N_2 stream and reconstituted in 250 µL with a mixture of water and methanol (9:1). All the samples were processed in triplicates.

The extracts were analysed by ultra-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (UPLC-QqQ-MS/MS). Chromatographic separation was achieved with an Acquity UPLC BEH C18 analytical column (2.1 x 50 mm, 1.7 µm particle size; Waters Corporation, USA) using the system Acquity UPLC H-Class (Waters Corporation). A pre-injection column PFCs isolator (Waters Corporation) was used, as well. Mobile phases consisted of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B) and injection was delivered at a flow rate of 0.4 mL/min. The elution programme was as follows: 20%B over a time period of 10 sec, then linear gradient to 80%B over another time period of 4 min and 50 sec, followed by a linear increase to 90%B during 2 min, followed by an isocratic hold at 90%B for a time period of 2 min and 50 sec, and then an isocratic hold was

implemented for 1 min more. At the minute 9:50, B was returned to 20% in 1 min. The total run time for each injection was 11 min and the injection volume was 10 µL.

After separation, the detection was carried out using a triple quadrupole analyser Xevo QqQ-MS (Waters Co.) with an electrospray ionisation (ESI) source operating in negative conditions.

2.4.2 Analysis of solid samples

Sediment sample analyses were carried out by the method that was previously developed and validated by our group (Llorca et al. 2012b). For the extraction of sediment samples, 1 g dried sediment was spiked with 20 µL of a mixture of internal standards (100 ng/mL) and left to reach equilibrium for 20 min. After this period, 10 mL of pure methanol was added, and the sediments were extracted by ultrasonic assisted extraction (UAE) for 1 hour. The extracts were then centrifuged for 20 min at 4000 rpm at 17 °C. After centrifugation, 4 mL of the supernatant was dried with a gentle stream of N_2, reconstituted in 100 µL of a mixture water:methanol (9:1) and directly injected in an on-line clean-up system. Extracts and the posterior analysis were performed in triplicates.

For the analysis of PFCs in fish, skin and muscle were processed separately, according to the validated procedure described by Llorca et al. (2012a). Briefly, 1 g of wet sample was spiked with 20 µL of a mixture of internal standards (100 ng/mL) and left at equilibrium for 20 min. The extraction procedure was based on alkaline extraction, mixing the sample with a solution of 10 mL of methanol with 10 mM sodium hydroxide and digesting for 2 hours in an orbital shaker. After digestion, the mixture was centrifuged at 4000 rpm and 17 °C for 20 minutes. Then, 4 mL of the supernatant was processed as described above for the analysis of PFCs in sediment samples (dried and reconstituted before on-line clean-up process). Due to the differences in weight and size of the selected species, the smallest fish samples were processed and analysed as a pool of individuals, whereas the biggest fish samples were treated as individuals. Whenever possible, the guts were removed from the fish body and only muscle and skin tissues were analysed, but whenever this was impossible, the whole fish body was extracted and analysed.

Extracts of sediments and fish were purified in an on-line clean-up system (Thermo Fisher EQuan™) based on turbulent flow chromatography (TFC). For the purification, two columns were used, Cyclone (50 mm × 0.5 mm, 60 µm particle size, 60 Å pore size) and C18 XL (50 mm × 0.5 mm, 60 µm particle size, 60 Å pore size), connected in tandem. Loading and eluting solvents are summarised in **Table S5.2**. After purification, the extracts were directly pumped to the analytical column Hypersil GOLD PFP (3 x 50 mm, 3 µm particle size; Thermo Fisher Scientific). Sample injection volume was set at 20 µl. Detection was carried out using a triple quadrupole analyser TSQ Quantiva (Thermo Fischer Scientific) equipped with an electrospray ionisation (ESI) source operating in negative conditions.

2.5 Quality assurance and quality control

In order to rule out any system contamination, instrumental blanks made of methanol:water (1:9) were run every three sample injections, while different points of the calibration curve were analysed before, during and after samples in order to check sensibility drifts. For water analyses, procedural blanks were prepared in parallel to samples in order to discard any contamination step during sample treatment.

In **Table S5.3** and **Table S5.4**, the method limits of detection and quantification, and the recoveries for different matrices, respectively, are presented.

2.6 Data analysis

Statistical analyses were performed with R software. All the values that were below the method limit of quantification (mLOQ) were substituted with half the limit of quantification. Variables with less than 10% of detections were removed from the dataset for statistical analyses. Correlation between variables in the different matrices was investigated using Spearman's rank-order correlation. Differences among the seasonal periods were studied through the non-parametric Kruskal-Wallis test. Reported values are means ± SD. The significance level was set at *p*-value (α) < 0.05.

3 RESULTS AND DISCUSSION

In water samples, among the 13 selected PFCs, only 5 compounds were detected in all of the sampling campaigns (autumn 2015; winter and spring-summer 2016), with perfluorocarboxylic acids (PFCAs) being the most abundant group. **Table S5.5** summarizes the main physical-chemical parameters of water samples, while **Table S5.6** provides a comparison between campaigns, along with summary statistics of the analysed PFCs. PFOA was the carboxylic acid detected at the highest frequency (67% in autumn 2015, 42%, in winter and 76% in spring), followed by PFPeA (30%, 17% and 66%, respectively) and PFNA (22%, 21% and 31%, respectively). Among perfluorinated sulfonates, PFOS was the most abundant compound with frequencies of 22% in autumn, 4% in winter and 86% during spring. Additionally, PFOS was almost the unique sulfonate compound detected, with the exception of PFHxS, found only in one sample during the autumn period, and PFDS, detected only during the spring period. PFOSA was not detected in any of the analysed samples. PFOA and PFOS were the compounds mostly found in river water, which is in agreement with previous studies (Campo et al. 2015; Llorca et al. 2012a; Valsecchi et al. 2015). Though, it is worth to be noted that PFOS annual average concentrations (0.52 ng/L in freshwater and 0.26 ng/L in seawater) were below the EQS set by EU Directive 2013/39 (EU 2013). Moreover, PFUdA, PFDA and PFDS were the only longer-chain compounds detected, at low frequencies. In detail, PFUdA was present in only two samples from the winter campaign, whereas PFDA and PFDS were recorded only in spring season. Lower

distribution of longer-chain compounds in water samples is not surprising, firstly because of their lower solubility, and secondly because of their current replacement in human production with shorter chain compounds, which have lower bioaccumulation potential (Onghena et al. 2012).

Table S5.7 reports concentrations of PFCs in surface waters of different published studies. Globally, the most abundant compound was once again PFOA, confirming the results showed in this work. Shorter chain compounds, especially PFHxA, were also quite abundant, as in Yangtze River (China) or in Ebro River (Spain), confirming their high solubility and their increase in use and production in spite of the longer chain PFCs. Sulfonates, and PFOS above all, showed a similar span of values, comparable to PFOA (for comparison of range of values, see **Table S5.7**).

In water samples we found that the concentrations of PFCs were minor during the winter period (**Figure 5.1**), coinciding with a higher dilution after the rainy season, as it can be inferred from the mean flow rate of Ebro River, which was c.a. 160 m^3/s during autumn and spring, and c.a. 450 m^3/s in winter (**Table S5.5**). However, the concentrations can be considered fairly constant along the course of the year. As expected, the major concentrations were reported in wastewater, with PFPeA being the compound reaching the highest concentrations, with 2329, 2775 and 345 ng/L in autumn, winter and spring, respectively, in agreement with its use as replacement compound (Wang et al. 2013). Nevertheless, effluents collected after the WWTPs showed a great efficiency in the removal of PFPeA from contaminated waters. In contrast, the two WWTPs revealed that they were inefficient in the removal of the other PFCs. For example, in the samples collected during autumn 2015, in spite of the significant removal of PFCs during the wastewater treatment, the final effluents of both WWTPs, Amposta and Sant Carles de la Ràpita, still showed notable concentrations of PFOA. The influents of Amposta and Sant Carles were 6.8 and 8.7 ng/L of PFOA while the final effluents were 3.5 and 6.0 ng/L of PFOA, respectively, indicating that more than 50% of this compound remains in the effluent. Higher concentrations of PFCs in effluents than in influents of WWTPs have already been reported by different studies as a result of the incomplete degradation of their precursors (such as polyfluoroalkyl phosphates and fluorotelomer alcohols) during water treatment processes with activated sludges (Guo et al. 2010; Lee et al. 2010; Loos et al. 2013a; Wang et al. 2005). Detection of PFHpA only at the effluents of the Sant Carles de la Ràpita WWTP is a further evidence of other related PFCs (e.g. fluorotelomers) partial degradation into shorter-chain PFCs.

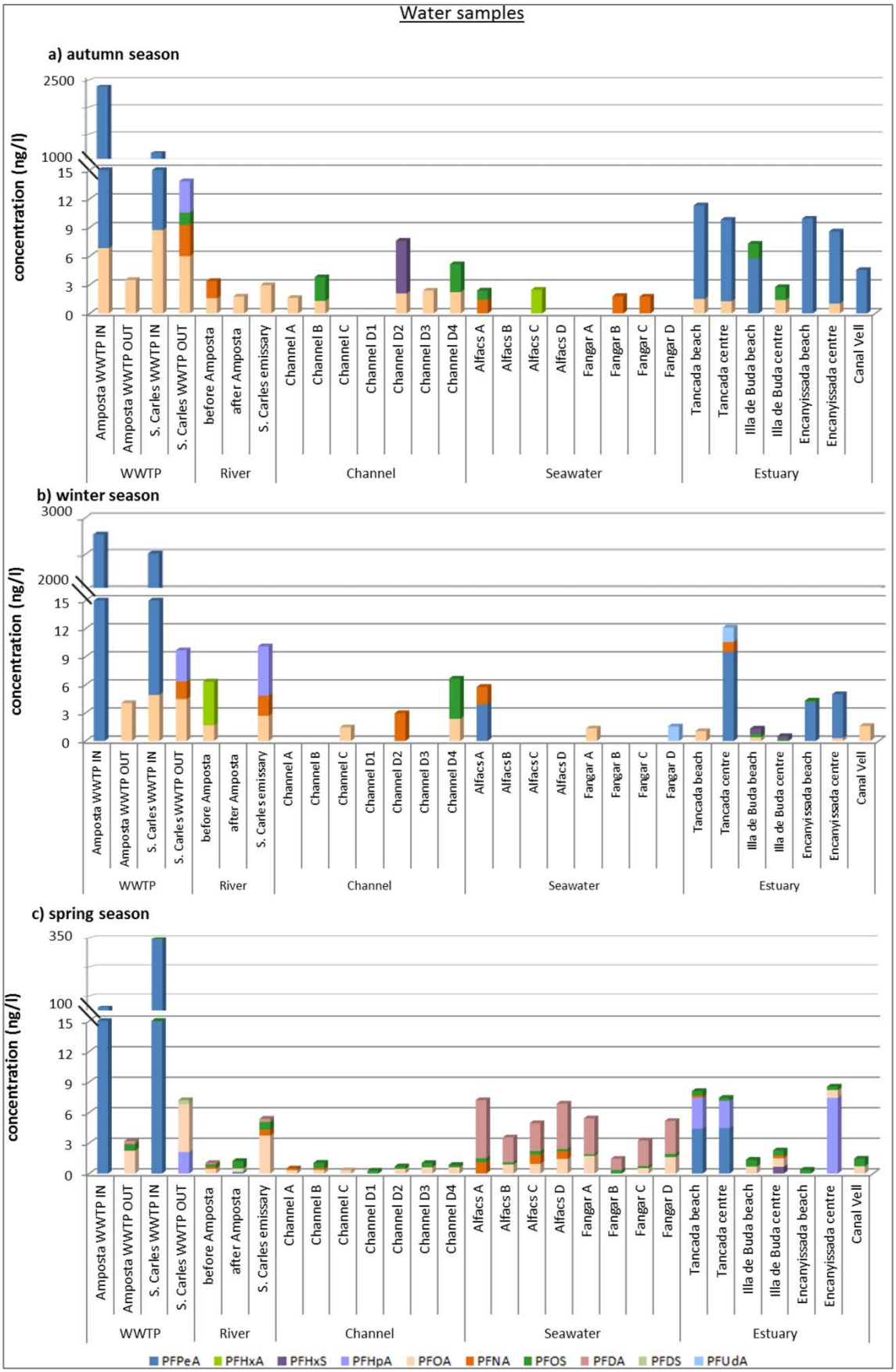


Figure 5.1 PFC concentrations (ng/L) detected in water samples during the autumn season (a), winter season (b) and spring-summer season (c)

In river waters, the concentrations were below 6 ng/L of PFOA as the most recalcitrant, followed by PFHxA and PFNA. It is noteworthy that the control site "before Amposta", located in the Ebro River far from the estuary area and selected as reference site, reported a slight contamination by PFCs, especially for PFCAs. This fact suggests that contamination of the estuary environment is not only due to the different surrounding human activities, which may impact on water quality, but reflects a contamination which originates at a far distance from the estuary. As it can be appreciated in **Figure 5.1**, the final part of the Ebro Delta (La Tancada, L'Encanyissada and Illa de Buda) was more affected by PFCs contamination, since it collects all waters from the surrounding irrigation channels. Comparing river water and seawater concentration patterns over the year, it is noteworthy that samples taken during the winter period showed lower concentrations than those samples collected during the autumn and spring-summer periods. Although PFOA still remained the most frequent compound among all PFCs, its frequency of detection decreased during the second sampling campaign, but was comparable between the first and third sampling campaigns (52% in autumn, 35% in winter, 55% in spring, excluding WWTPs data). In agreement with these data, the average concentrations for PFOA were 1.6, 0.97 and 0.87 ng/L in freshwaters (without WWTPs) for autumn, winter and spring, respectively (excluding WWTPs data).

No significant differences (p>0.05) were found regarding the total PFC concentrations over the year, stating their persistence in the water compartment. In addition, the specific features of non-polar compounds must be noted. For example, it is known that PFOS is partitioned into sediments (see next section) and that the concentrations detected in water are related to suspended organic material. In this case, the concentration of non-polar compounds can be influenced by heavy rain periods that redilute these compounds and increment their concentration in water, with a resulting constant trend of concentrations throughout the year.

The concentration data regarding sediment samples are summarised in **Figure 5.2** and **Table S5.8**. As in waters, for the first sampling campaign the most common compound was PFOA, detected in almost all analysed samples (96% frequency), along with PFHxA and PFHpA, recorded in 41% and 36% of samples, respectively. Among sulfonates, PFOS was predominant, with a maximum of 23 ng/g dw and mean value of 2.7±5.6 ng/g dw in autumn. It is noteworthy that during the winter period the only PFCs detected were PFOA and PFOS, and they were at lower concentrations compared to autumn sampling (mean value in winter of 0.84±1.10 ng/g dw for PFOA; 0.61±0.50 ng/g dw for PFOS) and lower frequencies (25% for PFOA, 15% for PFOS). PFDoA was also detected in both sampling periods, but at a higher frequency in winter (15%) than in autumn (5%), even if at low concentrations (mean value 0.91±0.29 ng/g dw). Samples collected during the spring period did not show any concentration of PFCs above the mLOQ.

Globally, sediment mean concentrations found in this work were in agreement with those ones recorded in the upstream section of Ebro River recorded by Lorenzo et al. (2016) and with other rivers of the Spanish

peninsula (Campo et al. 2016). Though, they were much higher than concentrations detected in Yangtze River sediments (Pan et al. 2014) and in Chinese river sediments (Lam et al. 2014), due to different sources of introduction and different environmental characteristics of rivers. For a better comparison of the ranges detected in those works, please refer to **Table S5.7**.

Figure 5.2 reports the distribution of PFC concentrations during autumn and winter periods. The most contaminated sites were the two lagoons of Illa de Buda and L'Encanyissada, along with Canal Vell, where PFOA and PFOS concentrations were accompanied by the detection of some of their replacement products in the autumn season (PFHxA, PFHpA, PFHxS, PFBS). Winter season displayed an evident decrease in PFCs detection, especially for samples taken from the open sea, where no compound was recorded; the estuarine environment showed a similar trend, with only some positive measurements in Illa de Buda and Canal Vell. Samples collected in the freshwater system, on the other hand, showed higher similarities between the two seasons. The Kruskal-Wallis test that was run on the samples of the first and second campaigns confirmed the different biogeochemical features between the two sampling periods, showing statistically significant differences for the occurrence of PFHxA, PFHpA, PFOA and PFNA ($p < 0.05$), which are reported in **Table S5.9**. This seasonal pattern suggests that PFC concentrations in sediments strongly depend not only on water-sediment interactions, but also on the surrounding environmental conditions, such as temperature, precipitations and water currents that may occur consequently to the higher rainfall rates. In this context, the decrease in concentrations of PFCs in sediments could be due to a resuspension of sediments in coincidence with the rainy season, leading to an increase of PFCs in the dissolved particulate matter and a corresponding decrease in sediments.

