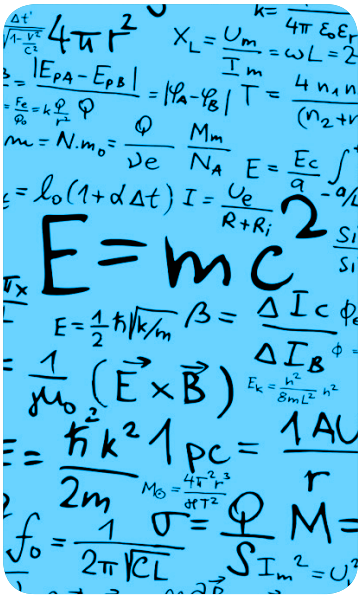
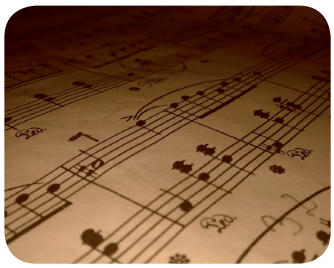




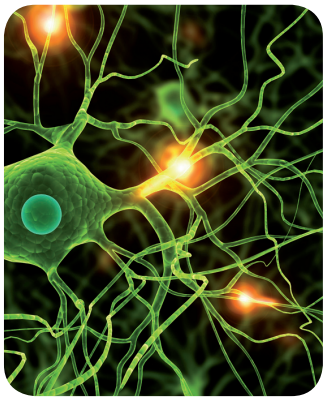
MÁSTERES de la UAM

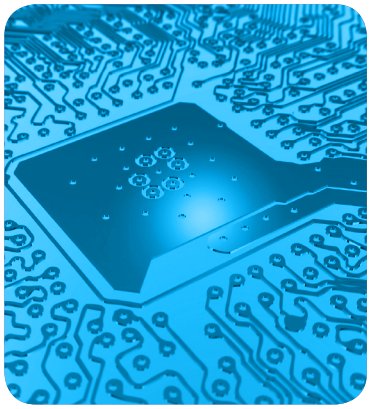
Facultad de Ciencias
/ 14-15

Calidad Aguas Continentales

UNIVERSIDAD AUTONOMA DE MADRID

excelencia Campus Internacional UAM CSIC





Presence, distribution and toxicity of diclofenac, estradiol and ethinylestradiol in Manzanares River

Cristina Corpa Santos

UAM EDICIONES

INLAND WATER QUALITY ASSESSMEN MASTER
2014/2015

Presence, distribution and toxicity of diclofenac, estradiol and ethinylestradiol in Manzanares River

Master Project

Cristina Corpa Santos

Coordinated by:

- Dra. Sonia Aguayo Balsas. Head of Water Pollution Unit, National Centre for Environmental Health, Institute of Health Carlos III.

- Dr Eugenio Rico Eguizabal. University Professor. Autonomous University. Madrid.

Collaborations:

- Dr Jesus Pablo García Cambero. Toxicology Area. National Centre for Environmental Health. Institute of Health Carlos III. (Zebrafish embryo toxicity assays).
- Work team Water Pollution Unit, National Centre for Environmental Health, Institute of Health Carlos III.

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratitude to my tutors, Dra. Sonia Aguayo and Dr Eugenio Rico, who have conveyed me their spirit of research and an excitement with regard to aquatic ecosystems preservation. Their enthusiasm and encouragement helped me to never give up and persevere in order to achieve my goals. They were always available for my questions and they were positive and gave generously of their time and vast knowledge. This dissertation would not have been possible without them guidance and help.

In addition, I am indebted to my family, my boyfriend and my friends, who have always stood by me in the bad moments, supporting me and making me believe that I was capable to do it. Moreover, I would like to thank to work team of Water Pollution Unit, Paloma, Pilar and Miguel Angel, for their effort, patience and affection because without them I would not have done this work. And finally, special thanks to Dr. Jesus P. García-Cambero for his selfless collaboration through which I learned a lot about research.

"We forget that the water cycle and the life cycle are one"

Jacques Yves Cousteau (1910-1997).

Table of contents:

List of Figures

List of Tables

Abstract

The increasing worldwide contamination of freshwater systems with pharmacological substances is one of the key environmental problems facing humanity. Despite the fact that most of these compounds are present at low concentrations, many of them raise considerable toxicological concerns. Wastewater treatment plants are considered the main emission sources of pharmaceutical products, although they not the only. Since 2013, under WFD three pharmaceutical products (diclofenac, 17-β-estradiol and 17-α-ethinylestradiol) have been included in the Watch List to consider their inclusion in the Priority Substances List by Member States, based on different variables. The aim of this study is to assess the occurrence of diclofenac (anti-inflamatory), 17-β-estradiol and 17-α-ethinylestradiol (hormones) in effluents, surface water and sediments of Manzanres River, which cross metropolitan areas of Madrid Region densely populated. To do so, an analytical method based on chromatographic techniques has been developed. The results have shown the presence of Diclofenac, mainly in effluents and river water, while 17-β-estradiol and 17-α-ethinylestradiol were found mostly in sediments. Furthermore, supplementary toxicity studies were carried out to assess the toxicity of a mixture containing these three compounds and the toxicity of the samples from surface water. According to the results obtained, it could be argued that the need to improve the processes of wastewater treatment plants seems crucial in order to increase the quality of discharges and reduce the ecological risk associated. In the same line, we consider important the inclusion of these three compounds in the Priority Substance List by the levels found in this study, and effect reported in literature. For future investigations, due to the significant concentrations of diclofenac, 17-β-estradiol and 17-α-ethinylestradiol detected in Manzanres Rivers, further studies in this region are required.

Acronyms and Abbreviations:

AA-EQS: Annual average - environmental quality standards

AOP: Advanced oxidation processes

ASE: Accelerate solvent extraction

DCF: Diclofenac

E.C.: Electrical conductivity

ED: Endocrine disruptor

E2: 17-β-estradiol

EE2: 17α-ethynylestradiol

EPA: Environmental Protect Agency

ESI: Electrospray Ionization

GAC: Granular activated carbon

GC: Gas chromatography

HT_{50}: Hatching time for 50% of the individuals

HPLC: High pressure liquid chromatography

KNAPPE: Knowledge Need Assessment on Pharmaceutical Products in Environmental Waters

LC_{50}: Lethal concentration for 50% of the individuals

LOEC: Lowest Observed Effects Concentration

LOQ: Limit of quantification

MR: Madrid Region

MS: Mass spectrometer

NSAIDs: Non-steroidal anti-inflammatory drugs

PAHs: Polycyclic Aromatic Hydrocarbons

Pf: post-fertilization (hpf: hours post fertilization)

PhACs: Pharmaceutically active compounds

PLE: Pressurized Liquid Extraction

PNEC: Predicted Non-Effect Concentration

SD: Standard deviation

SEM: standard error of the mean

SIM: Selected-ion monitoring

SPE: Solid phase extraction

Rt: Retention time

WFD: Water Framework Directive

WHO: World Health Organization

2,4-D: 2,4-Dichlorophenoxyacetic acid

1. Introduction

Aquatic environments are severely affected by human activity. Monitoring of aquatic ecosystems in Europe has become especially important after the implementation of the Water Framework Directive (WFD) in 2000 (EC, 2000). So, the presence in surface water of nutrients, pathogens, heavy metals, pesticides, polycyclic aromatic hydrocarbons (PAHs) is now regulated by WFD (EC, 2000). However, more recently, monitoring and research of "emerging pollutants" in different environmental compartments are the main focus of numerous studies (Barceló and Petrovic, 2006; Richardson and Ternes, 2011; Loos et al., 2013; Brack et al., 2015).

Emerging pollutants includes a great amount of substances such as pharmaceuticals, household and industrial chemicals, and flame retardants, among others. These emerging pollutants influence strongly in ecological balance, environmental quality, and public health. They are not included in any legislation, and therefore they are not generally monitored. They are present at very low concentrations (at µg/L, ng/L, even pg/L) and many of them lack methods of determination and quantification. Finally, there are a large knowledge gaps about their levels, fate and effects for wild organisms and human health. In addition, to cover these gaps, the Article 16 of WFD indicates the need to perform trials regarding the intrinsic risk of emerging pollutants, and particularly their environmental levels, aquatic ecotoxicity and human toxicity via aquatic exposure.

The main sources of aquatic pollutants in general are effluents of waste water treatment plants (WWTPs), but also industries, landfill, and some farming practices are important contributors of this load of pollutants. The control of wastewater release is regulated by National and International Regulations. European Directive 91/271, transposed to Spanish legislation in R.D. 11/1995 and R.D. 2116/1998, regulates aspects of collection, treatment and discharge of urban wastewater and some industrial waste at Community and National levels from an environmental point of view. So, the main objective is to protect the receiving environment by limiting the discharge of certain substances, and establishing a canon of issuance of these effluents. As an example, the cited legislation establishes the characteristics and limitations of discharges to public waterways, with explicit reference to the minimum treatment requirements, and the quality before discharge into the water body.

The traditional processes of water treatment are designed to reduce nutrients load (carbon, phosphorous and nitrogen) and pathogens (Loos et al., 2013). However, these processes are insufficient for the complete elimination of most of the anthropogenic pollutants (Zhang et al., 2008), and new techniques are being developed to enhance the treatment processes and to reduce the discharge and the environmental risks associated with their emission (Ternes et al., 2002; Estévez et al., 2005; Hartmann et al., 2008; Loos et al., 2013). Among them, membrane treatment, using both biological (membrane bioreactors) and non-biological processes (reversed osmosis, ultrafiltration, nano-filtration), and also advanced oxidation processes (AOP) are the most frequently considered as appropriate treatments to remove trace concentrations of polar emerging contaminants (Petrovic et al., 2002).

Consequently, WFD was reviewed and modified in 2013 (EC, 2013, amending EC, 2008) including the requirement of monitoring studies for some of the emerging pollutants. So, some substances, identified as potentially hazardous by the different EU members, were included in the first list to perform the monitoring programs (Watch List). Among the criteria or requirements to identify such substances were: i) the substance is suspected of posing a significant risk for aquatic environment, and ii) there is not enough information to assess the EU-wife exposure for the substances. The main purpose will be collecting monitoring data and toxicological information, particularly as regards emerging pollutants, to develop a

joint European strategy in order to improve the information basis for future identification of Priority Substances (EC, 2013).

Through Decision 495/2015 of the Commission, it was approved the first Watch List is approved, including ten substances or group of substances (Table 1). If the Risk Quotient (PEC/PNEC) ratio was $\geq$ 1 on the basis of monitoring data for at least three Member States, the substances was recommended for inclusion (Carvalho et al., 2014).

Table 1. List of substances of the first "Watch List" proposed to be defined as Priority Substances.

Substances for Watch List	Type of pollutant
Diclofenac	Non-Steroidal Anti-Inflammatory Drug
17-β-estradiol	Natural Hormone
17-α-ethinylestradiol	Synthetic Hormone
Oxadiazon	Pesticide
Methiocarb	Carbamate pesticide
2,6-ditert-butyl-4-methylphenol	Additive, Plasticizers
Tri-allate	Pesticide
Imidacloprid, Thiacloprid, Thiamethoxam, Clothianidin, Acetamiprid	Insecticide
Erythromycin Clarithromycin, Azithromycin	Antibiotics
2-Ethylhexyl 4-methoxycinnamate	UV filter

In order to increase the knowledge about environmental behaviour of emerging pollutants, several programmes and working groups have been also created in Europe; for instance, KNAPPE Program (Knowledge Need Assessment on Pharmaceutical Products in Environmental Waters, 2007) and SOLUTIONS (2008). These programs will develop a new generation of biological and chemical tools for identification, prioritisation and assessment of those water contaminants under a holistic point of view. Specifically, SOLUTIONS program is primary focussed to increased data on complex mixtures, metabolites and transformation products in large European basins (e.g. Danube), mainly based on early detection tools (Brack et al, 2015).

Apart from the monitoring programmes, EU members will be required to submit supplementary measures by 2018 to indicate how they will enforce the new requirements. In this sense, the reports shall indicate the monitoring matrices and evaluation of possible methods for each substance in order not to entail excessive costs. As suggested by Carvalho et al. (2014), the ecological risk of some compounds should be studied in surface water, sediment and drinking water, due to their low polarity and the Kow values. In this sense, it is expected that sorption onto sediments will be high when the log Kow value is greater than 3, and bioaccumulation also must be studied (Kuster et al., 2005; Johnson et al., 2013).

1.1. Pharmaceuticals in aquatic environment

Among the emerging contaminants, pharmaceutical are probably the group of growing concern and therefore more studied in recent years. The first publications appeared in United States in the late of 1970s (Hignite and Azarnoff, 1977), and increasing number of research studies have showed the presence of pharmaceutically active compounds (PhACs) residues in rivers, lakes, groundwater, drinking water and marine ecosystems (Ternes, 1998; Heberer et al., 2004; Hertzman, 2015; Gorga et al., 2015). About 3000 substances are registered in EU for pharmaceuticals purpose (Joss et al., 2006; Gros et al., 2010), and some of them are produced at high levels (more than 1 ton) (Figure 1). Consequently, more than 80 compounds, PhACs and several metabolites have been detected in the aquatic environment in different countries of

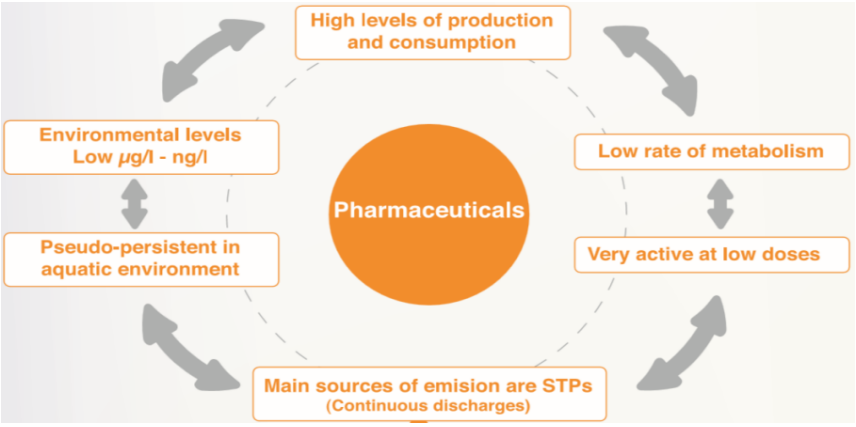
Europe, in Brazil, US, Canada and China (Ternes, 1998; Heberer et al., 2004; Zhang et al., 2008; Gros et al., 2010; Huerta et al., 2013).

Advances in analytical techniques have revealed a greater number of compounds previously undetectable. Different analytical methods, especially chromatographic techniques (liquid chromatography-mass spectrometer and gas chromatography-MS), in combination with different extraction method, mainly based on solid phase extraction (SPE), are being developed for the analysis of PhACs (Petrovic et al. 2003, Céspedes et al., 2004; Gibson et al., 2007; Huerta et al., 2013).

Nevertheless, in spite of progress of analytical techniques regarding environmental chemistry field, there is a great gap in standardized methods. In 2007, the last standardized methods to monitor levels of PhACs in environmental (EPA, method 1694 and method 1698) were created, but they were really complex. It is difficult to know differences statically relevant when data are obtained from different procedures, equipment and techniques, and limits of detections and quantifications are variables.

In Spain, it was not until the early 90s that the issue of PhACs in the environment has emerged strongly, as evidenced by the numerous articles published since then, which have generated great scientific and social interest, such as happened following the publication in the common press of some of the results obtained by the research team of Dr. Barceló (e.g. El Periódico, 26th October 2005). The first studies were conducted in the Ebro and Llobregat River (Petrovic et al. 2003; Céspedes et al., 2004; Barceló and Petrovic 2006). In the rest of Spain were less numerous studies, especially in Madrid where there is little information on levels of contaminants in aquatic ecosystems, despite being one of the most densely populated cities of Europe (Valcárcel et al., 2011).

The occurrence of PhACs in the environment is correlated with anthropogenic activities. As already mentioned, discharges from sewage treatment systems which collect water from urban and industrial uses are identified as the main source of PhACs to water bodies (Figure 1) (Ternes, 1998; Petrovic et al., 2003; Hartmann et al., 2008; Gros et al., 2010; Loos et al., 2013; Aris et al., 2014). Livestock and aquaculture practices are also identified as direct emission source of PhACs into the environment (Barceló and Petrovic 2006). Other minor emission sources could be pharmaceutical industry or inadequate disposal of pharmaceuticals (Valcárcel et al., 2011). Furthermore, indirect expositions pathways are reuse of effluents



from WWTPs as irrigation water, or recharges of aquifer and natural systems with scarcity problems (maintenance of ecological flow) (Hidalgo and Irusta, 2005).

Figure 1. General characteristics of PhACs.

Besides, PhACs are designed to be high biologically active at low levels and consequently they could be inducing diverse acute and/or chronic effects on environmental communities (Ferrari et al., 2003; Fent et al., 2006). Most of these substances have low rate of metabolism. This fact, make them to be assumed as persistent or "pseudo-persistent" in environment, further for their continual inputs into surface water from different sources, even though PhACs have relatively short environmental half-life (Hernando et al., 2006) (Figure 1).

Generally, based on the therapeutic class of the drugs, antimicrobials are the most frequently detected group. The second most frequently detected group was anti-inflammatory agents, followed by anti-hypertensive (Figure 2) (Report Knappe Project, 2007). Environmental levels from ng/L to low µg/L are described in the literature (Ternes, 1998; Hernando et al, 2006; Loos et al, 2013). Sex hormones, antibiotics and antineoplastic are the three most potentially dangerous groups for human health and aquatic environment (Estévez et al., 2005; Barceló and Petrovic 2006). Really, there are none known human health effects from such low-level exposures in drinking water, but special scenarios (such as human fete exposure) require more investigation. Despite of current environmental amount of PhACs do not constitute an imminent risk for public health, European Authorities carried out diverse activities to solve the environmental disturbs which could become human health problems even diseases (Valcárcel et al., 2011).

