Passive *in-situ* estrogenic potency sampling with DGT-YES

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HELENA IUELE

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ABSTRACT

The purpose of this research was to investigate the presence of bisphenolic plastics and synthetic estrogens in municipal water systems, as well as along the Waikato River. The concentration of these compounds was assessed using a novel combination of methods that allowed the actual levels of endocrine disruption to be ascertained. This was based on diffusive gradients in thin films (DGTs) coupled with high-performance liquid chromatography/mass spectrometry analysis (HPLC/MS) and the Yeast Estrogen Screen (YES).

DGT estimated time-integrated concentrations while HPLC/MS ensured a robust, automated and sensitive routine analysis. Most significantly, YES, a biomarker-based assay, was deployed to quantify the total estrogenic potency of the compounds adsorbed to the DGT matrix. The unique dimension of this research was to extend the application of DGTs to estrogen sampling and to combine the DGT and YES technique to provide in situ quantification of the estrogenic potency of water bodies through time.

DGTs were developed specifically for the sampling of BPA, BPAF, E2 and EE2. Several physical-chemical and chemical analyses were conducted to attest the suitability of the agarose (1.5%) and agarose (1.5%) – activated charcoal (1%) chosen respectively as diffusive gel and binding gel. They were found to be suitable for the detection of organic molecules with a bigger steric hindrance and agarose (1.5%) – AC (1%) appeared suitable for long deployments thanks to its high thermal resistance. An HPLC-MS method was optimised to quantify the compounds of interest. A methanol (MeOH, NH_4OH 0.06 M)/water (H_2O, NH_4OH 0.06 M) fast gradient elution program of 15 min was chosen to elute the analytes, MS parameters were optimized for each compound and the negative mode was selected to perform the fragmentation. LODs and LOQs of all targets resulted in the nanomolar range assuming 24 h as deployment time at 25°C with a 0.54 mm thick diffusive gel. The sensitivity of the method was increased in the sub-nanomolar range by adopting long deployment times (18 days) thus enhancing the accumulation of the targets.

The YES was successfully developed to quantify the total estrogenic potency of the DGTs eluted. The dose-response calibration curve of E2, employed as a control, demonstrated LOD and experimental EC_{50} values in the sub-nanomolar range. EE2, BPA and BPAF demonstrated agonistic endocrine activity, in particular EE2 being more potent than E2, while BPAF and BPA were less potent.

The environmental monitoring assessed the efficiency of removal of the selected ECs during the water treatment processes from river-to-tap and effluent-to-treated wastewater in Hamilton as well the water quality from source-to-outfall of the Waikato River. The HPLC-MS and YES results highlighted that primary treatments were not suitable for the removal of bisphenolic plastics and estrogens both at drinkable and wastewater treatment plants in Hamilton where they appear to worsen the water quality making these pollutants more available. At both treatment plants, the concentration and the estrogenic activity of the targets appeared to fluctuate around the same value, pointing out the inefficiency of removal of these compounds with the actual treatments. EE2 contributed the most to the estrogenic activity due to its higher concentration and higher potency at all sites.

The concentration and the estrogenic potency of all targets were found to be higher downstream at all sites monitored along the Waikato River. EE2 was the compound with the highest concentration at all sites. A moderate worsening of water quality was apparent moving from Taupo to Tuakau. The most polluted sites were Hamilton and Huntley after the outlets of wastewater treatment plants that serve the main conurbations in the area. The EDC concentrations recorded in November 2017 and February 2018 during the monitoring of the Waikato River at Hamilton upstream and downstream sites showed a significant dilution due to the weather conditions.

The data from a previous monitoring of the Waikato River adopting an SPE-HRGC/MS method[1] were compared with those of the DGT-HPLC/MS investigation. The water quality appeared slightly worsened at all sites from 2013 to 2018 due to increased concentrations of BPA and EE2.

The DGT-HPLC/MS quantification with the YES assay proved that not only active samplings but passive samplings as well can be coupled with a bioassay. This combined approach had the excellent potential to provide a reliable total estrogenic potency evaluation with time integrated concentrations. The use of the assay allowed estimation of the total estrogenic potency of the eluted DGT. The eluate appeared enriched by other compounds with estrogenic activity, suggesting this type of DGT could be suitable for the sampling and accumulation of other analytes that could be further investigated via HPLC/MS analysis. Compared to the traditional active approaches, DGT ensured sampling and concentration in one step, was easy to prepare and to use, required a very simple sample preparation step prior to analysis, and provided sensitivity in the sub-nanomolar range comparable to active sampling analyses. The YES assay was responsive, robust and easy to use compared to other assays that require laborious procedures and longer times for estrogenic potency evaluation and high sensitivity in the sub-nanomolar range comparable to other assays.

CONFERENCES

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School of Science General Assembly aka Graduate and Postgraduate conference. 19 October 2015, The University of Waikato, Hamilton, New Zealand.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4-MBC	3-(4-Methylbenzylidene)-camphor
AC	Activated Charcoal
ADP	Adenosine Diphosphate
ANZECC	Australian and New Zealand Environment and Conservation Council
ANOVA	Analysis of Variance
BCF	Bioconcentration Factor
BFRs	Brominated Flame Retardants
BMF	Biomagnification Factor
BPA	Bisphenol A
BPAF	Bisphenol AF
BP-3	Bexophenome-3
BPS	Bisphenol S
BTA	Benzotriazole
CE	Collision Energy
CPRG	Chlorophenol Red-β-Galactopyranoside
CID	Collision-induced Dissociation
CPP	Central Precocious Puberty
DBPs	Disinfection by-Products
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DEGME	Methoxydiglycol
DEHP	Di(ethylhexyl)phthalate
DGT	Diffusive Gradients in Thin-films
DMSO	Dimethyl Sulfoxide
DRP	Dissolved Reactive Phosphorus
E1	Estrone
α-E2	17α-Estradiol
β-E2	17β-Estradiol
E3	Estriol
EE2	17α-Ethinylestradiol
EEF	17β-Estradiol equivalency factor

EEQ	17β-Estradiol equivalency quantity
cEEQ	Calculated 17β-Estradiol equivalency quantity
ECs	Estrogenic Compounds
EC_{50}	Concentration of agonist (hormone) needed to reach the 50% of the signal
EC_{90}	Concentration of agonist (hormone) needed to reach the 90% of the signal
EDCs	Endocrine Disrupting Chemicals
EDX	Energy-Dispersive X-ray Spectroscopy
EGBE	2-Butoxyethanol
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
EREs	Estrogen Response Elements
ETC	Electron Transport Chain
FFA	Free Fatty Acids
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HABs	Harmful Algal Blooms
hER	Human Estrogen Receptor
HCHs	Hexachlorocyclohexanes
HMS	Homosalate
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
K_{OW}	n-Octanol/Water Partition Coefficient
L_D	Lipid on Dry Weight
L_W	Lipid on Wet Weight
LH	Luteinizing Hormone
LOD	Limit of Detection
LOQ	Limit of Quantification
Lsd	Least Significant Differences
MES	Mestranol
MMTV	Biological Carcinogen which induces somatic mutations as consequence to its integration into the host cellular genome[2].
MRM	Multiple Reaction Monitoring

MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
mtDNA	Mitochondrial Deoxyribonucleic Acid
nDNA	Nuclear Deoxyribonucleic Acid
NAs	Naphthenic Acid
NNK	Nicotine-Derived Nitrosamine Ketone
NPs	Nanoparticles
OD-PABA	Octyl-dimethyl-PABA
OMC	Octyl-methoxycinnamate
PAE	Phthalate Ester
PAH	Polycyclic Aromatic Hydrocarbons
PBBs	Polybrominated Biphenyls
PBDEs	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated Biphenyls
PCDDs	Polychlorinated Dibenzo-*p*-Dioxins
PCDFS	Polychlorinated Dibenzo Furans
PCOS	Polycystic Ovary Syndrome
PET	Polyethylene Terephthalate
PFA	Perfluoroalkoxy Alkane
PFCs	Perfluorinated Chemicals
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane Sulfonate
POCIS	Polar Organic Contaminant Integrative Sampler
POPs	Persistent Organic Pollutants
PPP	Peripheral Precocious Puberty
PVC	Polyvinyl Chloride
ROS	Reactive Oxygen Species
SD	Standard Deviation
SMF	Sulfonated Melamine Formaldehyde
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
t-NP	Technical Nonylphenols Equivalents
TBBPA	Tetrabromobisphenol A
TBT	Tributyltin

TCC	Tauranga City Council
TGC	Testicular Germ Cell Cancer
TIE	Toxicity Identification and Evaluation Procedures
U.S. EPA	Unite States Environmental Protection Agency
Vtg	Vitellogenin
WWTP	Waste Water Treatment Plant
YES	Yeast Estrogen Screen

1 INTRODUCTION

1.1 Outline of the thesis

Chapter one starts with a brief summary of the thesis. Then, the purpose of the study is investigated and an ethical upon the investigation is declared. After that, the chapter focuses on the literature review of the endocrine disruptors (EDCs) pollutants. The different classes of EDCs, their mechanism of action, their environmental and biological impact are discussed. Later, the chapter talks about the study location represented by the Waikato River. In this section the results arose from previous ECDs monitoring in the Waikato River are evaluated and summarizes. Finally, a state of the state of the art of the analytical techniques and in vitro bioassays available to monitor the EDCs concentration and disruption is presented. Pros and cons of the different approaches are discussed.

Chapter two discusses the diffusive gradients in thin films (DGTs) coupled with the high performance liquid chromatography-mass spectrometry (HPLC-MS) as analytical approach for the environmental monitoring. This section describes in detail all the protocols and then, the results obtained from the physical-chemical and chemical characterizations employed to optimized the DGT-HPLC/MS technique for the quantification of the targets endocrine disruptors. The chapter includes also the first environmental trials of the method thus implemented.

Chapter three talks about the *in situ* concentration monitoring of the selected EDCs through the drinking and wastewater treatment processes in Hamilton and in the fresh water of the Waikato River source-to-outfall. This chapter elucidates the protocols of the sampling and discusses the results of the analysis by making comparisons with the literature and with the results of previous monitoring of the same locations. DGT performances are also compared with those of a validated methodology.

Chapter four focuses on the correlation of the EDCs analytical dosages with their total estrogenic potency adopting the yeast estrogen screen (YES) assay as biomarker-based assay. The implementation and calibration of the YES assay and the protocol employed to analyse the DGT samples via this assay are described. The results of the DGT samples, analysed via YES assay, are discussed. The YES assay results are compared with the results arose from the monitoring of the same sampling locations using another validated bioassay.

Chapter five summarizes and discusses in detail all the funding of the monitoring of the drinking and wastewater treatment plants in Hamilton and of the Waikato River conducted adopting the DGT-HPLS/MS and DGT-YES methodologies. This chapter states in a critical way the new information arose from this study and the areas that need further investigation as well.

1.2 Target compounds and aim of the thesis

The New Zealand organic materials guidelines drafted by the Ministry for the Environment and the New Zealand Water Wastes Association are designed to safeguard the life-supporting capacity of soils and waters, promote their responsible use, protect public health and the environment and minimise risk to the New Zealand economy[3].

However, at this stage, there is not enough information to derive New Zealand specific limits for some classes of pollutants, in particular, endocrine disrupting chemicals (EDCs) such as steroids and pharmaceuticals, bisphenols, nonylphenols, flame retardants, antimicrobial and cleaning agents and persistent herbicides. Thus, the purpose of this research was to investigate the environmental concentration and the estrogenic potency of some potentially harmful unregulated classes of EDCs in fresh waters and throughout water process plants.