Only a few works focusing on PFCs seasonal trend in water and sediment have been conducted so far. Pan et al. (2014a) found no substantial variation of PFCs in sediments of Yangtze River in summer and winter seasons, even though the detected concentrations were very low (∑PFCs range of 0.05-1.44 ng/g dw), and most PFCs were not detected in the majority of the sampling sites. Pan et al. (2014b) in rivers of the Pearl River Delta region (South China) also showed comparable concentrations of PFCs in winter and summer, in contrast with the results obtained in this study. However, it should be highlighted that both studies were focused on river basins. The system of a delta environment is more complex since it is subjected to the influence of both inland waters and open seawaters. The anomalous behavior of concentrations in sediments of Ebro delta could be explained by sediment resuspension that is produced consequently to heavy rainfalls during winter and spring, which lead initially to a depletion of the shorter chain compounds, less hydrophobic than the longer chain ones, as is actually registered for PFC concentrations in winter season. Moreover, tidal events and strong water currents occurring during winter period in the Mediterranean Sea could cause a mobilization of superficial sediments, resulting in the removal and transport of sediments towards far distant areas along the coastline, as an effect of coastal erosion.

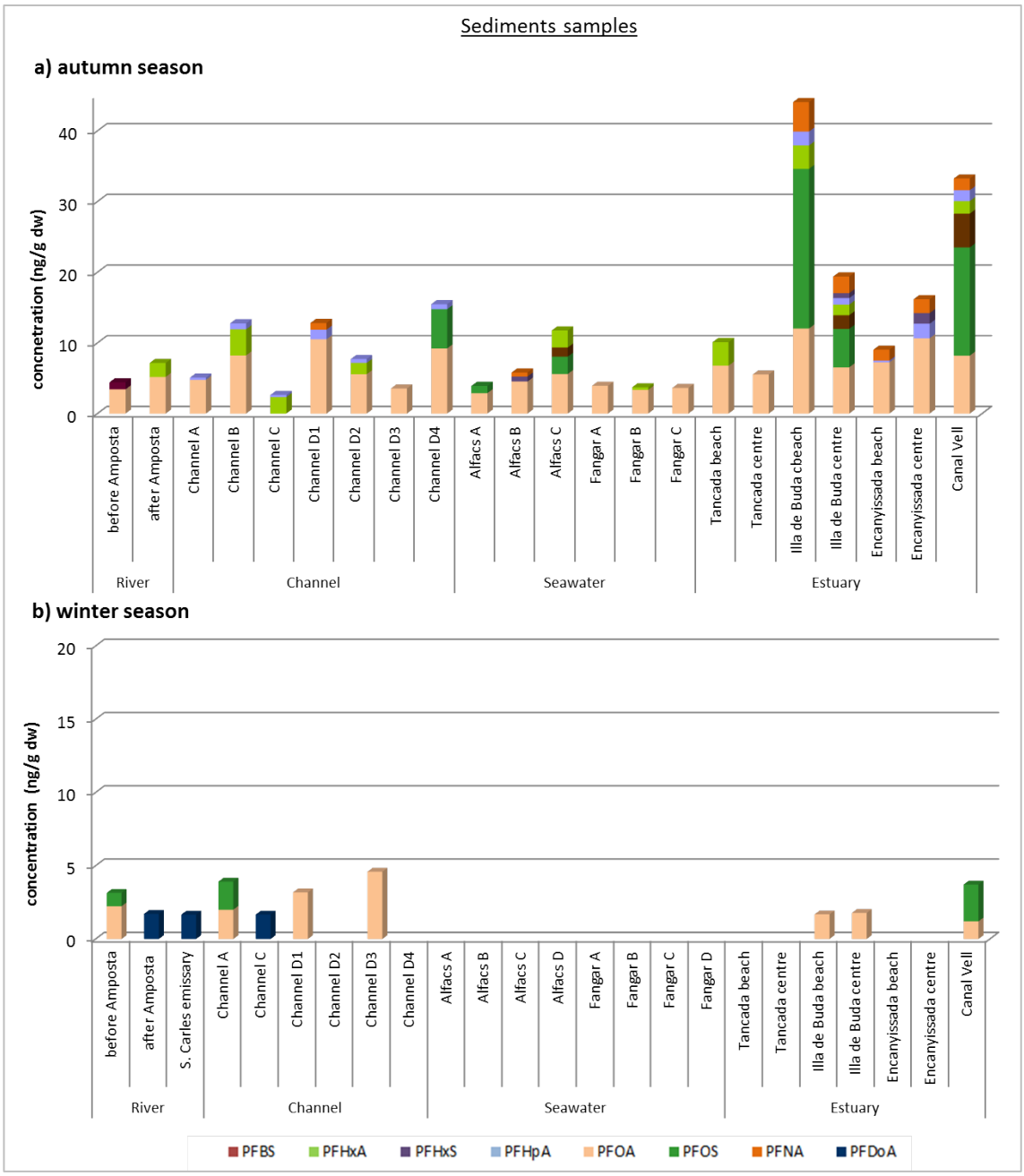


Figure 5.2 PFC concentrations (ng/g dw) detected in sediment samples during the autumn season (a) and the winter season (b)

Regarding the analysis of biota, the data which report PFCs accumulation in fish from Ebro River near the municipalities of Xerta and Tortosa are listed in **Table S5.10**, while the data of fishes that were collected in the Ebro Delta (estuarine and seawaters) are reported in **Table S5.11**. Notwithstanding the differences in sample preparation, PFOA was the most abundant compound, being detected both in the whole fish body and in muscle and skin tissues, and confirming its bioaccumulation potential (Llorca 2012). As could be expected, pool samples showed higher PFOA concentrations (from 94 to 330 ng/g ww) compared to samples for which only data on muscle and skin were available, since PFCs have been proven to bioaccumulate preferentially in liver and kidney, rather than in muscles or fat matter (Llorca, 2012). On the

other hand, PFDA and PFOSA showed the highest frequencies of detection, being detected in all samples, with relatively high mean concentration for PFDA (141±187 ng/g ww) and lower values for PFOSA (7.4±6.0 ng/g ww). In particular, PFDA reached 459 ng/g ww in European catfish (*Silurus glanis*), which is at the top of the aquatic food-chain, and 454 and 455 ng/g ww in mullet fish (*Liza* sp.) and bleak (*Alburnus alburnus*), respectively, which both feed on small molluscs, insect larvae, worms and small fishes. PFOS (range of values from <mLOQ to 154 ng/g ww) and the short chain PFHxA (range <mLOQ-122 ng/g ww) were also found at remarkable concentrations. Focusing, in more detail, on the individual contribution of PFCs in freshwater biota (**Figure 5.3**), concentrations of whole-body revealed a similar pattern in PFC bioaccumulation, both for pool samples (**Figure 5.3a**) and for individual samples (**Figure 5.3b**), showing the predominance of PFOA among all the PFCs.

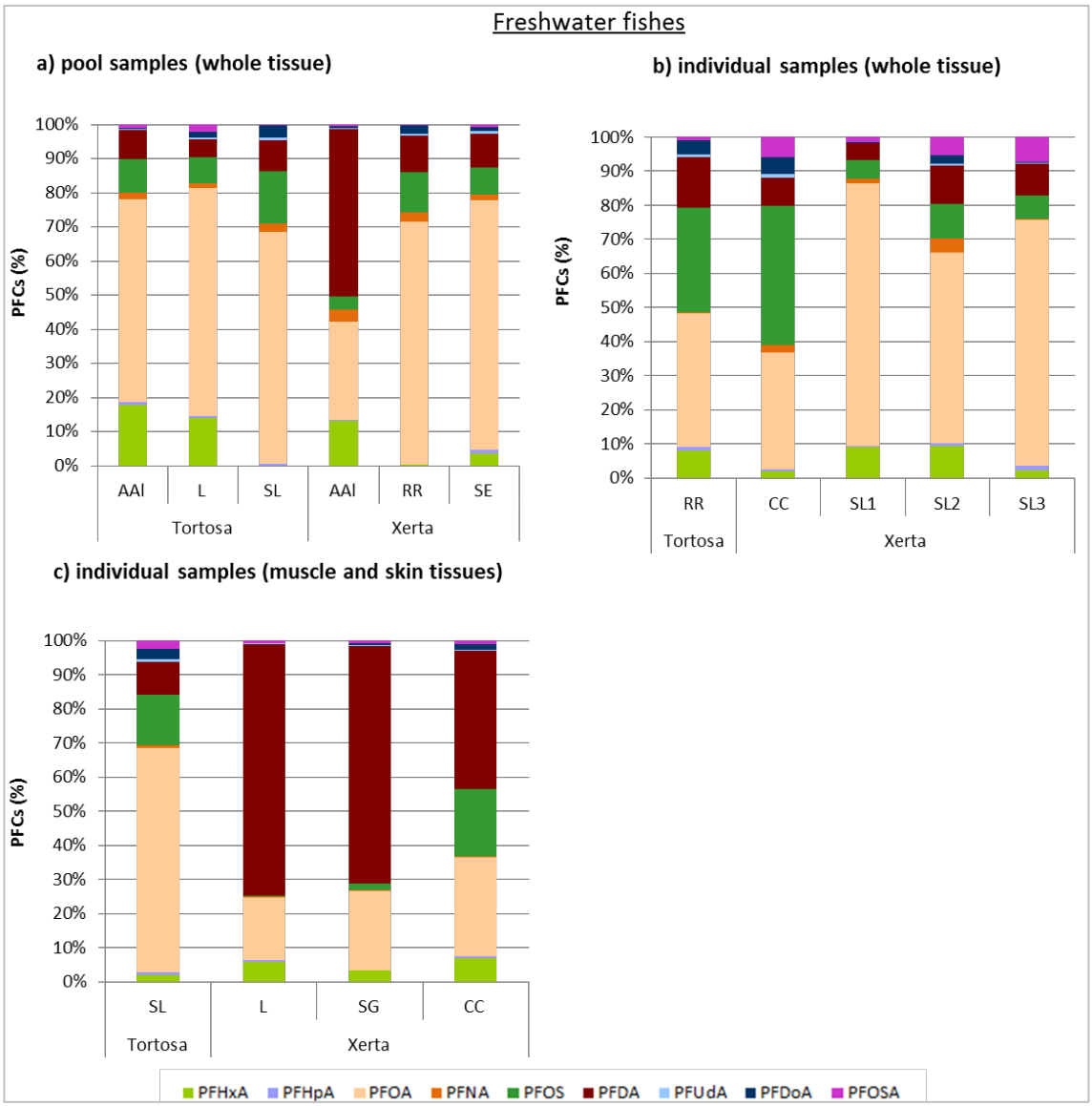


Figure 5.3 PFC concentration contributions (%) in freshwater fish samples. Data refer to the whole fish body concentration calculated from a pool of fishes (a) and from individuals (b), and muscle and tissue concentrations of individuals (c). The selected species are as follows: AAl: *Alburnus alburnus*; L: *Liza* sp.; SL: *Squalius laietanus*; RR: *Rutilus rutilus*; SE: *Scardinius erythropthalmus*; CC: *Cyprinus carpio*; SG: *Silurus glanis*

It is well-known that the longer chain compounds exhibit more bioaccumulative potential than the shorter chain compounds. In this study PFDA was the most frequently long-chain detected compound, contributing to the total PFCs amount of 9% on average, and comparable to the 6-carbon chain PFHxA (5% of total contribution). This suggests that, due to their higher use and replacement of 8-chain PFCs, short-chain compounds could lead over time to similar hazardous effects on organisms as those normally associated with long-chain compounds. PFOSA was detected in species such as *Cyprinus carpio* and *Silurus glanis*, even though no concentrations above the mLOQ were registered in water or sediment. These fishes are long-lived species, reaching large sizes, and moreover, *Silurus glanis* is a common predator at the top of the aquatic food chain; PFOSA detection only in these species could be a clear evidence of the biomagnification process through the trophic chain. In addition, detected concentrations of PFOSA can originate from amination of PFOS (Dimitrov et al. 2004) or from the metabolization of the N-ethyl-perfluorooctane-sulfonamido-ethanol (N-Et-FOSE) (Frömel Tobias 2010). Lower detection of PFOA in muscle and skin tissue (**Figure 5.3c**), in comparison to the concentration registered in the whole body (**Figure 5.3a** and 5.**3b**), reinforce its preferential partition in liver, whereas PFDA showed a higher detection frequency, reaching almost 60% of PFCs contribution in muscle-skin tissues.

PFCs accumulation in fish organisms has already been studied by several authors, although the high differences in fish preparation, as well as the wide variety of analysed species, make it difficult to compare the results. Nevertheless, a rough comparison of the results obtained in this study can be done with values taken from literature and reported in **Table S5.7**. As it can be seen, data regarding fish biota can be very different from one work to another, and are mostly dependent on the species selected and their habits. However, as a general pattern displayed in the majority of works, PFOS shows to be the most bioaccumulative compound, while among PFCAs, the longer chain compounds (>C8) are the most abundant and the most frequently detected ones. Data of freshwater biota were particularly compared to the results reported by Lorenzo et al. (2016) in the Ebro River, in order to compare the results obatined in different time periods but in the same river, even though the fishes that were analysed by the authors belong to the river upstream section, while fishes analysed in this work are closer to the Delta River mouth. Overall, both studies reported the occurrence of PFCAs such as PFOA and PFHxA as the main PFCs accumulated in biota. This is in contrast with the majority of other studies (Labadie and Chevreuil 2011; Houde et al. 2011; Xu et al. 2014; Ye et al. 2008) that assessed perfluorinated sulfonates, and PFOS among all of them, as the most bioaccumulative of the perfluoroalkyl group. The major content of PFCAs in fish could be due to the higher occurrence of perfluorocarboxylic acids than sulfonates found in waters. However, concentrations of almost all PFCs detected in this study were much higher than those obtained by Lorenzo et al. (2016). Moreover, these very high values seemed not to be related to water data, which in turn showed much lower concentrations (∑PFCs of Ebro River near Xerta: 3.4 ng/L in autumn, 6.4 ng/L in winter and 1.0 ng/L in spring-summer). Bioaccumulation is the result of long time interactions between organisms and the

contaminated environment, and can be thus considered the evidence of water contamination events occurred in the past. Furthermore, fish can move freely along the river and may have been affected by PFCs in different river transects far from the sampling area. Size and age of species, as well as gender, additionally influence the contaminants bioaccumulation and biotransformation process (Houde et al. 2011). All these aspects can thus explain such high PFCs concentrations detected in riverine fishes.

For each of the fish species collected in Xerta, a PFCs experimental bioaccumulation factor was calculated according to the formula $BAF=C_b/C_w$, and expressed as L/kg. C_b is the PFC concentration in fish and C_w its concentration in water, considering a mean value of water concentrations detected in Xerta throughout the year. BAF values were calculated only for PFHxA, PFOA, PFNA and PFOS because detections in water above the mLOQ were only available for these compounds in at least two sampling campaigns. LogBAF values are reported in **Table S5.12**. Among the four variables, PFNA showed the lowest logBAF values, while PFHxA, PFOA and PFOS showed comparable values (mean value of 4.2 for PFHxA, 5.1 for PFOA and 5.0 for PFOS), suggesting a higher bioavailability and uptake of these compounds in comparison to PFNA.

Contamination by PFCs was also found in fish collected from the estuarine and coastal areas (**Figure 5.4**). For these species, it was possible to separate skin from muscle, therefore results of these two tissues are displayed separately. In this case, PFOS was the predominant compound, with the highest concentrations detected in Illa de Buda lagoon: the common carp (*Cyprinus carpio*, 14.5 ng/g ww) and the European eel (*Anguilla anguilla*, 21.6 ng/g ww). PFUdA and shorter-chain PFCAs (PFNA, PFOA, PFHpA, PFHxA) were also detected, but at lower concentrations (<5 ng/g ww). On the other hand, the flathead grey mullet (*Mugil cephalus*) and the largemouth bass (*Micropterus salmoides*) showed lower concentrations (max value of 4.2 ng/g ww for PFOS). Among the marine fishes that were collected in the open sea (Alfacs Bay and Fangar Bay), only the salema (*Sarpa salpa*) did not present contamination by PFCs. This is consistent with its feed which is based on algae. Fishes taken at Alfacs Bay showed slightly higher concentrations of PFCs with respect to Fangar Bay, especially regarding muscle tissue. The fish species common torpedo (*Torpedo torpedo*) and bogue (*Boops boops*) were found to be very similar in their PFCs accumulation level, even though they are characterised by different behavioural habits (*Torpedo torpedo* is a benthic predator, while *Boops boops* is an omnivorous semi-pelagic organism). Anyway, the very low range (<2 ng/g ww) at which PFC concentrations were detected did not allow to distinguish any possible difference in the uptake of PFCs between the two species. No differences were found for PFCs distribution in muscle and skin tissues, except for PFHxA (*p*-value of Kruskal-Wallis test = 0.02), as can be seen in the graphs, where PFHxA is preferentially distributed in fish skin, rather than in muscle. It is well-known that PFCs tend to bind preferentially to protein and accumulate in liver and blood (Kannan et al. 2005); the higher concentrations found in the skin for PFHxA are thus not likely to be related to a bioaccumulation process of the organisms, but rather to skin contact with contaminated lagoonal sediments.