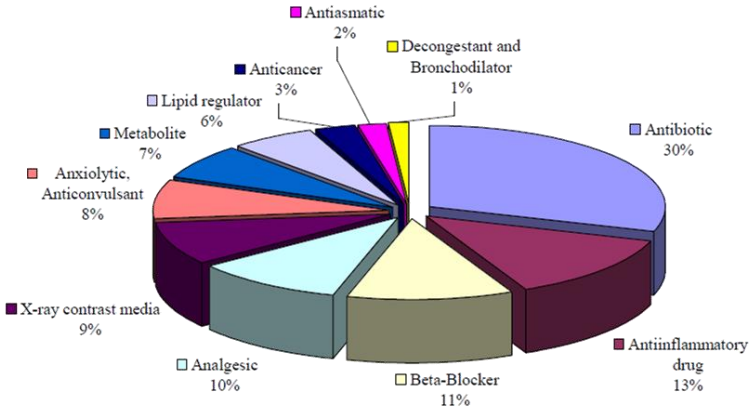


Figure. 2. Frequency of detection of the main PhACs groups in effluents (Source: Final Report Knappe Project, 2007).

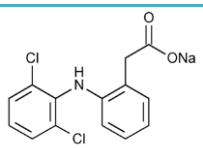
Although it took hard work to agree on the adoption of the first WatchList, three PhACs have already been selected for inclusion in the list: diclofenac (DCF) (anti-inflammatory), 17-α-Ethinyl estradiol (EE2) and 17-β-estradiol (E2) (synthetic and natural hormones, respectively). Due to their frequent occurrence and effects on aquatic organism and wild life in general, these PhACs have been identify as significantly hazardous by several European countries. Whether these PhACs included in the Watch List become Priority Substances, it is likely that other PhACs will follow.

1.1.1. Environmental presence, behaviour and toxicity of diclofenac

Diclofenac (DCF) is a non-steroidal anti-inflammatory drugs (NSAIDs) administrated to reduce inflammation and pain, especially in conditions such as arthritis or acute injury. Also it used to reduce fever and treat dysmenorrhea and some ocular diseases. The primary mechanism action is thought to inhibit the enzyme cyclooxygenase, which is essential in the biosynthesis of prostaglandins. Also, DCF can block the action of the neuromodulator glutamate which amplifies reflex response, and stimulates the release of endogenous opioid and serotonin (Altman et al., 2015). NSAIDs are the most consumed pharmaceuticals without medical prescriptions (Estévez et al., 2005).

DCF is available as a generic drug as the sodium or potassium salt. In Spain, NSAIDs are the second class of PhACs consumed, and DCF is mainly used in Voltaren medical products, such as sodium salt (Buser et al., 1998; Carballa et al., 2008). DCF sodium is known to be eliminated through metabolism, by urinary (65%) and biliary (35%) pathway as glucuronide and sulphate conjugates of the main metabolites (4'-Hydroxydiclofenac, 3'-Hydroxydiclofenac, 4'-5´-Hydroxydiclofenac) (Zhang et al., 2008); only less than 1% of parent compound is excreted (table 2) (Thomas and Hilton, 2004). It is estimated that 32.3 tons were consumed in 2003, only in Spain (Carballa et al., 2008); 179.8 tons is the annual Europe consumption, and about 940 tons are consumed all over the world (Al-Rajab et al., 2010). The main problem is its high consumption and low removal in WWTPs. Heberer (2002) emphasized that DCF is the most important medicine present in the water cycle.

Table 2. Physico chemical properties and other characteristics of diclofenac

	Diclofenac Sodium
Cas nº	15307-79-6
Therapeutic class	NSAIDs
Molecular formula	$C_{14}H_{10}Cl_2NNaO_2$
Molecular weight (g/mol)	318.14
Chemical structure	
Water solubility (mg/L)	4.82 [(1)]
Log K_{ow}	4.51[(2)]
Henry constant (atm.m^3/mol)	4.73 x 10^{-12}[(3)]
pKa (20ºC)	4
Half life (days)	5-0.5 [(4)] 8 [(5)]
Consumption (µg/cap/d)	449-2613 [(6)]
Excretion	65% urine[(7)] 35% bile
Removal rate	0-69 %[(2)]
Ecological effect	Hepatic effects[(8)]

1. Drugbank; 2. Ferrari et al., (2004); 3. Kujawa-Roeleveld, (2008); 4. Andreozzi et al, (2003); 5. Tixier et al., (2003); 6. Johnson et al., (2013); 7. Zhang et al. (2008); 8. Carvalho et al., (2014). SW: Surface Water; Sed: Sediment

Photodegradation seems to be the main elimination process of DCF in surface water (Buser et al., 1998; Heberer and Ternes, 2006; Salgado et al., 2013), and is really relevant in lentic systems (Johnson et al., 2013). Also abiotic transformation may occur via the processes of hydrolysis. Half-live ($t_{1/2}$) of DCF are in the range of 5-8 days (Table 3), however in summer the $t_{1/2}$ is reduced to about 0.5 day (Andreozzi et al, 2003); therefore it stands to reason the temperature is an important factor in DCF elimination. Vaporization is not regarded to be a significant mechanism for removal pharmaceutical due to low Henry's constant value (Table 2) (Kujawa-Roeleveld, 2008). Some researches about elimination of DCF indicated that

biodegradation cannot remove it efficiently. Nonetheless, Zhang et al (2008) studied the removal of DCF with different biological systems and affirm that DCF was better degraded in an anoxic biofilm reactor. This fact could explain the low removal rate of this compound in the WWTPs, which usually use oxygenic biological processes and sorption in suspended solids to purify micropollutants from water (Buser et al., 1998; Fent et al., 2006). This fact makes that level up to µg/L can be found in monitoring studies of effluents and surface waters (Ternes, 1998; Ferrari et al, 2003; Fent et al, 2006; Gros et al., 2010).

Actually, DCF is a compound which presents great variation in removal rates (0-90 %) (Ternes et al., 2002; Ferrari et al., 2003; Fent et al., 2006; Joss et al., 2006; Kujawa-Roeleveld, 2008; Gros et al., 2010). On the one hand, this fact could be due to its high solubility, but also it is known its high potential to be adsorbed onto suspended solids. This fact could be the reason of its high presence in both effluents and sludge, depending on the type of treatment (Kujawa-Roeleveld, 2008; Aguayo et al., 2010). Tertiary treatment has been described such as a good way of removal for PhACs, especially ozonisation treatment. Oxidation reaction can occur either by direct or indirect reaction with ozone or free radicals formed during spontaneous ozone decomposition. With ozone addition at 5 mg/L a removal of more than 90% could be achieved for most PhACs (even DCF) (Joss et al., 2006).

For instance, Ternes et al (2002) showed that sand filtration under aerobic and anoxic conditions, as well as flocculation using iron (III) chloride exhibited no significant elimination of DCF, while ozonation was quite effective in eliminating this polar compounds (DCF were reduced above 90%). In long term monitoring investigations of municipal sewage influents and effluents carried out in Berlin by Heberer (2002), DCF was found which average amount of 3.02 and 2.51 µg/L, respectively, which represent 83 % of DCF elimination. Other reports of WWTPs in Greece, Koutsouba et al (2003) found levels of DCF between 12 and 560 ng/L whereas, in the same WWTPs, effluent reaches levels between 10 and 365 ng/L, indicating that their removal during wastewater treatment was very low (35 %).

As it mentioned above, results of many studies carried out in Spain indicated that conventional WWTP are not capable to completely remove PhACs from wastewaters so they are consequently discharged into the aquatic environment (Kuster et al., 2005; Gros et al., 2010; Fernández et al., 2010; López-Roldán et al., 2010; da Silva et al., 2011; Valvárcel et al., 2011). Although levels found in WWTP effluents ranged from µg/L to high ng/L, DCF in river waters occurred at levels at least one order of magnitude lower (low ng/L range) because of dilution effect (Table 3). The distribution of DCF between surface water and suspended solids is an important issue, since some compounds are preferably bound to the solid phase. The sorption of PhACs depends on both, the properties of the PhACs and suspended solids. For instance, da Silva et al (2011) carried out a full study in Ebro basin detecting levels of DCF in effluents (from 218 to 808 ng/L), downstream surface water (from 4.1 to 148 ng/L), sediments (from 0.7 to 2.49 ng/g), and in suspended solids (from 3.84 to 468 ng/g). This study showed that it is important to consider the concentration of suspended solids since it can be even higher than in the sediments. Valcárcel et al. (2011) reported amounts of DCF up to 2000 ng/L in Manzanares River (Table 3). In the Jarama River which receives waters from Manzanares River, Fernández et al (2010) detected levels 156 ng/L. Some levels of DCF reported in literature are showed in follow table.

Table 3 Review of presence of diclofenac in different aquatic compartments

WWTP effluents (ng/L)		*Surface water (ng/L)*		*Sediment / Sludge (SL) (ng/g)*	
Amount	**Ref**	**Amount**	**Ref**	**Amount**	**Ref**
1600; 470-5450; 990; 170-2500; 1-529; 216–808; 49.5.	Ternes (1998); Ferrari et al, (2003); Tixier et al, (2003); Fent et al, (2006); Bueno et al, (2010); Da Silva et al, (2011); Loos et al, (2013).	1 – 800; 11-310; <1-12 Lake; 225; 212-3400; 4.1-148.	Ternes (1998); Buser et al, (1998); Buser et al, (1998); Hernando et al,(2006); Valcárcel et al,(2011); Da Silva et al, (2011).	9 µg/L (SL); <3.6 0.7-3.4; 9.5 ng/kg; 0.62–35.8;	Kujawa-R., (2008); Martín et al., (2010) Da Silva et al, (2011); Azzouz and B (2012); Vazquez-R et al., (2011).

The ecotoxicological data indicates that DCF may not produce toxicity at relevant environmental levels in primary producers, such as bacteria and green algae (Ferrari et al., 2003; Zhang et al., 2008). However, Schwaiger et al. (2004) demonstrated that DCF could cause effect in reproduction rate of some crustaceans such as *Daphnia magna* at concentrations of 200 µg/L; the same study revealed chronic effect in rainbow trout (*Oncorhynchus mykiss*) at low levels. The lowest observed effect concentration (LOEC) at which both renal lesions and alterations of the gills occurred was 5 µg/L. Triebskorn et al. (2004) also found that the cytological alteration on the liver, kidney and gills occurred even at 1 µg/L in fish. Another author, Gröning et al. (2007) carried out a research about adaptability of organism at different organic pollutants in German rivers revealing that the microflora of sediment can adapt to the different molecules available as energy source, DCF between them. This compound can be transported through the food chain, and severely harm species such as vultures (*Gyps*) or raptors leading to renal failure and death, after exposure to low concentrations, producing a significantly decline of these populations since 1990s (Fent et al., 2006; Zhang et al., 2008).

1.1.2. Environmental presence, behaviour and toxicity of estrogens

Natural and synthetic hormones are excreted by both human and animals, and can end up in surface water through effluents of WWTPs or through run-off from agriculture activities (Petrovic et al., 2003).

17-β-estradiol (E2) is a natural endogenous steroid, predominantly female, which is important for maintaining the health of the reproductive tissues, breast, skin, bone and brain. While estrogens levels in men are lower compared to women, estrogens have essential functions as well. Estradiol is produced especially within the follicles of the female ovaries, but also in other endocrine (i.e., hormone-producing) and non-endocrine tissues (e.g., including fat, liver, adrenal, breast, and neural tissues), where is biosynthesized from progesterone. E2 is found in most vertebrates as well as many crustaceans, insects, fish, and other animal species.

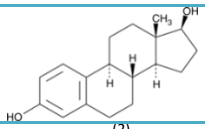
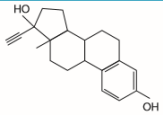
The natural excretion levels of this compound depend on women step life, reaching maximum levels of 250 µg/day in pregnant women (Table 4). Only a fraction about 2 % is free and biologically active (Ying et al., 2002). Estradiol is conjugated in the liver by sulphate and glucuronide formation and, as such, excreted via the kidneys. Some of the water-soluble conjugates and metabolites are excreted via urine (mainly as estriol metabolite), and partly reabsorbed after hydrolysis from the intestinal tract. This enterohepatic circulation contributes to maintaining estradiol levels (Nugent et al., 2003).

The main path of E2 environment degradation is biotic (Gomes et al., 2004). E2 can originate metabolites highly actives in WWTPs and aquatic ecosystems (Estévez et al., 2005). E2 is rapidly oxidized to estriol (E1) and then to estrone (E3). It was assumed that 50% of E2 will convert to E1 in the sewers before arriving at a WWTP (Johnson et al., 2013). E1 has lower estrogenic activity and therefore it is usually detected in higher concentrations than E2 in effluents of WWTPs. Nevertheless glucuronides conjugates of E2 are more frequently detected than original product (Kuster et al., 2005). In general, most of the conventional mechanical activated sludge treatment plants were effective at removing E2. For instance, Servos et al., (2005) found that in conventional mechanical treatment systems with secondary treatment (activated sludge and lagoon treatment systems) from Canada, the mean concentrations of E2 in final effluents were reduced more than 75% reaching levels between 0.2 and 14.7 ng/L (table 4). Regarding to the results obtained on WWTPs in France (Beausse et al., 2004), the concentrations of E2 can reach 40 ng/L in influents. For E2 the efficiency of French WWTPs was above 90% (table 4).

17-β-Ethinylestradiol (EE2) is a synthetic estrogen derived from E2 (an ethinil group is added in 17-β position to avoid oxidation) which is the main active compound of anticonceptive pills (Zhang et al., 2011). It can be used in estrogen replacement therapy, osteoporosis, and the treatment of breast and prostatic cancer (Aris et al., 2014). In aquaculture, it is used for population sex control due to its growth improvement (feminization).

The main environmental sources of EE2 are human excretes (bile, urine and faeces). EE2 is metabolized by humans, and released as parent compounds (faeces) and conjugates (urine), which end up in WWTPs, and then into water bodies. EE2 is absorbed in the small intestine and reaches serum. It undergoes extensive metabolism in the liver. EE2 and its metabolites are excreted with the bile. In circulation EE2 is almost fully bound to plasma albumin. It is metabolized by hydroxylation of the aromatic ring and excreted in both faeces and urine, in part as glucuronide and sulphate conjugate (Djerassi, 2006). According to Ying et al., (2002), the daily excretion of EE2 for a woman with contraceptive treatment is 35 µg/day (see table 4). The bioavailability of orally administered is about 60%.

Table 4 Physico chemical properties and other characteristics of 17-β-estradiol and 17-α-ethinylestradiol

	17-β-estradiol	17-α-Ethinylestradiol
Cas nº	50-63-6	57-63-6
Therapeutic class	Natural hormone	Synthetic hormone
Molecular formula	$C_{18}H_{24}O_2$	$C_{20}H_{24}O_2$
Molecular weight (g/mol)	272.36	296.41
Chemical structure		
Water solubility (mg/L)	3.6 [2]	4.8 [2] - 11.3 [2]
Log K_{ow}	3.62 [2]	3.84 [2]
Vaporization: Henry constant ($atm.m^3/mol$)	3.64×10^{-11} [2]	7.94×10^{-12} [2]
pKa (20ºC)	10.5 [3]	10.3 [2]
Half life (days)	2.8-4 sw [2] 0.3-8.7 sw [5] 5-10 sed. [4]	46 sw [2] 17.3 sw [5] 17 sed. [2]
Consumption (µg/cap/d)	4-8 [5]	0.84-2.59 [5]
Excretion	1.6-259 µg/day [6]	35 µg/day [6]
Removal rate	69-99 % [5,1]	10-75 % [1]
Ecological effect	Endocrine disruption (ED)	Endocrine disruption

1. Beausse et al, (2004); 2. Kuster et al., (2005); 3. Yoon et al., (2005); 4. Lee et al., (2003); 5. Johnson et al., (2013); 6. Ying et al., (2002); SW: Surface Water; Sed: Sediment.

Due to its nonpolar and hydrophobic properties, the synthetic estrogens presents low volatility (table 4) and seem to be more persistent than the natural estrogens both in water and sediment, even though EE2 and E2 have a similar chemical structure (Christiansen et al., 2002; Feng et al., 2010). The main removal mechanism in WWTPs is sorption onto particles, but not biotransformation processes (Huang et al., 2001; Feng et al., 2010). Vader et al. (2000) examined the degradability of EE2 by activated sludge under nitrifying and non-nitrifying conditions. They found that under non-nitrifying conditions, there was no degradation of EE2 while nitrifying sludge oxidized EE2 to more hydrophobic compounds. Vader et al. (2000) suggest that the seasonal and temperature effects on nitrification may therefore result in changes in the ability of treatment systems to remove EE2 and related compounds.

Therefore EE2 seems to be relatively stable during the activated sludge process in WWTP (Aris et al., 2014). Regarding to the results obtained on WWTPs in France (Beausse et al., 2004), the concentrations of EE2 are

lower than E2 (<10 ng/L) in influents. For EE2, concerning the efficiency of WWTPs, the elimination depends on plant treatment characteristics and can varies, on average between 10 and 75% (Table 4).

There are few studies that report data on levels of E2 in Spain. Esteban et al., (2014) reported levels < 0.04 ng/L of E2 and < 0.14 ng/L of EE2 in river waters from Tajo Basin. In a Spanish monitoring campaign, including river and sediments samples from Ebro, Llobregat, Júcar and Guadalquivir Basins, Gorga et al., (2015) showed higher amount of estrogens, reaching maximum levels in river waters of 7.8 and 2.2 ng/L for E2 and EE2, respectively, and maximum found in sediments of 1.6 and 2.2 ng/g for E2 and EE2, respectively. The highest levels reported in Spain rivers range from 71 to 130 ng/L for E2 and from 11.60 to 170 ng/L for EE2, mainly in Catalonia Region (Petrovic et al., 2002; Céspedes et al., 2004; Kuster et al., 2005; López-Roldán et al., 2010; Esteban et al., 2014). More data of those reported in literature are showed in table 5.

It is expected concentrations of E2 and EE2 up to 1000 times higher in bed sediment than in water column due to their hydrophobic properties. River sediment can act as sink of estrogens, most of the sorption occurring within the first 24 hours; eventually, these compounds may be released back into the water column and led bioavailable, reaching environmental levels really worrying (Kuster et al., 2005). However, the sorption process of estrogens compound in sediment is affected by organic matter (Sun et al., 2012). Additionally, Baronti et al., (2000) indicated that seasonal variations could be possible, with higher amounts of E2 and EE2 in spring and summer (from March to October).