Hormones and the bisphenolic plastics were chosen as selected targets because of particular concern. Synthetic steroid sex hormones, such as 17α-Ethynylestradiol (EE2), have the highest estrogenic potency among EDCs and their carcinogenic action has been proven[4,5]. Estrogens are metabolized in the body to sulfate and glucuronide conjugates and then eliminated in urine. The most prevalent

conjugates found in adult female urine are estriol (E3), 17β-estradiol (β-E2), and estrone (E1)[6] and their concentrations are in the order of μg mL^{-1}. Conjugated estrogens can also be found in prescription medications as sulfated estrogen salts to treat hormonal imbalances, post-menopausal symptoms, and osteoporosis, among other conditions[7]. These conjugates are less active but they are often converted back into their free forms during water treatment processes and regain their potency[8,9,10]. The relatively low polarity of these compounds also corresponds to high octanol-water partition coefficients, between 10^3 and 10^6, which enhance the adsorption of these pollutants to bed sediments[11]: between 13% and 92% of the estrogens entering a river system end up in the bed sediment compartment with the majority of adsorption occurring within the first 24 h of contact[12]. River sediments can therefore act as sinks where hormones may persist for long periods of time, be transported to other areas and be eventually released back to the water column[13]. Furthermore, synthetic estrogens such as mestranol and EE2 have been shown to partition to the sediment to a greater extent than natural estrogens[13]. Only a few studies have reported the occurrence of estrogens in river and marine sediments and according to these works they tend to accumulate in sediments reaching the pg g^{-1} or ng g^{-1} level[14,15]. It has been also observed that exposure to environmental estrogens results in high bioaccumulation: the bile of fish caged downstream of sewage treatment plants (STPs) contained estrogenic substances at concentrations 104-106 times higher than water levels[16,17].

Bisphenol A is used in the manufacture of certain plastics and epoxy linings of metal food containers and is therefore widely found in households and industry with more than 5 million tonnes of BPA used by manufacturers each year[18, 19]. It is found in raw sewage and surface waters because it is continuously released into the environment. BPA is found in sewage sludge at levels ranging from μg kg^{-1} to mg kg^{-1} levels. It has been included in the "dirty dozen list", in the OSPAR List drafted by the convention for the protection of the marine environment of the North-East Atlantic as a compound of possible concern because of its endocrine disrupting abilities[20] and its carcinogenic action[21,22] and it is no longer used in baby bottles in several countries including all EU countries[23]. BPA can be found in the human body and exposure primarily occurs in the domestic environment

and via direct ingestion[24]. Safety limits are defined as levels of dietary exposure without accounting for the appreciable risk for a lifetime of exposure, while regulatory limits are defined as the maximum amount of a substance permitted in a particular food. In New Zealand and Australia no safety limits and no regulatory limits have been set for BPA by the Joint FAO/WHO Expert Committee on Food Additives[25].

BPA works by imitating the natural hormone 17β-estradiol. It was initially considered a "weak" estrogen mimic based on a lower affinity for the estrogen receptor alpha relative to 17β-estradiol. Recent research instead shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane[26]. It is through these receptors that BPA stimulates rapid physiological responses at low pg ml^{-1} (parts per trillion) concentrations[27]. Due to its physicochemical properties that are similar to those of estrogens, it accumulates in bed sediments and bioaccumulates easily in animals[17].

Bisphenol AF (BPAF) is used as a BPA substitute; its domestic production in the U.S.A. was estimated to be between 4.5 and 227 tonnes per year[28] while production in China is unknown, however the larger manufacturers of BPAF in China have a production capacity of 100s tonnes per year[29]. BPAF is considered to have higher estrogenic activity compared to BPA due to the presence of the hydrophobic group[30], and experimental evidence has also shown it has higher bioaccumulative potential[30].

Furthermore it must be noted that both bisphenolic plastics and hormones may degrade under aerobic conditions, but persist for considerably long periods in water and sediments under anaerobic conditions[31]. Even if they degrade in the environment, they should still be considered and treated as persistent pollutants because they are constantly released in the environment due to their regular use.

The first aim of the investigation consists in developing a reliable, robust, fast, and cheap analytical method to monitor the selected endocrine disruptor chemicals in aquatic systems; the analytical approach chosen consists of diffusive gradients in thin films (DGTs) passive sampling coupled with high performance liquid chromatography/mass spectrometry (HPLC/MS) detection. DTG passive

sampling allows *in situ* integrated concentration measurements while HPLC/MS detection ensures a robust, fast and reproducible quantification of trace concentrations. DGTs are routinely used to monitor inorganic chemical species but few studies exploit this methodology to monitor organic species. Thus, the DGT application for the monitoring of EDCs would be beneficial in expanding the repertoire of this sampling technique. The performance of the DGT-HPLC/MS are implemented for the monitoring of target EDCs monitoring in the environment and compared with a validated methodology.

The second goal of this investigation is to apply the DGT methodology to *in situ* monitoring of endocrine disruptor concentrations throughout the drinking water and wastewater treatment plants in Hamilton, one of the main cities in New Zealand, and in the Waikato River, the longest river in New Zealand. The aim was to characterize the efficiency of removal of the selected compounds throughout the drinkable water treatment processes (river-to-tap) and the wastewater treatment processes (effluent-to-treated wastewater) in Hamilton, while the Waikato River monitoring was to evaluate its water quality from source to sea.

The final purpose of the study was to correlate the analytical concentrations of the EDCs collected on the DGT passive samplers with their total estrogenic potency determined by a bioassay. Thus, the DGT passive pre-concentration/sampling technique is coupled with an *in vitro* biomarker assay to correlate the environmental integrated concentration with its integrated estradiol equivalency quantity (EEQ). The Yeast Estrogen Screen (YES) assay was selected among all the assays available to evaluate the integrated EEQs.

1.3 Statement about Ethical Approval

This research DOES NOT involve the participation of animal subjects. Therefore ethics approval by the University Animal Ethics Committee is not required during the period of the research.

1.4 Endocrine Disruptors

1.4.1 Endocrine Disruptors (EDCs) and hormones: definitions

At the moment there are several definitions used to describe endocrine disruptors; the main definitions are shown below.

The U.S. Environmental Protection Agency (EPA)[32] defines an EDC as:

"an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process."

The International Program on Chemical Safety (IPCS)[33] has also developed the following definitions of an EDC and a potential EDC:

"an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" and

"a potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expressed to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations".

To understand in a better way the meaning of these definitions first of all it is necessary to define the hormones and the endocrine system and the way in which they work.

Hormones are *"molecules produced by an endocrine gland that travel through the blood to produce effects on distant cells and tissues"*[34,35].

The endocrine system can be, in this way, described as *"a series of ductless glands that secrete hormones directly into the blood to regulate various body functions"*[34,35].

Human endocrine glands (Figure 1.1) can be specialized or secondary. Specialized glands include the gonads and some areas of the pancreas, the adrenal glands in the abdomen next to the kidneys, the thyroid gland in the neck and the pituitary gland at the base of the brain.

Several organs that belong to other body systems secrete hormones and have secondary endocrine functions. Examples are the heart and the muscles, the body fat, the intestines the liver and the kidneys.

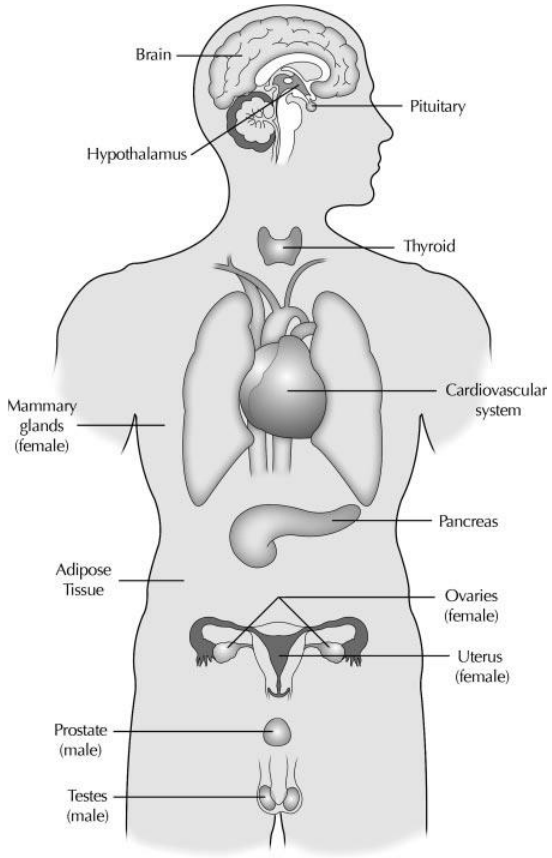


Figure 1.1
Model of the human endocrine systems and potential target of endocrine disrupting chemicals[36]. *Reproduced from Diamanti-Kandarakis (2009); used with publisher's permission.*

1.4.2 Endocrine Disruptors and hormones: properties, mechanisms of action and consequences

Hormones act in the same way in both vertebrates and invertebrates: they are necessary to manage and regulate a large number of physiological and behavioural processes in the body starting from cellular differentiation, during embryonic development and organ formation, to the control of tissue and organ function such as growth and development, movement, respiration, sensory perception, sleep, digestion, metabolism, excretion, reproduction, lactation, stress and mood in adulthood[34,35,37] (Table 1.1).

Table 1.1

Principal endocrine systems and hormones[38].

Hormone System	Hormone	Actions
Adipose tissue	Leptin	Decreases appetite, increases metabolism
Adrenal gland	Cortisol	Stimulates gluconeogenesis, fat metabolism, inhibits glucose uptake into cells
	Aldosterone	Stimulates water resorption, controls blood pressure & fluid balance
	Adrenaline/epinephrine	Boosts oxygen and glucose to brain & muscles, suppresses non-emergency body responses
	Noradrenaline/Norepinephrine	Boosts oxygen and glucose to brain & muscles
	Dopamine	Regulates heart rate & blood pressure
Hypothalamus	Thyrotropin releasing hormone (TRH)	Promotes secretion of TSH and prolactin by pituitary
	Growth hormone releasing hormone	Stimulates secretion of GH from pituitary
	Growth hormone inhibiting hormone (Somatostatin)	Inhibits release of GH from pituitary
	Gonadotropin releasing hormone (GnRH)	Stimulates secretion of FSH and LH from pituitary
	Corticotropin releasing hormone (CRH)	Stimulates ACTH secretion from the pituitary gland
	Vasopressin	Pressor effect on the cardiovascular system
	Oxytocin	Major anti-diuretic hormone
	Dopamine	Causes smooth muscle contraction including the uterus during parturition and in milk let-down
Liver	Insulin-like growth factor (IGF)	Regulates cell growth, has insulin-like properties
Kidney	Renin	Regulates blood pressure & fluid balance
	Erythropoietin	Stimulates production of red blood cells
Ovary	Progesterone estrogens (converted from androstenedione)	Timing of ovulation, Supports pregnancy
		Ovulation
		Secondary sex characteristics Uterine growth

Pancreas	Insulin	Uptake of glucose, regulates glycolysis
	Glucagon	Release of glucose, regulates gluconeogenesis
	Somatostatin	Inhibits release of insulin and glucagon
Parathyroid	Parathyroid hormone (PTH)	Regulates blood calcium levels
Pituitary gland	Growth hormone (GH)	Stimulates growth
	Thyroid stimulating hormone (TSH)	Stimulates T4 production by thyroid gland
	Follicle stimulating hormone (FSH)	Stimulates follicle maturation in ovary Stimulates Spermatogenesis in testes
	Luteinizing hormone (LH)	Stimulates ovulation (females) Testosterone synthesis (males)
Placenta & uterus (during pregnancy)	Progesterone	Supports pregnancy
	Chorionic gonadotropin	Promotes maintenance of corpus luteum Coordinates thyroid function
	Prolactin	Promotes growth of mammary gland & milk production
Testes	Androgens	Maturation of sex organs Secondary sex characteristics Body size
Thyroid	Thyroxine (T4)	Major product of thyroid gland metabolism development
	Triiodothyronine (T3)	Hormonally active form of T4
	Calcitonin	Regulates blood calcium levels stimulates bone construction
Stomach	Gastrin	Causes secretion of gastric acid
	Ghrelin	Stimulates appetite

The hormonal signal is articulated in different steps[39]. First of all a particular tissue biosynthesizes a certain hormone that is stored and secreted. The hormone is then transported to the target cell/cells where the associated cell membrane or intracellular receptor protein recognizes it. The received hormonal signal is transmitted and amplified by a signal transduction process which leads to a cellular response.At this point the original hormone-producing cells are able to recognize the cellular response, leading to a down-regulation in hormone production, after which the hormone breaks down.