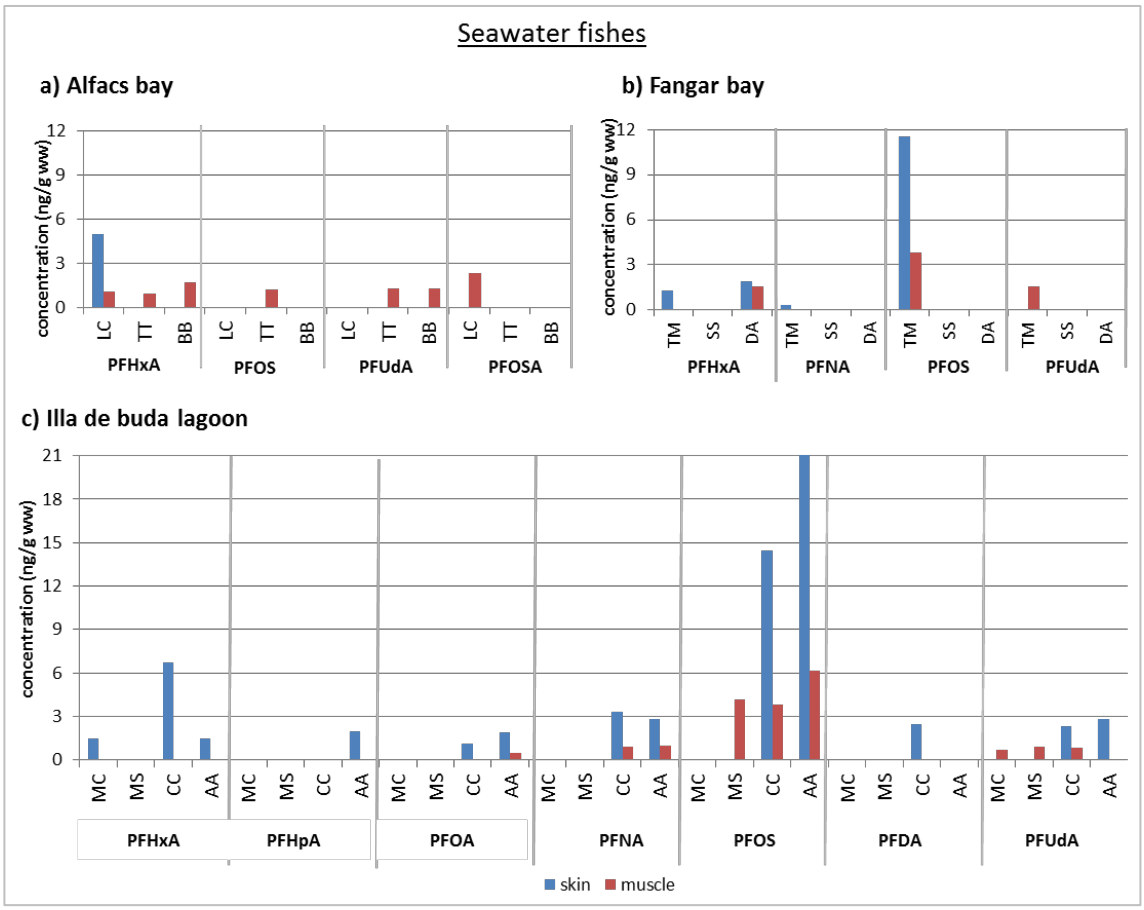


Figure 5.4 PFC concentrations (ng/g ww) in skin and muscles of coastal fishes collected in Alfacs Bay (a), Fangar bBay (b) and Illa de Buda lagoon (c). Fishes species are as follows: LC (*Mugil cephalus*), TT (*Torpedo torpedo*), BB (*Boops boops*), TM (*Trachurus mediterraneus*), SS (*Sarpa salpa*), DA (*Diplodus annularis*), MC (*Mugil cephalus*), MS (*Micropterus salmoides*), CC (*Cyprinus carpio*), AA (*Anguilla anguilla*)

Overall, the estuarine and marine biota analysed in this study showed an accumulation of perfluorinated compounds that are not found in waters or sediments, such as the short-chain PFHxA and PFHpA (see **Figure 5.1** and **5.2** for comparison). This is consistent with the fact that these compounds in waters and sediments are influenced by a high variability, due to the continuous change of environmental conditions (e.g. temperature, pH, precipitation rates, water currents), while bioaccumulation through the aquatic food chain is the product of a longer time period exposure of organisms to contaminants. Seawater fishes showed lower concentrations compared to freshwater fishes, in contrast to what would be expected from water and sediments results, which highlighted a greater contamination of Illa de Buda lagoon, located in the final stretch of the Ebro Delta, with lower salinity than seawater sampling points, but much higher salinity than freshwater zones (**Table S5.5**). PFC biota concentrations that are higher in freshwater than in seawater ecosystems have already been reported, and a possible explanation can be related to the lower solubility, and thus lower bioavailability, of PFCs in marine water (Zhao et al. 2011b). It is noteworthy, however, that the majority of the species that were captured in the estuarine area and in river were different. Among the common species that were captured in both environments, such as the common carp,

those from Illa de Buda were younger. Caution should thus be exercised in comparing these results. Anyway, in this study the common carp (*Cyprinus carpio*) was the most affected species with respect to PFCs accumulation in tissues, both in river and estuarine environments. Bioaccumulation of contaminants is strictly species-specific, being mostly dependent on the metabolism of the selected organism. The results of this study point out that the common carp is a good marker of PFCs contamination in biota, as it is also well known for the many POPs and metal pollutants. These results should be taken into consideration when addressing future research on PFCs bioaccumulation.

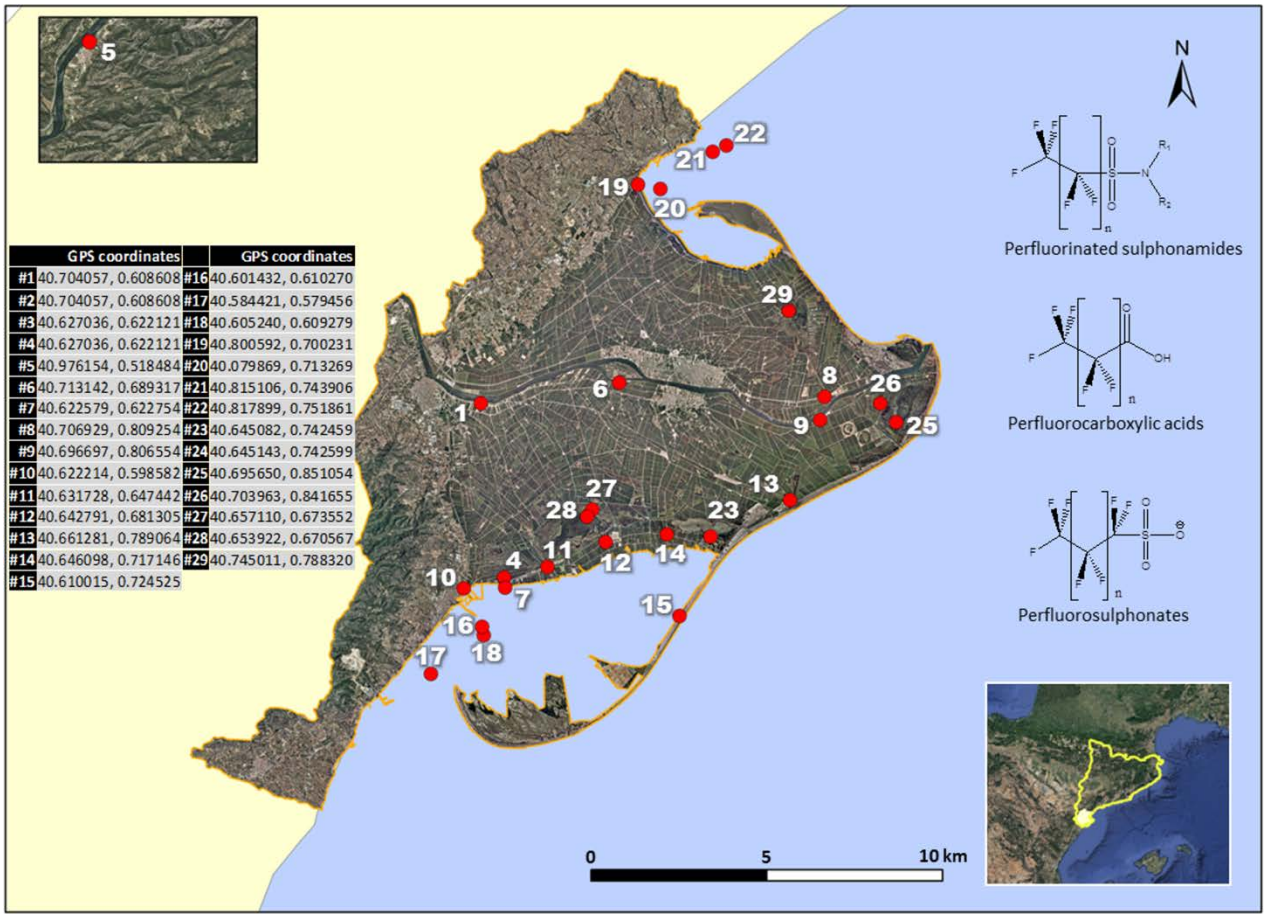
PFCs accumulation in fish is an issue of great concern, since it can be related to human expoure to PFCs through intake of contaminated fish. In this context, Directive 2013/39/EU set an Environmental Quality Standard (EQS) for PFOS in fish biota of 9.1 μg/kg, in order to safeguard ecosystem and human health (EU 2013); no EQS for the other PFCs have been set yet, as a consequence of the lack of information about their real toxicity levels. Concentrations detected in this study in the edible muscle tissue of seawater fishes were acceptable according to the EU Directive; on the contrary, almost all freshwater fishes exceeded the EU threshold for PFOS (range <mLOQ-154 μg/kg ww; mean value 49±51 μg/kg ww), thus revealing a very anomalous situation in Ebro River. To conclude, PFCs accumulation in freshwater fishes should thus be better analysed, considering that PFOA and PFDA showed even higher concentrations than PFOS, in order to understand if PFC levels detected in fish of Ebro River can pose a risk for human beings or the ecosystem.

4 CONCLUSIONS

This study focused on the occurrence and biogeochemical features of PFCs in waters, sediments and fishes of the Ebro Delta region (Catalonia, Spain). Sampling campaigns in different time periods were carried out in order to investigate seasonal trends of PFCs. The study revealed a difference in the sampling campaigns that is likely due to the different environmental conditions, with the main influencing factors being temperature and rainfall regimes. PFOA was confirmed to be the predominant compound among all the perfluorinated compounds, both in water and in sediment. With respect to waters, PFPeA was the most abundant compound, reaching very high concentrations, especially in the WWTPs, as a consequence of its widespread use as an alternative to PFOA and PFOS. Concerning sediments, PFOS was found to be the most abundant perfluorinated compound, being detected at higher concentrations than those found in waters, and revealing its preferential behaviour to be adsorbed onto sediment particles rather than staying in a water-dissolved phase. Sediments registered a very different pattern of PFCs than did water, consisting of a progressive decrease in the occurrence of PFCs throughout the year. This decrease reflects a very high influence of the environmental conditions on PFCs distribution in sediments and suggests water-sediment partitioning is happening over a long-term time scale. Seawater fishes showed PFC concentrations higher in

their skin than in their muscle tissues; PFOS was once again the most abundant and the most detected compound. Results on waters, sediments and biota confirmed Illa de Buda to be the most contaminated site of the Ebro Delta. On the other hand, freshwater fishes showed very high concentrations of both sulfonates and carboxylic acids, in contrast with those ones registered for seawater fishes. Such high differences in concentrations could be due to a different uptake mechanism between freshwater and seawater fishes and to the different behavioural habitats of the two fish types.

SEASONAL VARIATIONS IN THE OCCURRENCE OF PERFLUORINATED COMPOUNDS IN WATER, SEDIMENT AND FISH SAMPLES FROM EBRO DELTA (Catalonia, Spain)



GPS coordinates		GPS coordinates
#1 40.704057, 0.608608	#16	40.601432, 0.610270
#2 40.704057, 0.608608	#17	40.584421, 0.579456
#3 40.627036, 0.622121	#18	40.605240, 0.609279
#4 40.627036, 0.622121	#19	40.800592, 0.700231
#5 40.976154, 0.518484	#20	40.079869, 0.713269
#6 40.713142, 0.689317	#21	40.815106, 0.743906
#7 40.622579, 0.622754	#22	40.817899, 0.751861
#8 40.706929, 0.809254	#23	40.645082, 0.742459
#9 40.696697, 0.806554	#24	40.645143, 0.742599
#10 40.622214, 0.598582	#25	40.695650, 0.851054
#11 40.631728, 0.647442	#26	40.703963, 0.841655
#12 40.642791, 0.681305	#27	40.657110, 0.673552
#13 40.661281, 0.789064	#28	40.653922, 0.670567
#14 40.646098, 0.717146	#29	40.745011, 0.788320
#15 40.610015, 0.724525		

Table S5.1 Sampling location and information about the three sampling campaigns

Acronym	Sample Name	Tipology	X Coordinates*	Y Coordinates*	1st campaign		2nd campaign		3rd campaign	
			* WGS84		*waters*	*sediments*	*waters*	*sediments*	*waters*	*sediments*
IC.1	Amposta WWTP IN	wastewater influent	0.608608	40.704057	✔	🎗	✔	🎗	✔	🎗
IC.2	Amposta WWTP OUT	wastewater effluent	0.608608	40.704057	✔	🎗	✔	🎗	✔	🎗
IC.3	Sant Carles de la Ràpita WWTP IN	wastewater influent	0.622121	40.627036	✔	🎗	✔	🎗	✔	🎗
IC.4	Sant Carles de la Ràpita WWTP OUT	wastewater effluent	0.622121	40.627036	✔	🎗	✔	🎗	✔	🎗
IC.5	Xerta town	freshwater	0.518484	40.976154	✔	✔	✔	✔	✔	✔
IC.6	after Amposta	freshwater	0.689317	40.713142	✔	✔	✔	✔	✔	✔
IC.7	Sant Carles de la Ràpita emissary	freshwater	0.622754	40.622579	✔	✔	✔	✔	✔	✔
IC.8	channel A	freshwater	0.809254	40.706929	✔	✔	✔	✔	✔	✔
IC.9	channel B	freshwater	0.806554	40.696697	✔	✔	🎗	🎗	✔	✔
IC.10	channel C	freshwater	0.598582	40.622214	✔	✔	✔	✔	✔	✔
IC.11	channel D1	freshwater	0.647442	40.631728	✔	✔	✔	✔	✔	✔
IC.12	channel D2	freshwater	0.681305	40.642791	✔	✔	✔	✔	✔	✔
IC.13	channel D3	freshwater	0.789064	40.661281	✔	✔	✔	✔	✔	✔
IC.14	channel D4	freshwater	0.717146	40.646098	✔	✔	✔	✔	✔	✔
IC.15	Alfacs bay A (beach)	sea water	0.724525	40.610015	✔	✔	✔	✔	✔	✔
IC.16	Alfacs bay B (centre)	sea water	0.610270	40.601432	✔	✔	✔	✔	✔	✔
IC.17	Alfacs bay C (open sea)	sea water	0.579456	40.584421	✔	✔	✔	✔	✔	✔
IC.18	Alfacs bay D (open sea)	sea water	0.609279	40.605240	🎗	🎗	✔	✔	✔	✔
IC.19	Fangar bay A (beach)	sea water	0.700231	40.800592	✔	✔	✔	✔	✔	✔
IC.20	Fangar bay B (centre)	sea water	0.713269	40.079869	✔	✔	✔	✔	✔	✔
IC.21	Fangar bay C (open sea)	sea water	0.743906	40.815106	✔	✔	✔	✔	✔	✔
IC.22	Fangar bay D (open sea)	sea water	0.751861	40.817899	🎗	🎗	✔	✔	✔	✔
IC.23	La Tancada lagoon (beach)	estuary	0.742459	40.645082	✔	✔	✔	✔	✔	✔
IC.24	La Tancada lagoon (centre)	estuary	0.742599	40.645143	✔	✔	✔	✔	✔	✔
IC.25	Illa de Buda lagoon (beach)	estuary	0.851054	40.695650	✔	✔	✔	✔	✔	✔
IC.26	Illa de Buda lagoon (centre)	estuary	0.841655	40.703963	✔	✔	✔	✔	✔	✔
IC.27	L'Encanyissada lagoon (beach)	estuary	0.673552	40.657110	✔	✔	✔	✔	✔	✔
IC.28	L'Encanyissada lagoon (centre)	estuary	0.670567	40.653922	✔	✔	✔	✔	✔	✔
IC.29	Canal Vell lagoon (beach)	estuary	0.788320	40.745011	✔	✔	✔	✔	✔	✔