Table 5 Review of presence of estradiol and ethinylestradiol in different aquatic compartments

Therapy class	*WWTP effluents (ng/L)*		*Surface water (ng/L)*		*Sediment / Sludge (SL) (ng/g)*	
	Amount	**Ref**	**Amount**	**Ref**	**Amount**	**Ref**
E2	<0.1-88; 4.5-8.6; 0.01-0.02; < 10.	Christiansen et al, (2002); Cargouet et al, (2004); Gibson et al, (2007); Loos et al, (2013);	<0.05-15; 1.7-3.2; 2.3; 134; <0.037.	Christiansen et al, (2002); Cargouet et al, (2004); Alvarez et al (2008); Zhou et al., (2011); Esteban et al., (2014)	<0.03-1.20; <1.9; 2.35; 1.20; 0.87-41.	Labadie and Hill, (2007); Martín et al., (2010); Zhang et al, (2011); Zhou et al., (2011); Froehner et al, (2012);
EE2	<0.05-62; 2.7-4.5; 7; 0.06; < 10;	Christiansen et al, (2002); Cargouet et al, (2004); Hernando et al, (2006); Gibson et al, (2007); Loos et al, (2013);	<0.053-31; 1.1-2.9; 2.4; 8.1; 7-24 <0.14.	Christiansen et al, (2002); Cargouet et al, (2004); Hernando et al,(2006); Alvarez et al (2008); Zhou et al., (2011); Hernando et al., (2014).	0.05 –1.5; <0.04; 48.1; 2.18; 5.10; 133.64.	Petrovic et al, (2003); Labadie and Hill, (2007); Martín et al., (2010); Zhang et al, (2011); Zhou et al., (2011); Froehner et al, (2012).

In spite of low ng/L levels detected in aquatic compartment, effects on aquatic environments have been reported in literature (Christiansen et al, 2002; Cargouet et al, 2004; Alvarez et al, 2008; Esteban et al., 2014). The estrogenicity of these substances may affect aquatic organisms by altering their normal hormone functions (Céspedes et al., 2004). Some of the main effects in aquatic organisms are the appearance of abnormalities in vitellogenin production and develop of oocytes in testicles in male fish (Segner et al., 2003). Though some authors as Caldwell et al (2012) did not consider that there is evidence to consider VTG production in male a negative effect on reproduction except in case of extremely high concentrations (resulted in kidney failure) and it can be very useful as a biomarker. The first study in Spain, which shows the existence of feminization in fish (carp), was in the Llobregat River Basin (Solé et al., 2000; Petrovic et al., 2002). Céspedes et al., (2004) observed hormonal alteration in fish exposed to extremely low levels of E2 and EE2 (0.1 to 10 ng/L). Others authors described effects such as a decrease in egg and sperm production, reduction of gamete quality, behavioural changes, and also early hatching, among others (Aris et al., 2014).

EE2 is considered to be very toxic to a large number of organisms, directly or indirectly (Aris et al., 2014). It has been also referred to be more sensitive to early life stage of aquatic organism. EE2 presents the same estrogenic activity than its precursor, however its endocrine disruptor (ED) potential for different organisms

become more than 10 times higher than others natural estrogens appeared in WWTP effluents, such as E2 (Segner et al., 2003; Aris et al., 2014).

E2 and EE2 are involved in a group of organic substance called Endocrine Disruptor Compounds (EDCs). Since Purdom et al (1994) detected the first estrogenic signal in aquatic environment; many definitions are described depending on the working groups. For World Health Organization (WHO), EDCs are "*Exogenous substances that alter the function of the endocrine system and consequently cause adverse health effects in an intact organism, its progeny, or their populations*" (WHO/IPCS, 2002). The European Commission adopted in 1999 the Community Strategy for Endocrine Disruptors (COM (1999) 706) due to the gradual increase of evidence on various health and environmental problems attributed to endocrine disruption (Jobling et al., 2004). EDCs included natural and synthetic hormones, as well as industrial chemicals such as dioxins, nonyphenols, pesticides among others (Aris et al., 2014).

1.2. Environmental Toxicity: toxicological assays

The WFD (EC, 2000), requires an integrated approach to the monitoring and assessment of the quality of surface water bodies. As it is mentioned above, the analysis of emerging pollutants levels as DCF, E2 and EE2 in different aquatic compartments must be joined with increase information about toxicological effects, in order to get a deep knowledge to perform a specific evaluation based on human health risk posed by them (EC, 2000). An additional difficulty to the lack of standardized chemical methods is the scarcity of information regarding the toxicity of the compounds. Due to the enormous number of chemicals with different modes of action, many efforts have been undertaken to develop toxicological methods. For this purpose, many toxicologists agree to perform toxicological tests on zebrafish.

Nowadays, there is growing interest in the use of fish embryos as an alternative tool to examine the presence and potency of aquatic toxicants (Lammer et al., 2009). Due to several inherent advantages, zebrafish (*Danio rerio*) are being utilized to assess the physiological effects of chemical compounds directly in living vertebrate organisms. This model system possesses several features that make it ideal for in vivo compound including: ease of maintenance, small size, short reproductive cycle, high fecundity, transparency (enabling non-invasive imaging) and permeability to small molecules (allowing for drug administration by immersion). Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug screening (Mathias et al., 2012). In a relatively short time, zebrafish chemical screening has evolved from visual observation of arrayed embryos to an advanced system in which individual larvae can be moved in and out of multiwell plates for manipulation and high-resolution imaging (Pardo-Martín et al., 2010). Recent tests have shown that zebrafish perform very well when existing human drugs are tested for conservation of phenotypic effects (Mathias et al., 2012).

In toxicology, apart from being an alternative to fish acute toxicity test in routine wastewater control, the zebrafish early life stage (ELS) test is one of the most popular tools for evaluating the acute or chronic toxic effects of aquatic pollutants on fish (Mathias et al., 2012). The model provides a valuable tool to assess toxicological endpoints, such as those related to development effects (somites, eyes, otolith, nervous and muscular tissue, etc…), but also the model provides assessment of the circulatory system (heart rate, beat variability, circulation, presence of edema), nervous tissue (spontaneous movement, locomotion, alteration in acetylcholinesterase) and general growth (biometric parameters) (Fraysse et al., 2006).

Also in the last years, zebrafish are becoming a widely used model in neurobehavioral research due to the physiological and anatomical similarities to the vertebrates (Miklósi and Andrew, 2006). Thus, larvae models are being currently used for high-throughput exploratory-based models, such as locomotion among others, in order to evaluate neurodevelopment effects of chemicals (Selderslaghs et al., 2010) and surface waters (García-Cambero et al., 2012).

1.3. Objectives

Manzanares River is the main river of Madrid metropolitan area, crossing one of the most densely populated areas in Europe (Madrid Region). In spite of fact that the first Watch List includes the necessity of improve the knowledge of DCF, E2 and EE2 levels, there are few data about the presence of DCF, E2 and EE2 in Manzanares River. Considering the initial hypothesis that WWTPs are not able to remove such pollutants, DCF, E2 and EE2 are expected to be eliminated to the surface waters of Manzanares River, which can produce adverse effects on aquatic environment.

Therefore, the present study aims to study the presence, distribution as well as the toxicity of DCF, E2 and EE2 conveyed by Manzanares River when cross through Madrid Region.

For this main purpose, the following objectives are proposed:

1. Optimization of an analytical method for determination of diclofenac, 17-β-estradiol and 17-α-ethinylestradiol in different environmental matrices (effluents, surface water, and sediment).

2. Determine the presence of DCF, E2 and EE2 in emission source (effluents), and downstream (surface waters and sediment) of Manzanares River.

3. According Watch List, increase the knowledge about the relevance of these three substances in Manzanares River to suggest their inclusion in Priority Substance List.

4. Assess the toxicological effects of Manzanares River water to aquatic vertebrates, in this case, in *Dario Rerio* (Zebrafish) embryos. Evaluate whether the toxicity of surface water of Manzanares River to Zebrafish embryos can be potentially ascribed to the presence of diclofenac, 17-β-estradiol and 17-α-ethinylestradiol in the River.

2. Material and methods

2.1. Sampling

Madrid Region (MR), which is Spanish Capital, is one of the most densely populated areas in Europe. It is estimated that MR has about 6.5 million inhabitants in an area of 8028 km^2 (INEbase, 2014). Manzanares River flows through MR, born in Ventisquero de la Condesa the southern slope of the Sierra de la Cuerda Larga, goes through Manzanares el Real, El Pardo, Madrid City and after traveling 92 km ends at the Jarama River, which is the main tributary of the Tajo River.

Manzanares River Basin has a total of 16 WWTPs, some of them discharges effluents in our stream of interest and other in tributaries streams (C.H. Tajo, 2000). Large part of Manzanares River flow rate come from emissions of these WWTPs. The stretch of the river under study involves 5 WWTPs: Viveros de la Villa, La China, Butarque, Sur and Arroyo Culebro Cuenca Baja (Table 6). These sampling points were selected according to the population served and due to they are located in the stretch of the river more densely populated (about 20 km). The WWTPs selected receive and treat sewage urban and industrial waters from both the city and its suburbs (54.2% total population of MR), and towns lying in the Greater Madrid metropolitan.

All these WWTPs have secondary treatment by biological pathway. Additionally, Viveros de la Villa and La China have tertiary treatment of sewage, based on sand bed. La China also has nutrient reduction by biological pathway. Furthermore, Viveros de la Villa and La China have facilities for the production regenerated water for irrigation. Treatment consists of decantation, filtration, microfiltration and UV disinfection (www.madrid.org). As a curiosity data is noteworthy that La China was the first sewage treatment plant in Spain.

Table 6 Treatment plants under study. Source: Canal Isabel II, 2013.

Name of WWTP	Population equivalent	Treatment	Flow Rate Emission
Viveros de la Villa	700.000	Biological activated sludge. Tertiary treatment.	190.080 m^3/d
La China	1.335.000	Biological activated sludge with nutrients reduction. Tertiary treatment	280.000 m^3/d
Butarque	1.612.800	Biological activated sludge with nutrients reduction.	276.480 m^3/d
Sur	2.937.600	Biological activated sludge with selectors	410.000 m^3/d
Arroyo Culebro Cuenca Baja	1.353.600	Biological in two stages. Anaerobic sludge digestion and sludge dehydration.	172.800 m^3/d

This stretch is encompassed in the Tajo Hydrological Plan to carry out actions of protection, conservation and recovery of the functions of the water system as it is considered to be at high risk because of its high human influence (C.H. Tajo, 2000). We have differentiated three sections: i) Manzanares River prior to its passage by Madrid city (El Pardo), ii) passing through Madrid city, iii) Arroyo Culebro (12 km), tributary of Manzanares which collecting spills of the two largest WWTPs the southwest of Madrid (Figure 3).

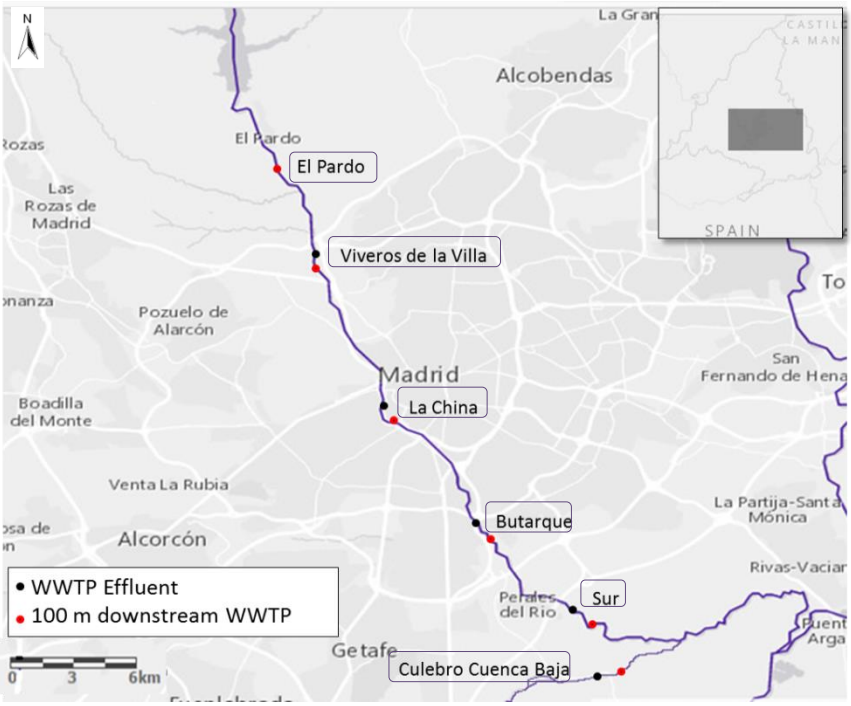


Figure 3. Sampling points in Manzanares River.

Sampling plan was carried on according to the standardized procedure ISO 5667-1, 2, 3 and ISO 5667-12. A total of 10 effluents samples and 12 surface water and sediment samples were collected in two different sampling campaigns performed in March and April 2015. Sampling points were selected as follow:

- Effluents discharges of five WWTPs mentioned before,
- Downstream (from 100 to 200 meters) of the WWTPs discharge point: surface water and sediment.
- Reference sampling point were selected in El Pardo town because is located upstream any WWTPs selected for this study and Madrid city.

Grab and punctual liquid samples were collected in clean amber glass bottles, from WWTPs effluents (2 L) and from river water 100 m after these WWTPs (4 L). Before sample collection, each bottle was pre-rinsed with sample three times. Sediment samples were taken in recipients of polypropylene (250 ml). Samples were stored at 4 ° C in the dark until treatment before 48 hours. Also, physico-chemical parameters were measure “in situ” with a multi-parametric probe to give additional information (Annex 1).

2.2. Pharmaceutical and reagent selected

Standards of diclofenac sodium, 17-β-estradiol (99.2% purity) and 17-α-ethinilestradiol (99.4% purity) were purchase in Fluka. Derivatization regeant MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide synthesis, 97%) was from ACROS. The solvents, High pressure liquid chromatography (HPLC)-grade methanol, acetonitrile, ethyl acetate and formic acid (98 %) were provided by Scharlab, Sigma - Aldrich and J. Baker. High Quality Water was obtained from Milli-Q-gradient equipment, Millipore. Nitrogen used for drying from Air Liquid (Spain) was of 99.99% purity. The cartridges used for SPE were Scharlab EBH (200 mg, 6 ml).

Individual standard solutions of the analytes were initially prepared at 100 mg/L in methanol and subsequently diluted also in methanol in order to obtain an appropiate analyte concentration. These solutions were stored at -35 ºC and renewed monthly.

2.3. Sample preparation

For this work, a method for the detection and quantification of DCF (HPLC/MS) and estradiols (Gas chromatography, GC/MS) was developed. Treatment of surface water and effluent samples were based on 1694 EPA method. Liquid samples were filtered through membrane filters as they arrived at the laboratory. Then, it was extracted by SPE process. The procedure used for sediment extraction is based on PLE (Pressurized Liquid Extraction) method described by Céspedes et al., (2004). Extractions were carried out using a Dionex ASE 350 (Dionex, Idstein, Germany). Estradiols (E2 and EE2) suffered the same extraction method, and additionally a derivatization step previous their chromatographic analysis, according to method described by Zhou et al., (2007).

2.3.1. Treatment of surface water and effluent samples:

River Water (2 L) and effluent samples (1 L) were passed through a glass fibre filter (GF/F, Whatman, no 1828-047). The pH of sample was adjusted to 2 $\pm$ 0.5 with acid HCl (0.1 M). The extraction was performed in a vacuum system (Visiprep-DL), where is possible to process 12 samples simultaneously. Prior to extraction, the SPE cartridges (200 mg, 6 cc) were sequentially conditioned with 6 ml methanol, 6 ml high quality water and 6 ml high quality water adjusted to pH 2 $\pm$ 0.5. Then, samples were passed through a SPE column at a flow rate of approximately 5 ml/min. The EBH cartridges were dried under vacuum for 30 minutes. Wrapped into aluminium foil, and stored in a self-seal plastic bag at −30º C until elution.

Columns were allowed to defrost before elution of the analytes. The elution was also performed in the vacuum system. The analytes were eluted with 8 ml methanol. Before it open the flow (flow rate 1 ml/min), methanol was maintained in the column for 5 minutes. Then, the extracts were evaporated near to dryness under N_2 stream at 25 ºC (Turbo-vap LV 103199, Biotage). The dry residues were resolved in 1 ml methanol and transferred to chromatographic vials after a filtration step through 0.22 µm PTFE filters. The extracts were stored at -30º C until HPLC/MS analysis. All samples were evaluated by triplicates.

2.3.2. Treatment of sediment samples:

Sediment samples were dried in oven at 100 ° C for 48 hours (BK500, Heraus). Then the sample particle size was reduced with a grinder and homogeneous samples with similar particle size were obtained. Each sediment sample was analyzed by triplicates. Sediment samples were extracted by PLE using a Dionex ASE 350 system (Thermo Fisher, Germering, Germany). Each sample (5 g dry weight $\pm$ 0.0001 g, XS-204 Balance, Thermo Mettler) was thoroughly mixed with hydromatrix (diatomaceous earth from Thermo fisher Ssc.) and the mixture was put into a 22 mL stainless steel extraction cell containing a glass-fibre filter (27 mm diameter, type D28, Dionex) in the cell inlet and outlet. The extracting solvent was 1:1 methanol: HPLC-quality water, and the operating conditions were as follows: extraction temperature, 100 °C; preheating, 10 min; static time extraction, 10 min; nitrogen purge, 60 s; 40 % rinse volume; and two static cycles.

Each PLE extract was diluted with high quality water to a final volume of 250 ml. Then, the analytes were extracted in EBH cartridges (200 mg, 6 cc), previously conditioned with 6 ml of methanol and 6 ml of high quality water. The sample was introduced at a flow rate of approximately 5 ml/min. The cartridges were dried under vacuum for 30 minutes, and then, the analytes were eluted with 8 ml methanol. Before start the flow, methanol (flow rate 1 ml/min) was maintained in the column for 5 minutes. Then, extracts were evaporated near to dryness under a nitrogen stream at 25 ºC. Next, the residues were dissolved in 1 ml methanol, filtered through 0.22 µm PTFE filters and transferred in a vial for analysis. The extracts were stored at -30º C until HPLC/MS analysis.

2.3.4. Derivatization

Derivatization steps were carried out evaporating the extracts of 1 ml methanol under N_2 stream. Then, they were resolved in ethyl acetate (100 µl) and added derivation agent MSTFA (200 µl). Lastly, vials were closed and maintained at 65ºC for 30 minutes in an ultrasonic bath (Ultrasonic H 3000839, Selecta). Derivatized samples were analyzed immediately.