EDCs may cause abnormalities and dysfunctions of the endocrine system at several points in this process.Exogenous chemicals usually exert their hormonal interference using one of two pathways:

- they exert a direct action on a specific protein that controls the place and the time of the hormone delivery[40]. This protein could be involved in hormone production (e.g. aromatase), or be an important transporter (e.g. sodium/iodide symporter), or be a carrier protein (e.g. cortisol binding protein). Thus, a chemical that blocks/stimulates the synthesis of a hormone causes a decline/increase of the hormone level in the blood. The downstream result would be the same as the case in which hormone levels are altered because of disease or a genetic defect in which the hormone synthesis is inhibited/stimulated;
- they act directly on a hormone receptor protein complex. Thus, a chemical that interacts directly with a hormone receptor can cause quite complex effects and should interact following the mechanism adopted by hormones when they interact with receptors[27].

  EDCs can act as agonist or antagonist binding to the receptor. An agonist is a compound that mimics the natural ligand, the hormone, and produces a similar biological effect binding the receptor. The agonist binds at the same binding site of the hormone leading in the absence of those to either a partial or full response.

  An antagonist inhibits the effects of the hormone, the agonist, inhibiting receptor activity.

As mentioned above, hormonal effects are mediated by receptors that are specific proteins. The step in which the receptor recognizes the hormone is crucial because without receptors, hormones cannot exert their hormonal effects[41].

There are different classes of receptors related to the various type of hormones, for example:

- Nuclear receptors act directly to regulate gene expression; they bind steroid and thyroid hormones.

  Some of the environmental chemicals, mentioned above as EDCs, are able to interact directly with some nuclear receptors changing their ability to regulate gene processes and thereby producing unexpected effects. These effects need to be identified and considered when thinking about endocrine disruptors.
- Membrane receptors produce effects inside the cell by a second messenger system which is, in some cases, directly linked to the membrane receptor; membrane receptors bind to protein and amine hormones.
- "Co-regulator" proteins, which differ depending on the cells, affect the way a nuclear hormone receptor can function; co-regulator proteins link the hormone receptor to the transcriptional apparatus.

Three different hormonal classes based on the chemical composition can be identified[42]:

- Steroid hormones are converted from their parent compound, cholesterol. They need to be carried by specific "carrier" proteins through the blood, and then they passively enter cells and interact with receptors inside the cells[41]. It is important to remember that steroid hormones are also able to act through cell membrane receptors.
- Peptide hormones and protein hormones consist of 3 to more than 200 amino acid residues. They need specific mechanisms to get to their target organs; these mechanisms usually involve interactions with receptors on the outside of the cell in turn associated with the cellular membrane[41].
- Amine hormones are derived from single amino acids, usually tyrosine. Amines, like protein hormones, need to interact with cell membrane associated receptors on the outside of the cell to reach their target organs[41].

Thyroid hormones make up a subset of this class and to gain access to the inside of a cell require specific transport proteins[41].

It is necessary to keep in mind all the ways, described above, in which hormones act in order to understand their effects and the ways in which exogenous chemicals are able to interfere with their actions.

1.4.3 Endocrine Disruptors and hormones: very low concentrations required for activity

Hormones are powerful compounds and in general very low concentrations are needed to perform their function. This is due to the fact that hormones act using high affinity receptors; so hormones can bind to the receptor population and initiate important biological effects at very low concentrations.

For this reason even extremely low, indeed any level of exposure at all may cause endocrine or reproductive abnormalities, especially if exposure occurs during a critical developmental window[43].

1.4.4 Endocrine Disruptors and hormones: dose-response dynamics

Generally hormones produce a monotonic sigmoidal dose-response curve[36,41] (Figure 1.2).

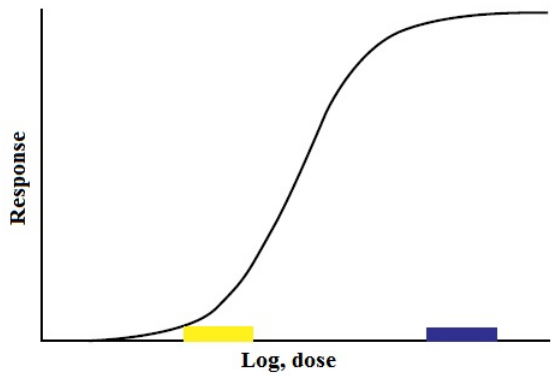


Figure 1.2

Dose-response curve for hormones. The response increases in a logarithmic manner until the point of saturation as the dose of hormone increases[44].

As shown in Figure 1.2 small variations in hormone concentration in the yellow zone at the low end of the dose-response curve cause greater differences in effect

than similar variations in hormone concentration in the blue zone at the high end of the dose-response curve.

This means that very low concentrations of EDCs may produce a response that is much greater than those that would be predicted based on the endogenous hormone by itself. This also means that low doses of EDCs may even exert more potent effects than higher doses[45].

Hormone receptors are commonly expressed at different concentrations in a single cell; this variable significantly affects the various characteristics of the dose-response curve[44] (Figure 1.3).

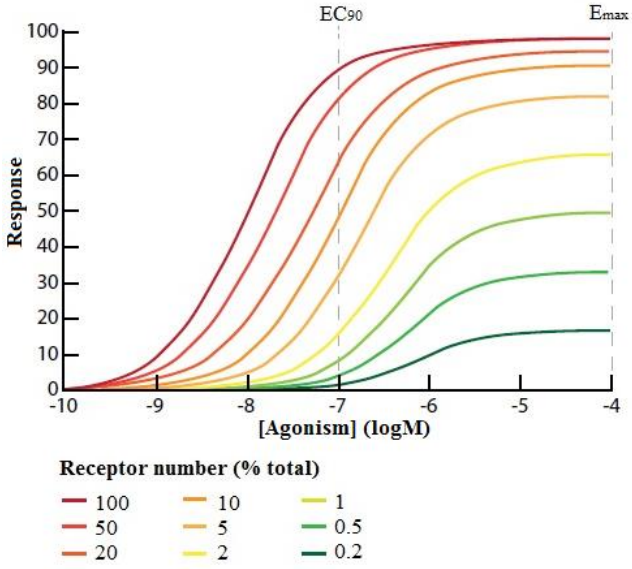


Figure 1.3
Comparison of the dose-response curve for hormones in which the receptor concentration is different. Hormone concentration [Agonism] vs signal abundance [Response] with the variation of the receptors number[44]. Re-edited from Charlton (2009).

The trends in Figure 1.3 show that the dose-response curve shifts to the left when the receptor concentration is higher thus lower hormone concentrations produce the same biological effect.

In this way, hormones associated with cells with a higher number of receptors can be considered "more potent" than the others because significantly lower hormonal concentrations are required to produce the same response. EC_{90} is defined as the concentration of agonist needed to reach 90% of the maximum signal and hormones with low receptor level do not achieve the EC_{90} maximum response shown at high receptor levels. These trends explain why some target cells are more sensitive to exogenous chemicals than others and why some endpoints of hormone are more sensitive to hormones than others.

Hormones interact with and activate their receptors not only in a non-linear sigmoidal way, but also in a more complex way, including non-monotonic responses[46]. Non-monotonic responses can be produced by a variety of mechanisms[45].

One of these mechanisms, for example, requires the integration of two or more monotonic responses that affect a common endpoint. A significant example of this type of mechanism is given by studies conducted on prostate cell lines. Only in the presence of intermediate concentrations of androgen are prostate cells able to proliferate to the highest degree. Receptor down regulation also produces non-monotonic dose responses.

The downregulation of receptor number occurs when hormones are present in high concentrations and are bound to their receptors. In fact when the hormone is abundant the receptors are degraded more rapidly and the cell's ability to replace them is slower than the rate at which they are removed from the system. Thus, fewer receptors are available when there are high concentrations of hormone and this causes a natural shift in the receptor-mediated response.

These concepts have been known for more than a dozen natural hormones and neurotransmitters, but in the past decade they have begun to be appreciated for EDCs too. More than 60 exogenous chemicals classified as EDCs, analyzed in both cell culture and animal experiments, have in fact reported non-monotonic dose-response curves[27,45] .

1.4.5 Endocrine Disruptors: latency of effect

Most hormones only act at a specific time during the life cycle because their receptors are available only during development or in adulthood, so the damage caused by EDCs can be sometimes irreversible. In some cases, multiple receptor types mediate the actions of the same hormone so if one does not work there are other receptors that ensure the hormone recognition.

An adult exposed to EDCs may have very different consequences from a developing *fetus* similarly exposed. In fact there is scientific evidence that, from the fetal period to the early postnatal developmental period during which organs

continue to undergo substantial development, the environment, including the maternal environment in mammals and the egg in other vertebrates, and the external environment after birth interacts with the individual's genes to determine the propensity of that individual to develop a disease or dysfunction later in life. Exposure to EDCs during early life can be described as "the developmental basis of adult disease"[47].

The consequences of exposure to EDCs may not be immediately apparent early in life but may be manifested in adulthood or during aging, so there is a latent time between the exposure and the manifestation of a disorder[36]. Latency may also occur in adult exposure.

1.4.6 Endocrine Disruptors: heritability and transgenerational effects

EDCs may also exert transgenerational effects because they act not only on the exposed individual but also on subsequent generation both in humans and non-human fauna. For some classes of EDCs, such as diethylstilbestrol (DES), there is experimental evidence of heritable diseases both in human and animal studies[48,49,50].

The mechanism of transmission may be nongenomic for example context-dependent transmission (the casual factor persists across generations[51]) or germline-dependent (the germline itself is affected[52,53,54]). The effect may also be transmitted though modifications to factors that regulate gene expression rather than mutation of the DNA sequence.

1.4.7 Endocrine Disruptors: exposure to mixtures

Individuals and populations may be exposed simultaneously to mixtures of these compounds and other environmental pollutants, in fact the contamination of environments is rarely due to a single compound. Simultaneous exposure to different EDCs and other pollutants may cause unexpected effects on the endocrine system because the coaction of various classes of EDCs may be additive or even synergistic[55].

1.4.8 Endocrine Disruptors: persistence

Many of the chemicals classified as EDCs are synthetic and many were expressly designed to have long half-lives because they were especially used in industrial applications, for this reason EDCs are often referred to as POPs, that is persistent organic pollutants.
Even if their molecular design was useful for their industrial use, it has turned out to be detrimental to humans and to wildlife: these substances do not decay easily, they may not be metabolized, or they may be metabolized or broken down into more toxic compounds than the parent molecule.

Different substances banned decades ago remain stable, despite their limited emission, and in high levels in the environment. Banned EDCs, in fact, may be still detected in water, sediments[56], plants, animals and humans[57,58].

A famous example of a persistent EDC is DDT: one of the first chemicals widely used as a pesticide. Today, nearly 40 years from the ban on use in the U.S.A., DDT continues to exert effects. It can still be detected in human milk[59,60] and is found in stable concentration in sediments and in the leaves of the plants[56]. Other EDCs, for example BPA, may not be as persistent, although recent evidence[61] suggest longer half-lives, but they are so widespread in their use that there is a significant human exposure that cannot be ignored.

Even so-called “pristine” environments are threatened by these persistent interfering chemicals. In fact some EDCs are detectable unexpectedly in these environments even if they are situated at remote distances from the place in which EDCs were produced, used, or released. The reason is that air currents, water and migratory animals, that spend part of their life in a contaminated area and that are then incorporated into the food chain in an otherwise uncontaminated region, may easily move these contaminants to new areas.