✔ available sampling points 🎗 not available sampling points

Table S5.2 Loading and eluting LC pump conditions used for on-line LC-MS/MS analysis of sediment and fish samples

Loading pump							Eluting pump			
Time	**Flow**	**%**				**Step**	**Flow**	**Grad**	**%**	
(min:sec)	*(ml/min)*	(A)	(B)	(C)	(D)		*(ml/min)*		(E)	(F)
00:00	1.5	100	-	-	-	Loading sample	0.4	Step	90	10
00:33	0.2	-	-	100	-	Cleaning matrix effects	0.4	Ramp	90	10
00:50	0.2	70	-	-	30	Transfer step	0.2	Ramp	90	10
01:00	0.4	-	100	-	-	Cleaning column I	0.4	Ramp	20	80
02:50	0.4	-	-	-	100	Cleaning column II	0.4	Ramp	10	90
07:50	0.4	-	-	-	100	Loading loop step	0.4	Step	10	90
08:00	0.4	20	-	-	80	Cleaning column III	0.4	Step	90	10
09:00	0.4	100	-	-	-	Cleaning column III	0.4	Step	90	10
09:50	0.4	100	-	-	-	Cleaning column III	0.4	Step	90	10

Loading pump:
solvent A: water (pH 3.4, with formic acid)
solvent B: acetone:isopropanol:acetonitrile (10:45:45)
solvent C: water
solvent D: methanol

Eluting pump:
solvent E: water (20 mM NH_4Ac)
solvent F: methanol (20 mM NH_4Ac)

Table S5.3 Method limit of detection (mLOD) and quantification (mLOQ), expressed in ng/L, for water, sediment and fish samples

	waters						sediments		fish	
	sea	river	WWTP	sea	river	WWTP				
	mLOD			***mLOQ***			***mLOD***	***mLOQ***	***mLOD***	***mLOQ***
PFPeA	-	-	-	-	-	-	0.54	1.80	0.91	3.03
PFBS	0.31	0.52	2.70	1.03	1.75	9.00	0.08	0.27	0.09	0.31
PFHxA	1.22	0.18	1.71	4.06	0.60	5.69	0.29	0.97	0.27	0.90
PFHpA	0.68	0.40	0.76	2.25	1.35	2.54	0.23	0.70	0.27	0.91
PFHxS	0.09	0.12	0.23	0.30	0.38	0.75	0.28	0.72	0.38	1.27
PFOA	0.08	0.06	0.11	0.26	0.21	0.37	0.23	0.77	0.29	0.97
PFNA	0.05	0.05	0.08	0.16	0.17	0.26	0.43	1.44	0.46	1.54
PFOS	0.04	0.07	0.08	0.14	0.25	0.27	0.27	0.9	0.64	1.13
PFDA	0.04	0.02	0.10	0.15	0.08	0.33	0.21	0.70	0.21	0.69
PFDS	0.06	0.04	0.30	0.19	0.14	0.99	2.66	8.87	2.69	8.97
PFUdA	0.03	0.02	0.15	0.11	0.08	0.51	0.27	0.91	0.24	0.82
PFOSA	0.03	0.04	0.60	0.10	0.13	1.99	0.36	1.19	0.36	1.19
PFDoA	0.02	0.01	0.07	0.07	0.02	0.23	0.48	1.59	0.41	1.38

Table S5.4 Recoveries for water, sediment and fish samples

	waters			sediments (16 ng/L)	fish (16 ng/L)
	sea (13 ng/L)	**river** (8 ng/L)	**WWTP** (4 ng/L)		
PFPeA	-	-	-	87	48
PFBS	70	79	129	89	44
PFHxA	109	39	110	98	65
PFHpA	132	87	113	101	65
PFHxS	81	68	86	108	65
PFOA	74	62	103	103	68
PFNA	61	52	76	78	52
PFOS	83	56	91	99	88
PFDA	51	42	64	100	65
PFDS	39	34	46	141	148
PFUnA	54	42	90	87	64
PFOSA	35	33	41	44	59
PFDoA	92	61	54	120	87

Table S5.5 Physical-chemical parameters of water samples

Sampling site	1st sampling campaign						2nd sampling campaign						3rd sampling campaign					
	T (°C)	pH	O_2 (mg/L)	Conductivity (µS/cm)	Salinity (ppt)	Flow* (m^3/s)	T (°C)	pH	O_2 (mg/L)	Conductivity (µS/cm)	Salinity (ppt)	Flow* (m^3/s)	T (°C)	pH	O_2 (mg/L)	Conductivity (µS/cm)	Salinity (ppt)	Flow* (m^3/s)
Amposta WWTP IN	21.7	7.78	8.99	1362	0.68		11.9	8.15	8.56	1290	0.60		23.5	7.85	9.56	682	0.34	
Amposta WWTP OUT	20.3	7.60	7.78	2690	1.39		12.6	7.68	9.56	1344	0.60		24.7	8.50	N/A	1433	0.70	
Sant Carles de la Ràpita WWTP IN	20.9	7.36	7.37	20046	11.9		14.6	7.78	12.9	3837	2.59		28.0	7.68	N/A	3940	2.10	
Sant Carles de la Ràpita WWTP OUT	21.8	7.77	8.03	1190	0.59		13.7	8.39	9.1	1332	0.60		25.8	8.71	N/A	671	0.30	
before Amposta (Xerta town)	21.9	7.94	9.73	1189	0.59	161	N/A	N/A	N/A	N/A	N/A	447	25.9	8.45	N/A	672	0.30	166
after Amposta	21.3	7.50	12.88	2933	1.52	161	19.0	7.64	15.1	2791	1.65	447	23.6	7.70	12.8	2151	1.13	166
Sant Carles de la Ràpita emissary	17.1	7.14	5.06	2100	1.07	N/D	15.3	7.76	10.9	2765	1.79	N/D	23.7	7.64	7.14	2595	1.37	N/D
channel A	17.0	7.27	6.60	1800	0.91	19	20.1	7.94	20.9	27049	18.5	19	22.7	7.47	6.40	3801	2.14	19
channel B	14.9	7.24	6.17	1500	0.75	25	12.0	7.25	10.4	8762	6.67	0.00	24.6	7.82	7.35	1451	0.73	30
channel C	14.9	7.42	4.20	1476	0.74	N/D	16.8	8.23	25.6	13499	9.48	N/D	25.2	7.62	13.3	17502	10.3	N/D
channel D1	17.3	7.60	7.40	35971	22.7	N/D	N/A	N/A	N/A	N/A	N/A	N/D	31.6	8.21	7.70	65759	38.4	N/D
channel D2	N/A	N/A	N/A	N/A	N/A	N/D	10.7	7.85	11.2	37256	33.5	N/D	28.1	8.04	6.37	59303	37.1	N/D
channel D3	19.2	7.78	7.00	45332	29.3	N/D	12.2	7.85	10.5	39462	34.3	N/D	28.0	8.03	6.11	59344	37.2	N/D
channel D4	N/A	N/A	N/A	N/A	N/A	N/D	12.6	7.84	10.1	40346	34.7	N/D	27.5	7.98	6.52	59939	37.9	N/D
Alfalcs bay A (beach)	20.4	7.78	8.01	47732	31.1		12.8	7.83	10.9	40073	35.1		28.0	8.02	7.22	60021	37.7	
Alfalcs bay B (centre)	17.9	7.75	8.08	41647	26.7		12.0	7.81	10.5	40554	35.3		28.2	8.02	8.40	49691	30.5	
Alfalcs bay C (open sea)	19.9	7.75	7.90	48564	31.7		12.5	7.65	10.4	40719	35.2		28.1	8.04	7.26	50621	31.8	
Alfalcs bay D (open sea)	N/A	N/A	N/A	N/A	N/A		12.6	7.74	12.0	40892	35.3		27.9	8.01	8.00	55036	34.6	
Fangar bay A (beach)	17.3	8.29	9.06	32900	20.6		14.7	7.82	15.0	40460	33.0		29.2	8.37	12.6	47320	28.1	
Fangar bay B (centre)	17.3	8.32	10.9	32423	20.2		14.0	7.78	12.3	40227	33.4		28.9	8.14	8.33	47068	28.2	
Fangar bay C (open sea)	13.3	7.73	11.6	14907	8.66		20.7	7.92	25.0	7266	4.40		27.2	7.94	7.30	5133	2.63	
Fangar bay D (open sea)	13.2	7.75	10.1	14978	8.70		20.3	8.23	43.5	7713	4.74		25.5	8.29	9.32	8560	4.90	
La Tancada lagoon (beach)	10.1	7.50	10.5	21800	13.1		18.7	8.48	37.2	44222	33.0		24.5	8.01	5.60	35990	23.1	
La Tancada lagoon (centre)	10.4	7.90	12.6	22775	13.7		18.7	8.33	27.9	44059	32.9		23.5	8.17	8.06	35088	22.8	
Illa de Buda lagoon (beach)	16.9	8.14	14.7	27426	16.8		17.3	8.42	22.3	32431	24.2		27.5	8.08	13.9	20725	11.8	

*Media flow values obtained from SAIH Ebro ; N/A: not analysed. N/D: no data available.

Table S5.6 Summary statistics of the selected PFCs in water samples collected in autumn (1st sampling campaign), winter (2nd sampling campaign) and spring-summer (3rd sampling campaign) seasons. Values are expressed in ng/L

	Min			Max			Mean			SD			n. detected			Frequency (%)		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
Perfluorinated carboxylic acids (PFCAs)																		
PFPeA	<mLOQ	<mLOQ	<mLOQ	2329	2775	346	2.1[a]	1.2[a]	0.77[a]	3.2[a]	2.1[a]	1.1[a]	8	4	19	30	17	66
PFHxA	<mLOQ	<mLOQ	<mLOQ	2.5	4.7	<mLOQ	-	-	-	-	-	-	1	1	-	4	4	-
PFHpA	<mLOQ	<mLOQ	<mLOQ	3.3	5.3	7.4	<mLOQ	<mLOQ	-	<mLOQ	<mLOQ	<mLOQ	2	2	4	7	8	14
PFOA	<mLOQ	<mLOQ	<mLOQ	8.7	4.9	4.7	1.6	0.97	0.87	2.2	1.4	1.1	18	10	22	67	42	76
PFNA	<mLOQ	<mLOQ	<mLOQ	3.3	3.0	1.1	0.41	0.41	0.21	0.79	0.78	0.27	6	5	9	22	21	3
PFDA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	5.7	-	-	0.94	-	-	1.6	-	-	11	-	-	38
PFUdA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	1.6	<mLOQ	-	0.14	-	-	0.39	-	-	2	-	-	8	-
PFDoA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
Perfluorinated sulfonates(PFSAs)																		
PFBS	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
PFHxS	<mLOQ	<mLOQ	<mLOQ	5.5	<mLOQ	0.67	-	-	-	-	-	-	1	-	1	4	-	4
PFOS	<mLOQ	<mLOQ	<mLOQ	2.9	4.3	2.5	0.42	-	0.43	0.87	-	0.45	6	4	25	22	4	86
PFDS	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	0.39	-	-	-	-	-	-	-	-	1	-	-	4
Perfluorinated sulfonamides (PFASA)																		
PFOSA	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Mean and SD for PFPeA have been calculated while excluding WWTP data

Table S5.7 Concentrations of PFCs in water, sediment and biota of other previous studies

Compound	Country	Matrix	Concentrations	Reference
PFPeA	Spain	Surface water	0.08-2.82 ng/L	Campo et al. (2016)
PFHxA			1.44-18.7 ng/L	
PFHpA			0.64-20.1 ng/L	
PFOA			0.07-52.2 ng/L	
PFNA			0.85-19.8 ng/L	
PFDA			0.09-213 ng/L	
PFHxS			12.07-36.7 ng/L	
PFOS			0.01-128 ng/L	
PFHxA		River sediments	bdl	
PFOA			0.15-6.69 ng/g dw	
PFNA			3.63-3.63 ng/g dw	
PFDA			0.37-1.65 ng/g dw	
PFBS			2.17-29.2 ng/g dw	
PFHxS			bdl	
PFOS			0.06-9.83 ng/g dw	
PFPeA		Biota (river fish)	9.84-946 ng/g ww	
PFHxA			bdl	
PFOA			bdl	
PFDA			bdl	
PFHxS			0.63-0.63 ng/g ww	
PFOS			0.56-8.13 ng/g ww	
PFPEA	USA	WWTP Effluent	12 ng/L	Houtz et al. (2016)
PFHxA			26 ng/L	
PFHPA			4.4 ng/L	
PFOA			21 ng/L	
PFNA			8.4 ng/L	
PFDA			3.5 ng/L	
PFHxS			4.8 ng/L	
PFOS			13 ng/L	
PFHxA	China	Biota (fish)	bdl-0.40 ng/g ww	Xu et al. (2014)
PFHpA			bdl-0.72 ng/g ww	
PFOA			0.38-2.47 ng/g ww	
PFNA			0.42-2.86 ng/g ww	
PFDA			1.63 -10.29 ng/g ww	
PFUdA			1.27-9.41 ng/g ww	
PFDoA			0.61-2.62 ng/g ww	
PFOS			2.26-20.96 ng/g ww	
PFPeA	Hong Kong, China	Surface water	1.24-4.59 ng/L	Loi et al. (2011)
PFHxA			1.93-5.62 ng/L	
PFHpA			1.30-8.49 ng/L	
PFOA			4.74-13.3 ng/L	
PFNA			0.43-2.76 ng/L	
PFDA			bdl-0.91 ng/L	
PFUdA			bdl-0.70 ng/L	
PFHxS			0.84-2.34 ng/L	
PFOS			1.66-12.0 ng/L	
PFOA		River sediments	bdl -109 pg/g ww	
PFDA			bdl -44.5 pg/g ww	
PFOS			141-232 pg/g ww	
PFOA		Biota (fish,	0.13-0.14 ng/g ww	
PFDA		*Mugil cephalus*)	0.75-0.98 ng/g ww	
PFUdA			0.87-1.11 ng/g ww	
PFDoA			0.37-0.50 ng/g ww	
PFOS			4.36-8.01 ng/g ww	
PFDS			0.13-0.14 ng/g ww	

Table S5.7 (continued)