2.4. Quantification by chromatographic analysis

The proposed method was validated using matrix-spiked with standard solutions as calibration curves. This quantification work meets three commitments:

1. know matrices interferences for detection and quantification of PhACs selected. It is complex matrices where it cannot exclude the presence of other organic pollutants which can interfere with the analysis of our compounds of interest;

2. recoveries studies for each analytes after extraction procedure in those matrix with high organic content (sediment y effluent); and

3. obtain a calibration curve for each matrix with six known and growing levels of DCF, E2 and EE2, validated through statistical analysis to obtain coefficients of variation, appropriate standard deviations (SD) and thus obtaining the limits of quantification (LOQs) for each matrix and each product.

These calibration curves were analyzed simultaneously with real samples. The standard curves and spiked matrices curves were carried out as follow:

- Effluents curves were performed spiking sample from La China WWTP, due to that showed the lowest amount of our compounds of interest in a previous screening analisys. Six portion samples containing known concentrations were extracted as effluent sample procedure (showed in 2.3.1.), and then each curve was analyzed. Three replicates were carried on for each point of the curve.

- River samples were quantified with interpolation of the chromatographic results in a calibration curve performed with standard solutions for each analyte.

- Finally, levels of DCF, E2 y EE2 present in sediment samples were quantified by using the regression equation obtained by spiking sediment at six levels. Sediment samples considered as reference matrix were from El Pardo sampling point. Six known and growing concentrations of the three analytes under study were added at dried sediment samples. Three replicates were carried on for each point of the curve. Then, the spiked replicates reposed 24 hours, and finally they were extracted as sediment sample procedure described in 2.3.2 section.

2.4.1. Liquid chromatography for diclofenac

Liquid chromatographic method for DCF analysis was performed by an Alliance 2695 separation module coupled to a single quadrupole mass spectrometer (MS) model 3100 of Waters, using Empower 2.0 software. Analytes were separated on a 2.1 cm × 50 mm (2.5 µm, 80 Å) X-bridge B6H-C8 column (Waters). A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to achieve chromatographic separation. The mobile phase gradient goes from 90/10 acetonitrile/HPLC-quality water with 0.1 % formic acid to 90/10 HPLC-quality water/acetonitrile in 15 minutes. Additional chromatographic parameters were as follows: injection volume, 20 µL; column temperature, 30 °C; flow rate, 250 µL/min.

The MS was operated in positive ESI (Electro spray ionization) mode. All control of HPLC and MS parameters and analysis data were performed by Empower 2.0 Waters Software.

Selected Ion Monitoring (SIM) and mass spectra of analytes were obtained from infusion of 0.5 µg/L DCF standard solution at a flow rate of 20 µl/min. DCF showed an abundant $[M+H]^+$ ion. It selected specific voltage parameters of MS detector in order to optimize the detection of our compounds. The most sensitive SIM transition of DCF was used for quantification. Retention time (Rt) of DCF standard solution was within ± 15 seconds of variability criteria stablished by 1694 EPA Method. Dwell times of 0.1 seconds were selected to assure enough data points per chromatographic peak (at least 100 pints) to have satisfactory peak shape.

2.4.2. Gas chromatography for E2 Y EE2

Derivatized samples were analysed for estrones using an Agilent GC-6890 Gas Chromatograph and 5973 Quadrapole MS equipped with a nonpolar HP-5MS 30 m × 0.25 mm capillary column with 0.25 µm film (Agilent, USA). The injector was set at 300°C in splitless mode (1 µl, 20 ml/min, 1 min), and the oven temperature was programmed at 60°C for 1 min, ramped at 25°C/min to 220°C, and then ramped at 10°C/min to 300°C and maintained at this temperature for 10 min. The carrier gas was helium with a constant flow rate of 1 ml/min. The MS was operated in the electron impact ionization mode at 70 eV in selected-ion monitoring (SIM) mode. All control of GC and MS parameters and analysis of data were performed by the MSD Productivity Chemstation Software Rev. E. 02.00.493.

2.5. Toxicological Assays

Zebrafish progenitors were wild-type, obtained from a local pet store and maintained at standard laboratory conditions of 26ºC on a 14:10 dark/ligth photoperiod in a recirculation system for at least 2 months, before using them as progenitors. The water was prepared according to UNE-EN ISO 7346-3:1998. Fish were fed twice a day with a commercial diet (Zeigler), and every two days the diet was complemented with living daphnia magna.

One day before the toxicity experiment, parent animals were separated from the rest and caged in tanks (one female and two males) overnight. Spawning was induced when then light was turned on the following morning.

The toxicological assays with surface waters were carried out according method described by García-Cambero et al., (2012). Briefly, the egg medium was prepared according to Water UNE-EN ISO 7346-3:1998. After several washes with egg medium, zebrafish eggs, staged in 4-8 cells, were selected carefully to discard unfertilized eggs and then transferred to glass beakers (15 eggs/beaker) containing egg medium.

Thereafter, the egg medium was discarded and 30 ml of the undiluted sampling water was added to the groups of exposure. Surface water sample from Viveros de la villa, La China and Butarque were used for the toxicological assay. The negative control group was incubated with the same volume of egg medium, while the positive control group was exposed to a solution of 4 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) in the egg medium. In order to test the toxicity of the mixture DFC + E2 + EE2 evaluated/quantified in the river water samples, another group of exposure was prepared. The group consisted on 2 beakers (15 embryos/beaker) exposed to a mixture of DFC + E2 + EE2 prepared in egg water. The concentration of the mixture was based on the maximum values of the compounds found in surface water during the second sampling Viveros de la Villa. So, the concentration of each component was 160 ng/L (DFC), 1.5 ng/L (E2), 5 ng/L (EE2).

The beakers were then incubated in a climate chamber at 28.5 ºC with a 14:10 ligth: darkness cycle and for 6 days with medium renovation every day. Two replicates of 15 embryos/beaker/water sample were evaluated, and the experiment was repeated the following week (n=60 embryos per sample).

2.5.1. Toxicological endpoints evaluated in the zebrafish embryos

Mortality and development effects were assessed in all zebrafish embryos at 3 h, 10 h, 24 h and thereafter, every 24 h until the end of the experiment or, 144 hours post-fertilization (hpf).

At 75 hpf, tail length and curvature was assessed in about eight embryos/replicate/sample/experiment (n=30/sample) following Fraysse et al. (2006) procedure. For that purpose, embryos were transferred to 6-well plates containing a buffered solution of tricaina (0.08 %). After 3 min, they were positioned on the lateral side and photographed (Axiocam HRC, Zeiss). Then, transferred to a new beaker with fresh medium water (samples or egg water, three changes), and maintained separated in order not to be used for the locomotion evaluation. The tail length was measured from the beginning of the first somite to the end of the most posterior one. The values were obtained in pixels and converted in micrometres. Hatching was evaluated by visual inspection of all zebrafish embryos from 48 hours pf to 80 hpf.

To evaluate the heartbeat frequency, 10-seg videos were carried out between 51 and 53 hpf to 5-6 embryos per replicate of the sample in each experiment, previously acclimated to 28.5 ± 0.2 ºC (n= 30/sample). Heartbeats were counted automatically by using Danioscope software (Noldus®)

The evaluation of larvae locomotor activity was achieved using the Noldus Behavior Recording System (Noldus Information Technology, Inc. The Netherlands) consisting of a video camera (Ikegami B/N) and a tracking software (Ethovision XT, V.5). Videotrack system has been previously validated and successfully used for assessment of zebrafish larvae locomotion exposed to neurotoxicants (Selderslaghs et al., 2010; Padilla et al., 2011; García-Cambero et al., 2012). Briefly, larvae were located into 6-well plates (1 larva/well), containing 4 ml egg medium and allowed for 3 min. Subsequently, 10-min videos were recorded by the video camera (60 frames/second) with the lights turned on (200 lux). Swimming movements were captured within predefined arenas (one arena corresponded to one well, with an internal diameter approximating 35 mm), by means of subtraction. The videos were simultaneously processed by the tracking software, and the analysis of the parameters here presented (total distance travelled and mean velocity) was obtained. After tracking, a quality control measure was applied as wells showing incorrect tracking (e.g. detection of the edge of the well instead of the larva) were discarded from the final data set. Locomotor activity was assessed in 10-15 larvae/sample in each experiment (n=25-30/sample), at day 6 pf, and each session started at 10:00 am and ended at 13:00 pm.

2.5.2. Statistics

Statistical analysis was performed using the statistical package SPSS 22.0 for Windows. All the replicates from both independent experiments for the same water sample were analyzed simultaneously. They are presented as the mean $\pm$ standard error of the mean (SEM). Mortality findings and mean hatching time were assessed by Wilcoxon-Lichfield to give the LC_{50} or HT_{50} (hatching time for 50% of the individuals) with 95 % CI, respectively. The data were tested for homogeneity and normality. When these assumptions were met- t-Student test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was performed; otherwise non parametric U Mann-Witney or Kruskal-Wallis was applied. The significance level was set at $p<0.05$.

3. Results and Discussion:

3.1. Method validation

The identification of the compounds was based on two variables: the Rt and their the precursor ions (SIM). The SIR for DCF was 296, while for E2 and EE2 had as representative ion 386 and 425, respectively. The identification and obtaining of E2 and EE2 spectra was performed with a standard solution of 0.5 µg/L in scan mode (mass spectra for E2 is showed in Figure 4a), and then it was compared with the database system, obtaining a similarity of 96 %. Subsequently it confirmed and quantified in SIM mode (pure peak of E2 showed in Figure 4b). All data selected for quantification are showed in table 7. Also spiked samples were used to confirm the identification of each analyte.

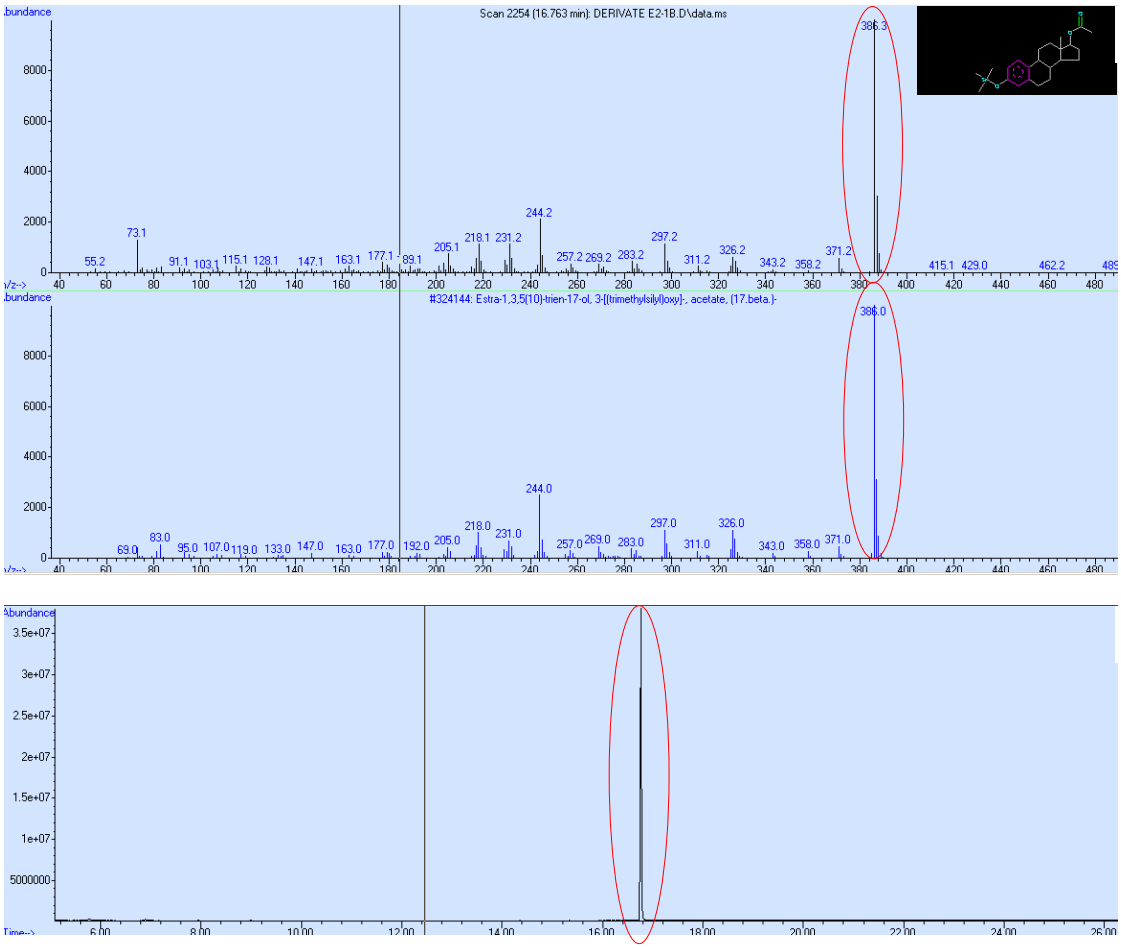


Figure 4 a) Scan mass spectra of standards E2 (upper image) compared with spectra of E2 from library (image below). Red circle shows ion selected; 4b) Chromatogram in SIM mode for derivatized standard solution of E2.

The quantitation limit (LOQs) were estimated as the relation between the y-intercept of the line and the slope of each regression equation. Estimated LOQs for the quantitation of environmental levels of DCF, E2 and EE2 presents in each environmental matrix are showed also in table 7. Due to the complexity of the effluent matrix, LOQs in effluents are higher than surface water. Suspended solids are the main interference for quantification in this matrix.

Linearity of the chromatographic method was achieved, in triplicate, six levels of concentration of single standard (DCF, E2 and EE2), and was observed in different ranges depending on the complexity of the

emission and environmental matrices. The ranges of linearity for our three compounds in effluents, surface water, and sediment, respectively, are compiled in table 7. Calibration curves showed satisfactory correlation coefficients (also showed in table 5). The method denoted low dispersion of data, low uncertainty of the estimated regression coefficients and ideal adjustment data for estimated regression line, taking into account the great of the matrices composition.

Recoveries percentages of the different procedures were calculated at low and high levels (10 and 100 ng/L). Spiking matrices before and after extraction step, at concentrations selected based on levels showed by samples in a previous screening analysis. Accuracy studies were performed in terms of recovery. Accuracy also was evaluated at two concentration levels selected for each matrix. Highest recovery percentage was for DCF in sediments (95%), and much lower recoveries were obtained for estrogens in this matrix. Contrarily, hormones had better recoveries percentages than DCF in effluents. This is a very important factor that needs to be improved. Validation data are available in follow table.

Table 7 Validation parameters for DCF, E2 and EE2 in each type of matrix.

Matrix	SIM (m/z)	Rt (min)	Linearity Range	R^2	LOQ	Recoveries (%)
DICLOFENAC						
Effluents	296	10.02	5-1000 ng/L	0.9695	67 ng/L	65
Surface water			0.62-620 ng/L	0.9977	2 ng/L	n.a.
Sediment			2.5-80 ng/g	0.9986	0.78 ng/g	95
17-β-ESTRADIOL						
Effluents	386	16.75	5-1000 ng/L	0,9966	10.9 ng/L	71
Surface water			0.1-500 ng/L	0,9940	4.44 ng/L	n.a.
Sediment			2.5-80 ng/g	0,9997	0.32 ng/g	58
17-α-ETHINILESTRADIOL						
Effluents	425	17.20	5-1000 ng/L	0,9968	12.2 ng/L	68
Surface water			0.5-500 ng/L	0,9885	7.0 ng/L	n.a.
Sediment			2.5-80 ng/g	0,9893	2.1 ng/g	48

* n.a.: Not available

3.2. Presence of PhACs under study in Manzanares River

This work reports the results obtained in the study of the presence of DCF, E2 y EE2 in the most densely and disturbed stretch of Manzanares River. All results presented in this work must be interpreted taking into account that punctual samples were taken during the study. As a consequence different variables could be affecting the variability of the results such as the day of the week or time of sampling.

3.2.1. Occurrence of diclofenac

DCF was detected in the 76 % of the total samples analysed. DCF was detected in all effluents and river water samples, and in 45 % of the sediment samples. As expected, the concentration of DCF is higher in the emission source (effluent) than in surface waters (Manzanares River samples). Levels of DCF in effluents ranged from 284.4 to 1667.2 ng/L including two sampling campaigns, whereas in surface waters the range of concentrations were from 2.9 to 16.7 ng/L. Levels of DCF in sediment samples were from 1.6 to 15.0 ng/g (all values in Table 11, Annex 2).

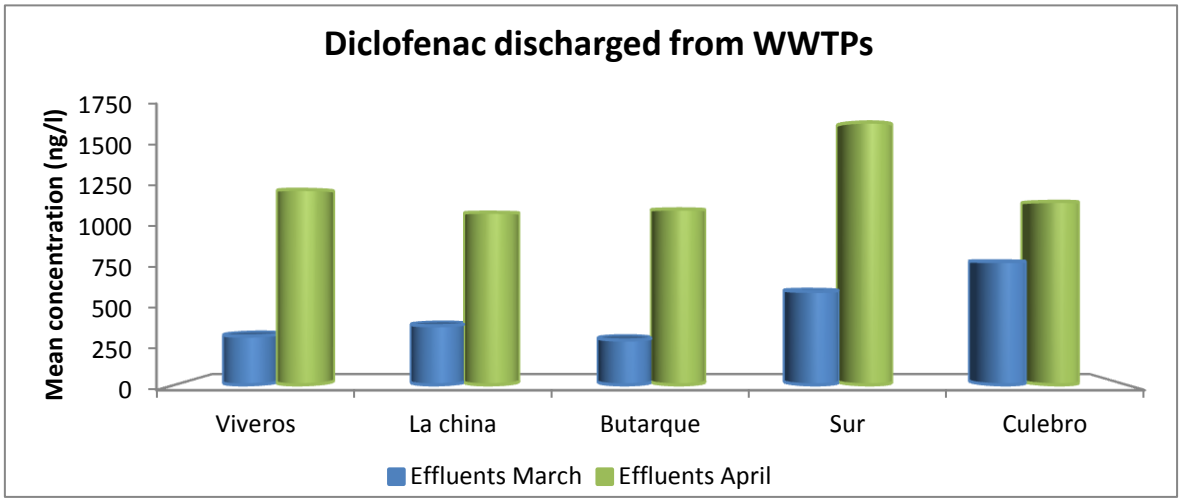


Figure 5. Diclofenac mean concentrations measured in effluents emitted into the stretch of the Manzanares River under study. Blue columns represent DCF concentrations in effluents during first Sampling Set (S.S.). Green columns represent DCF concentrations in effluents during second S.S. Table containing values ± SD are showed in Annex 2 Table 11.