1.4.9 Endocrine Disruptors: bioconcentration

The result of direct uptake of a chemical by an organism only from water defines bioconcentration. Experimentally, the result of this process is reported as the bioconcentration factor (BCF). Thus, the ratio of steady state concentration of the chemical in aquatic organisms (C_F) and the corresponding freely dissolved chemical concentration in the surrounding water (C_W) defines the BCF value[62].

$$BCF_W = \frac{C_F}{C_w} = \frac{[ng\ kg^{-1}]}{[ng\ L^{-1}]} \qquad 1.1$$

Three different BCF can be defined for aquatic organisms[63]: on a wet weight basis (BCF_W), on a lipid basis (BCF_L) and/or on a dry weight basis (BCF_D).

All three BCF values can be viewed as essentially unitless because 1 L water has a mass of 1 kg; so the dimensions of the chemical concentration in water are equivalent to the dimensions of the chemical concentration in the organisms[63,64,65,66].

It was also shown that the lipid content of the organism influences the BCF_W value of lipophilic organic chemicals[67,68,69]. Therefore, the bioconcentration factor on the lipid basis (BCF_L) is the most important BCF value of a lipophilic chemical in an organism. To easily calculate the BCF_L values from the BCF_W it is only necessary to know the lipid content (L in % on a wet weight basis: L_W (%)) of the organism:

$$BCF_L = \frac{BCF_W \cdot 100}{L_W\ (\%)} \qquad 1.2$$

The water content (%) of the organism must also be measured if the lipid content of the organisms is given on a dry weight basis (L_D in %). The most important parameter is the lipid content on a wet weight basis (L_W in %) of the organisms since it is most relevant to the living organism.

1.4.10 Endocrine Disruptors: bioaccumulation and biomagnification

Bioconcentration should not to be confused with the terms bioaccumulation, biomagnification and ecological magnification which refer to indirect contamination[70,71].

The uptake of substances from both food and water defines the bioaccumulation. The term biomagnification is used for dietary uptake via contaminated food. Biomagnification is the effect produced by the inability of the cells to get rid of these harmful compounds: EDCs may not be metabolized or may be metabolized to even more dangerous compounds. The direct consequence is that they accumulate in the tissues during the life of the organism so the amount in adult plants or animals will always be higher than the amount detected in young ones. Biomagnification, like bioconcentration, can be expressed using the biomagnification factor (BMF) on wet, dry, or lipid basis. The ratio between the concentrations in organism and food at a steady state defines the BMF of a chemical[72]. Increasing chemical concentrations along the food chain is called ecological magnification[73].

1.4.11 Endocrine Disruptors: classes of pollutants and emerging contaminants

In general hormone receptors have a stronger affinity for their natural ligand than for non-natural EDCs, but there are some exceptions.
An example of an EDC that has a similar and even greater affinity than that of the natural ligands is tributyltin (TBT). TBT has in fact an affinity for RXR (retinoid-X-receptor) and PPARγ(peroxisome proliferator activating receptor subtype gamma) in the low nanomolar[74] range becoming in this way the most potent agonist known for these receptors.

However, it is important not to confuse affinity for the receptor, that is the ability to bind, with potency of action, that is the ability to cause effects[75].
Some examples of EDCs with different estrogenic potency are shown in Table 1.2.

Table 1.2

Relative estrogenic potency of several EDCs determined by different bioassays (1-3)[11].

Compounds	1 YES	2 MCF7 assay	3 ER-CALUX
Steroid sex hormones			
17β-estradiol	1	1	1
Estriol	3.7×10^{-1}		
Ethinyl estradiol	1.9×10^{-1}–1.2	1.25–1.9	1.2
Diethylstilbestrol	4.5×10^{-2}–1.1	2.5	
Estrone	1.9×10^{-2}–1.0×10^{-1}	1.0×10^{-2}	5.6×10^{-2}
Alkylphenolic compounds			
Nonylphenol	7.2×10^{-7}–4.1×10^{-4}	1.3×10^{-5}–1.0×10^{-4}	2.3×10^{-5}
Octylphenol	1.0×10^{-5}–4.9×10^{-4}	1.0×10^{-4}–2.5×10^{-4}	1.4×10^{-6}
Nonylphenol monoethoxylate	4.0×10^{-6}–1.3×10^{-5}		3.8×10^{-6}
Bisphenolic compounds			
Bisphenol A	1.0×10^{-5}–8.1×10^{-5}	2.5×10^{-5}–6.0×10^{-5}	7.8×10^{-6}
Phthalates			
Di(ethylhexyl)phthalate	2.5×10^{-5}		
Dibutylphthalate	1.0×10^{-7}–1.0×10^{-5}		1.8×10^{-8}
Benzylbutylphthalate	1.0×10^{-6}–8.9×10^{-6}		1.4×10^{-6}
Dimethylphthalate	1.0×10^{-6}–7.0×10^{-6}		1.1×10^{-5}
Diethylphthalate	5.0×10^{-7}–4.7×10^{-6}		3.2×10^{-8}
Fragrances			
Musk xylol		3.3×10^{-5}	
Musk ketone		7.0×10^{-5}	

Moreover the part of the chemical that controls the receptor activation is not necessarily the same part of the chemical that controls its ability to bind to the receptor; additionally the dose at which a hormone activates the receptor will be impacted by the receptor abundance, as well as the dose in which an endocrine disruptor acts.

All these factors may underlie why an EDC does not generate the same effects in different tissues or cells. Thus EDCs will be receptor and tissue specific just like the hormone with which they interfere.

The group of compounds classified as EDCs is highly heterogeneous and they exert their interference with different mechanisms: acting upon hormone receptors, hormone synthesis or hormone conversion. Several classes of EDCs act as antiandrogens and as thyroid hormone receptor agonists or antagonists: in recent times some classes of EDCs have also been identified as androgenic also[36].

EDCs are quite different and it seems that they do not share any structural similarity other than being small molecular mass (< 1000 Daltons) compounds[36].

Thus it is difficult to predict whether a compound may or may not exert endocrine-disrupting actions.

Some EDCs often contain a phenolic moiety, to mimic natural steroid hormone shape and this may enable endocrine disruptors to interact with steroid hormone receptors as analogs or antagonists, and/or halogen group substitutions[41,76]. Examples of EDCs with this structure are the dioxins, PCBs, PBBs, and pesticides.

Heavy metals and metalloids also show estrogenic activity[41], suggesting that these compounds are EDCs as well as more generalized toxicants. Twelve EDCs are known colloquially as the "dirty dozen"[77,78] because they are powerful EDCs able to cause harmful effects on the human health, wildlife and environment. They were commonly used in industry but, after the Stockholm Protocol, an international treaty signed in December 2000 by about 120 countries, they have been fully or partially banned. Altogether close to 800 chemicals are documented as endocrine disruptors; these can be grouped in different clusters:

- heavy metals (lead, mercury, cadmium, arsenic).

  Lead is regularly encountered in mining, smelting, refining, leaded petrol (gasoline), lead-acid batteries, paints, jewellery, children's products and in many other products. Children are potential candidates of lead poisoning, since the amount they ingest per unit body weight is obviously higher and they do not have a fully developed blood-brain barrier. Thus neurological effects of lead are always higher in children compared to adults[79]. Mercury is a potent environmental pollutant when it is in the form of methylmercury. It was formerly produced directly or indirectly as part of different industrial processes such as the manufacture of acetaldehyde. Actually the burning of fossil fuels, in particular coal, and the burning of wastes containing inorganic mercury are the only anthropogenic sources of methylmercury. Although inorganic mercury is only a trace constituent of fuels such as coal, large scale combustion in utility and commercial/industrial boilers releases into the environment hundreds of tons of elemental mercury to the atmosphere each year in the USA[80] alone. There are also natural processes that transfer mercury to the atmosphere

such as forest fires, volcanoes, volatilization from the ocean[81] and weathering of mercury-bearing rocks[82].

Cadmium has many common industrial applications: it is a key component in battery production, it is present in pigments[83] and coatings[84] and it is commonly used in electroplating.

Arsenic has multiple applications. Metallic arsenic is mainly used for strengthening alloys of copper and especially lead needed in car batteries. It is also a common n-type dopant in semiconductor electronic devices, in fact the optoelectronic compound gallium arsenide is the most common semiconductor in use after doped silicon. Arsenic and its compounds, especially the trioxide, have been used to produce pesticides, herbicides and insecticides and to treat wood products[85]. Arsenic also enters the New Zealand environment from geological activity.

- Industrial solvents or lubricants and their byproducts (glycol ethers, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo furans (PCDFs)); Glycol ethers are common solvents in paints, cleaning products, brake fluid and cosmetics such as the wide spread methoxydiglycol (EGBE) and (DEGME) 2-butoxyethanol. Dioxins and furans come from varied sources[86,87]. Combustion sources can be municipal waste[88] or medical waste incinerators and private backyard barrel burning. Dioxins are especially generated by the combustion of chlorine-containing substances such as polyvinyl chloride (PVC). Other sources are metal smelting, refining and process sources and the chemical manufacturing industry where dioxins occur as by-products in the manufacture of some organochlorides or in the chlorine bleaching of paper. There is also production of dioxins from natural sources such as volcanoes and forest fires or from environmental reservoirs.

- Flame retardants (polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs) such as PBBs, polybrominated diphenyl ethers (PBDEs)).

Flame retardants are widely used in several products ranging from computers, electronics and electronic equipment, textiles, foam furniture, insulating foams and other building materials. Flame retardants are not bound to the products and, as a result, they may easily be released into the environment. Although these compounds have been prohibited in many countries they are still considered sources of pollution because of their long half-life in the environment[79].

Because of their high lipophilicity (log K_{OW}>6) and resistance to degradative processes, PBDEs are expected to bio-accumulate easily. The higher brominated compounds tend to end up in sediments at high residue levels near their emission sources but not as much in marine organisms because they are less mobile in the environment; they have low water solubility and strong adsorption to sediments. Less substituted bromine compounds on the contrary are predicted to be more volatile and more easily water soluble and so they bio-accumulate in biota[11].

- Surfactants (alkyphenol, perfluorinated chemicals (PFCs)).

  Alkylphenols have been used in industry for over 40 years. Alkylphenols, especially the long-chain ones[89], are used extensively as precursors to the detergents, as additives for fuels and lubricants, polymers, and as components in phenolic resins. They are also used as building block chemicals needed to make fragrances, thermoplastic elastomers, antioxidants, oil field chemicals and fire retardant materials. Through the downstream use in making alkylphenolic resins, alkylphenols are also found in tires, adhesives, coatings, carbonless copy paper and high performance rubber products. Fluorosurfactants[90] are widely used in the production of teflon and related fluorinated polymers. They have also been used to confer hydrophobic, stain-resisting properties to fabrics.

  Alkylphenols are lipophilic compounds (log K_{OW} = 4.48) so they partition preferentially to the organic fraction of sediments and show considerable potential to bioaccumulate in aquatic organisms[11].

- Plastics (Bisphenols such as bisphenol A (BPA), bisphenol AF (BPAF), bisphenol S (BPS)).

  Bisphenols are used frequently in many plastic-based containers. Approximately 5 million tonnes of bisphenol A (BPA) are used by manufacturers each year[19]. BPA is employed to make certain thermosetting and thermoplastic polymers: it is a key monomer in production of epoxy resins[91,92] and in the most common form of polycarbonate plastic[93,94,95]. BPA is also used in the synthesis of polysulfones and polyether ketones, as an antioxidant in some plasticizers, and as a polymerization inhibitor in PVC. BPA is a precursor to the brominated flame retardant tetrabromobisphenol A (TBBPA), and formerly was used as a fungicide[96]. BPA is a preferred colour developer in carbonless copy paper and thermal point of sale receipt paper[97,98].