Compound	Country	Matrix	Concentrations	Reference
PFPeA	China	Surface water	bdl-2.59 ng/L	Pan et al. (2014a)
PFHxA	(Yangtze River)		0.11-22.7 ng/L	
PFHpA			bdl-2.61 ng/L	
PFOA			0.52-18 ng/L	
PFNA			bdl-0.86 ng/L	
PFDA			bdl-0.33 ng/L	
PFHxS			bdl-4.50 ng/L	
PFOS			bdsl-3.93 ng/L	
PFHxA		Sediment	bdl-0.32 ng/g dw	
PFOA			0.03-0.72 ng/g dw	
PFNA			bdl-0.06 ng/g dw	
PFDA			bdl-0.06 ng/g dw	
PFOS			bdl-0.59 ng/g dw	
PFHxA	China	Surface water	bdl-7.94 ng/L	Lam et al. (2014)
PFHpA			bdl-3.43 ng/L	
PFOA			bdl-8.34 ng/L	
PFNA			bdl-4.49 ng/L	
PFDA			bdl-4.80 ng/L	
PFHxS			bdl-3.97 ng/L	
PFOS			bdl-15.07 ng/L	
PFHxA		Sediment	bdl-0.05 ng/g dw	
PFOA			bdl-0.28 ng/g dw	
PFNA			bdl-0.15 ng/g dw	
PFDA			bdl-0.08 ng/g dw	
PFOS			0.01-0.48 ng/g dw	
PFHxA		Fish	bdl-0.36 ng/g ww	
PFOA		(blood+liver)	bdl-0.33 ng/g ww	
PFNA			bdl-13.22 ng/g ww	
PFDA			0.06-20.58 ng/g ww	
PFDoA			bdl-19.18 ng/g ww	
PFOS			bdl-145.23 ng/g ww	
PFPeA	Spain	Surface water	0.1-12.5 ng/L	Lorenzo et al. (2016)
PFHxA	(Ebro River)		9.6-31.4 ng/L	
PFHpA			13.7-17.2 ng/L	
PFOA			2.0-125 ng/L	
PFNA			4.8-7.9 ng/L	
PFDA			0.1-6.5 ng/L	
PFHxS			1.1-5.8 ng/L	
PFOS			0.1-27 ng/L	
PFHpA		River sediment	0.4-0.6 ng/g dw	
PFOA			0.4-32.2 ng/g dw	
PFDA			0.1-0.4 ng/g dw	
PFBS			0.5-6.8 ng/g dw	
PFOS			0.01-2.2 ng/g dw	
PFHxA		Biota (river fish)	53.5-1280 ng/g ww	
PFHpA			14.5-14.5 ng/g ww	
PFOA			18.8-29.9 ng/g ww	
PFNA			15.1-19.9 ng/g ww	
PFDA			19.4-19.4 ng/g ww	
PFHxS			0.01-0.1 ng/g ww	
PFOS			14.9-19.4 ng/g ww	

Table S5.7 (continued)

Compound	Country	Matrix	Concentrations	Reference
PFOA	Sweden	Biota (river fish/seawater fish)	bdl-0.25/bdl-0.39 ng/g fw	Berger et al. (2009)
PFNA			bdl-0.71/bdl-0.47 ng/g fw	
PFDA			0.15-0.81/bdl-0.34 ng/g fw	
PFUdA			0.09-0.89/bdl-0.61 ng/g fw	
PFDoA			bdl-0.53/bdl-0.15 ng/g fw	
PFHxS			bdl-0.80/bdl-0.20 ng/g fw	
PFOS			0.97-23.1/0.47-2.92 ng/g fw	
PFNA	USA	Biota (river fish)	bdl-2.48 ng/g ww	Stahl et al. (2014)
PFDA			bdl-28.5 ng/g ww	
PFOs			bdl-127 ng/g ww	
PFOSA			bdl-63.1 ng/g ww	
PFOA	Canada	Biota (freshwater fish)	1.0-90 ng/g ww	Martin et al. (2004)
PFNA			0.8-57 ng/g ww	
PFDA			1.3-32 ng/g ww	
PFDoA			1.8-14 ng/g ww	
PFOS			13-450 ng/g ww	
PFOSA			4.0-180 ng/g ww	

bdl: below detection limit

Table S5.8 Summary statistics of the selected PFCs in sediment samples collected in autumn (1st sampling campaign), winter (2nd sampling campaign) and spring-summer (3rd sampling campaign) seasons. Values are expressed in ng/g dw

	Min			**Max**			**Mean**			**SD**			**n. detected**			**Frequency** (%)		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
Perfluorinated carboxylic acids (PFCAs)																		
PFPeA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
PFHxA	<mLOQ	<mLOQ	<mLOQ	3.7	<mLOQ	<mLOQ	1.3	-	-	1.1	-	-	9	-	-	41	-	-
PFHpA	<mLOQ	<mLOQ	<mLOQ	2.1	<mLOQ	<mLOQ	0.7	-	-	0.56	-	-	8	-	-	36	-	-
PFOA	<mLOQ	<mLOQ	<mLOQ	12.0	4.6	<mLOQ	6.0	0.84	-	2.9	1.1	-	21	5	-	96	25	-
PFNA	<mLOQ	<mLOQ	<mLOQ	4.2	<mLOQ	<mLOQ	1.1	-	-	0.82	-	-	5	-	-	23	-	-
PFDA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
PFUdA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
PFDoA	<mLOQ	<mLOQ	<mLOQ	4.0	1.7	<mLOQ	-	0.91	-	-	0.29	-	1	3	-	5	15	-
Perfluorinated sulfonates(PFSAs)																		
PFBS	<mLOQ	<mLOQ	<mLOQ	4.8	<mLOQ	<mLOQ	0.48	-	-	1.1	-	-	3	-	-	14	-	-
PFHxS	<mLOQ	<mLOQ	<mLOQ	1.5	<mLOQ	<mLOQ	0.44	-	-	0.26	-	-	3	-	-	14	-	-
PFOS	<mLOQ	<mLOQ	<mLOQ	22.6	2.5	<mLOQ	2.7	0.61	-	5.6	0.50	-	6	3	-	23	15	-
PFDS	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-
Perfluorinated sulfonamides (PFASA)																		
PFOSA	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	-	-	-	-	-	-	-	-	-	-	-	-

Table S5.9 *p*-values of the Kruskal-Wallis test run on sediment samples (autumn and winter period). Values reported in bold are related to variables which are statistically different between the two seasons; values in grey refer to variables which do not differ significantly between the two seasons

Variable	***p*-value** comparison 1^{st}-2^{nd} campaigns
PFBS	0.06
PFHxA	**$4.6*10^{-4}$**
PFHpA	**$3.1*10^{-3}$**
PFHxS	0.06
PFOA	**$2.9*10^{-8}$**
PFNA	**0.01**
PFOS	0.15
PFDoA	0.38

Table S5.10 PFC concentrations (ng/g ww) detected in freshwater fishes

Species	Sample treatment	PFHxA	PFHpA	PFOA	PFNA	PFOS	PFDA	PFUdA	PFDoA	PFOSA
Xerta										
C. carpio	individual (whole fish body)	6.8	1.4	116	7.3	137	28.0	3.9	16.1	20.6
C. carpio (muscle)	muscle tissue	78.5	3.0	298	1.1	154	401	2.3	8.4	10.5
C. carpio (skin)	skin tissue	33.3	3.2	171	5.1	137	231	2.6	13.9	5.5
Liza sp.	individual (muscle + skin tissues)	35.7	3.1	113	1.8	<mLOQ	454	<mLOQ	0.69	4.8
R. rutilus	pool sample	<mLOQ	<mLOQ	166	7.0	27.2	25.1	1.5	5.4	0.70
S. erythropthalmus	pool sample	4.7	1.3	94.2	1.9	10.1	12.9	0.88	1.4	1.2
S. glanis	individual (muscle + skin tissues)	21.4	<mLOQ	154	<mLOQ	12.9	459	1.5	3.1	5.2
S. laietanus (1)	individual (whole fish bosy)	21.7	<mLOQ	185	3.1	13.5	11.5	<mLOQ	<mLOQ	3.7
S. laietanus (2)	individual (whole fish bosy)	16.3	0.98	96.9	6.8	17.7	19.5	0.97	4.4	9.3
S. laietanus (3)	individual (whole fish bosy)	4.9	2.9	161	<mLOQ	15.0	20.7	0.92	0.69	16.1
S. laietanus (4)	individual (whole fish bosy)	13.1	1.6	<mLOQ	3.4	5.5	20.3	0.92	2.2	16.3
A. alburnus	pool sample	122	3.0	265	32.3	35.9	455	2.1	5.7	3.9
Tortosa										
S. laietanus	individual (muscle + skin tissues)	1.7	1.4	174	5.2	69.0	24.0	2.1	9.0	2.2
R. rutilus	Individual (whole fish body)	17.5	2.6	87	0.77	67.8	33.0	2.1	9.1	2.2
A. alburnus	pool sample	98.6	4.5	330	9.9	55.1	47.9	1.5	<mLOQ	6.0
Liza sp.	pool sample	66.3	3.7	318	6.7	37.5	24.8	1.8	8.2	10.4

Table S5.11 PFC concentrations (ng/g ww) detected in seawater fish

	PFHxA		PFHpA		PFOA		PFNA		PFOS		PFDA		PFUdA		PFOSA	
	skin	*muscle*	*skin*	*muscle*	*skin*	*muscle*	*skin*	*muscle*	*skin*	*muscle*	*skin*	*muscle*	*skin*	*muscle*	*skin*	*muscle*
Alfacs bay																
M. cephalus	5.0	1.1	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	2.3
T. torpedo	<mLOQ	0.88	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	1.2	<mLOQ	<mLOQ	<mLOQ	1.3	<mLOQ	<mLOQ
B. boops	<mLOQ	1.7	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	1.3	<mLOQ	<mLOQ
Fangar bay																
T. mediterraneus	1.3	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	0.30	<mLOQ	11.5	3.8	<mLOQ	<mLOQ	<mLOQ	1.6	<mLOQ	<mLOQ
S. salpa	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ
D. annularis	1.9	1.5	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ
Illa de Buda lagoon																
M. cephalus	1.4	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	0.70	<mLOQ	<mLOQ
M. salmoides	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	<mLOQ	4.2	<mLOQ	<mLOQ	<mLOQ	0.92	<mLOQ	<mLOQ
C. carpio	6.7	<mLOQ	<mLOQ	<mLOQ	1.1	<mLOQ	3.3	0.86	14.5	3.8	2.5	<mLOQ	2.3	0.80	<mLOQ	<mLOQ
A. anguilla	1.5	<mLOQ	2.0	<mLOQ	1.9	0.46	2.8	0.95	21.6	6.2	<mLOQ	<mLOQ	2.8	<mLOQ	<mLOQ	<mLOQ

Tab S5.12 LogBAF values calculated for freshwater fish collected in Xerta

	PFHxA	PFOA	PFNA	PFOS
C. carpio	1.4	2.2	0.79	3.0
Liza sp.	1.3	2.0	0.42	0.68
R. rutilus	-	2.1	1.0	2.3
S. erythropthalmus	0.42	1.9	0.45	1.9
S. glanis	1.1	2.1	0.06	2.0
S. laietanus	0.90	2.0	0.71	2.0
A. alburnus	1.8	2.3	1.7	2.4

Chapter 6

ECOLOGICAL AND HUMAN HEALTH RISK ASSESSMENT

1 INTRODUCTION

Ecological Risk Assessment is a powerful tool in the frame of chemical policy and regulation and is mostly used to predict the potential risk for the environment related to the exposure of organisms to individual chemicals.

Assessing the ecological risk of a specific substance is very complex, due to the complex structure of the ecosystem itself and to the different responses of organisms according to species and their life-stage sensitivity.

Directive 93/67/EEC and Regulation 1488/94 have pointed out the need of this procedure to safeguard the environment and human health, and with this purpose the European Commission in 2003 provided Technical Guidelines (TGD) for Risk characterization (EC 2003).

One approach for risk assessment proposed by the TGD of the European Commission is to calculate risk by comparing the predicted environmental concentration of a substance (PEC) with the concentration at which adverse effects are not likely to occur (PNEC) and estimating the ratio PEC/PNEC. Representing the concentration below which adverse effects are not likely to occur, the PNEC can be considered a reference value which ensures a general protection of the environment, if not exceeded. A PEC/PNEC ratio ≥ 1 is thus an index of potential risk for organisms exposed to that kind of chemical. The PEC can be derived from available measured data or from model calculation. PNEC values can be derived from a batch of single species laboratory tests or from model ecosystem tests, taking into account adequate assessment factors to correct for the toxicological data (EC 2003). When sufficient data are available, PNEC values can also be obtained from statistical extrapolation methods. Whatever the method used to derive PNECs, when assessing the ecological risk of a chemical compound, great attention is paid to the most sensitive species of an ecosystem, since ecosystem health is highly dependent on them; hence, protecting them ensures the protection of the entire community.

In the following sections PNEC derivation for water and sediment will be discussed and results regarding risk associated to exposure of organisms to EDCs in the Romagna area and in the Ebro delta will be presented. Finally, human health risk as regards fish and drinking water consumption in the Spanish and in the Romagna population will also be assessed and discussed.

2 RISK ASSESSMENT DERIVATION METHODS

2.1 Derivation of PNEC in the water phase

There are different approaches that can be applied to calculate the PNEC value.

If a large dataset on chronic effects obtained from long-term tests on different taxonomic groups is available, statistical extrapolation methods may be used to derive the PNEC. The most commonly used statistical method is the Species Sensitivity Distribution (SSD). This method can be applied only if the distribution of species sensitivity follows a theoretical distribution function. As a minimum sample size for a SSD, it is advisable to use toxicity data of at least 10 species belonging to at least 8 taxonomic groups; the use of toxicity data of more than 15 species is highly encouraged, though (EC 2003). For an optimal estimation of the toxicity of a particular substance, TGD guidelines propose the selection of species belonging to different categories: a fish; a second family of the *phylum* Chordata (amphibian, fish, etc.); a crustacean; an insect; a family in any order of insect or any *phylum* not already considered; algae; higher plants. Generally, the SSD is based on the selection of No-Observed Effect Concentration (NOEC) values as endpoint.

However, such rich datasets are available only for few chemicals. When only short-term toxicity data or data of few different species are available, it is better to use assessment factors (AF) to derive the PNEC value. The assessment factor approach consists in dividing the lowest LC_{50} or EC_{50} of the available toxicity data by an AF of 1000; a lower AF value can be applied if NOEC data derived from long-term studies are available. In particular, an AF of 10 can be applied when long-term NOECs from at least three species representing three trophic levels are available. The use of assessment factors has the key role to correct the final risk value for the uncertainty of extracting toxicity data from laboratory to the real environment. The higher the uncertainty of such extrapolation, the higher the value of the assessment factor.

Additionally, EC guidelines provide a proposal for risk assessment related to substances with intermittent release, that is for substances which are not continuously introduced into the environment, but are rather emitted a few times a year, being dependent on point sources of contamination. In this case, organisms are not continuously exposed to such substances, and the exposure event may be of short duration. When deriving PNEC for these substances, generally only short-term effects are preferred. Extrapolation of PNEC in this case will be based on an assessment factor of 100 applied to the lowest LC_{50} or EC_{50} of at least three short-term tests from three trophic levels (EC 2003).

In this work, PNEC values of each EDC were calculated from toxicity data obtained from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/), which is a comprehensive database of toxicity data of both aquatic and terrestrial organisms available for a lot of chemicals. This database was used because only

reliable and relevant toxicity tests results are contained in it; data were handled following the recommendations published on the website. In the following section greater details about the derivation of PNEC values for the different EDCs analysed in this study are provided.

2.1.1 PNEC derivation using statistical extrapolation methods

Only BPA and NP datasets concerning freshwater species and extrapolated from the US EPA ECOTOX database accomplished to the minimum sample size requirements of EC guidelines (EC 2003) to apply the SSD method; for this reason, SSD approach to derive PNEC was used only for these two compounds. PNEC derivation for OP was not considered, since its occurrence in the study area was <LOQ in all samples.

Table S6.1 and **Table S6.3** provide toxicity data selected for BPA and NP in freshwater, respectively. As suggested by TGD guidelines, NOEC values were preferred and selected. To ensure that the data fitted to a distribution function, the statistical Anderson-Darling test was run on log- transformed data, confirming the log-normal distribution of both populations. For each chemical, the Hazardous Concentration to five percent of the species (HC_5) in the SSD was then calculated and the final PNEC was obtained following the formula:

$$PNEC = \frac{HC_5 \ (50\%\ c.i.)}{AF}$$

where HC_5 represents the value at which 5% of the species exhibit an effect; AF is an assessment factor used to correct the derived value and can run from 1 to 5. In this study an assessment factor of 5 was chosen for both BPA and NP for the precautionary principle, in order to correct the final result for the error of deriving a PNEC from non-local toxicity data. The 50% c.i. stands for the 50% confidence interval relative to HC_5 which has to be derived, as well, according to TGD guidelines. **Table 6.1** reports the final PNEC calculated for both BPA and NP, along with the 50% of confidence interval of the HC_5.

2.1.2 PNEC derivation using assessment factors

The toxicity database for PFOA and PFOS, along with BPA and NP toxicity for seawater species, were not as exhaustive as those ones available for BPA and NP for freshwater species, and the minimum sample size requirement recommended by TGD guidelines was not fulfilled. Hence, the statistical extrapolation method could not be applied on the datasets, and the Assessment Factor approach was used. **Table S6.2** and **Table S6.4** provide information about BPA and NP toxicity for seawater organisms; **Table S6.5** and **Table S6.6** concern PFOA toxicity in freshwater and seawater species, while **Table S6.7** and **S6.8** PFOS toxicity in freshwater and seawater organisms. NOEC values of different species belonging to different taxonomic groups were selected. Following EC Guidelines, PNEC of PFOA for freshwater species and BPA and NP for seawater species were obtained by applying an AF of 10 to the lowest NOEC extrapolated from a dataset of

at least three species of three trophic levels (EC 2003). Conversely, for PFOS in both freshwater and seawater species, and PFOA toxicity for seawater species, the lowest NOEC was divided by an AF of 100, since the datasets comprised a more limited number of species and/or taxonomic groups. The obtained PNEC values are reported in **Table 6.1**.