In WWTP effluents data showed highest DCF level (1667.2 ng/L) in the effluents of Sur, followed by Viveros, Culebro Cuenca Baja, Butarque and La China, the average value of effluent concentration was 901.0 ng/L (Figure 5). Sur WWTP was initially designed to hand 2,937,000 population equivalents. This greater capacity of initial design is reflected in their highest flow rate emission (see Table 6, section 2.1) and could be the main reason for the highest levels observed showed. In spite of that the relation between flow rate emission and amount of DCF discharged is not clear. Butarque seems to be the WWTP with lower DCF emissions, despite being the second largest WWTP respect to flow rate emission. There is a slight trend according distribution of emission sources, i.e., increasing concentration can be related to the course of the river.

All samples showed highest amount of DCF in April sampling campaign than in March. The seasonality of the emissions is a parameter which must to be measured trough an intensive monitoring study; therefore this goal could not cover in this work. It is expected that pharmaceuticals such as DCF might be consumed more frequently in winter (Heberer et al., 2002). Variations in photodegradation as a result of seasonally changing light intensity might (partly) explain the seasonal variation of DCF loads, as this chemical is known to be rapidly degraded when exposed to light (Buser et al., 1998; Salgado et al., 2013). Also variation might

therefore be explained by increased biological degradation as a result of higher temperatures in the waste water treatment and in the environment (Andreozzi et al., 2003; ter Laak et al., 2010).

As it is mentioned above, It is not fully defined the removal percentage of DCF which ranged between 0 and 90 % of elimination (Ferrari et al., 2003; Fent et al., 2006; Kujawa-Roeleveld, 2008). Suspended solids seem to be very an important receptor of DCF residues (Aguayo et al., 2010). In present study we have not measured concentrations in the suspended fraction thus there may be an underestimation of the presence of DCF in effluents. It has been shown that the most effective tertiary treatment involves applying AOP techniques, specifically ozonation techniques. Moreover, it has demonstrated that sand filtration exhibited no significant elimination of DCF (Ternes et al., 2002; Joss et al., 2006). Viveros de la Villa and La China WWTPs are equipped with a tertiary treatment mainly based on sand bed, specifically, Viveros has a system called depth filtration textile recommended for elimination of micropollutants (teqma.com). However, no significant decreases in the levels of DCF have been shown because of the tertiary treatment.

Moreover, Zhang et al (2008) study the removal of DCF with different biological systems and affirm that DCF was better degraded in an anoxic biofilm reactor. Also include the acidic condition as influencing factor. They show that pH 4.4 was preferable for removal of acidic pharmaceuticals such as DCF, increasing water-sludge partition coefficient. Biological oxygenic treatment is usually employed in WWTPs under study, only Arroyo Culebro Cuenca Baja has anaerobic sludge digestion and not showed significant reduction of emission. Probably these are the reasons why DCF levels in effluent studied are higher than data collected from other parts of Spain (da Silva et al., 2013).

DCF were detected in effluents samples in a range of concentration similar to literature of PhACs that show levels ranged from 49.5 to 5450 ng/L (Table 3, section 1.1.1). The study conducted by da Silva et al., (2012) in Ebro River reported less amount of DCF in effluents ranged from 216 to 808 ng/L. Even lower levels are reported by Loos et al., (2013) with 50 ng/L, which is closed to results obtained by Bueno et al., (2012) in a two-years monitoring program in 5 Spanish WWTPs (1-529 ng/L). Moreover, López-Roldán et al., (2010) exposed that less amount of DCF is emitted in Catalonia WWTPs which employ tertiary treatment and the concentration reported (421.50 ng/L) is also lower than the range of our mean results in La China and Viveros de la Villa. Despite of this, we can assume that our results are within the median detected in Europe WWTP (Ternes, 1998; Ferrari et al., 2003; Tixier et al., 2003; Hernando et al., 2006; Fent et al., 2006).

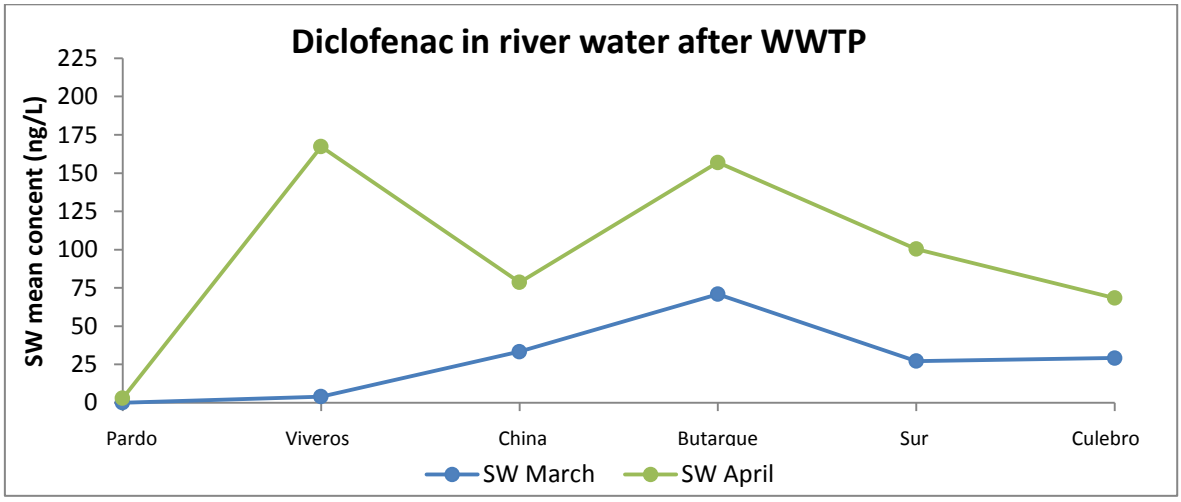


Figure 6. Diclofenac mean concentrations measured in SW 100 m downstream of effluent discharge from WWTP. Blue line represents DCF concentrations in SW during first Sampling Set (S.S.). Green line represents DCF concentrations in SW during second S.S. Table containing values ± SD are showed in Annex 2 Table 11. SW: Surface Water.

After WWTPs, reference sampling point (El Pardo) showed the lowest DCF level in surface water (2.90 ng/L during the second sampling set), the highest concentration was measured in Viveros de la Villa (167.3 ng/L), and the average value was 67.2 ng/L (Figure 6). DCF were detected in river samples taken downstream the point of emission of El Pardo, Culebro Cuenca Baja, La China, Sur, Butarque followed of Viveros, from lower to higher concentrations, respectively. It estimated that amount of DCF detected in surface water corresponds to 0.92 ± 0.4 % of concentration in effluents. Viveros de la Villa is the sampling point upstream from the rest, so less anthropogenic influence or accumulation of pollutants is assumed. Despite this, chemical analyses show a larger quantity of DCF at this point, which matches with the high emissions produced by this WWTP. It is also important to consider physical-chemical variables, such as pH, conductivity (E.C.) or suspended solids, among others, that are important when considering chemical quality of this area. For instance, E.C. increase along the river (Annex 1 table 10), which is indicative that greater amount of ions are able to catch or interact with DCF.

The low amount of DCF in receiving waters may be due to attenuation in water related to the dilution process after the emission from WWTP (Aris et al., 2014). Dilution effect produces reduction of one order of magnitude from µg/L to low ng/L. Nonetheless, direct photolysis is the predominant removal process in freshwater, exhibiting an average elimination rate of 0.082 µg/day, corresponding to half-life of eight days (Tixier et al., 2003). As a whole, DCF properties such as half-life and high photodegradation rate, among others, lead us to think about the concept of pseudo-persistence.

Although DCF concentration in the environment is low, there is a steady input of the drug into the environment, considered as a pseudo-persistent compound, so organisms are continuously exposed. Regarding the results, we also consider that the natural ability of self-purification capacity of the river is exceeded and cannot remove DCF residues totally. It is important to say that in most of the cases our levels detected are below of predicted non-effect concentration (PNEC) (10 µg/L) recommended by Gamarra et al., (2015), despite adverse effects are described at lower levels (Ferrari et al., 2003; Triebskorn et al., 2004; Schwaiger et al., 2004; Zhang et al., 2008). Furthermore, thousands of chemicals co-exist in nature, and organisms are exposed to a combination of stressors. Hence a negative effect of chronic exposure in the environment remains possible.

In this study, concentrations of DCF are within the lower range reported in the literature (from 1 to 3400 ng/L in surface waters, see Table 5). Our results are in line with those reported by other studies in the literature, in which DCF was obtained at ng/L level (Ternes, 1998; Hernando et al., 2006; da Silva et al. 2011). However in our study, DCF was detected below levels reported in similar studies in the same river with 2000 ng/L as median value (Valcárcel et al., 2011). This could be explained by seasonal variations or different sampling points selected in different studies, while to define patterns of DCF in environmental samples and potential associations with their sources, transport pathways, seasonal variations or sampling point selection is complicated. Moreover, comparison of concentrations data measured throughout the two different sampling campaigns revealed highest DCF level April than March once again.

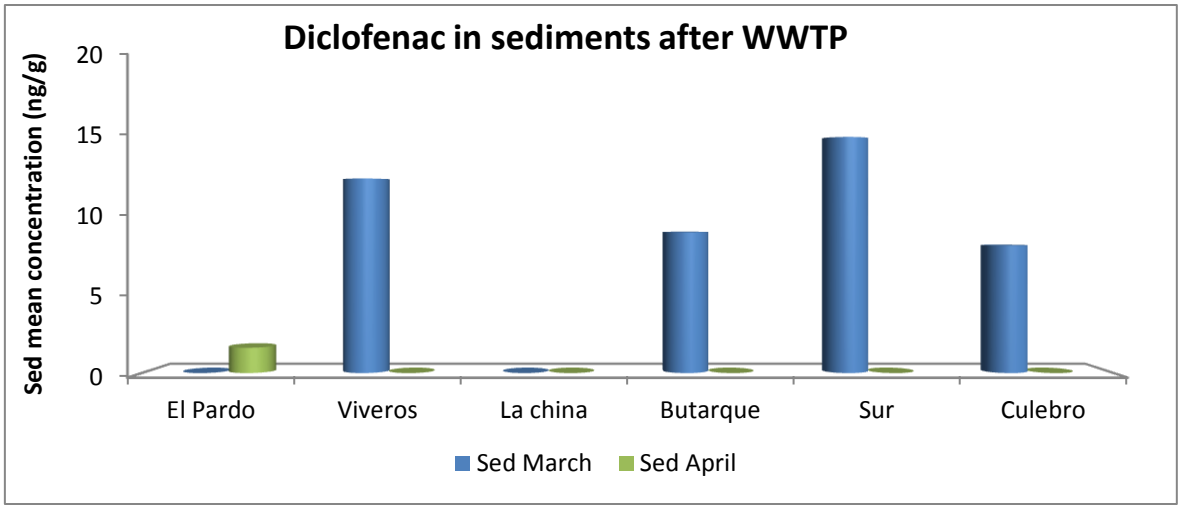


Figure 7. Diclofenac mean concentrations measured in sediments 100 m downstream of effluent discharge from WWTP. Blue columns represent DCF concentrations in sediments during first Sampling Set (S.S.). Green columns represent DCF concentrations in sediments during second S.S. Table containing values ± SD are showed in Annex 2 Table 11. Sed: sediments.

Sediment data showed lower DCF level (1.6 ng/g during second sampling set) in El Pardo sampling point, followed by the Arroyo Culebro, Butarque, Viveros and Sur (Figure 7). Data were consistent with those findings in effluents and surface water samples (rivers) due to Sur and Viveros de la Villa seem to be the most polluted areas under study. However, DCF were not detected in sediment taken downstream emission point of the La China WWTP which it could expect to be one of the most polluted areas because receive sewage from great populated area, including hospitals and industry. Notably, DCF were extensively more detected in March than in April campaign in sediments, in contrast to happen in the others matrices.

Some studies establish that the interaction of acidic PhACs between sediment and water was regulated, to some extent, by their hydrophobicity (Kow) and molecular weight (Gibson et al., 2007). DCF presents a log Kow of 4.51 (Ferrari et al., 2004). Due to its low polarity, sorption to sediments appears quite likely to be cumulative process (Hernando et al, 2006). There are other PhACs detected in waters with higher log Kow that were not present in sediments. Probably, it could be due to their pKa value (Pka=4.15, Table 2 section 1.1.1), and then the pH of the water sample affect the ionization of the DCF molecules and then their sorption coefficients (Osorio et al., 2012). Then the presence of DCF in sediment of Manzanares River could be correlated to the organic content of the sediment, its pH value, environmental temperature (seasonal variability), and inputs from WWTP (consumption pattern and type of treatment system in WWTP).

The level encountered by the proposed method for DCF was higher than those found in others studies. For instance, da Silva et al (2011) reveal levels ranged from 0.7 to 3.4 ng/g in Ebro River sediments (Table 3 section 1.1.1). Also, Martín et al (2010) and Azzouz and Ballesteros (2012) showed much lower DCF levels in the sediments of several Spanish rivers, with <3.6 and $9.5 \cdot 10^{-3}$ ng/g, respectively (Table 3 section 1.1.1). We can compare our results with concentrations showed by Vazquez-Roig et al., (2011) in Valencia River sediments with highest amount of 35.8 ng/g.

In short, DCF presence in Manzanres River is evidenced by the results shown in our study. The worst data is that, all WWTPs emissions and most of the river samples showed concentrations above AA-EQS suggested by European Commission for DCF (100 ng/L). Generally, higher concentrations were found in areas of the river where the flow rate emission is higher. It can also observed increasing amount of DCF in effluents along the river. However, the trend in river water and sediments is unclear.

3.2.2. Occurrence of estrogens

E2 were detected in the 52 % of the total samples analysed. E2 was quantified only one effluent sample (10%) and two river water samples (18%), and in 100 % of the sediment samples. On the other hand, EE2 was quantified in a lower percent of samples (46 %). Half the effluent samples contained EE2 (50 %), which corresponds to all samples analyzed in the second sampling. Only 20 % of the river water samples contained EE2, and 64 % of sediments samples showed concentrations of EE2 up to LOQ. As expected, the concentration of estrogens is higher in sediments than in surface waters. Surprisingly, the emission sources not showed higher concentration than surface water, to neither of our estrogenic compounds under study. Most of the E2 detected in effluents were below LOQ (<10 ng/L), whereas in surface waters (Manzanares River samples) the range of concentration was from 56.5 to 229 ng/L. Levels of E2 in sediment samples were from 0.7 to 6.2 ng/g. EE2 in effluent samples only showed levels upper the LOQ in second sampling set ranging from 19 to 115 ng/L. In river water samples analyzed had values between 86 and 226 ng/L, and sediments contained concentration from 2.7 to 21.6 ng/g (all values in table 12, Annex 2).

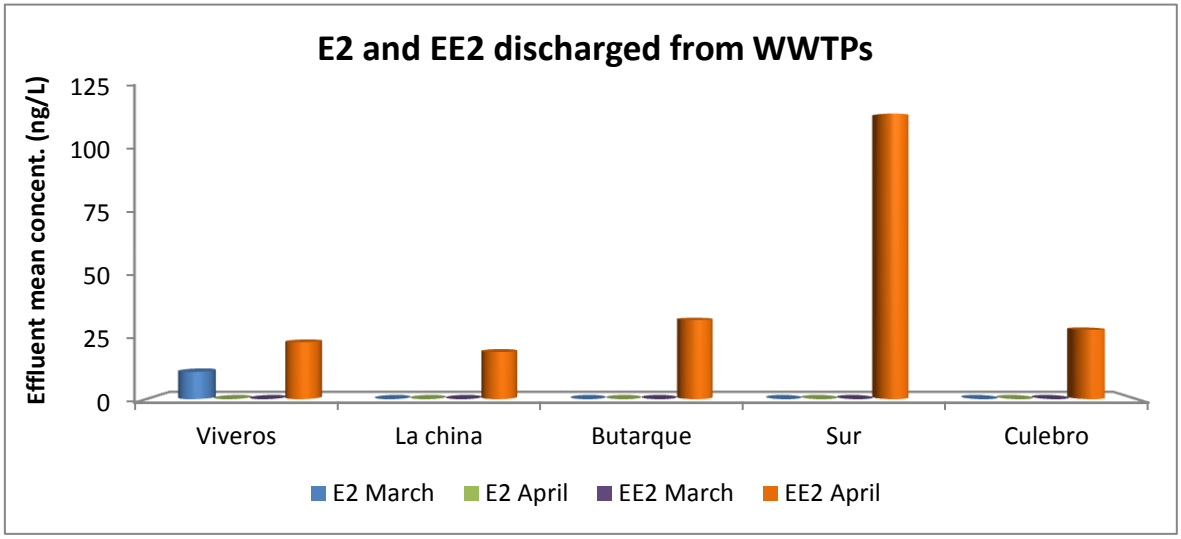


Figure 8. 17-β-estradiol and 17-α-ethinylestradiol mean concentrations of effluents found in the stretch of the Manzanares River under study, in different sampling campaigns. Blue columns represent concentration of E2 in effluents during first Sampling Set (S.S.); green columns represent E2 concentrations in effluents during second S.S.; purple columns represent EE2 concentrations in effluents during first S.S.; and orange columns represent EE2 concentrations in effluents during second S.S. Table containing values ± SD are showed in Annex 2 Table 12.

In WWTPs effluents data showed highest level in Viveros de la Villa (11 ng/L) for E2, while most of the concentrations of E2 detected ranged values below the LOQ (10 ng/L). Sur had the highest level for EE2 (115 ng/L) followed by Butarque, Arroyo Culebro, Viveros de la Villa and La China, with a mean value of 43.5 ng/L (Figure 8). Once again as it happened in the DCF case, Sur seems that produce more emissions for EE2, but it is necessary taking into account that its population equivalent and flow rate emission are higher than the others WWTPs. Moreover, La China showed the lowest emission levels for both compounds.

The results obtained are in accordance with degradation information from literature. Estrogens are the major contributor of estrogenic activity, present in effluents discharging in rivers (Aris et al., 2014). Biodegradation is the main degradation pathway of E2 (Feng et al., 2010). However, EE2 can be highly resistance to the process of biodegradation. EE2 trends to absorb and accumulate in organic matter, sediment and biota, in fact, sorption onto particles seem to be the best elimination pathway (Huang et al., 2001). Even despite of good removal rate show by ozonisation treatment, research has shown that EE2 becomes extremely stable against oxidation due to the introduction of the ethynyl group in 17-position (Zhang et al., 2011). In short, EE2 seems to be relatively stable during the activated sludge process in WWTP

(Aris et al., 2014). It would be interesting whole study of E2 and E1, or other degradation products from E2, since during wastewater treatment great quantity of E2 is oxidized which is reflected in higher amount of E1 release in effluents (Petrovic et al., 2002; Jonhson et al., 2013). The activated sludge treatment is applied in the WWTPs under study. This technique should be good to remove these compounds, but EE2 could not be entirely. According to the study carried out by Beausse et al., (2004), the amount of E2 may be reduced up to 90% in the WWTP with activated sludge treatment. However, this author indicates that the effectiveness regarding EE2 can reach maximum elimination rate of 75 %.