  It must be said that BPA used in these applications is present as free monomer: discrete and non-polymerized. This means that the BPA is more available than BPA polymerized into a resin or plastic. BPA-based plastics are clear, shatter-proof and tough and are made into a variety of common consumer goods such as water bottles, sports equipment, medical and dental devices, dental fillings sealants, CDs and DVDs, household electronics, eyeglass lenses[94], foundry castings, and the lining of water pipes[99]. Epoxy resins containing BPA are used as coatings on the inside of almost all the metal containers for canned foods and beverage[100]. The lining is used to give protection from pathogens but since it is in direct contact with the food, BPA may find its way into food and finally into human beings[79]. Because of the hazardous effects on human beings, the compound is no longer used in baby bottles in several countries and in all EU countries[23].

- Plasticizers (phthalates).

  Phthalates are esters of phthalic acid mainly used as plasticizers. These plasticizers are globally widespread and approximately seven million tonnes of phthalates are consumed every year world-wide[101]. These substances are added to plastics in order to increase their flexibility,

transparency, durability, and longevity. In all reported studies di(ethylhexyl)phthalate (DEHP) was found to be the predominant phthalate ester, due to its high production (nearly 90% of European plasticizer use) and its physico-chemical properties (low solubility and relatively high K_{ow})[11]. Phthalates are also used as viscosity control agents, gelling agents, film formers, stabilizers, dispersants, lubricants, binders, emulsifying agents, and suspending agents. Their common applications include adhesives and glues, agricultural adjuvants, building materials, personal care products, medical devices and pharmaceuticals, detergents and surfactants, packaging, toys, modelling clay, waxes, paints and pigments, printing inks and coatings, food products, household applications, and textiles.

- Pesticides: insecticides (organochlorine compounds such as methoxychlor, chlorpyrifos, DDT; organophosphate compounds; carbamates), herbicides (triazines such as atrazine, simazine), fungicides (azole such as triazoles, imidazoles; dicarboxamides such as vinclozolin).

  Pesticide compounds are used to control organisms that are considered to be harmful[102] and have been designed in order to be highly sensitive towards the reproductive and neural systems of these organisms. However the similarity of these processes with normal human physiological systems indicates that these chemicals can also affect humans, for this reason a huge amount of studies, conducted on this field, are available. Organochlorine hydrocarbons were the first pesticides with widespread use. They act by disrupting the sodium/potassium balance of the nerve fiber in insects, forcing the nerve to transmit continuously. They have been phased out because of their great toxicity, persistence and high bioaccumulation potential[103]. Organophosphates and carbamates have largely replaced organochlorines. Both operate through inhibiting the enzyme acetylcholinesterase, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis in the pests.

- Pharmaceutical agents and hormones (such as diethylstilbestrol (DES))[36]. Synthetic hormones and nonsteroidal xenobiotics and their metabolites can all act as EDCs. This class of EDCs is the most powerful among the ECD classes and their potency in fact is the highest of any ECD class[11]. Thus even an extremely low concentration in the environment may stimulate rapid physiological responses. Free hormones and nonsteroidal xenobiotics are rarely detected in urine and in general their metabolites or conjugates exhibit a less significant biological activity. However, they are converted back into their free forms during water treatment techniques and regain their potency[8,9,10].

- Phytoestrogens (such as genistein, coumestrol).
  Phytoestrogens can be found in human and animal food. These substances, are generally thought to have relatively low binding affinity to endocrine receptors, are widely consumed and are even components of infant formula[104, 105].

The list of EDCs is continuously updated and also includes suspected endocrine disruptors in the category of emerging contaminants. These compounds are pollutants recently introduced into the environment, which need to be studied and monitored, to fully understand their effect in the short and long term, their mechanism of action and the best methodology to remove them from environmental waters (e.g., through reverse osmosis, microfiltration, advanced oxidation, photolysis, microbial degradation, etc.). For this reason emerging contaminants are unregulated and they may be candidates for future regulation depending on the data collected from research on their potential health effects and monitoring regarding their occurrence[11].

For this purpose an ongoing trend in research for most of these emerging contaminants is to investigate them by combining analytical chemistry with toxicology. Moreover intermediates and degradation products are also identified and their toxicity evaluated because often the treatments used are not suitable to completely remove these contaminants. New emerging contaminants include pollutants that potentially might enrich existing EDC classes or create new ones[106].

The latest contaminant candidate list drafted by the U.S. Environmental Protection Agency (EPA) was published in April 2015. This list is a drinking water priority contaminant list for regulatory decision making and information collection. The listed contaminants are known to occur or anticipated to occur in drinking water systems and will be considered for potential regulation[107]. This final CCL-4 contains 100 chemicals. The microbial contaminants are included because their toxins may be found to be endocrine disruptors.

This final CCL-4 now includes PFOA and PFOS, three pharmaceuticals (erythromycin, 17α-ethinylestradiol (EE2), and nitroglycerin), eight hormones (17α-estradiol (αE2), 17β-estradiol (βE2), equilenin, equilin, estriol (E3), estrone (E1), mestranol (MES), and norethindrone), and several DBPs (chlorate, formaldehyde, acetaldehyde, and five nitrosamines), as well as pesticides, pesticide degradation products, metals, industrial solvents/ingredients.

1.4.12 Endocrine disruptors: effect upon humans

A wide variety of developmental problems and common adult diseases are known to be caused by abnormal endocrine function. All hormone-sensitive physiological systems are vulnerable to EDCs and their contributions have changed the incidence of some disease and disorders.

Reproductive effect

Reproductive hormones, steroids (estrogens, androgens, progestins) and proteins (luteinizing hormone (LH), follicle stimulating hormone (FSH)) control the complex physiological processes associated with reproduction. EDCs with intrinsic sex hormone activity are typical exogenous agents causing reproductive health diseases and alterations.

EDCs may be involved in precocious puberty; the average age of menarche 200 years ago occurred around 17 years of age but during the last several decades it has moved to 13 years of age[108,109]. Improvements in general health and nutrition have certainly affected this advancement[110]. About 80% of the variance in pubertal timing can likely be explained by genetic factors[110,111] but the other 20%

is explained by the increased body mass index and childhood obesity[112,113] and by other environmental factors involved[108,114].

There is convincing evidence from experimental studies with both rodents and primates that prenatal and/or neonatal treatment with estrogen receptor agonists accelerates pubertal onset (gonadotropin-releasing hormone (GnRH) release) in a dose-dependent fashion. Epidemiological evidence also attests that exposure to chemicals such as dioxins[115,116], metabolites of pesticides[117,118], PCBs[116,119,120], lead[119,121] and pharmaceutical estrogens, in both animals and humans will affect sexual development. Children from developing countries who move to industrialized and enriched environments have an increased risk of developing central precocious puberty (CPP)[110] and endocrine disruptors have been hypothesized to contribute[117]. There are many case reports of peripheral precocious puberty (PPP) in children exposed to pharmaceutical drugs or ointments or food containing sex steroids[122].

EDC exposure is also related to the capacity to conceive a child: couples that live in developed countries, more exposed to EDC pollution, experience the inability to conceive with a higher rate (3.5 to 16.7%) than in couples that live in less developed countries (6.9 to 9.3%)[123]. Paternal exposure to EDCs, such as pesticides[124], dioxins[125] or lead[126], reduce fertility altering semen quality and thus the male ability of conceiving. Maternal exposure to EDCs may cause sub fertility/infertility with increased risks of adverse pregnancy outcomes[127] such as spontaneous abortion, preterm delivery, low birth weight, premature birth and fetal death.

The sex ratio, defined as the numbers of boys divided by the numbers of girls born, in humans is slightly greater than one[128]. Even if the sex ratio is correlated with the age of mothers in some countries[129], exposure to chemicals is a potential causative factor in declines in male births[130]. High occupational or accidental exposures to dioxin[131,132], to contaminated trichlorophenate[133], to pesticides[134], or to PCBs[135,136] have all been associated with changes in the sex ratio of human populations.

There is evidence of EDC contribution to female reproductive system illness. EDCs may affect menstrual cyclicity and menopause age in women: shorter menstrual cycles have been observed under lead exposure[137], in women exposed to chlorodibromomethane in drinking water[138], or those exposed to DDT[139,140]; longer menstrual cycles have been observed in association with exposure to dioxins[141], pesticides[142] and elevated serum PCBs[143] ; early menopause age has been observed with DDT/DDE or dioxin exposures[140,144]. Polycystic ovary syndrome (PCOS) is a disorder affecting both metabolism and reproduction and occurs in 3 to 15% of women of reproductive age. PCOS has been associated with BPA exposure: studies of adults found a relationship between serum BPA levels and women with this disorder[145,146]. Uterine fibroids, known also as leiomyomata, are the most common benign tumour of the female reproductive tract[147]. Fetal exposures to both DES and BPA during particular periods in development have been shown to cause increased risk of fibroids in adulthood[21,148]. Higher levels of the metabolites of the phthalate plasticizers were found in women with fibroids compared to controls, and a higher risk for women who were glutathione S-transferases M1 null, suggesting genetic susceptibility to phthalate effects[149].
Prenatal exposure to the estrogenic DES was also linked with the development of a rare form of vaginal cancer in the adult[48,150,151,152]. Endometriosis is a major cause of infertility and chronic pelvic pain in women. It has also been linked to increased risk of endometrioid and clear cell ovarian cancer, non- Hodgkin lymphoma, and atopic disorders[153]. Endometriosis can be promoted by many organochlorines, including the dioxin TCDD, the pesticides methoxychlor and DDT, or many PCBs with dioxin-like effects[154]. In several studies, fetal exposure has been found to promote future endometriosis.

Breast cancer incidence rates are increasing in almost all industrialized countries[155] and several studies have highlighted the importance of environmental factors including chemical exposures[156, 157]. The breast is particularly vulnerable to cancer-causing influences during development in the womb and during puberty[158]. Women born from mothers who used DES during pregnancy to avoid the risk of miscarriages, show a high breast cancer risk[159]. Natural and therapeutically used estrogens strongly contribute to breast cancer risks[160].

Worldwide studies concluded that among the hormone replacement therapeutics (HRT), estrogen-HRT[161] and estrogen-progesterone combined-HRT[162,163] are associated with breast cancer. It has also been shown that breast cancer risks increased with rising total estrogen load in adipose tissue[4] suggesting that lipophilic EDCs in combination contribute to breast cancer risks, just as do natural and therapeutically used estrogens. Epidemiological studies confirmed that environmental pollutants with estrogenic activity such as PCDD/F, PCBs, organic solvents[164] or DDT/DDE exposures during puberty[165] undoubtedly contribute to breast cancer risks. Exposure to the estrogen mimic cadmium, is also associated with breast cancer[166]. BPA is associated with aggressive breast cancer suggesting that more polar xenoestrogens, like this plastic, develop tumour progression and cause poorer patient outcome[22].

Endometrial cancer is another common cancer which afflicts the female reproductive system. It occurs especially in industrialized countries and during the postmenopausal period. There are two types of endometrial cancer: an estrogen dependent variety, and one not dependent on estrogen. The increases in incidence are limited to the estrogen dependent type[167]. As seen with breast cancer, elevated levels of endogenous sex hormones including total and free estradiol, estrone, and total and free testosterone are associated with increased risk of endometrial cancer[168]. Pharmaceutical estrogens in combination with progestogen, used as hormone replacement therapy during menopause[5], and combined with long-term cadmium intake[169] increase endometrial cancer risks.

Male reproductive endocrine disorders are caused by male hormone (androgen) insufficiency and/or by an imbalance between female and male hormones during critical times during the life cycle[41]. This can lead to malformations such as cryptorchidism, hypospadias and changes in anogenital distance. These symptoms originate at the same time during fetal development[170], so the extent and severity to which they are manifested is dependent on the degree to which normal developmental processes have been perturbed. Moreover, any perturbations occurring during the male programming window are irreversible and have lifelong implications for the affected individual and also for his offspring.

Congenital cryptorchidism is a condition in which one or both testes are not located at the bottom of the scrotum at the time of birth. Epidemiological data indicate that cryptorchidism is developed in offspring after EDC exposure in occupational settings[171,172] but there are difficulties in deciding what chemical, or suite of chemicals, are of significance. It may be assumed that persistent and anti-androgenic organochlorine pesticides and PBDEs are connected to this disease[173], also higher levels of heptachloroepoxide and hexachlorobenzene have been found in fat samples of cryptorchid boys than in controls[174].