2.1.3 PNEC derivation for substances with intermittent release

The previous *Chapters 3* and *4* pointed out that estrogens occurrence in the Romagna area was not continuous, but was dependent on sporadic and intermittent introductions in surface waters. Therefore, for PNEC derivation of this class of contaminants the procedure suggested by TGD guidelines for substances with intermittent release was followed. For substances that are intermittently introduced into the environment, LC_{50} and EC_{50} toxicity data are preferred; an AF of 100 is applied to the lowest end-point value of at least three toxicity tests from three trophic levels (EC 2003). Toxicity data in freshwater and seawater species are reported in **Tables S6.9-S6.10** (E1) and **Tables S6.11-S6.12** (E2). PNEC for EE2 was not calculated because the compound was not detected at concentrations higher than the LOQ in any sampling campaign. PNEC values obtained with this approach are reported in **Table 6.1**.

2.2 PNEC derivation in the sediment compartment

Contaminant analysis on sediment can be very useful, especially to track contamination happening over a long time period. Sediments can act both as sink for contaminants through adsorption and accumulation, and as source with their release into the aquatic phase through resuspension as the environmental conditions change. Sediment contamination can thus be hazardous for both pelagic and benthic aquatic communities; moreover, sediment-dwelling organisms affected by contamination can represent a further risk considering the contaminant bioaccumulation and magnification through the food chain.

The best approach to assess sediment risk would be to use a method similar to the one adopted for the aquatic compartment, calculating a PNEC value from toxicity tests on sediment-dwelling organisms. However, very few toxicity data are available for new substances. Hence, to assess the ecological risk associated to EDCs presence in sediments, the equilibrium partitioning method was used (EC 2003).

In this method, $PNEC_{water}$ derived for aquatic organisms and the sediment/water partition coefficient are required for the calculation of the $PNEC_{sed}$. The formula proposed by TGD Guidelines was applied:

$$PNEC_{sed} = \frac{K_d}{RHO_{sed}} \cdot PNEC_{water} \cdot 1000$$

Where $PNEC_{sed}$ is the Predicted-No-Effect-Concentration to be derived; K_d is the sediment/water partition coefficient (L/kg), calculated as the median of K_d values obtained from the experimental work; RHO_{sed} the

bulk density of sediment (estimated to be 1300 kg/m^3; Janssen et al. 2004), $PNEC_{water}$ the Predicted-No-Effect-Concentration derived for aquatic organisms in the water column. $PNEC_{sed}$ values are reported in **Table 6.1**. Please, note that a $PNEC_{sed}$ for estrogens could not be derived since K_d values could not be calculated from the experimental data collected in this work.

It should be kept in mind, however, that this approach has many limitations, since it is based on the assumption that sediment-dwelling organisms and aquatic species share a similar sensitivity to contaminants, even though this is barely realistic. Moreover, the formula takes into consideration only ingestion through the water phase; the uptake of contaminants by sediment organisms, however, can occur through other different pathways, as well, such as ingestion or direct contact with contaminated sediment. In this sense, the uptake of contaminants can often be underestimated, especially for hydrophobic substances ($3 < \log K_{ow} < 5$) which have the potential to bioaccumulate. For this reason, the equilibrium partitioning method should be used only as a trigger for further analyses on organisms using spiked sediment.

Table 6.1 Calculated PNEC for each of the analysed EDCs

Compound	HC_5 (50%c.i.)	$PNEC_{water}$ freshwater	$PNEC_{water}$ seawater	$PNEC_{sed}$ river sediment	$PNEC_{sed}$ marine sediment
BPA	1216* (379-2436)*	243*	117*	112**	54**
NP	1930* (1000-2000)*	386*	100*	175**	101**
E1	-	4.6*	6.3*	-	-
E2	-	2*	5*	-	-
PFOA	-	50,000*	15,000*	113,000**	119,000**
PFOS	-	5,000*	150*	4,000**	740**

* ng/L; ** ng/g

2.3 Risk Quotient calculation in water and sediment

Once all PNEC values for each compound had been calculated both in water and sediment, the final Risk Quotient (RQ) was assessed at each sampling site as the ratio between the Measured Environmental Concentration (MEC) of each EDC (compound "i"), and its derived PNEC:

$$RQ_i = \frac{MEC}{PNEC}$$

RQ_i, which stands for the Risk Quotient obtained for each of the *i* EDC compounds, was calculated at each sampling point. The obtained RQ_i were then ranked according to the following division: $0 < RQ_i < 0.5$ as a low risk; $0.5 < RQ_i < 1$ as medium risk; $RQ_i > 1$ as high risk.

Regarding RQ_i derived for the sediment compartment, all the limitations that are involved in the derivation of $PNEC_{sed}$ should be taken into consideration. Since risk assessment of sediment was based on toxicity data extrapolated from the organisms in the water column, the final result may not represent the real situation of risk associated to EDCs occurrence in sediment. The ratio calculated for sediment, however, can be considered a useful screening tool to assess the risk of sediment organisms to contaminants exposure. A $RQ_i > 1$ can represent a situation of possible concern, and tests with sediment organisms using spiked sediment need to be conducted to support a refined risk assessment (EC 2003).

In both water and sediment of the coastal environment of Ebro delta, in which 13 PFCs were analysed, the associated risk was calculated taking into account only mean concentrations of PFOA and PFOS over the year of sampling. For the other compounds belonging to the perfluorinated class, on the contrary, it was not possible to derive a PNEC, since only few data about toxicity of these compounds are available, and many of them are not reliable or relevant for a risk derivation (Valsecchi et al. 2017).

2.4 Human health risk related to fish consumption

In addition to the ecological risk assessment, human health risk associated to exposure of contaminants through food will be discussed in the following section. To this purpose, only data on PFCs accumulated in potentially edible species of fish discussed in *Chapter 5* will be accounted for.

Food, and especially fish consumption, has been recognized to be the main pathway of human exposure to PFCs (Berger et al. 2009; Zhao et al. 2011b; Pérez et al. 2014). To assess the risk of human exposure to fish contaminated by PFCs, Estimated Dietary Intake (EDI) of each of the PFCs encountered in freshwater and seawater fish samples was calculated according to the formula (Wang et al. 2017):

$$EDI_{fish} = (C_{fish} \cdot Q_{fish} / M_{bw})$$

where EDI stands for the Estimated Dietary Intake (ng/Kg bw/d); C_{fish} stands for the concentration for each of the PFCs detected in fish (ng/g ww), Q_{fish} represents the amount of fish consumed daily (g/day) and M_{bw} is the human average body weight (kg).

Fish consumption data were taken from the European database provided by EFSA (EFSA 2011); only data regarding fish consumption in Spain were selected. Data of fish consumption were subdivided into four classes based on age of population: toddlers (1-3 years), children (4-12 years), adolescents (13-18 years) and adults (> 18 years). For the estimation of PFCs dietary exposure, the model reported by Wang et al. (2017) was followed. The model defines three scenarios of exposure to contaminants: low exposure (1), represented by the 5^{th} percentile of PFCs concentration in fish and fish consumption; intermediate exposure (2), calculated from the median of PFCs concentration in fish and fish consumption; high exposure

(3), accounting for the 95th percentile of PFCs concentration in fish and fish consumption. Low exposure scenario represents the best case of human exposure to PFCs as regards fish consumption, whereas the high exposure scenario depicts the worst case of exposure to PFCs.

Only for PFOA and PFOS the European Food Safety Authority has defined provisional Tolerable Dietary Intake (TDI) concentrations, which are concentrations in food below which a health risk for human is not likely to occur. These values are set as 1500 ng/kg bw/d for PFOA and 150 ng/kg bw/d for PFOS (EFSA 2008). Considering these concentrations as reference value of absence of risk for human, the Hazard Quotient (HQ) was then calculated according to the following formula:

$$HQ = \frac{EDI}{TDI}$$

A HQ ratio > 1 is considered as potential harmful for human beings.

For the other PFCs detected in fish samples it was not possible to calculate any HQ since still little is known about their toxicity (Valsecchi et al. 2017).

2.5 Human health risk related to drinking water consumption

Another major pathway of human exposure to EDCs is through contaminated drinking water. Different studies have pointed out the non-completely efficiency in the removal of contaminants of the most common treatment water processes, that lead to the detection of EDCs in drinking water (Maggioni et al. 2013; Esteban et al. 2014b).

Many of the surface water bodies of the Romagna area are used to produce drinking water; therefore the human health risk related to drinking water consumption was taken into consideration for this study area and assessed according to the formula:

$$HQ_{dw} = \frac{C_w}{RfD}$$

where HQ_{dw} stands for Hazard Quotient in drinking water; C_w is the concentration of contaminants in water, and RfD is the reference dose for each of the analysed EDCs. Since data regarding EDC concentrations in drinking water of the Romagna area were not available, concentrations found in rivers in proximity to the water abstraction points for drinking purposes were used, assuming no efficiency of the treatment techniques used in the drinking water treatment plants to remove contaminants (worst-case scenario). RfD values were taken from literature. The Italian Institute of Health proposed a *long-term minimum quality goal* to achieve in drinking waters of 0.5 µg/L for PFOA and 0.03 µg/L for PFOS (Valsecchi et al. 2017, and references therein), so these thresholds were set as reference dose. Regarding phenolic and estrogen

compounds, no guideline in drinking water has still been proposed by public authorities. Hence, to derive the Reference Dose for these chemicals, the method suggested by Valsecchi et al. (2017) was followed. Briefly, this method consists in deriving a quality water standard from the available TDI data, according to the formula:

$$QS_{dw} = \frac{0.1 \cdot TDI \cdot 70}{2}$$

where QS_{dw} is the drinking water Quality Standard (µg/L); 70 stands for the average human body weight (kg); 0.1 is the default fraction of the TDI allocated to drinking water, and 2 (L/d) is the estimated daily uptake of drinking water. TDI has been set at 4 µg/kg bw/d for BPA (EFSA 2015a); 5 µg/kg bw/d for NP (FSA 2010); 0.0013 µg/kg bw/d for E2 and 0.005 µg/kg bw/d for E1 (Achene et al. 2011b). According to the equation, QS_{dw} was 14 µg/L for BPA and 18 µg/L for NP, 5 ng/L for E2 and 18 ng/L for E1; these QS_{dw} were set as Reference Dose.

3 RESULTS

3.1 Ecological risk assessment of water and sediment compartments

Table 6.2 summarizes the RQ values for each contaminant, in both water and sediment of the Romagna area.

Table 6.2 Risk Quotient values calculated for each EDC in water and sediment of the Romagna area: range of values (minimum-maximum), median (med), mean value and standard deviation (sd)

	Water										Sediment				
	summer 2015					summer 2016					summer 2016				
	Min	**Max**	**Med**	**Mean**	**SD**	**Min**	**Max**	**Med**	**Mean**	**SD**	**Min**	**Max**	**Med**	**Mean**	**SD**
E1	0	6.1	0.06	1.5	2.2	0.05	1.5	0.06	0.16	0.29	-	-	-	-	-
E2	0	20	0.37	2.3	5.1	-	-	-	-	-	-	-	-	-	-
BPA	0	0.7	0.12	0.17	0.19	0	1.0	0.11	0.24	0.29	0	0.27	0.02	0.05	0.07
NP	-	-	-	-	-	0	0.47	0.10	0.14	0.13	0	1.3	0.17	0.22	0.28
PFOA	0	$4{\cdot}10^{-4}$	$2{\cdot}10^{-6}$	$6{\cdot}10^{-5}$	$9{\cdot}10^{-5}$	0	$1{\cdot}10^{-3}$	$2{\cdot}10^{-4}$	$3{\cdot}10^{-4}$	$3{\cdot}10^{-4}$	-	$7{\cdot}10^{-6}$	-	-	-
PFOS	0	$1{\cdot}10^{-3}$	$3{\cdot}10^{-5}$	$2{\cdot}10^{-4}$	$3{\cdot}10^{-4}$	0	$2{\cdot}10^{-2}$	$1{\cdot}10^{-4}$	$9{\cdot}10^{-4}$	$3{\cdot}10^{-3}$	-	-	-	-	-

The contribution of EDCs to the ecological risk as regards the water compartment was in the order estrogens > phenolic compounds > perfluorinated compounds, in both monitored periods. Estrogens are biologically active hormones, and they can exert their effects at very low concentrations; therefore PNEC values for these compounds are lower than for the other xenochemicals and range in few ng/L (2 ng/L E2 and 4.6 ng/L E1; see **Table 6.1**). It is hence to be expected that their detection in the environment might exceed this low thresholds. A situation of high concern was in fact registered in summer 2015, with E2

reaching up to a RQ value of 20 and E1 a value of 6; such high values of risk could be expected considering the analytical data previously reported in *Chapter 3*. On the contrary, the almost absence of estrogens in summer 2016 was reflected in a decrease of potential risk; in those samples in which estrogens were detected, however, the associated ecological risk was slightly higher than one.

BPA was the compound that secondly accounted for the ecological risk in water after estrogens, showing maxima RQ values around unity, in both monitored periods. These situations of high anomaly (and thus high risk) were recorded in the southern portion of the Romagna area, and more specifically in the Marecchia river, which has already been reported to be affected by a high level of BPA contamination (see *Chapters 3* and *4* for details on chemical data). A lower degree of BPA contamination in the other water bodies is highlighted by the lower RQ median and mean values, which are both below 0.3, and are thus indicative of a situation of no or low risk.

The contribution of PFCs (PFOA and PFOS) to the ecological risk of the Romagna water bodies was rather negligible, as could be assumed by comparing the concentration levels found in the two monitoring periods (see *Chapters 3* and *4*) and the PNEC values of these two compounds that are higher than the other EDCs (**Table 6.1**); combined together, these two factors give the very low RQ values reported in **Table 6.2**. This result suggests that PFCs occurrence in the aquatic environment of Romagna area is not of great concern, since the detected concentrations are much lower than the associated threshold of adverse effects on organisms.

Sediments were primarily affected by contamination of phenolic compounds, while estrogens and PFCs registered only some local measurements (see *Chapter 4* for discussion). Concerning the ecological risk associated to sediments, NP showed a higher contribution with respect to BPA, in contrast with water RQ results. This is mainly related to NP higher partitioning into the sediment compartment compared to BPA, due to its hydrophobic characteristics. Notwithstanding the higher occurrence of NP and the higher RQ values, NP concentrations were below the threshold level at which adverse effects on organisms can occur, registering a RQ<1 in the majority of sampling stations, and only one sample at around unity (RQ=1.3). This sample is located in Pialassa Baiona coastal lagoon (sample CG1, see previous Figure 4.1 of *Chapter 4*), and the resulting RQ value was mainly dependent on the lower $PNEC_{sed}$ obtained for the saltwater environment with respect to the freshwater one. In addition, the higher RQ_{NP} values in the transitional environment of Pialassa Baiona were also due to the higher concentrations detected in sediments with respect to riverbed sediments, as a consequence of the lower NP solubility in saltwater.

Concerning the ecological risk of the Ebro delta area, in Spain, the obtained RQ values are reported in **Table 6.3**. Please note that, since this study area was focused only on the analysis of the behavior of PFCs in the environment, risk ecological values were calculated only for this class of contaminants, and more

specifically only for PFOS and PFOA, due to the lack of toxicological data for the other PFCs, required for PNEC derivation.