It is remarkable that La China and Viveros de la Villa WWTPs exhibit generally low values, probably due to both have additional filtration treatments, as already mentioned. Despite of no relevant differences have been observed regarding DCF emissions, tertiary treatment seems to be suitable to estrogens compounds. More than 95% of estrogenic compounds can be removed by reverse osmosis (Estévez et al., 2005), due to that we recommend this kind of treatment to obtain better quality of emissions. La China WWTP is also equipped for nutrients reduction. An efficient nitrogen removal by nitrification process has been associated with high removal of biodegradable PhACs (Vader et al., 2000; Osorio et al., 2012), although seasonal and temperature could affect de efficiency of treatment (Vader et al., 2000).

Effluents levels reported in literature for E2 and EE2 (Table 5, section 1.1.2) are from 0.01 to 88 ng/L (Gibson et al., 2007; Christiansen et al., 2002) and from 0.06 to 62 ng/L, respectively (Christiansen et al., 2002). In general, the presence of E2 and EE2 in effluents is in line of other studies, except in the case of the Sur WWTP during second sampling set where levels of EE2 are much higher.

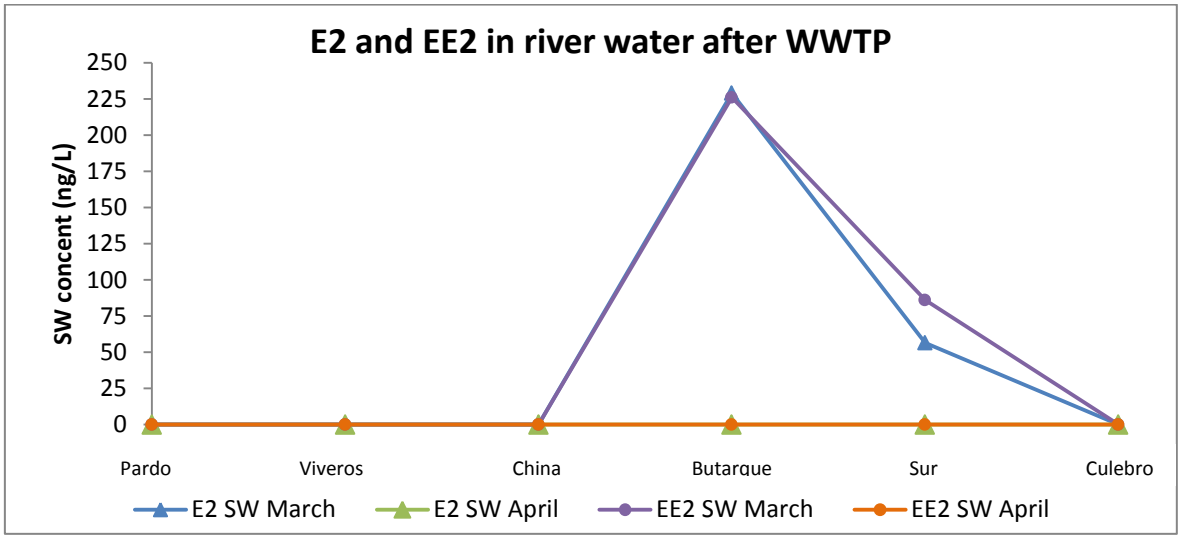


Figure 9. E2 and EE2 mean concentrations measured in SW 100 m downstream of effluent discharge from WWTP. Blue line represents E2 concentrations in SW during first Sampling Set (S.S.). Green line represents E2 concentrations in SW during second S.S. Purple line represents EE2 concentrations in SW during first S.S. Orange line represents EE2 concentrations in SW during second S.S. Values ± SD are showed in Annex 2 Table 12. SW: Surface Water.

Relative to river water samples, E2 detection is relevant only in lower stretch of the Manzanares River, downstream of Butarque and Sur WWTPs. In any other section of the river these compounds were detected. In fact, Butarque showed the highest amount of E2 with 229 ng/L (Table 12, Annex 2). This trend can also be observed in the case of EE2 occurrence. EE2 only was quantified in river water from Butarque and Sur, with higher amount in Butarque stretch (226 ng/L). Both cases the estrogens have been detected in first sampling campaign (Figure 9).

Once again, the amount of estrogens in Manzanares River is mainly due to direct discharges from WWTPs. But contamination of the environment by estrogens may also occur through runoff from manure and sewage sludge that have been used in agriculture field (Kuster et al., 2005). This fact could explain the great

amount of estrogens detected in Butaque and Sur since both are areas with strong presence of crops and livestock. Additionally, direct inputs into natural waters are also possible through storm water overflow and leakages in the sewer system. It so important take into account that it likely seasonal variation (Baronti et al., 2000). In spite of in many cases quantification of estrogens under study had not been got because levels are below LOQ, their presence could induce endocrine disrupting effects in aquatic organism present in Manzanares River. In fact, some of the levels of E2 measured in Manzanares environment are above PNEC proposed by Caldwell et al, (2012) (2 ng/L). For EE2, levels are much higher than PNEC (0.002 ng/L) recommended by Aris et al, (2014) and some other of magnitude higher than PNEC of 0.35 ng/L proposed by Caldwell et al (2012). Regarding these data it is likely that aquatic organisms are affected by the presence of E2 and EE2.

The results obtained from this study are much higher than other reported in literature. E2 and EE2 in Germany surface water reach concentration of 15 ng/L and 31 ng/L, respectively (See table 5 section 1.1.2.) (Christiansen et al., 2002). In China, Zhou et al., (2011) reported 134 ng/L for E2 and 24 ng/L for EE2. As it mentioned before, levels reported in Catalonia Region (Spain) ranged from 71 to 130 ng/L for E2 and from 11.60 to 170 ng/L for EE2 (Petrovic et al., 2002; Céspedes et al., 2004; Kuster et al., 2005; López-Roldán et al., 2010). In the Manzanares River, Esteban et al. (2014) showed level much lower than our results. According to AA-EQS suggested by European Community (COM (2011)876) (0.4 ng/L for E2 and 0.035 ng/L for EE2), most of the data registered in Spanish studies indicated that aquatic ecosystem could be affected by the hormones exposure.

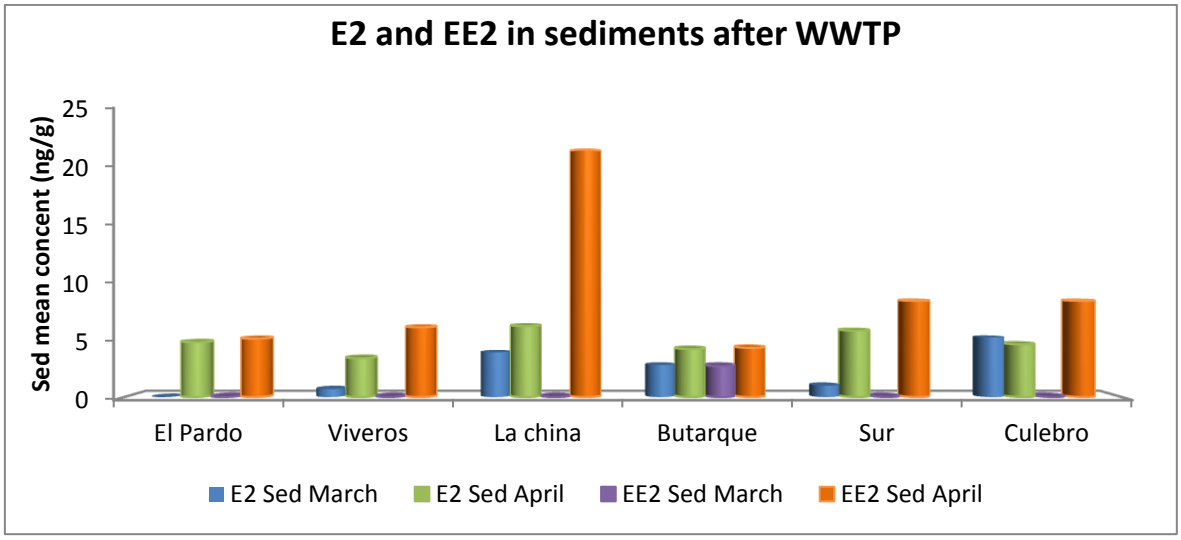


Figure 10. E2 and EE2 mean concentrations measured in sediments 100 m downstream of effluent discharge from WWTP. Blue columns represent concentration of E2 in sediments during first Sampling Set (S.S.); green columns represent E2 concentrations in sediments during second S.S.; purple columns represent EE2 concentrations in sediments during first S.S.; and orange columns represent EE2 concentrations in sediments during second S.S. Values ± SD are showed in Annex 2 Table 12.

Regarding sediments results, expectations have been met indicating greater adherence of E2 and EE2 in sediments. Most of the sediment samples analysed showed presence of natural and synthetic estrogens up to LOQ. Once again, it can observe an increased presence of estrogens in April, although the difference is not so marked. La China seems to be the most polluted area reaching maximum level 6.2 ng/g for E2 and 21.6 ng/g for EE2, following in decreasing order by Sur, Culebro, Butarque and Viveros (Figure 10).

As already mentioned, bed sediment can accumulate concentration up to 1000 times higher than in the overlying water column due to its hydrophobic properties (log Kow > 3). Sorption is affected by composition of sediments, mainly by organic matter content (Sun et al., 2012). Time of sorption is estimated occurs in the first 24 h (Kuster et al., 2005), and this fact could be indicated that the river bed-sediments under study

have the potential to be an environmental reservoir for E2 and EE2 which can slightly vary several factors such as according additional inputs. Given the relative low polarity of these compounds which present octanol-water partition coefficients of 3.62 for E2 and 3.84 for EE2 (Table 4 section 1.1.2.), sorption to bed sediments appears a quite likely cumulative process from where estrogens can eventually become bio-available specially when they are anaerobic (Beausse et al., 2004; Zhou et al., 2011). Therefore, we consider that the natural self-cleaning capacity of the river in not enough to eliminate these compounds and prevent their effects on aquatic organisms. In addition, the half-life of E2 and EE2 in sediments is quite high, 10 and 17 days, respectively (Table 4, section 1.1.2). This fact makes that exposure time of organisms is high and may affect different generations of several species. Studies carried out over the time also allowed us to elucidate seasonal variations in the levels of these potentially harmful compounds, showing higher incidence in spring time (Baronti et al., 2000), this could be confirmed with a more intensive monitoring program.

The occurrence of estrogen compounds in the sediments has been detected in many studies. Levels of estrogens detected in our study are in line of similar studies. In Spanish studies, EE2 sediments reach levels from 0.05 to 1.5 ng/g (Petrovic et al., 2003). Martín et al., (2010) reported higher level in Sevilla Region with 48.1 ng/g, which is twice time higher than our maximum level detected in La China (second sampling set).Other monitoring campaign carried out in several Spanish basins reported 1.6 and 2.2 ng/g for E2 and EE2 (Gorga et al., 2015). In Europe, Labadie and Hill (2007) reported that the estrogenic compounds levels in bed sediment from UK ranged between <0.03 to 1.20 ng /g for E2, and EE2 below 0.04 ng /g (see Table 5 section 1.1.2.). From Brasilian, Froehner et al, (2012) reported the highest amount of E2 and EE2 found in sediments in the literature with 41 ng/g and 133.6 ng/g, respectively.

As a whole, the results obtained about presence of E2 and EE2 in Manzanares River showed a clear trend to sorption onto bed sediments. It is note that E2 and EE2 were not found in Butarque and Sur effluents (in April campaign) while corresponding with the highest levels in surface water in these sampling points. Possibly, this is a clear handicap of punctual sampling. Also reflected when it is compared the amount in surface water from these areas in March campaign with levels in sediments. To avoid these bias, whenever possible the samples must be taken continuously. In upper areas (El Pardo, Viveros de la Villa, La China), estradiols were detected at very low levels or non-detected while sediments showed considerable amount of them, especially EE2.

Taking into account the results globally, the inability of WWTPs to completely remove of DCF, E2 and EE2 is reflected. Although the levels detected not imply an imminent risk to aquatic life or human health, chronic effects cannot be ruled out. Sur stretch seems to be the most disturbed area because of WWTP discharges. Even more troubling is that the area was initially supposed less affected (El Pardo) also contains considerable signs of contamination by the compounds object of this study.

3.3. Toxicological assays

The zebrafish embryo test has become a tool widely used to assess toxic effects of chemicals (Fraysse et al., 2006; Selderslaghs et al., 2010), and more recently to effluents (Hallare et al., 2004), because they are sensitive indicators of toxic components in industrial or municipal waste and landfill leachates. Additionally, Praskova et al., (2011) demonstrated a statistically higher sensitivity to PhACs such as DCF in embryonic stages compared to the juvenile fish. This toxicity assay tries to join both the embryo and larvae stages into a combined model to assess neurodevelopmental toxicity.

The EDCs (E2 and EE2) can interact with physiological systems and cause hormone system effects but also alterations in development, growth and reproduction in wildlife that are exposed to them (Jobling et al., 2004). On the other hand, some studies in the literature show that DCF has sublethal effects in fish in the range of waste water concentrations (Fent et al., 2006). For instance, Ferrari et al., (2003) showed long term effects in zebrafish at 4 mg/L concentration.

3.6.1. Mortality

The first apical endpoint in toxicity is the assessment of mortality. In this experiment, the exposure of zebrafish embryos to surface water from Viveros de la Villa, lab mixture based on Viveros de la Villa (Mixture Viveros) and Butarque did not induce relevant mortality at any time of the observation period. However, surface waters from China produced 100% mortality in both experiments (Table 8). Even, diluted samples (50% and 10%) produced a high index of mortality (100 and 42%, respectively). Although some mortality was observed in the second experiment for Viveros and Butarque, these values fell into the range of historical control data for this laboratory (n=20 experiments), which is around 7%. Positive control, exposed to 2,4-D, recorded a mortality of 100 %. In this sense, this positive control group should produce >30% mortality within 96h according to OECD 236 guideline. Finally, negative control group did not record any mortality.

Table 8. Mean % mortality (± SEM) data after different assay times.

Samples	N	8 h	24 h	48 h	72 h	144 h
			% Mean ± SEM			
Control -	68	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Control +	34	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0
Viveros	49	0 ± 0	3 ± 0.7	0 ± 0	0 ± 0	0 ± 0
China 100 %	64	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0
China 50 %	49	0 ± 0	4 ± 0.9	4.8 ± 0.4	23.5 ± 4.8	67.3 ± 5.3
China 10 %	49	0 ± 0	4 ± 0.9	0 ± 0	14.7 ± 3.0	22.9 ± 3.4
Butarque	68	0 ± 0	4 ± 0.8	0 ± 0	0 ± 0	0 ± 0
Mix Viveros	68	0 ± 0	1 ± 0.3	0 ± 0	0 ± 0	1.5 ± 0.3

The 144-h-LC_{50} value determinated for *D. renio* embryos was 6.11 mg/l. In comparison, the 96-h-LC_{50} value for juvenile *Danio rerio* was 166.6 mg/l (Praskova et al., 2011). The concentration of DFC in this experiment is far lower than those producing mortality for zebrafish embryo, therefore no mortality is expected when embryos are exposed to the sample lab-mixture.

3.6.2. Developmental effects

Hatching time was similar between groups of exposure. In this sense, the exposure of embryos to Manzanares river samples did not produce adverse effects on hatching. Even, exposure to surface waters shortened the time at which embryo hatched. By contrast, those embryos exposed to the lab mixture were not able to hatch during the observation period (48-80 h) or even later (144h). HT_{50} values are showed in follow table (Table 9).

In general, the exposed embryos developed normally or at least similar to controls, and no gross malformations were observed at observation through the stereomicroscope. Embryos exposed to China showed developmental delay, and even some cases of multiple malformations were observed. Also, none of the embryos exposed to the environmental water samples developed tail curvature or malformations.

Table 9. HT_{50} obtained in toxicological assay after 144 h

Samples	HT_{50} hours (95% CI)
Control -	63 (range)
Cont + (2,4-D)	NA
Viveros	56 (range)
Mix-Viveros	Later than 144h
China 100%	NA
China 50%	52 (range)
China 10%	61 (range)
Butarque	56 (range)
NA = Not applicable due to the high mortality **CI = Confident interval (95%)**	

In zebrafish embryos, no effect of DCF on embryonic development was observed by Hallare et al (2004), except delayed hatching at 1 and 2 mg/l. In the same line, Van den Brandhof and Montforts (2010), found that DCF concentrations above 1 mg/l produce specific effects on hatching, with an EC_{50} (72 h) value for DCF 5.3 mg/l. These values are far from the environmental concentration in this study or the concentration prepared in the mixture. However, our mixture produced an adverse effect on hatching, therefore, other components of the mixture or the interaction produce such an adverse effect.

Apart from gross morphology, the biometric parameters evaluation has been successfully used to indicate sublethal effects on development (Fraysse et al., 2006). No edemas were observed in the exposed embryos at visual inspection through the microscope. In this study, the tail length (Figure 11) in the exposed embryos was similar to that of the control group, and no statistical differences were evidenced. Even, larvae exposed to Viveros showed a statistical significant increase in the tail length ($p < 0.05$) in comparison with controls and mixture Viveros. This is not a strange effect, since those larvae grown in surface waters usually are larger than those living in a laboratory water (distilled water plus salts), and it was seen in another studies with surface waters (García-Cambero et al., 2012). In short, the assessment of the tail length did not indicate a negative impact over the growth of the exposed larvae.