Hypospadias is the condition in which the urethra opens on the ventral side of the penis or in the perineum instead of the tip. It results from an incomplete closure of the urethral folds, leaving a split on the penis[175]. Estrogens and anti-androgens may cause this condition. Children exposed to DES during pregnancy[176] showed hypospadias as well as men exposed to pesticides[177].

Since the middle of the twentieth century, so in a couple of generations, the incidence of testicular germ cell cancer (TGC) has increased up to 400%[178]. The increasing rates of TGC clearly suggest environmental causes beyond genetic susceptibility, moreover these rates are also associated with impaired semen quality[179] and lower fertility, even prior to the development of cancer[180]. Prenatal exposures of the mother to POPs has been demonstrated to be a risk factor for TGC[181].

Prostate cancer is one of the most common cancers diagnosed in European and USA men[182]. Environmental factors, including diet and chemical exposures[156,183] contribute to its appearance. Several pesticides, in particular certain organochlorines[184,185, 186], are linked with increased prostate cancer risks.

Metabolic effects

Thyroid hormones control metabolic processes and coordinate these with the many hormones involved in appetite and body weight regulation and metabolism. A large list of environmental synthetic chemicals may cause a reduction in circulating levels of thyroid hormone[187]. Although not all of the EDCs produce goitre, many reduce serum concentrations of thyroid hormone interfering directly with the receptor for thyroid hormone[188] or with other processes controlling

thyroid hormone action[189]. Exposure to PCBs depresses thyroid function in humans reducing circulating levels of thyroid hormone[190,191].

Obesity is an endocrine-related disease/dysfunction so it is potentially sensitive to endocrine disrupting chemicals[192,193,194]. Obesogen EDCs may cause not only weight gain, acting on the endocrine pathways responsible for control of adipose tissue development and for the growth of the number of fat cells, but also may alter lipid metabolism and glucose sensitivity[195] or alter food intake and metabolism via effects on sexual dimorphism and appetite and reward centers in the brain. Chemical exposures during vulnerable windows of development may affect adult weight[196,197]. EDCs such as tributyltin[198], BPA[21], organochlorine[199] and organophosphate[200] pesticides, dioxin[199], PCBs[201] can lead to altered cholesterol metabolism and weight gain later in life.

Epidemiological evidence prove that adult exposures to EDCs may contribute to the development of type 2 diabetes[202]: people with the high exposure to persistent EDCs are forty times more likely to develop this illness than the people with the lowest levels of exposure[203]. The EDCs implicated in the development of type 2 diabetes include BPA[204], flame retardants such as PCBs[205], organochlorine pesticides[206], phthalates and arsenic.

1.4.13 Endocrine disruptors: interaction with other non-human fauna

Wild mammals, non-mammalian vertebrates and also invertebrates show adverse effects after EDC exposure including: female[207,208]/male[209,210] reproductive illness, thyroid dysfunctions[211,211], cancers[212,213], metabolic disorders[194,214,215,216].

One of the first studies that described endocrine disruptor actions on animals was the one conducted on alligators exposed to organochlorine pesticides that presented many reproductive and endocrine problems[217]. Birds exposed to the organochlorine pesticide experienced reproductive failure[218]. Sheep, that consumed clover with phytoestrogens, showed impaired fertility[219]. Exposure of rodents to DES at the perinatal period produced developmental toxicity, neoplasia, and more subtle endpoints of reproductive dysfunction[150,151,152].

The vast majority of EDCs do not affect DNA directly but some induce genetic mutations in rats[220,221]. An important example of germline transmission of an epigenetically modified trait was shown in rats exposed to a fungicide that manifested a higher likelihood of metabolic disorders, tumors, and reproductive dysfunctions in the next four generations[52,53,54,222,223,224].

The fact that multi-component mixtures act to produce additive effects has been confirmed by studies conducted on animal models. Fishes exposed to estradiol and xenoestrogens suffered from hyper-production of Vtg[225,226]. Rats exposed to combinations of androgen receptor antagonists showed changes in anogenital distance and retained nipples, effects considered to be the hallmarks of disruption of androgen action in fetal life[227]. Similar observations were made with combinations of antiandrogens that work by a variety of different mechanisms in animals [228,229]. Studies with thyroid disrupting chemicals also showed additive combination effects at low doses in animals[230].

The trophic transfer and the biomagnification potential of EDCs are particularly visible in aquatic ecosystems[71]. Organic chemicals such as fungicides, organochlorine pesticides, PCBs, PAHs, dioxins, and inorganic compounds have been studied: the majority of chemicals evaluated do not biomagnify in aquatic food webs; for many of the compounds examined, trophic transfer does occur but does not lead to biomagnification in aquatic food webs; organochlorine pesticides and methyl mercury have the potential to biomagnify in aquatic ecosystems; the lipid content of predators directly influences the biomagnification potential of lipophilic chemicals; even those compounds for which evidence for biomagnification is strongest show considerable variability and uncertainty regarding the magnitude and existence of food web biomagnification in aquatic ecosystems.

For most chemicals the bioconcentration factor[231,232] is of great importance: the uptake from food contributes significantly if the concentration of the chemical in food is 10^5 times higher than the concentration of the chemical in water[233]. For very hydrophobic chemicals with log K_{OW} greater than 6.3 bioaccumulation is of relevance[231].

1.5 Analytical procedures and methods to qualify and quantify endocrine disruptors in fresh water, sediments and biota

1.5.1 Sampling strategies

In aquatic systems, most anthropogenic chemicals and waste materials, particularly POPs and inorganic chemicals, disperse in the water and could accumulate in sediments. In this way sediments become repositories for many of the more toxic chemicals, especially lipophilic ones, introduced into surface waters. Sediments provide essential habitat for many freshwater, estuarine, and marine organisms, so their contamination means also potential contamination of the organisms that proliferate within them. The simultaneous collection of water and sediments could therefore be beneficial in understanding if chemical accumulation occurs within the sediments. Water and sediments can be sampled adopting different strategies such as active or/and passive samplings. One approach is preferred to the other according to the purpose of the sampling and the parameters that need to be monitored, the timeframe available for the sampling, the location of the sampling as well the budget and easiness of sampling.

1.5.1.1 *Active sampling*

Active sampling is a rapid approach that gives information about the concentrations of the analytes in the specific moment in which the sampling happens. Water samples can be collected one-time or a series of samples can be pooled over a period of time. The choice of container material and the volume of sample is predetermined based on the potential of the chemical to adsorb to the container material, the likely concentration of the analytes and its potential to decompose overtime as consequence of the exposure to the visible light or temperature (in this case amber containers and refrigeration after the sampling are recommended). Filtering of the sample to remove suspended particulates is usually undertaken prior to analyte concentration depending on whether the total concentration or dissolved phase concentration needs to be determined.

1.5.1.2 *Passive sampling*

Passive sampling is a class of techniques in which the target molecules freely flow from the sampled medium to the collecting medium, where they accumulate over time, as a consequence of the different chemical potentials[234].

Passive samplers are preferred nowadays for several reasons[235]:

- affordability;
- are generally robust, small and light;
- do not need power;
- are relatively simple to operate;
- are suitable to monitor analytes of interest present in very low concentrations.

Traditional sampling strategies require large volumes of sample (water/sediments/soils) to detect trace level of chemicals and necessarily require extraction/preconcentration thereby adding another step to the sample preparation prior to the analysis. On the contrary, passive sampling strategies concentrate the analytes *in situ*, reducing in this way the limit of detection (LOD) and limit of quantification (LOQ), and offer time-weighted average concentrations of pollutants over deployment periods ranging from days to weeks to months[236].

All passive samplers are based on the principle of the accumulation of the analytes by passive diffusion in the device. In general these tools are made up of a receiving phase (solid/liquid absorbent or a chelating gel), selective for a specific class of contaminants and a diffusion-limiting layer (porous/non-porous membrane or a gel) that separates the receiving phase from the sampled matrix[235]. Two main examples of passive samples are the polar organic contaminant integrative sampler (POCIS) and the diffusive gradients in thin films (DGT).

Polar organic contaminant integrative (POCIS) sampler

POCIS samplers are designed to trap hydrophilic organic micropollutants in aquatic environments, in particular polar organic compounds (log K_{OW} <4)[235]. This kind of sampler (Figure 1.4) is made up of a solid sorbent located between two diffusion-limiting membranes of microporous polyethersulfone (pore size 100 nm).

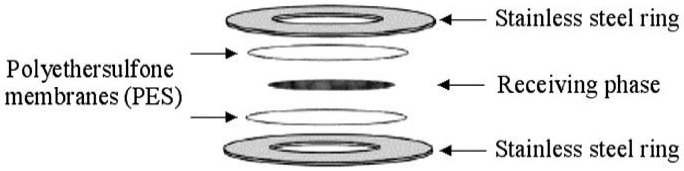


Figure 1.4 *Schematic representation of POCIS assembly. Re-edited from Morin (2012)*[235].

Different sorbent phases[235] are available specifically designed to trap pesticide compounds and some hormones (triphasic mixture) or pharmaceutical compounds (Oasis HLB phase). Analytes are then extracted from the solid sorbent by solvents and the eluent is then analysed.

Diffusive gradients in thin films (DGT) samplers

DGTs are versatile samplers employed to trap different kinds of microcontaminants both in water, sediments and soils. These samplers accumulate dissolved substances in a controlled way and allow the estimation of average concentrations in seawater and freshwater, to evaluate the bioavailable fraction (effective solution concentrations), to measure fluxes and concentrations in soils and sediments, and to quantify at high spatial resolution (microns to centimetres).

In general all DGT units are made up of a plastic probe housing. In sequence they comprise a receiving phase that consists of a resin gel layer, a diffusive gel disc and a specific filter membrane, which are arranged on the moulded base and then contained with a cap (Figure 1.5).

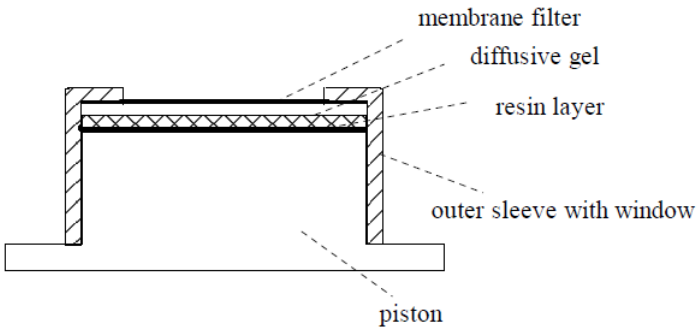


Figure 1.5 *Typical section of a DGT assembly.*

The deployment of a gel, as diffusive and sensing layer, also has the advantage of a very long shelf life. It is possible to store gel sheets for up to 12 months, using

the appropriate solution and temperature, while it is possible to store DGT units already assembled for up to 6 months. After the deployment the resin-gel is retrieved from the probe, eluted in an appropriate solvent and subsequently analysed.

DGTs are suitable to quantify dissolved species for which there is a selective binding agent. The type and number of binding agents tested is always increasing making these devices suitable and validated for a broad range of analytes. The actual resin gels available allow:

- to quantify trace metals[237, 238] (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn), including rare earth elements and radionuclides;
- to detect micronutrients such as calcium, magnesium and macronutrients such as phosphate[239] and nitrate[240];
- to evaluate the presence of other inorganic species such as sulphide[239]
- to quantify organic species such as plastics[241].

DGT probes accumulate solutes on a binding agent after passage through a hydrogel of known thickness which acts as a well-defined diffusion layer. A selective binding agent, usually a resin, is immobilised in a thin layer of hydrogel. Within a few minutes of immersion in the bulk solution, a steady state linear concentration gradient is established between the solution and the resin gel (Figure 1.6).