Table 6.3 Risk Quotient values calculated for PFOA and PFOS in water and sediment of the Ebro delta area: range of values (minimum-maximum), median, mean value and standard deviation (sd)

	Water					Sediment				
	Min	**Max**	**Median**	**Mean**	**SD**	**Min**	**Max**	**Median**	**Mean**	**SD**
PFOA	0	$1{\cdot}10^{-4}$	$3{\cdot}10^{-5}$	$3{\cdot}10^{-5}$	$3{\cdot}10^{-5}$	$3{\cdot}10^{-6}$	$9{\cdot}10^{-5}$	$4{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$3{\cdot}10^{-5}$
PFOS	0	0.01	$8{\cdot}10^{-4}$	$1{\cdot}10^{-3}$	$2{\cdot}10^{-3}$	$2{\cdot}10^{-4}$	$9{\cdot}10^{-5}$	$4{\cdot}10^{-4}$	$2{\cdot}10^{-3}$	$3{\cdot}10^{-3}$

Risk values reported in **Table 6.3** were calculated considering the average concentration of PFOA and PFOS throughout the year of sampling. As can be easily noticed, PFOA and PFOS occurrence in both water and sediment compartments of the Ebro delta area showed comparable RQ values well below 0.1, indicating a situation of no ecological risk in the study area. This is not surprising, given the low concentrations of PFCs detected in both waters and sediments (see *Chapter 5* for data discussion) and their associated high PNEC thresholds. Moreover, PFOA and PFOS RQ values were comparable to the PFC risk values encountered in the Romagna area, suggesting that the concentration values at which these compounds are detected in environments with different characteristics are though not of concern in terms of adverse effects that may occur on organisms. It has to be reminded that risk assessment in the Ebro delta was calculated only for PFOA and PFOS, since no PNEC thresholds could be calculated for the other perfluorinated compounds that were analysed in this area. However, considering that PFOA and PFOS were the two PFCs detected at the highest frequency and highest values in the area, and that the short chain PFCs that were detected are expected to have a lower toxicity than PFOS and PFOA, it can be concluded that at the levels recorded in the Ebro delta area, PFCs did not represent a risk for the aquatic biota.

3.2 Human health risk related to fish consumption

Figure 6.3 displays the EDI calculated for each of the three scenarios of PFCs recorded in freshwater fish, divided by age of Spanish population. The highest EDI values were registered for PFOA in all three scenarios, suggesting PFOA as the compound of greatest concern as regards human health exposure through fish consumption. In the intermediate- and high exposure scenarios, PFDA, PFHxA and PFOS were the other PFCs for which EDI values increased; PFDA particularly recorded very high EDI values in the third scenario, in contrast to its lower values in low- and intermediate scenarios, as a consequence of the asymmetric distribution of its concentration in fish (27 ng/g ww as median; 456 ng/g ww as 95th percentile in fish). Regardless of the type of scenario, toddlers within the age 1-3 years old resulted to be the most vulnerable group to PFCs exposure through fish consumption; PFCs intake decreased with the increase of

age, depending on the growth of human body weight. As a result, it can be concluded that PFCs exposure of children through the food is of major concern than for adults.

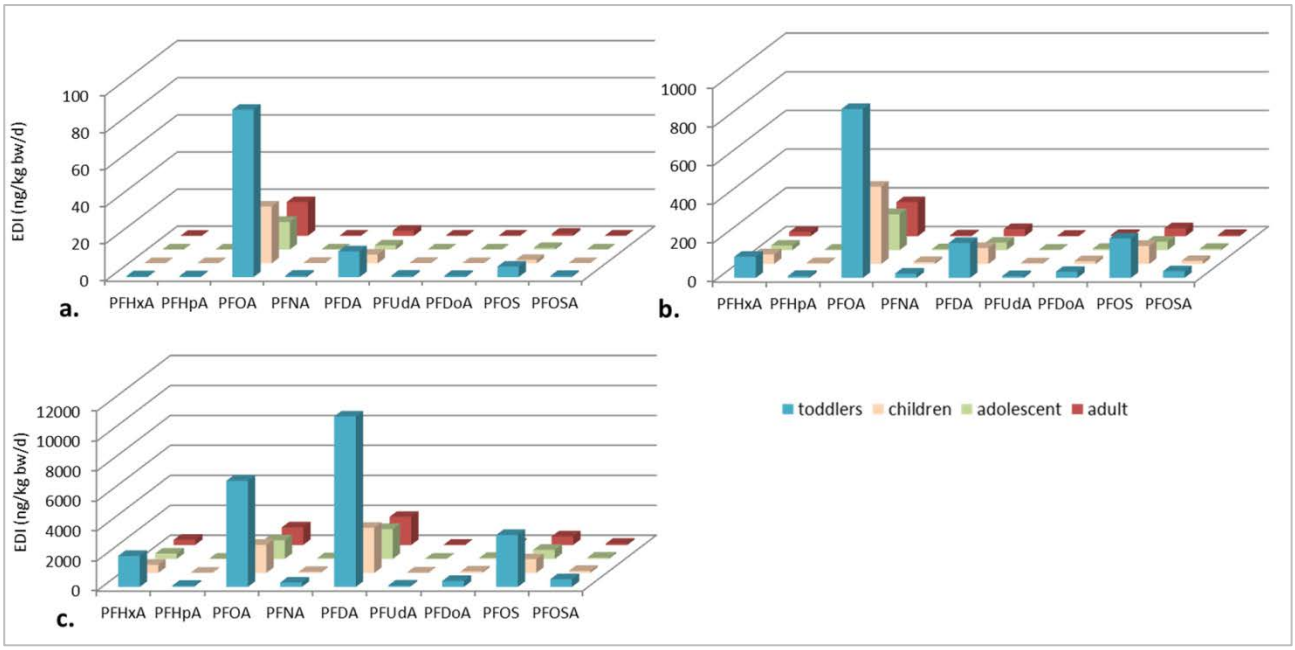


Figure 6.5 EDI (ng/kg bw/d) values of Ebro River freshwater fish consumption, subdivided by different exposure scenarios: low exposure (a); intermediate exposure (b); high exposure (c).

Table 6.2 reports the Hazard Quotient calculated for PFOS and PFOA, obtained from the comparison of EDI values with the maximum acceptable values of 1500 ng/kg bw/d (PFOA) and 150 ng/kg bw/d (PFOS) suggested by EFSA (2008).

From the data obtained in this study, PFOS in fish could represent a potential risk for highly consumers of highly contaminated fish (high-exposure scenario), since the provisional TDI of 150 ng/kg bw/d was exceeded in all age categories. Toddlers were once again the most vulnerable group as regards PFOS exposure, with 20-times higher concentrations than the precautionary reference dose. PFOA concentration in fish was of minor concern, registering a slight exceedance of the maximum acceptable TDI only in the youngest categories of Spanish population (< 12 years old). However, these results regarded only the "worst-case" scenario, which accounted for high consumption of highly contaminated fish, that would happen only in extreme circumstances, since it is based on the 95th percentile of both PFC concentrations in fish and fish consumption rate. The more likely intermediate scenario reported lower risk values for human exposure to PFCs, with no exceedance for PFOA of the TDI proposed by EFSA, and only one slight exceedance for PFOS estimated for the youngest population (1-3 years; HQ = 1.4).

Even though in the intermediate and low exposure scenarios no great risk for the population exposure to PFOA and PFOS in fish was estimated, the two scenarios pointed out how younger people can be more

affected by PFOS occurrence in fish products and are hence more sensitive to PFCs exposure. Therefore, special attention should be paid to this group of population.

Table 6.2 Hazard Quotient (HQ) values of PFOS and PFOA dietary intake through fish. Values in red represent a HQ>1

		Hazard Quotient							
		Freshwater species				Seawater species			
		Toddlers	*Children*	*Adolescents*	*Adults*	*Toddlers*	*Children*	*Adolescents*	*Adults*
PFOS	Low	0.04	0.01	0.01	0.01	0.005	0.002	0.001	0.001
	Intermediate	1.4	0.62	0.29	0.28	0.03	0.01	0.01	0.01
	High	23	6.1	4.1	3.8	0.87	0.23	0.15	0.14
PFOA	Low	0.06	0.02	0.01	0.01	-	-	-	-
	Intermediate	0.58	0.27	0.12	0.12	-	-	-	-
	High	4.7	1.3	0.83	0.78	-	-	-	-

Human health risk was assessed also in relation to the consumption of seawater fish collected from the Ebro delta. For the risk characterization, only concentrations of PFOS and PFUdA in muscle tissue were considered, since only these two compounds were detected in at least 50% of samples. The corresponding EDI values are reported in **Figure 6.6**.

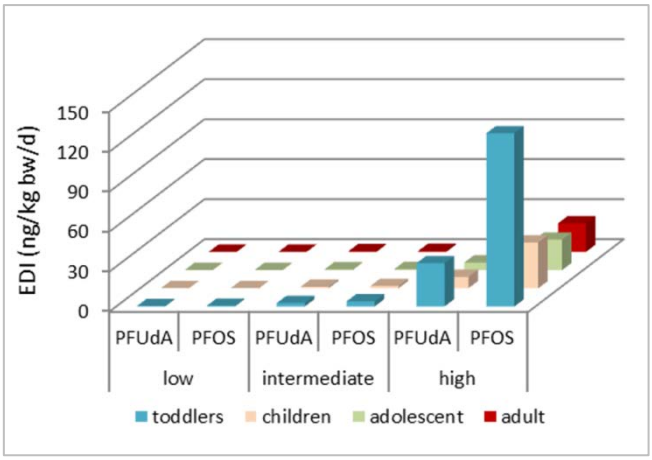


Figure 6.6 EDI (ng/kg bw/d) values of Ebro Delta seawater fish consumption, subdivided by the three exposure scenarios (low, intermediate and high)

The high exposure scenario was found to be the only scenario showing higher EDI values; this is mainly due to the fact that the 5th percentile and median PFOS and PFUdA concentrations (on which the low and intermediate exposure scenarios depend) were half the LOQ. Therefore, only high consumption of (relatively) high contaminated fish of Ebro delta could represent a potential threat to human health. However, the Hazard Quotient obtained from PFOS dietary intake states there is no risk in consuming this kind of fish, even for the high exposure scenario (**Table 6.2**). Nevertheless, results of seawater fish consumption revealed the youngest group (<3 years old) to be the most sensitive among the local population, confirming human health risk results related to freshwater species consumption.

A similar situation was reported in a German study, which investigated PFC levels found in wild fish of German rivers and the Baltic Sea (Schuetze et al. 2010). In their study, there was no risk associated to PFCs in seawater fish; on the other hand, freshwater fish showed quite high PFOS concentrations, up to 225 ng/g ww, which could pose a risk for high consumers health.

3.4 Human health risk related to drinking water consumption

Human health risk related to drinking water consumption was assessed in the Romagna area, where both surface waters (Lamone and Fiumi Uniti rivers) and groundwaters are currently used to provide drinking water. In *Chapter 3* groundwater quality related to EDCs contamination was discussed, and the aquifers used for drinking water purposes were proved to be safe for the local population, since no compound was detected at concentrations higher than its corresponding LOQ. As regards the Ebro delta area, none of the analysed water bodies are used for drinking water purposes; hence, in this section only surface water bodies used to provide drinking waters in the Romagna area will be considered.

Human health risk assessment was performed considering only three sampling points, corresponding to the locations from which water is abstracted for drinking water purposes: sample point L5, at the Lamone river mouth; sample L6 of the CER channel; sample F1, corresponding to the Ridracoli lake, which is an artificial dam which supplies drinking water for almost 50% of the whole area. For a better spatial understanding of the location of these sampling sites, please refer to any of the maps reported in *Chapter 4* of this thesis.

Each of the three sites showed a HQ ratio <0.1 of low risk for all the analysed EDCs, hence indicating that, even in the worst case scenario of absence of contaminants removal during water treatment processes, the local human population should be safe from exposure to EDCs through drinking water consumption.

4 CONCLUSIONS

Ecological risk assessment was performed in the Romagna area and in Ebro delta in order to evaluate the risk of possible adverse effects on organisms exposed to the EDCs levels recorded in this work. The Romagna area showed some concerns regarding risk in the water column, mostly related to high concentrations of estrogens. However, since these high concentrations were proven to not continuously occur in the environment, the resulting associated risk was not constant over time; toxicological studies are though suggested in order to assess ecosystem safety. BPA was the main contributor of a potential high risk in Marecchia River in both sampling campaigns, and this suggests the need for further research aimed at an in-deep analysis on the real risk for organisms in this river. Nevertheless, the river sections from which water is abstracted for drinking purposes did not represent a risk for the local human population. On the other hand, PFCs were proven to be the least harmful compounds for biota, in both the freshwater system

of the Romagna area and the saltwater one of Ebro delta. Concerning human health risk related to the potential consumption of fish of the Ebro delta area, this study found that the Spanish population should not be exposed to a high risk, though the youngest population is more vulnerable to PFCs exposure through fish consumption.

Overall, data on risk assessment point out how currently the freshwater system is the most vulnerable environment as regards exposure to emerging contaminants; further research should thus be addressed in order to find the best management actions to limit risk in this compartment.

It is worth to remark, however, that many limitations are present in the risk assessment approach presented in this work.

First of all, PNEC water values have been calculated from an eco-toxicological database which accounts for a lot of species, whose habitats can be far different from those ones studied in this work; these data are also based on laboratory tests whose conditions can not necessarily depict the real environmental conditions. Conducting field-based studies is suggested for a better and real risk characterization. In addition, toxicological databases on which the PNEC derivation is based are not exhaustive for all the compounds considered in this study: estrogens and PFCs toxicological data, for example, are available only for few species (not necessarily the most sensitive ones), and this could lead to an inaccurate estimation of the real effects that could be expected from exposure of organisms to these chemicals. Moreover, PNEC values for the sediment compartment were extrapolated from the PNEC values in waters, but this could also lead to a bias, since sediment dwelling- and water column organisms are not likely to show the same sensitivity to the chemicals. Toxicology studies on sediment organisms is thus strongly needed. Furthermore, other factors such as bioaccumulation and biomagnification potential should also be considered, as secondary ways of exposure to hydrophobic contaminants.

It should be also taken into account that risk values are referred to individual chemicals, but in the environment organisms are exposed to a mixture of known and unknown chemicals and the effects coming from the interaction of all contaminants are still not well understood. As an instance, calculating the sum of the individual chemicals risk could not be the realistic pathway through which these compounds act.

Risk assessment results stand out the importance of assessing a standardized approach for the ecological risk evaluation, which does not consider only contaminants taken individually; the assessment of an index which accounts for a mixture of different contaminants is advised, in order to better depict the global effects that might affect the environmental health. To do so, it is of great importance to understand the multiple effects of organisms exposure to a mixture of the contaminants that mostly occur in the environment.

That being said, the above mentioned risk assessment methodology provides only an estimate of the real risk resulting from exposure to chemicals, that needs to be integrated with a continuous long-term monitoring, combined with biological and toxicological tests aimed at detecting the real estrogenic effects in the different environmental matrices.

Chapter 6 ECOLOGICAL AND HUMAN HEALTH RISK ASSESSMENT

Table S6.1 BPA toxicity data for freshwater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Chlorella pyrenoidosa	5,000	Population growth rate	10 d
Chlorolobion braunii	2,000	Chlorophyll A concentration	4 d
Cochlodinium polykrikoides	3,470	Population abundance	3 d
Scenedesmus acutus var. *acutus*	5,000	Population growth rate	4 d
PLANT			
Lemna gibba	7,800	Biomass; growth rate	7 d
FISH			
Carassius auratus	4.5	Increase in vitellogenin	30 d
Danio rerio	500	Heart rate	21 d
Gasterosteus aculeatus	1	Gonad morphology	165 d
Melanotaenia fluviatilis	500	Growth length	4 d
Oryzias latipes	0.1	Tissue/organ development	44 d
Pimephales promelas	160	Progeny counts/number	122 d
Salmo trutta	1.75	Cellular malformation	30 d
Sebastes schegelii	100,000	Change in population sex ratio	29 d
CRUSTACEAN			
Asellus aquaticus	500	Growth rate	21 d
Ceriodaphnia dubia	940	Progeny counts/number	7 d
Daphnia magna	30	Progeny counts/number	21 d
Gammarus fossarum	500	Change in sex ratio	103 d
Gammarus pulex	8,400	Changes in reproductive behavior	36 h
Hylalella azteca	490	Progeny counts/number	42 d
AMPHIBIANS			
Rhinella arenarum	15,000	Blastula mortality	2 d
Xenopus laevis	1,800	Juveniles mortality	2 d
MOLLUSCS			
Marisa cornuarietis	100	Hatch mortality	12 d
Physella acuta	100	Hatch mortality	21 d
Potamopyrgus antipodarum	4.83	Progeny counts/number	28 d
Valvata piscinalis	100	Progeny counts/number	28 d
INVERTEBRATES			
Brachionus calicyflorus	1800	Intrinsic rate of population increase	2 d
WORMS			
Dugesia japonica	50	Limb/body part regeneration	7 d
Lumbriculus variegatus	5	Size growth	103 d

* Values are expressed in µg/L; ** d: days; h: hours

Table S6.2 BPA toxicity data for seawater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Skeletonema costatum	400	Cell count	4 d
FISH			
Oryzias melastigma	200	Heart rate; growth length	8 d
Poecilia reticulata	549	Growth length and size	21 d
Sebastes schegelii	100,000	Change in population sex ratio	29 d
CRUSTACEAN			
Charybdis japonica	1,415	Mortality	21 d
Tigriopus japonicus	10	Change in sex ratio; fecundity	21 d
MOLLUSCS			
Haliotis diversicolor ssp. *supertexta*	50	Embryo abnormality	8 h
Mytilus galloprovincialis	23	Hatch mortality	12 h
Perna viridis	1.17	Phagocytosis	7 d
INVERTEBRATES			
Ciona intestinalis	228	Body weight	2 d
Psammechinus miliaris	9,804	Fertilization	2 d