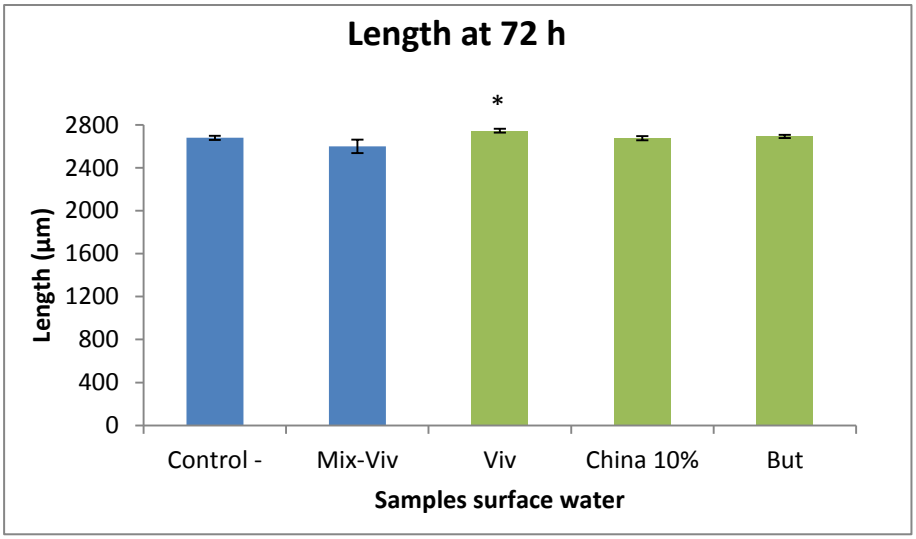


Figure 11. Tail length evaluated in embryos exposed for 3-days. Bars represent means values ± SEM (95 % confidence interval). N = 29, 29, 32, 19 and 38 for the control, Mixture Viveros, Viveros, China 10 and Butarque, respectively. Blue column: lab solution; Green column: real sample *: p<0.05

Many authors consider the moderately reduced growth of zebrafish exposed to concentrations in range of µg/L not a repeatable, treatment-related effect of DCF (Memmert et al., 2013; Chen et al., 2014). Both morphological abnormalities and body deformations in *D. rerio* after DCF exposure were also observed by Van den Brandhof and Montforts (2010) and Praskova et al. (2011) in concentrations above 1.5 mg/L.

3.6.3. Cardiotoxicity

Effects on the heart beat frequency may impact negatively in the embryo respiratory process and thus on the development, hatching success and finally on growth (García-Cambero et al., 2012).

The heartbeat frequency seemed not to be affected in embryos exposed to the Manzanares River waters, and there was not any macroscopic indication of pericardial edema presence. The heart rate was not significantly different ($p<0.05$; t-student test) in embryos exposed to surface waters in comparison to that of the control group. Zebrafish embryos showed an average of 222,5 ± 11, 218.9 ± 19, 218.8 ± 16, 225.0 ± 12, 216.9 ± 15 and 200.0 ± 28 beats/min for control, Butarque, Viveros, Mixture Viveros, China 10 and China 50, respectively (Figure 12). Among the exposed embryos, it is noticeable that those exposed to Mixture Viveros showed the highest value, while those exposed to China 10% and China 50% showed the lowest ones. As expected, embryos incubated with China 50% are highly affected as confirmed by cardiotoxicity and mortality data ($p < 0.01$). Moreover, statistical differences (p <0.05, t-test) were observed in embryos incubated with Viveros regarding those incubated in Mixture Viveros which are highest. In spite of no statistical differences were showed comparing with controls, this fact could mean that in Viveros de la Villa sample exist pollutants which affect heartbeats (decreasing it) which were not taken into account when preparing Mixture Viveros.

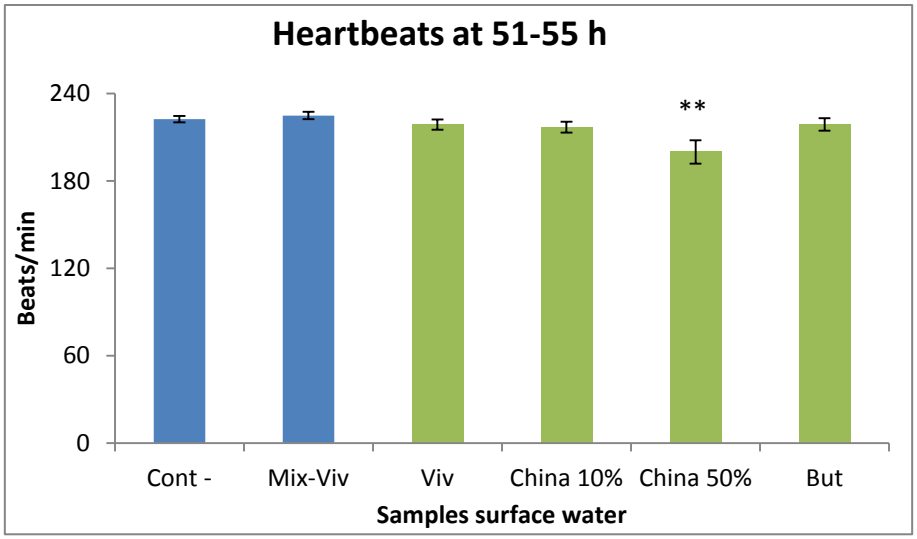


Figure 12 Heartbeat in 2-day larvae exposed to surface waters from Manzanares River. Bars represent means values ± SEM (95 % confidence interval). N = 26, 24, 22, 17, 12 and 20 for the control, Mixture Viveros, Viveros, China 10, China 50, and Butarque, respectively. Blue column: lab solution; Green column: real sample. **p<0.01.

3.6.4. Effects on behaviour (locomotion)

Response to touch is usually observed at 48 hpf onwards. In this experiment, embryos exposed to Viveros and Butarque had low response to stimulation, and embryos exposed to China 50 hardly had any response to touch.

On the other hand, the pattern of locomotion was measured at day 6 of development, time at which the pro-larva has developed autonomous movements. This endpoint has been very sensitive to other surface water exposure (García-Cambero et al., 2012). In this experiment, the total distance travelled by the larvae decreased statistically in Butarque sample regarding control (p < 0.01) (figure 13). Also, a significant difference was observable when compared to embryos exposed to Viveros de la Villa, which is upstream surface water (p < 0.05). No significant differences were evidenced between other exposed groups. Larvae mean velocity also decreased statistically in larvae exposed to Butarque surface water (p < 0.01) (Figure 14). In contrast, larvae exposed to Viveros, Mixture Viveros and China 10 did not show such a significant difference. Therefore, it seems that Butarque induced a negative effect on neurodevelopment in the zebrafish larvae.

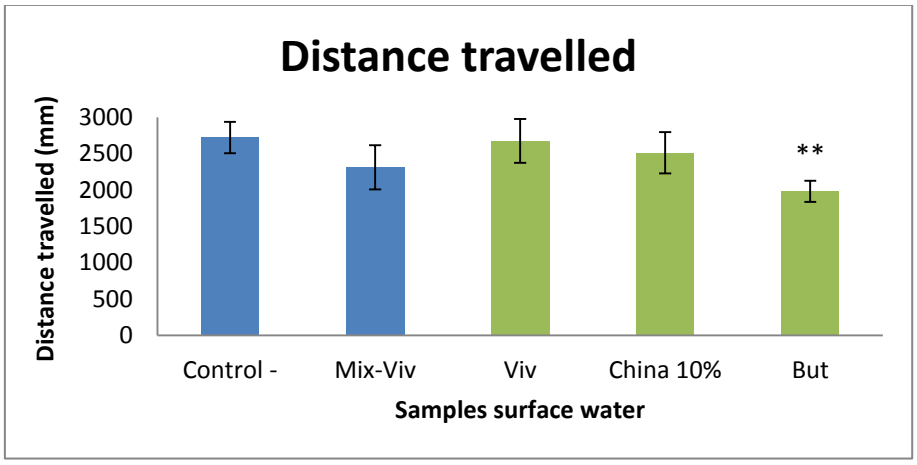


Figure 13 Mean distance travelled in 6-day larvae exposed to surface waters from Manzanares River. Bars represent means values ± SEM (95 % confidence interval). N = 19, 21, 13, 10 and 27 for the control, Mixture Viveros, Viveros, China 10 and Butarque, respectively. **p<0.01

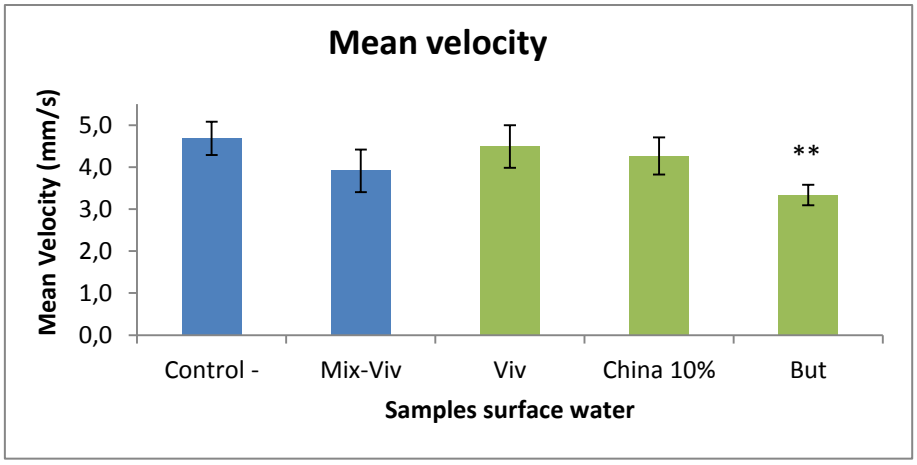


Figure 14 Mean velocity in 6-day larvae exposed to surface waters from Manzanares River. Bars represent means values ± SEM (95 % confidence interval). N = 19, 21, 13, 10 and 27 for the control, Mixture Viveros, Viveros, China 10 and Butarque, respectively. **p<0.01

As a whole, the first point of sampling of Manzanares River, Viveros did not produce any adverse effect on the development of the zebrafish embryo. However, when the zebrafish embryos were exposed to water samples from La China, mortality and developmental effects were evidenced. Even, when La China water was diluted to 10%, as high as 42% of mortality could be still observed. Finally, when embryos were exposed to water samples from Butarque, they exhibited neurodevelopmental effects based on a decrease in the mean velocity and the total distance travelled.

Considering the low toxicity of DFC, E2 and EE2 for zebrafish found in literature, toxicity exerted by waters from La China was due to mixtures of other substances. This WWTP receives water from Madrid households, hospitals and its depurative ability is saturated. When compared to Butarque, this latter has a higher capacity and the emission flow is lower which indicates more effectiveness.

On the other hand, the direct comparison between Viveros and Mix-Viveros effects showed no differences, except for hatching. The mixture prepared in the laboratory have the same composition than the 2nd sampling of Viveros, however it may not have the same bioavailability of the toxic compounds. The sample mixture prepared in the lab contained free DFC, EE2 and E2 dissolved in egg water (distilled water plus salts), but Viveros surface water contained similar levels of the active ingredients but probably not bioavailable totally.

4. Conclusions:

This research was set out to analyse the occurrence and distribution of diclofenac, estradiol and ethinylestradiol included in Watch List according WFD in Manzanares River crossing metropolitan area of Madrid. This kind of study is needed for the evaluation of the risk posed by them for aquatic organism and human health. The most noteworthy results of this study are:

The analytical method appears to be suitable for routine screening of DCF, E2 and EE2 in effluents, sediments and surface water. Further improvements are needed in order to lower recoveries percentages and LOQs. The complex nature of aquatic samples (effluents, river water and sediments) has been demonstrated to give rise to a number of issues, which need to be considered for further developing tools of gathering data and tracing contaminants by LC or GC/MS. Thus, the composition varies considerably between sample type and origin and can affect the method recovery.

Furthermore, it has demonstrated the presence of DCF, E2 and EE2 in most of the samples studied. DCF had important presence in effluents and hormones has been detected mainly in sediments, provided the initial expectations. It would expect higher levels of DCF in sediments, while the study of presence of DCF in suspended solids fraction to a better definition of its distribution is needed.

In the same vein, the influence of emissions from WWTPs has been clear, due to incomplete removal of these substances. Complementary treatment steps such as ozonation to remove PhACs from WWTP effluents could improve water quality. Since continuous release from WWTP occurs, the pseupersistence of the compounds under study is very important factor to take account for their inclusion in Priority Substance List. It is considered that the self-cleaning capacity of the river is compromised by human action.

Regarding the results obtained in this study, it seems evident that a deep monitoring program of these substances in Manzanares' River is needed. Also, we consider their inclusion in the legislation through the Priority Substance List should be seriously considered.

Moreover, supplementary toxicity studies show that DCF, E2 and EE2 contained in surface water do not seem to be the primary cause toxicity to zebrafish embryos, as a parallel study reflects not toxicity occurred. In the same line, toxicological tests showed that La China WWTP produces emissions that are highly toxic to aquatic vertebrates (neurotoxicity, cardiotoxicity, and developmental effects), whereas samples of surface water from Viveros de la Villa and Butarque showed no significant toxicity to aquatic vertebrates. Hence, we consider that the toxicity in some sections of the Manzanares River is due to a complex and diverse mix of pollutants.

References:

- Aguayo, S., De La Torre, A., Carballo, M., Cabrera, R., & Méndez, J. (2010). A review of the most relevant residues of pharmaceuticals products of human use in effluents and sludges from waste water treatment plants. Toxicology Letters, 196.
- Al-Rajab, A. J., Sabourin, L., Lapen, D. R., & Topp, E. (2010). The non-steroidal anti-inflammatory drug diclofenac is readily biodegradable in agricultural soils. Science of the total environment, 409(1).
- Altman R, Bosch B, Brune K, Patrignani P, Young C. Advances in NSAID Development: Evolution of Diclofenac Products Using Pharmaceutical Technology. Drugs. 2015;75. doi:10.1007/s40265-015-0392-z.
- Alvarez, D., Cranor, W., ... & Brent, R. (2008). Reconnaissance of persistent and emerging contaminants in the Shenandoah and James River basins, Virginia, during spring of 2007. U. S. Geological Survey.
- Andreozzi, R., Raffaele, M., & Nicklas, P. (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere, 50(10).
- Aris, A. Z., Shamsuddin, A. S., & Praveena, S. M. (2014). Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. Environment international., 69.
- Azzouz, A., & Ballesteros, E. (2012). Combined microwave-assisted extraction and continuous solid-phase extraction prior to gas chromatography–mass spectrometry determination of pharmaceuticals, personal care products and hormones in soils, sediments and sludge. Science of the Total Environment, 419.
- Barcelo, D. and Petrovic, M. (2006) Reducing the Environmental Risk from Emerging Pollutants. TrAC. Trends Anal. Chem., 25.
- Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., & Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in receiving river water. Environmental Science & Technology, 34(24).
- Beausse, J. (2004). Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. TrAC Trends in Analytical Chemistry, 23(10).
- Brack, W., Altenburger, R., Schüürmann, G., Krauss, M., Herráez, D. L., van Gils, J., ... & de Aragão U., G. (2015). The SOLUTIONS project: challenges and responses for present and future emerging pollutants in land and water resources management. Science of the Total Environment, 503.
- Bueno, M. M., Gomez, M. J., Herrera, S., Hernando, M. D., Agüera, A., & Fernández-Alba, A. R. (2012). Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: two years pilot survey monitoring. Environmental Pollution, 164.
- Buser, H. R., Poiger, T., & Müller, M. D. (1998). Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake. Environmental Science & Technology, 32(22).
- Caldwell, D. J., Mastrocco, F., Anderson, P. D., Länge, R., & Sumpter, J. P. (2012). Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. Environmental Toxicology and Chemistry, 31(6).
- Carballa, M., Omil, F., Lema, J.M., 2008. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. Chemosphere 72.
- Carvalho, R., Ceriani, L., Ippolito, A. and Lettieri, T. , 2014. Development of the 1st Watch List under Environmental Quality Standars Directive. JRC Science and policy reports. Directive 2013/39/EU in the field of water policy.
- Céspedes, R., Petrovic, M., Raldúa, D., Saura, Ú., Piña, B., Lacorte, S., ... & Barceló, D. (2004). Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediment by use of the biological technique recombinant yeast assay and chemical analysis by LC–ESI-MS. Analytical and bioanalytical chemistry.
- Chen, J. B., Gao, H. W., Zhang, Y. L., Zhang, Y., Zhou, X. F., Li, C. Q., & Gao, H. P. (2014). Developmental toxicity of diclofenac and elucidation of gene regulation in zebrafish (Danio rerio). Scientific reports, 4.
- Christiansen, L. B., Winther-Nielsen, M., & Helweg, C. (2002). Feminisation of fish. The effect of estrogenic compounds and their fate in sewage treatment plants and nature, Environmental Project, 729.
- COM (1999) 706. EC (1999): Communication on the Community Strategy on Endocrine Disrupters. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:1999:0706:FIN:EN:PDF

- COM (2011) 876. European Community document. Directive of the European Parliament and of the council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Available:http://ec.europa.eu/smart-regulation/impact/ia_carried_out/docs/ia_2012/com_2011_0876_en.pd
- Confederación Hidrográfica del Tajo. Capítulo III. Agua Comunidad de Madrid. Available: http://www.madrid.org/cs/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobkey=id&blobtable=MungoBlobs&blobwhere=1119126717339&ssbinary=true.
- da Silva, B. F., Jelic, A., López-Serna, R., Mozeto, A. A., Petrovic, M., & Barceló, D. (2011). Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediment of the Ebro river basin, Spain. Chemosphere, 85(8).
- Commission implementing decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council
- Directive, C. (1991). 91/271. EEC of, 21, 40. COUNCIL DIRECTIVE of 21 May 1991 concerning urban waste water treatment.
- Djerassi, C. (2006). Chemical birth of the pill. American journal of obstetrics and gynecology, 194(1).
- European Commission (EC, 2000). Directive 2000/60/EU of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Offic. J. Europ. Union L327.
- European Commission (EC, 2008). Priority Substances Daughter Directive - Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy.
- European Commission (EC, 2013). Directive 2013/39/EU of the European Parliament and of the council of 12 August 2013 amending Directive 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. European Union (2013). Official Journal of the European Union.
- Esteban, S., Gorga, M., Petrovic, M., González-Alonso, S., Barceló, D., & Valcárcel, Y. (2014). Analysis and occurrence of endocrine-disrupting compounds and estrogenic activity in the surface waters of Central Spain. Science of the Total Environment, 466.
- Estévez, M. C., Font, H., Nichkova, M., Salvador, J. P., Varela, B., Sánchez-Baeza, F., & Marco, M. P. (2005). Immunochemical determination of pharmaceuticals and personal care products as emerging pollutants. In Water Pollution (pp. 181-244). Springer Berlin Heidelberg.
- Feng, Y., Zhang, Z., Gao, P., Su, H., Yu, Y., & Ren, N. (2010). Adsorption behavior of EE 2 (17α-ethinylestradiol) onto the inactivated sewage sludge: Kinetics, thermodynamics and influence factors. Journal of hazardous materials, 175(1).
- Fent, K., Weston, A. A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. Aquatic toxicology, 76(2).
- Fernández, C., González-Doncel, M., Pro, J., Carbonell, G., & Tarazona, J. V. (2010). Occurrence of pharmaceutically active compounds in surface waters of the Henares-Jarama-Tajo River system (Madrid, Spain) and a potential risk characterization. Science of the Total Environment, 408(3).
- Ferrari, B., Paxeus, N., Giudice, R. L., Pollio, A., & Garric, J. (2003). Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicology and Environmental Safety, 55(3).
- Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxēaus, N., Giudice, R. L., ... & Garric, J. (2004). Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environmental toxicology and chemistry, 23(5).
- Fraysse, B., Mons, R., & Garric, J. (2006). Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotoxicology and environmental safety, 63(2).
- Froehner, S., Machado, K. S., Stefan, E., Bleninger, T., da Rosa, E. C., & de Castro Martins, C. (2012). Occurrence of selected estrogens in mangrove sediment. Marine pollution bulletin, 64(1).
- García-Cambero, J. P., Catalá, M., & Valcárcel, Y. (2012). River waters induced neurotoxicity in an embryo–larval zebrafish model. Ecotoxicology and environmental safety, 84.
- Gibson, R., Becerril-Bravo, E., Silva-Castro, V., & Jiménez, B. (2007). Determination of acidic pharmaceuticals and potential endocrine disrupting compounds in wastewaters and spring waters by selective elution and analysis by gas chromatography–mass spectrometry. Journal of Chromatography A, 1169(1).
- Gomes, R. L., Avcioglu, E., Scrimshaw, M. D., & Lester, J. N. (2004). Steroid-estrogen determination in sediment and sewage sludge: a critique of sample preparation and chromatographic/mass spectrometry

considerations, incorporating a case study in method development. TrAC Trends in Analytical Chemistry, 23(10).