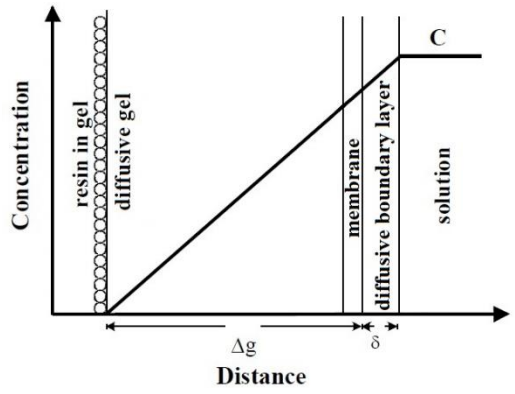


Figure 1.6
Cross-section through a DGT probe in contact with solution showing the steady state gradient concentration.

Exploiting the steady state condition it is possible to measure concentrations *in situ*. Experimentally the DGT device is deployed for a fixed time, *t*. On

retrieval the binding gel layer is peeled off and the mass of the accumulated target analyte is measured. The mass of the target can be evaluated:

- directly in the binding gel layer by drying it and using a beam technique (example, laser ablation ICP-MS or direct counting);
- by eluting the binding gel layer with a known volume (V_e) of solution. The concentration of analyte in the eluent (C_{DGT}), is then measured by any suitable analytical technique. The elution is realized in batch mode so only a fraction of the bound analyte is retrieved. The ratio of the eluted to bound analyte is known as the elution factor (f_e). The elution factor needs to be evaluated and considered, as well the volume of the gel (V_g, mL) in the binding layer, to calculate the accumulated mass (M, moles) of analyte in the binding layer[237] as shown in the equation (1.3).

$$M = \frac{C_{DGT}\,(V_g + V_e)}{f_e} \qquad 1.3$$

The mass (M) can be then used to calculate the concentration in the bulk solution (C_{DGT}) with a knowledge of the physical area of the exposure window (A), the thickness of the diffusive layer and filter membrane (Δg), the diffusion coefficient of the analyte in the gel at the specific temperature (D) and the deployment time (t)[237,242] as shown in the equation (1.4).

$$C_{DGT} = \frac{M\,\Delta_g}{DAt} \qquad 1.4$$

The use of this simplified equation requires several assumptions[242]: under well stirred conditions the thickness of the diffusive layer and filter membrane (Δg) apply with a negligible diffusion boundary layer; interactions of solution species with the gel and the membrane filter comprising (Δg) are negligible; measured species interact at the binding layer surface without penetration; the time needed by the initial transient before steady-state is negligible compared with the deployment time.

DGTs exploit diffusive processes so the diffusional characteristics of the hydrogels, employed as diffusive and binding layers, influence the performance of the devices and the quality of the measurements. Different gels have been studied and characterized (Table 1.3**Error! Reference source not found.**)[243].

Table 1.3

Characteristics of several types of gels used as diffusive layer in DGTs samplers[243]. Re-edited from Zhang (1999).

Gel type	Monomer (%)	Cross-linker (%)	% T total monomer concentration	% C percentage of crosslinking	Pore size (nm, radius)	Expansion factor	Water (%)
APA agarose cross-linked polyacrylamide gel	15	0.3			>5	3.2	95
BPA1 bis cross-linked polyacrylamide gel	15	0.3	15:3	2:0	>2	1.6	91
BPA2 bis cross-linked polyacrylamide gel	5	1.0	6:0	16:7	>5	1.0	94
CGa polyacrylamide gel (supplied by DGT Research Ltd., Lancaster, UK)	15	0.8	15:8	5:1	<1	1.0	84
AGE pure agarose gel	1.5	0			>20	1.0	98

The dimension of the pores as well as the swelling, that represents the final percentage of water in the gel, are the main factors regulating the diffusional efficiency. The water within the gel can be bound, becoming part of the structure, or can be unattached and free[243]. High swelling polymers are more likely to have a greater proportion of free water which contributes to the free diffusion of solutes. The diffusion of large molecules may be affected by the hydrogel in the DGT assembly, while simple ions diffuse with an effective diffusion coefficient indistinguishable from that for the ion in water[244]. Thus DGT is suitable only to measure dissolved species with molecular size sufficiently smaller than the pore size of the hydrogel to allow them to diffuse freely as well as a fraction of larger molecules which will be partially impeded.

1.5.2 Extraction

Water and sediments next to sources of pollution such as WWTP and agricultural areas are rich in conventional pollutants, such as phosphorus and nitrogen, that reach concentrations above mg L^{-1} and non-conventional pollutants, such as POPs and heavy metals, at concentrations in the order of μg L^{-1} or ng L^{-1}.
The direct measurement of concentrations of the order of parts per billion (ppb) or parts per trillion (ppt), especially in complex environmental matrices, is difficult, sometimes not sufficiently specific, and very expensive[245].

As regards the analysis of organic pollutants, such as EDCs, the analytical approach involves first the use of extractive techniques in case of the use of traditional active sampling strategies. The sample preparation step is considered to be the most polluting step of the entire analytical procedure[246]: traditional technologies such as Soxhlet extraction[247,248] and liquid–liquid extraction (LLE)[249,250] are labour intensive, time consuming and often require the use of solvents harmful to the operator, as well as expensive and environmentally hazardous[251]. To avoid these disadvantages, other extractive approaches that ensure satisfactory recovery (70 to 130 %) have been developed.

Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method

The QuEChERS approach is designed to perform both extraction and purification; it is economic, simple, rapid, requires a minimum amount of organic solvents, can be applied to multicomponents analysis and leads to high recoveries of very polar and volatile compounds[252]. This technique prepares high-water content solid samples and includes several steps[253]: the extraction of the sample by organic solvents and the partitioning to organic phase by exploiting salting out effects (Figure 1.7).

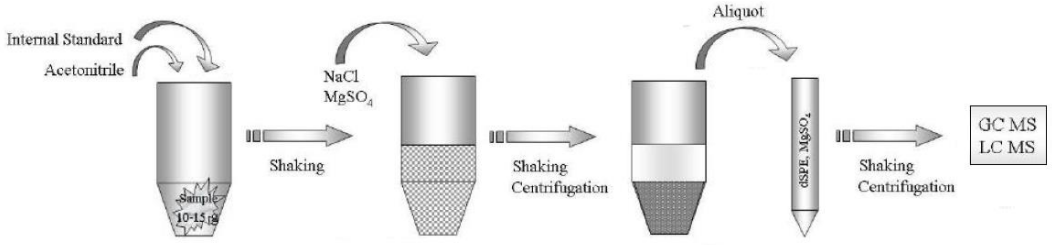


Figure 1.7

Basic steps of the QuEChERS technique[253]. Re-edited from Švarc Gajić (2012).

Solid sorbents used are of general use and are intended only roughly to clean-up, thus extracts still contain matrix components and it is best to use this technique in conjunction with another extraction technique and/or powerful analytical tools. Compounds with substantial differences in their physico-chemical properties require different extraction solvents, because of their different solubilities, and different sample preparation protocols for optimum performance[253] so this methodology realizes optimum performance if used with analytes of the same class. The original QuEChERS method has been constantly improved to comminute the sample and extract pH-dependent molecules. Therefore, it can be successfully applied to different types of pollutants such as pesticides[254], PAHs[255], antibiotics[256] and different matrices, such as milk[257], sugarcane juice[258], soil samples[259], animal tissues[260, 261,256] and banana leaves[262].

Solid-phase extraction (SPE)

SPE plays an important role in a broad range of applications from trace levels to industrial scales and often is used as a reference method[263]. This technique uses an adsorbent material to extract trace organic compounds from aqueous samples. SPE cartridges can be conveniently stored thanks to the stability of the adsorbed analytes[264] but its use is limited to semivolatile or nonvolatile compounds with boiling points higher than the desorption solvent temperature[265]. It offers reduced processing time and important solvent saving but the method still requires multiple steps. SPE presents disadvantages such as losses in the evaporation step, risks of contamination and losses in sensitivity due to the injection of only a small aliquot of the sample. On-line methods, which couple SPE sample preparation to

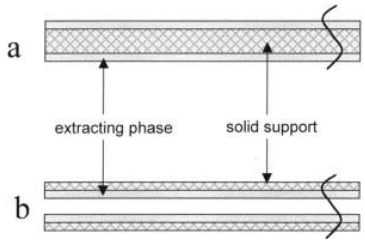
GC/LC separation, ensure more accurate results and prevent most of the problems previously mentioned[266]. SPE C18/NH_2 disks[267,268] or Oasis HLB cartridges[269] with organic eluents, such as acetonitrile, methanol, acetone, are generally used to extract EDCs from environmental matrices. Solid phase extraction is a robust, relatively low cost and well validated extraction method. SPE cartridges have a longer life and are less likely to be saturated and poisoned compared to solid phase microextraction (SMPE) fibers. Moreover, the same type of cartridge can be used with different extraction schemes according to the type of sample being analysed[270]. Therefore it was selected in many official EPA protocols for routine EDC analysis[263,271,272].

Solid-phase microextraction (SPME)

The SPME device is characterized by a very small geometry that allows fast mass transfer during extraction/desorption and prevents plugging. The device is made up of a fine rod[273], either solid or hollow[274], made of fused-silica on which is placed a very small quantity of the extracting phase[275] (Figure 1.8).

Figure 1.8

Two different implementations of the SPME technique: a) polymer coated on outer surface of fiber; b) polymer coated on internal surface of capillary tube.



This miniaturization, however, makes the device fragile, thus an integrated protection system is required: the fiber is exposed only during extraction/desorption and after is lodged in a protective case.

SPME allows an extreme customization[276]: a wide variety of fibers is commercially available or can be realized *ad hoc* in the laboratory to ensure the best recovery for each type of compound.

The principle behind SPME extraction[273] is the distribution of the analyte between the phase in which is initially contained and the polymer film of which the fiber is coated. The extraction is complete when it has reached an equilibrium between the analyte concentrations in the initial phase and in the polymer film that coats the

fiber. The amount of analyte extracted by the fiber is proportional to the initial concentration present in solution through the partition coefficient between the two phases. Thus, it is necessary to take account of the partition coefficient between the fiber and the solution of the desired molecules during the choice of the type of fiber because it determines the efficiency of the extraction.

In general, volatile compounds require a thick polymer coat and a head-space extraction, whereas semi-volatile and non-volatile compounds require a thinner coat and a deep immersion extraction (Figure 1.9). Analysis of high boiling-point and non-volatile analytes in complex matrices are also possible[274].

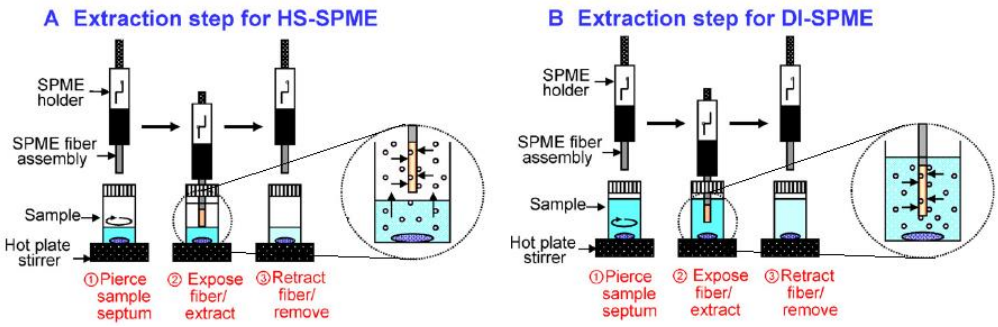


Figure 1.9

Schematic diagram of SPME extraction modes[276]: a) HS –SPME head space; b) DI-SPME deep immersion. Re-edited from Kataoka (2011).