* Values are expressed in µg/L; ** d: days; h: hours

Table S6.3 NP toxicity data for freshwater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Pseudokirchneriella subcapitata	694	Population dry biomass	4 d
PLANTS			
Lemna minor	901	Biomass	4 d
FISH			
Danio rerio	330.5	Gene expression	3 d
Melanotaenia fluviatilis	5,000	Fertilization; gamete production	10 d
Oncorynchus mykiss	6	Growth length	91 d
Oryzias latipes	3	Imposex	100 d
Oryzias latipes	2	Gene expression	7 d
Oryzias latipes	10	Organ formation	4 d
Xiphophorus helleri	2	Growth length	60 d
CRUSTACEAN			
Ceriodaphnia dubia	125	Progeny counts/number	7 d
Daphnia magna	24	Reproduction	21 d
Daphnia magna	77.3	Progeny counts/number	21 d
Daphnia magna	3	Hemoglobin mRNA	1 d
Daphnia magna	30	Growth weight	1 d
ROTIFERA			
Trichocerca sp.	23		20 d
AMPHIBIANS			
Triturus carnifex	19	Cytoplasmic inclusion	30 d
Xenopus laevis	25	Changes in growth	14 d
INSECTS			
Chironomus riparius	1	Genotoxicity	1 d
Chironomus tentans	100	Growth; survival	1 d
INVERTEBRATES			
Brachionus calicyflorus	5	Reproduction	4 d

* Values are expressed in µg/L; ** d: days

Table S6.4 NP toxicity data for seawater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Skeletonema costatum	12	Population dry biomass	4 d
FISH			
Acanthogobius flavimanus	6.37	Ubiquitin carboxy-terminal hydrolase	21 d
Gadus morhua	29	Metabolic rate	21 d
Oncorynchus mykiss	6	Growth length	91 d
Oryzias latipes	3	Imposex	100 d
Oryzias latipes	2	Gene expression	7 d
Oryzias latipes	10	Organ formation	4 d
Psetta maxima	29	P450 aromatase activity	21 d
Rivulus marmoratus	300	Metallothionein mRNA	4 d
CRUSTACEAN			
Americamysis bahia	10	Maturity	13 d
Neomysis integer	1	Electron transfer system activity	4 d
Tisbe battagliai	20		28 d
MOLLUSCS			
Tapes philippinarum	100	Growth length	14 d
Pacific oyster	1	Sperm motility	4 d

* Values are expressed in µg/L; ** d: days

Table S6.5 PFOA toxicity data for freshwater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Anabaena sp.	5,000	Cell viability; population abundance	3 d
FISH			
Cyprinus carpio	55,565	Growth length and weight	4 d
Danio rerio	500	Heart rate	5 d
Danio rerio	75,000	Growth length	5 d
Danio rerio	200,000	Curvature	5 d
Gobiocypris rarus	3,000	Gene expression; vitellogenin mRNA	28 d
Oncorhynchus mykiss	500,000	Gene expression	14 d
Oryzias latipes	1,000	Hatch mortality	28 dph
Oryzias latipes	10,000	Organ weight	14 d
Pimephales promelas	74,100	Organ weight; progeny counts/number	39 d
CRUSTACEAN			
Daphnia magna	6,250	Time to first progeny	21 d
Daphnia magna	207,000	Immobility	2 d
Moina macrocopa	3,125	Progeny counts/number	7 d
INVERTEBRATES			
Brachionus calyciflorus	4,000	Intrinsic rate of increase	4 d

* Values are expressed in µg/L; ** d: days; dph: days post hatch

Table S6.6 PFOA toxicity data for seawater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Isochrysis galbana	25,000	Population growth rate	3 d
FISH			
Psetta maxima	1,500	Growth length	4 d
CRUSTACEAN			
Siriella armata	5,000	Survival	4 d
INVERTEBRATES			
Paracentrotus lividus	10,000	Growth length	2 d

* Values are expressed in µg/L; ** d: days

Table S6.7 PFOS toxicity data freshwater

Species	NOEC*	Effect	Exposure period**
FISH			
Carassius auratus	8,000	Standard Metabolic Rate	2 d
Carassius auratus	32,000	Oxygen transfer	2 d
Cyprinus carpio	620	Blood genetic damage	4 d
Cyprinus carpio	48,242	Growth length; liver weight	4 d
Danio rerio	500	Survival	6 dpf
Danio rerio	1,000	Heart rate	4 dpf
Danio rerio	5,000	Apoptosis	1 dpf
Oryzias melastigma	1,000	Hatch mortality	8 d
Oryzias melastigma	4,000	Estrogen receptor gene	2 d

* Values are expressed in µg/L; ** d: days; dpf: days post fertilization

Table S6.8 PFOS toxicity data in seawater species

Species	NOEC*	Effect	Exposure period**
ALGAE			
Skeletonema costatum	3,200	Growth	5 d
FISH			
Psetta maxima	15	Growth length	4 d
Salmo salar	100	Growth length; estrogen receptor beta mRNA	35-49 d
CRUSTACEAN			
Siriella armata	1,250	Survival	4 d

* Values are expressed in µg/L; ** d: days

Tabe S6.9 E1 toxicity data in freshwater species

Species	LC_{50}/EC_{50}*	Effect	Exposure period**
MACROPHYTES			
Medicago sativa	50,000	Plant growth	2 d
FISH			
Danio rerio	0.46	Organ weight in relation to body	21 d
Pimephales promelas	98	Egg production	21 d
WORMS			
Dugesia japonica	5,000	Mortality	2 d

* Values are expressed in µg/L; ** d: days

Table S6.10 E1 toxicity data in seawater species

Species	LC_{50}/EC_{50}*	Effect	Exposure period**
FISH			
Oryzias latipes	100	Feminization	24 d
Salmo trutta	0.63	biochemistry	10 d
CRUSTACEAN			
Acartia tonsa	410	Slowed, delayed or non-development	5 d
Neomysis integer	1,000	Mortality	4 d
Tisbe battagliai	100	Mortality	10 d
INVERTEBRATES			
Strongylocentrotus purpuratus	604	Abnormal development	4 d

* Values are expressed in µg/L; ** d: days

Tabe S6.11 E2 toxicity data in freshwater species

Species	LC50/EC50*	Effect	Exposure period**
FISH			
Danio rerio	2.17	Ovarian weight	21 d
Pimephales promelas	0.2	Progeny counts/number	19 d
CRUSTACEAN			
Daphnia magna	648	Mortality	21 d
AMPHIBIANS			
Lithobates pipiens	681	Mortality	14 d
INVERTEBRATES			
Strongylocentratus purpuratus	14.2	Abnormal development	4 d
WORMS			
Dugesia japonica	1500	Mortality	3 d

* Values are expressed in µg/L; ** d: days

Tabe S6.12 E2 toxicity data in seawater species

Species	LC50/EC50*	Effect	Exposure period**
FISH			
Fundulus heteroclitus	5000	Mortality	4 d
Oryzias latipes	460	Mortality	3 d
Rivulus marmoratus	620	Mortality	4 d
CRUSTACEAN			
Acartia tonsa	720	Slowed, delayed or non-development	5 d
Americamysis bahia	890	Mortality	4 d
Eurytemora affinis	45	Mortality	4 d
Nitocra spinipes	1600	Mortality	4 d
Tigriopus japonicus	3500	Mortality	2 d
Tisbe battagliai	100	Mortality	10 d
INVERTEBRATES			
Dendraster excentricus	10,000	Fertilization; abnormal development	2 d
Strongylocentrotus purpuratus	0.5	Abnormal development	19 hpf

* Values are expressed in µg/L; ** d: days; hpf: hours post fertilization

Chapter 7

CONCLUSIONS

This PhD thesis was aimed at understanding environmental fate and behavior of EDCs in aquatic environments. To this purpose, both freshwater and saltwater environments were analysed. Three classes of EDCs were studied in the freshwater environment of the Romagna area, NE Italy (estrogens, phenolic compounds and perfluorinated compounds). In the saltwater system of the Ebro delta (NE Spain) the study was focused on the analysis of 13 PFCs, including long-chain and short-chain PFCAs, PFSAs and PFOSA, which are still not regulated or included in environmental monitoring.

From the results of these analyses, different conclusions can be drawn, that help understand the behavior of estrogens, phenolic and perfluorinated compounds in the environment.

1. In the freshwater environment, both PFCs and phenolic compounds were proven to be persistent in the water compartment, since they showed constant concentrations over the two sampling campaigns conducted in the Romagna area. Hence, their occurrence in the water phase can be related to the presence of continuous sources of contamination. Wastewater treatment plants played a major role in their release in the water phase, as in the case of PFCs; the local industrial activities could also contribute to the introduction of phenolic compounds in the surrounding river bodies. Estrogens, on the other hand, did not show constant concentrations in surface water over time. Their occurrence in rivers was likely dependent on the surrounding stockbreeding; runoff from farming areas that use animal manure as fertilizer can also be an important source of introduction, considered the land use of the study area.

2. Analysis on groundwater collected from confined aquifers in the Romagna area showed no contamination by EDCs, proving that dilution by rainfalls, spatial distance from the recharge areas, and natural attenuation during water infiltration are factors that altogether contribute to the removal of contaminants through the aquifers.

3. EDCs in sediments were also analysed in the Romagna area in order to assess their partition between the solid phase and water. Among the three classes of EDCs selected, phenolic compounds were the only group that displayed a higher partition in sediment. In particular, BPA and NP concentrations revealed to be higher in the fine-grained fraction of sediments (<180 μm),

being mostly dependent on hydrophobic interactions that may happen between the contaminants and organic carbon content in sediments. Organic carbon was in fact the main component of sediment that mostly controlled phenolic compounds distribution in the solid compartment. Salinity was also found to influence their occurrence and behavior, determining a lower solubility, and hence a higher partition of the compounds in sediments, as the content of salts in water increased. Regarding estrogens, in the second sampling campaign they were detected only at local stations in water and sediment; PFCs, on the other hand, showed a wide distribution in the water compartment, in contrast with a very low frequency in sediment. The low detection of PFCs in sediment was related to the lower hydrophobicity of these contaminants with respect to phenolic compounds; their presence in water as anionic compounds can also limit the interactions with sediments, which are mainly characterized by negative surface charges.

4. PFCs partition in sediments was enhanced under salinity conditions. Sediments of Ebro delta, in fact, registered higher concentrations of PFCs, with PFOS being the compound detected at the highest concentrations. However, PFCs values showed a high seasonal fluctuation over time. Concentrations significantly differed from one season to another, with the highest values recorded in late summer-autumn period, and the lowest ones in the following spring period. This decreasing pattern suggested resuspension of surface sediment as a consequence of the stronger water currents that occur in winter season that lead to a remobilization of contaminants adsorbed onto the sediment surface. This hypothesis was confirmed by the constant concentrations of PFCs detected in the water phase, that otherwise should have shown a significant decrease of values in the winter period, as a consequence of dilution due to the increase of the water level in river channels or in the lagoons. Hence, sediments can remove contaminants through adsorption processes, but can also represent an important secondary source of contamination as soon as the surrounding environmental conditions change.

5. Analysis on PFCs in biota highlighted high accumulation rates of the contaminants mostly in freshwater fish, whereas seawater species displayed only a slight accumulation of PFOS in their tissues. Such differences could be explained by the different behavioral habits of species. Generally, freshwater fish have a larger home range than lagoonal species; hence, they have a greater chance to be exposed to contaminants, potentially coming into contact with PFCs in areas far from the sampling one. Size and age of species are additional factors to be taken into account. In addition, the lower solubility of hydrophobic compounds in saltwater systems can lead to a lower bioavailability of contaminants, resulting in a lower bioaccumulation.

6. Ecological and human health risk assessment were also performed, in order to evaluate if the environmental concentrations of the detected EDCs in both freshwater and saltwater environments

could harm organisms and human health. The study pointed out some concerns regarding risk in the water column of the Romagna area; situations of higher concern were related to the detection of estrogens, which are known to have the highest estrogenic potential, among all EDCs. A potential high risk was also recorded in the southern section of the Romagna area (Marecchia River), and was related to the exposure to high concentrations of BPA. On the contrary, EDCs in sediments revealed to be safe for the sediment- dwelling organisms. No risk associated to PFCs exposure in aquatic and sediment organisms of the coastal area of Ebro delta or in the freshwater environment of the Romagna area was registered. Results on human risk exposure to PFCs through Ebro River fish consumption showed that the Spanish population should not be exposed to a high risk considering low and median consumption rates of fish.

CURRENT GAPS AND FUTURE RESEARCH NEEDS

This thesis elucidated different ways of behavior of EDCs in the environment, in which salinity and organic carbon in sediment play a significant role in controlling organic contaminants distribution in the environment. However, the study also elucidated different aspects, that need to be studied further:

1. Sampling campaign in the Romagna area was conducted only in the summer season (dry season), which is the period in which higher concentrations of contaminants are expected to be detected, owed to the environmental conditions. The choice of focusing the study only in the summer season was made in order to better assess behavior of contaminants and partitioning effects, and conclusions have already been mentioned in the previous section and Chapters. However, it is also of environmental interest to study their behavior in different seasons, in order to better depict their fluctuations over time. As shown for PFCs in the Ebro delta, in fact, sediment can be important sink and source of contamination, being highly dependent on the environmental conditions.

2. The study of EDCs in the Romagna area arose the concern that EDCs contamination can also affect coastal environments. Further research should thus be addressed to better assess their occurrence in the coastal area, since more than 30% of local inhabitants live along the coastline, so assessing human levels of exposure to contaminants of possible concern is of crucial importance.

3. A continuous monitoring in both freshwater and saltwater environments is needed in order to check for possible variations of the concentration ranges over time, in line with Water Framework Directive purposes.

4. Ecological risk assessment pointed out a situation of possible concern in the water compartment of the Romagna area related to estrogens exposure (in the northern section of the study area) and BPA exposure (in the southern part). Even though the non-constant concentrations of estrogens

should reduce the risk associated to their exposure for aquatic organisms, more information on the temporal pattern of estrogens is needed bor a better evaluation of the real risk. Further toxicological research is also needed in order to assess the real harmful effects of BPA in aquatic organisms of Marecchia River.

5. From the ecological risk assessment approach performed in the freshwater and saltwater environments some limitations arose, and they have been already mentioned in *Chapter 6*. In particular, more toxicological data on organisms exposure to EDCs are strongly needed, and tests should be performed on real environmental concentration ranges (ng/L) to deepen understand the real risk exerted by EDCs on organisms, instead of using assessment factors to extrapolate the corresponding concentration values of no adverse effect. It should be kept into consideration, in fact, that these contaminants are characterized by non-linear dose-response curves from a toxicological point of view, hence they are expected to have more adverse effects at the lower concentrations than at the higher ones.

6. The development of specific requirements for the assessment of a global risk which takes into consideration EDCs exposure as a mixture of compounds is urgently needed. Some authors have proposed the use of an index that sums all the individual risk values of contaminants, but there is still no clear evidence about the real mechanisms of interaction of joint contaminants in the environment. Hence, research should be focused on the understanding of the global effects that can occur when organisms are exposed to a mixture of compounds, focusing on those chemicals that are more likely to occur in the environment.

7. Regarding EDCs exposure risk, different authors have suggested the use of EEF values to assess the estrogenicity potential of contaminants, as reported in *Chapter 4*. However, different methodologies have been proposed to derive such EEF values, obtaining different values for the same contaminant. Notwithstanding this limitation, this approach could be useful for EDCs estrogenicity potential assessment, and more research focused on the derivation of a standardized methodology for EEF values identification is advisable, in order to obtain final universal threshold values for each EDC. Such values could then be integrated in current and future legislation, in a similar way of Toxic Equivalent Factors (TEFs) currently used to regulate dioxins, furans and polychlorobiphenyls (PCBs) in the environment.

8. Chemical analyses should be combined with biological or toxicological studies in order to check for the real adverse effects on organisms consequently to EDCs exposure. For example, in-vitro bioassays are becoming increasingly an useful tool for the identification and assessment of endocrine disruptors. The recombinant yeast assay (RYA) and the E-SCREEN bioassay are only two

biological tools examples useful to evaluate the potential of endocrine disruption of an environmental sample.

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