- Gorga, M., Insa, S., Petrovic, M., & Barceló, D. (2015). Occurrence and spatial distribution of EDCs and related compounds in waters and sediment of Iberian rivers. Science of the Total Environment, 503.
- Gröning J. Held C., Garten C., Claussnitzer U., Kaschabek S.R., Schlömann M., 2007, Transformation of diclofenac by the indigenous microflora of river sediment and identification of a major intermediate, Chemosphere69.
- Gros, M., Petrović, M., Ginebreda, A., & Barceló, D. (2010). Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment international. 36(1).
- Hallare AV, Köhler HR, Triebskorn R (2004) Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56.
- Hartmann, J., Bartels, P., Mau, U., Witter, M., Tümpling, W. V., Hofmann, J., & Nietzschmann, E. (2008). Degradation of the drug diclofenac in water by sonolysis in presence of catalysts. Chemosphere, 70(3).
- Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology letters, 131(1).
- Heberer T. , Mechlinski A., Franck B., Knappe A., Massmann G., Pekdeger A., Fritz B., (2004), Field Studies on the Fate and Transport of Pharmaceutical Residues in Bank Filtration, Ground Water Monitoring & Remediation 24.
- Heberer, T., & Ternes, T. (2006). Residues of pharmaceuticals from human use. Partition and Adsorption of Organic Contminants in Environmental Systems.
- Hernando, M. D., Mezcua, M., Fernández-Alba, A. R., & Barceló, D. (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediment. Talanta, 69(2).
- Hertzman, H. (2015). Pharmaceuticals in groundwater: a literature review. Dissertations in Geology at Lund University.
- Hidalgo, D., & Irusta, R. (2005, July). The cost of wastewater reclamation and reuse in agricultural production in the Mediterranean countries. In IWA Conference on Water Economics, Statistics and Finance, Rethymo.
- Hignite, C., & Azarnoff, D. L. (1977). Drugs and drug metabolites as environmental contaminants: chlorophenoxyisobutyrate and salicylic acid in sewage water effluent. Life Sciences, 20(2).
- Huang, C. H., & Sedlak, D. L. (2001). Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environmental Toxicology and Chemistry, 20(1).
- Huerta, B., Jakimska, A., Gros, M., Rodríguez-Mozaz, S., & Barceló, D. (2013). Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1288.
- Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. Widespread sexual disruption in wild fish. Environ Sci Technol 1998.
- Johnson, A. C., Dumont, E., Williams, R. J., Oldenkamp, R., Cisowska, I., & Sumpter, J. P. (2013). Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards?. Environmental science & technology, 47(21).
- Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C. S., ... & Siegrist, H. (2006). Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. Water research, 40(8).
- Koutsouba, V., Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., & Hiskia, A. (2003). Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography–mass spectrometry. Chemosphere, 51(2).
- Kujawa-Roeleveld, K. (2008) Biodegradability and fate of pharmaceutical impact compounds in different treatment processes. Sustainable Water Management in the City of the Future. 018530 – SWITCH.
- Kuster, M., de Alda, M. J. L., & Barceló, D. (2005). Estrogens and progestogens in wastewater, sludge, sediment, and soil. In Water Pollution (pp. 1-24). Springer Berlin Heidelberg.
- Labadie, P., & Hill, E. M. (2007). Analysis of estrogens in river sediment by liquid chromatography–electrospray ionisation mass spectrometry: comparison of tandem mass spectrometry and time-of-flight mass spectrometry. Journal of Chromatography A, 1141(2).
- Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., & Braunbeck, T. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test?. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 149(2).

- Lee, L. S., Strock, T. J., Sarmah, A. K., & Rao, P. S. C. (2003). Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soils and sediment. Environmental science & technology, 37(18).
- Loos, R., Carvalho, R., Antonio, D. C., Comero, S., Locoro, G., Tavazzi, S., ... & Gawlik, B. M. (2013). EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water research, 47(17).
- López-Roldán, R., de Alda, M. L., Gros, M., Petrovic, M., Martín-Alonso, J., & Barceló, D. (2010). Advanced monitoring of pharmaceuticals and estrogens in the Llobregat River basin (Spain) by HPLC–MS/MS in combination with UPLC–TOF-MS. Chemosphere, 80(11).
- Martín, J., Santos, J. L., Aparicio, I., & Alonso, E. (2010). Multi-residue method for the analysis of pharmaceutical compounds in sewage sludge, compost and sediments by sonication-assisted extraction and LC determination. Journal of separation science, 33(12).
- Mathias JR, Saxena MT, Mumm JS. Advances in zebrafish chemical screening technologies. Future medicinal chemistry. 2012;4(14).
- Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J. P., & Hartmann, A. (2013). Diclofenac: new data on chronic toxicity and bioconcentration in fish. Environmental Toxicology and Chemistry, 32(2).
- Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. December 2007. U.S. Environmental Protection Agency. Office of Water. Office of Science and Technology. Engineering and Analysis Division.
- Miklósi, Á., & Andrew, R. J. (2006). The zebrafish as a model for behavioral studies. Zebrafish, 3(2).
- Nugent AG, Leung KC, Sullivan D, Reutens AT, Ho KK (2003). "Modulation by progestogens of the effects of oestrogen on hepatic endocrine function in postmenopausal women". Clin. Endocrinol. (Oxf) 59 (6).
- Osorio, V., Marcé, R., Pérez, S., Ginebreda, A., Cortina, J. L., & Barceló, D. (2012). Occurrence and modeling of pharmaceuticals on a sewage-impacted Mediterranean river and their dynamics under different hydrological conditions. Science of the Total Environment, 440.
- Padilla, S., Hunter, D. L., Padnos, B., Frady, S., & MacPhail, R. C. (2011). Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicology and teratology, 33(6).
- Pardo-Martín C, Chang T, Koo BK, Gilleland CL, Wasserman SC, Yanik MF. High-throughput in vivo vertebrate screening. Nat. Methods. 2010;7.
- Petrovic, M., Solé, M., López De Alda, M. J., & Barceló, D. (2002). Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environmental Toxicology and Chemistry, 21(10).
- Petrovic, M., Gonzalez, S., & Barceló, D. (2003). Analysis and removal of emerging contaminants in wastewater and drinking water. TrAC Trends in Analytical Chemistry, 22(10).
- Praskova, E., Voslarova, E., Plhalova, L, Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Kruzikova K., Hostovsky, M., Skoric, M., Svobodova, Z. Subchronic effect of diclofenac to zebrafish (Danio rerio). Pol. J. Vet. Sci., submitted-b.
- Purdom, C. E., Hardiman, P. A., Bye, V. V. J., Eno, N. C., ... (1994). Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology.
- Richardson, S. D., & Ternes, T. A. (2011). Water analysis: emerging contaminants and current issues. Analytical chemistry, 83(12).
- Salgado, R., Pereira, V. J., Carvalho, G., Soeiro, R., Gaffney, V., Almeida, C., ... & Noronha, J. P. (2013). Photodegradation kinetics and transformation products of ketoprofen, diclofenac and atenolol in pure water and treated wastewater. Journal of hazardous materials.
- Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., & Negele, R. D. (2004). Toxic effects of the nonsteroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquatic Toxicology, 68(2).
- Segner, H., Caroll, K., Fenske, M., Janssen, C. R., Maack, G., Pascoe, D., ... & Wenzel, A. (2003). Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicology and environmental safety, 54(3).
- Selderslaghs, I. W., Hooyberghs, J., De Coen, W., & Witters, H. E. (2010). Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity. Neurotoxicology and teratology, 32(4).
- Servos, M. R., Bennie, D. T., Burnison, B. K., Jurkovic, A., McInnis, R., Neheli, T., ... & Ternes, T. A. (2005). Distribution of estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. Science of the Total Environment, 336(1).

- Solé, M., Porte, C., & Barcelo, D. (2000). Vitellogenin induction and other biochemical responses in carp, Cyprinus carpio, after experimental injection with 17α-ethynylestradiol. Archives of environmental contamination and toxicology, 38(4).
- Sun, K., Jin, J., Gao, B., Zhang, Z., Wang, Z., Pan, Z., ... & Zhao, Y. (2012). Sorption of 17α-ethinyl estradiol, bisphenol A and phenanthrene to different size fractions of soil and sediment. Chemosphere, 88(5).
- Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. Water research, 32(11).
- Ternes, T. A., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H. J., Haist-Gulde, B., ... & Zulei-Seibert, N. (2002). Removal of pharmaceuticals during drinking water treatment. Environmental science & technology, 36(17).
- http://teqma.com/noticias/tratamiento-terciario-avanzado-en-la-edar-de-viveros-de-la-villa
- ter Laak, T. L., van der Aa, M., Houtman, C. J., Stoks, P. G., & van Wezel, A. P. (2010). Relating environmental concentrations of pharmaceuticals to consumption: a mass balance approach for the river Rhine. Environment international, 36(5).
- Thomas, K. V., & Hilton, M. J. (2004). The occurrence of selected human pharmaceutical compounds in UK estuaries. Marine Pollution Bulletin, 49(5).
- Tixier, C., Singer, H. P., Oellers, S., & Müller, S. R. (2003). Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environmental science & technology, 37(6).
- Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H. R., & Schwaiger, J. (2004). Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (Oncorhynchus mykiss). Aquatic toxicology, 68(2).
- UNE-EN ISO 7346-3:1998. Calidad del agua. Determinación de la toxicidad letal aguda de las sustancias frente a un pez de agua dulce. [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprimidae)]. Parte 3: Método de flujo continúo.
- Vader, J. S., Van Ginkel, C. G., Sperling, F. M. G. M., De Jong, J., De Boer, W., De Graaf, J. S., ... & Stokman, P. G. W. (2000). Degradation of ethinyl estradiol by nitrifying activated sludge. Chemosphere, 41(8).
- Valcárcel, Y., Alonso, S. G., Rodríguez-Gil, J. L., Gil, A., & Catalá, M. (2011). Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere, 84(10).
- Van den Brandhof, E.J., Montforts, M., 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotoxicol. Environ. Saf. 73.
- Vazquez-Roig, P., Segarra, R., Blasco, C., Andreu, V., & Picó, Y. (2010). Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1217(16).
- WHO/IPCS. 2002. Global assessment of the state-of-the-science of endocrine disruptors. World Health Organization/International Program on Chemical Safety. WHO/PCS/EDC/02.2.
- Ying, G. G., Kookana, R. S., & Ru, Y. J. (2002). Occurrence and fate of hormone steroids in the environment. Environment international, 28(6).
- Yoon, Y., Westerhoff, P., & Snyder, S. A. (2005). Adsorption of 3H-labeled 17-β estradiol on powdered activated carbon. Water, Air, and Soil Pollution, 166(1-4).
- Zhang, Y., Geißen, S. U., & Gal., C. (2008). Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere, 73(8).
- Zhan, J., ... & Peng, J. F. (2009). Determination of Multi-residue of Anabolic Steroids by Gas Chromatography-Mass Spectrometry [J]. *Chinese Journal of Analytical Chemistry*.
- Zhang, X., Gao, Y., Li, Q., Li, G., Guo, Q., & Yan, C. (2011). Estrogenic compounds and estrogenicity in surface water, sediment, and organisms from Yundang Lagoon in Xiamen, China. Archives of environmental contamination and toxicology, 61(1).
- Zhou, y. q., Wang, z. j., & Ning, J. I. A. (2007). Formation of multiple trimethylsilyl derivatives in the derivatization of 17α-ethinylestradiol with BSTFA or MSTFA followed by gas chromatography-mass spectrometry determination. Journal of Environmental Sciences, 19(7).
- Zhou, X., Lian, Z., Wang, J., Tan, L., & Zhao, Z. (2011). Distribution of estrogens along Licun River in Qingdao, China. Procedia Environmental Sciences, 10.

Annex 1:

Table 10. Physico chemical parameter from sampling points downstream of WWTPs measured *in situ*.

First sampling campaing							
Sampling points	**pH**	**E.C. (µS/cm)**	**O_2 (mg/l)**	**O_2 (% sat)**	**Tª (ºC)**	**Sampling Date**	**Hour**
Pardo (Referencia)	7,58	153	9,4	83	7,8	05/03/2015	11
Viveros de la Villa	7,1	391	8,5	84	12,4	05/03/2015	12
China	7,34	696	7,45	81	16,1	03/03/2015	15
Butarque	7,45	892	6,7	71	14,9	03/03/2015	13
Sur	7,41	1051	7,53	81	17,3	05/03/2015	13
Arroyo Culebro	7,09	1242	7,84	85	15,9	03/03/2015	11
Second sampling campaing							
Sampling points	**pH**	**E.C. (µS/cm)**	**O_2 (mg/l)**	**O_2 (% sat)**	**Tª (ºC)**	**Sampling Date**	**Hour**
Pardo	7,1	166	8,25	81,6	11,7	07/04/2015	10
Viveros de la Villa	6,7	422,2	8,3	91,6	13,4	07/04/2015	11
China	7,2	610	7,37	78,1	13,5	08/04/2015	10
Butarque	7,4	907,3	7,15	74,7	16,5	07/04/2015	13
Sur	7,33	1022	7,05	80,7	17,6	08/04/2015	12
Arroyo Culebro	7	1351	8,79	101,5	18,9	08/04/2015	14

Annex 2:

Table 11 Concentration of diclofenac measured in different environmental compartment of Manzanares River

DICLOFENAC												
SAMPLES	EFFLUENTS (ng/L)				SURFACE WATER (ng/L)				SEDIMENT (ng/g)			
LOQ	67 ng/L				2 ng/L				0.78 ng/g			
SAMPLING DATE	March		April		March		April		March		April	
SAMPLING POINT	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
El Pardo	NA	NA	NA	NA	NA	NA	2,9	0,9	NA	NA	1.6	0.3
Viveros	309.3	73.0	1241.0	9.9	4,0	1,7	167.3	7.9	12.4	7.5	ND	NAp
La china	751.5	7.5	1096.4	38.4	33.4	14,7	78.6	1.3	ND	NAp	ND	NAp
Butarque	284.7	59.2	1117.1	177.6	70.9	11,1	157.0	63.9	8.9	1.5	ND	NAp
Sur	594.2	42.3	1667.2	11.7	27.2	11,8	100.4	33.8	15.0	1.7	ND	NAp
Arroyo Culebro	784.1	76.6	1164.9	504.4	29.2	6,04	68.4	59.8	8.1	0.3	ND	NAp

ND: Non detected; NA: Not available; Nap: Not Applicable; LOQ: Limit of Quantification.

Table 12 Concentrations of estradiols measured in different environmental compartment of Manzanares River

17-β-ESTRADIOL												
SAMPLES	EFFLUENTS (ng/L)				SURFACE WATER (ng/L)				SEDIMENT (ng/g)			
LOQ	10 ng/L				4.44 ng/L				0.32 ng/g			
SAMPLING DATE	March		April		March		April		March		April	
SAMPLING POINT	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
El Pardo	NA	NA	NA	NA	NA	NA	ND	NAp	NA	NA	4,8	2,7
Viveros	11	3	<LOQ	NAp	ND	NAp	<LOQ	NAp	0,7	0,3	3,4	1,6
La China	<LOQ	NAp	<LOQ	NAp	ND	NAp	ND	NAp	3,9	3.0	6,2	4,2
Butarque	<LOQ	NAp	ND	NAp	229	182.9	ND	NAp	2,8	1.4	4,2	1.1
Sur	<LOQ	NAp	<LOQ	NAp	56,5	28.8	ND	NAp	1,0	0,7	5,8	0,8
Arroyo Culebro	<LOQ	NAp	<LOQ	NAp	ND	NAp	ND	NAp	5,2	5.0	4,6	1,5
17-α-ETHINILESTRADIOL												
SAMPLES	EFFLUENTS (ng/L)				SURFACE WATER (ng/L)				SEDIMENT (ng/g)			
LOQ	10 ng/L				6.96 ng/L				2.16 ng/g			
SAMPLING DATE	March		April		March		April		March		April	
SAMPLING POINT	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
El Pardo	NA	NA	NA	NA	NA	NA	ND	NAp	NA	NA	5,1	1,5
Viveros	ND	NAp	23	NA	ND	NAp	<LOQ	NAp	<LOQ	NAp	6,1	1,8
La China	<LOQ	NAp	19	NA	ND	NAp	ND	NAp	<LOQ	NAp	21,6	14,3
Butarque	<LOQ	NAp	32	NA	226	173,1	ND	NAp	2,7	2,9	4,3	2,3
Sur	ND	NAp	115	NA	86	23.2	ND	NAp	15.3	5,9	8,4	2,3
Arroyo Culebro	<LOQ	NAp	28	NA	ND	NAp	ND	NAp	<LOQ	NAp	8,4	3,5

ND: Non detected; NA: Not available; Nap: Not applicable; LOQ: Limit of Quantification.