The SPME approach can be successfully applied in environmental[277,278] and food analysis[279,280] and also in bioanalytical and clinical applications[281]. Using this technique EDCs can be quantified in every type of matrix, such as sediments[282] and milk[283] and also *in vivo* testing[276] due to the ability of SPME to non-lethally sample tissues of living organisms, in which it is not possible to use conventional techniques. Validated methods for the measurement of a variety of emerging contaminants in animals are available[284] and also for pharmacokinetic studies[285]. Several official methods for the environmental analysis of persistent EDCs employ SPME[282,286,287]. The use of a small amount of liquid phase in microextraction techniques provides better performance over the large volume approach[288] and it provides a "green" sample preparation[289]. The SPME process has two steps: partition of analytes between the coating and the sample matrix, followed by desorption of the concentrated extract into the analytical instrument.

It can be easily automated and successfully hyphenated especially with GC. A clean-up step is not necessary in the SPME technique because of the selective nature of coatings[290].

1.5.3 Characterization and quantification

The detection, identification and quantification of analytes in the order of hundreds of ng L^{-1} presupposes the development and the application of analytical methods that are extremely sensitive and reliable. Thus sophisticated instruments and trained personnel are needed.

The choice of the analytical approach is linked to several factors:

- the limit of detection (LOD) of the method and the quality of the analyte, presumably present in the sample, on which depends the choice of the most suitable instrumental technique (analytical and instrumental sensitivity);
- the sensitivity required by the legal limit (which, however, in the case of emerging contaminants, has not yet been established, so the only reference is the literature);
- the quality of analytical data obtained (certainty of the analytical result);
- the possibility of using methods recognized and/or validated;
- the cost-benefit ratio.

The main toxicity identification and evaluation (TIE) procedures, used for the effect-based analysis of the EDCs in environmental samples, employ extraction/fractionation and preconcentration and then fast-GC or fast-LC analysis using short, narrow bore columns, high mobile phase flow-rates and ultra-high pressures which shorten the analytical run times and ensure high sample throughput required in monitoring studies[11]. Isotopically labeled internal standards such as deuterated compounds are generally used during the analysis to evaluate matrix effects[291].

The detection limits for the monitoring of the EDCs are being pushed from the μg L^{-1} to ng L^{-1} range and even to below the ng L^{-1} range. Tandem systems (MS-MS) such as time-of-flight MS (ToF-MS), quadrupole-time-of-flight (Q-ToF-MS),

triple quadrupole (TQMS), ion trap and orbitrap, have improved analytical performance in terms of reliability and sensitivity and allowed a gradual shift from the detection of parent compounds to the analysis of metabolites and transformation products. Tandem systems have added power but also add complexity to the analysis making available a variety of scan functions and modes such as product ion scan, precursor ion scan, neutral loss, multiple reaction monitoring; and they also provide multi-residue methods in which different compound classes can be determined in a single analysis. Using LC-MS the LOD for the alkylphenolic compounds[292] achieves the low ng L^{-1} range and the LOD for the steroid hormones in complex environmental matrices[293] in order are LC-MS LOD 200 pg μL^{-1} > GC-MS-MS limit of quantification (LOQ) 20 pg μL^{-1} > LC-MS-MS LOQ 5 pg μL^{-1} .

To evaluate EDCs, LC is generally performed in reversed phase, using electrospray ionization (ESI) in negative mode[291]. Impurities coming from the matrix can affect ionization efficiency and, in co-elution with compounds of interest, result in signal suppression or signal enhancement. HPLC coupled with MS-MS detection reaches LODs under or in the range of 1 ng L^{-1}, depending on matrices and analytes. LC is less used than GC for the analysis of solid matrices[291].

LC-MS-MS has been often chosen as the preferred technique for EDCs analysis[293] because it provides good sensitivity and selectivity, requires short analysis times, it can be easily on-line coupled with SPE, it does not require derivatization, and allows simultaneous analyses in the same run of both free and conjugated estrogens without an intermediate hydrolysis step. Chemical identification and structure elucidation of compounds analysed through LC with electrospray ionization (ESI) and detected by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are made faster thanks to the availability of updated spectra database collected by university consortia[294].

To evaluate EDCs, GC is generally performed using various columns and typical temperature programs (45 - 300°C) with helium carrier gas[295,296]. The GC-MS analysis implies the use of electronic ionization (EI) which also has the advantage

of the availability of extensive libraries of mass spectra for identification of unknown peaks in estrogenically active fractions[291].

To increase volatility and peak shape of the target compounds, a derivatization step, such as silylation is generally performed with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)[13,14] or acylation[297]. Derivatisation is time-consuming, increases the risk of analyte losses and requires in some case the use of harmful reagents[291]. A method for the determination of steroid hormones in tap and sewage water samples based on GC-MS combined with derivatisation and hollow-fiber liquid-phase microextraction (HF-LPME)[298] obtained enrichment factors over 1400 and LODs of 1.6 - 10 ng L^{-1} while GC-MS-MS combined with derivatisation and SPE[10] was capable of detecting natural and synthetic estrogens in water at trace levels reaching LODs of 0.25 - 5 ng L^{-1}.

The possibility of direct GC-MS analysis of trace levels of underivatized steroid estrogens in river sediments, even if challenging and often not successful, has also been demonstrated[269]: low pressure (LP)-GC-MS is a suitable option for routine analysis if only a low-resolution single-quadrupole mass analyser is available, because it ensures rapid separation of sample components at low temperatures and lower LODs, between 1.5-5 ng g^{-1}, compared with conventional systems. For further reduction of detection limits, reduction of the risk of false positives, and/or non-target screening of steroids and other contaminants present in sample extracts, TOF-MS is an excellent tool: GC-TOF-MS reaches LOD of 12 ng g^{-1} while GC×GC-TOF-MS LOD reaches 0.4 ng g^{-1}. TOF instruments offer resolving power superior to quadrupole and ion-trap detectors by minimizing matrix interferences and increasing the signal-noise ratio (S/N)[299].

For aqueous matrices, LODs obtained by GC-MS and GC-MS-MS are generally under 1 ng L^{-1} while for solid matrices, the LODs with GC-MS are 1 - 5 ng g^{-1}, while the LOQs obtained by GC-MS^2 are under 0.5 ng g^{-1} for sediments and 2 - 4 ng g^{-1} in sludge[291.]. The matching and the identification of unknown compounds analysed using GC analysis with electron impact ionization (EI) and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection is made easier and faster thanks to the availability of reliable, comprehensive and updated mass spectra databases[300].

1.5.4 Validation methods

The validation step proves the consistency of the method developed to detect and quantify hormones and EDCs with the validation criteria drafted by the International Conference on Harmonization (ICH)[252] and United States Environmental Protection Agency (USEPA)[263]. The criteria used for validation are the limit of detection (LOD), limit of quantification (LOQ), linearity, extraction recovery, repeatability, intermediate precision, and accuracy. The LOD represents the lowest concentration of the analyte that can be detected but not quantified and LOQ is the smallest concentration that can be quantified.

There are multiple approaches for determining the detection limits[301, 302]:

- based on standard deviation of the blank. This method of limit determination uses the mean and standard deviation of blank samples to set the limits; 10 or more determinations are made at no concentration in the appropriate matrix.

$$\mathrm{LOD} = \overline{blank} + 3.3\,\sigma\,(\text{one sided } 95\% * 2) \qquad 1.5$$

$$\mathrm{LOQ} = \overline{blank} + 10\,\sigma\,(\text{one sided } 95\% * 6) \qquad 1.6$$

  Where $\overline{blank}$ is the mean of the blank and σ is the standard deviation of the blank.

- Based on standard deviation of the response and the slope. This approach can be used if the method does not exhibit background noise of any magnitude. A standard curve may be used to determine LOD and LOQ. Samples need to be taken in the range of the LOD and LOQ and not extrapolated into the range. Six or more determinations are usually made at five concentrations.

$$\mathrm{LOD} = 3.3\,\sigma\,/\mathrm{slope} \qquad 1.7$$

$$\mathrm{LOQ} = 10\,\sigma\,/\mathrm{slope} \qquad 1.8$$

Where σ is the standard deviation of the of the response and slope is the slope of the calibration curve.

- Based on signal to noise. This method is used only when the analytical method exhibits background noise when nothing is in solution. Five to seven concentrations are used with six or more determinations for each concentration. Signal to noise is calculated at each concentration. Signal is the measurement at each concentration, and noise is the blank control from the method. LOD and LOQ are determined for signal-to-noise ratios of 3.3 and 10.

The validation strategy is generally performed on five concentration points and extended over a period of 3 days. Each point corresponds to a sample spiked with a standard solution of the targeted analytes. The calibration curve for each analyte is based on five concentration points adopting the internal standard where possible, otherwise the external standard calibration. Depending on the compound, LOQs could be different. Thus, it is necessary to establish a range of concentrations for linearity, taking into account the sensitivity of each compound. The response of each compound should be linear over the entire concentration range with a correlation coefficient greater than 0.99. Recoveries and RSDs are usually calculated on three replicates[301]. It is also necessary to obtain and estimate a matrix blank. Intermediate precision is evaluated at the same levels as repeatability after 3 days[261]. Accuracy is determined as the bias between the theoretical and calculated concentrations, expressed as a percentage and it is assessed using 3 replicate measurements for 3 concentrations[252].

1.6 *In vitro* biochemical assays for assessing the total hormonal activity in environmental matrices

In vitro bioassays have been extensively use to explore chemicals that can elicit estrogenic responses and could further compromise human and wildlife health. Generally it is recommended to associate complementary *in vivo* studies to the *in vitro* bioassays to accurately assess the effect of any chemical or a complex mixture downstream, upon a specific living being.

As mentioned, xenoestrogens are a broad range of substances that do not necessarily share any structural resemblance to the prototypical estrogen, βE2, but produce agonist or antagonist responses using comparable mechanisms of action.
It is possible to identify ECs according to their ability to bind to the estrogen receptor (ER) and to induce or attenuate a response. Once an estrogen binds to ER it causes the dissociation of the HSP90, allowing occupied ERs to homodimerize. This homodimer complex exhibits a high affinity for specific DNA sequences known to be estrogen response elements (EREs). Then the homodimer complex bonded to ERE recruits transcription factors to the target gene promoter, which leads to increased gene expression. The increased level of mRNA transcribed corresponds in turn to the production of proteins, the ultimate effectors of the observed responses[303]. ECs act as ER ligand mimics that bind to the receptor, thus modulating endocrine response via a receptor-mediated process. To identify and assess alleged xenoestrogens, several *in vitro* bioassays exploit this reception-mediated mechanism of action, even though ECs could exert their effect using pathways independent of the ER.

1.6.1 Cell assays

The competitive ligand binding assay consists of a biologically specific binding agent that competes for radioactively labeled or unlabeled compounds[304]. This assay has been widely used to investigate ER-ligand interactions. This assay may be adopted to investigate the ability of a chemical to bind to ER but it cannot distinguish between receptor agonist and antagonist, moreover it does not provide enough evidence to conclude that the compound will adversely affect human health and environmental quality[303].

Cell proliferation assays, often referred as E-Screen, use ER-positive, estrogen responsive MCF-7 or T47-D human breast cancer cells[305]. The E-Screen compares the number of cells present following a 6 day incubation period in a medium supplemented with steroid-stripped dextran-coated charcoal (DCC) serum in the presence or absence of alleged xenoestrogens. Also this essay suggests but does not unequivocally demonstrate that a substance is estrogenic. It also has limited screening applicability for both agonistic and antagonistic

activities due to its modest responsiveness and relatively long incubation periods. Despite these disadvantages the MCF-7 E-Screen results in one of the most sensitive *in vitr*o assays to evaluate the estrogenicity of a chemical with a reported detection limit of 10 pg of βE2/mL (30 pM βE2)[305].

The estrogen-dependent ability of confluent MCF-7 human breast cancer cells to generate multicellular clusters has been proposed as an ER-mediated response that could be used to identify and evaluate the potency of a supposed EC[303]. Foci are nodules of cellular outgrowth generated by a piling up and overlapping of cells on a confluent monolayer background. This assay exhibits reasonable selectivity and sensitivity compared to other bioassays, but it may be less widely used since the foci formation is only observed after 9-10 days in the presence of 1 nM βE2 and 15 days in the presence of 10 pM βE2[303].

1.6.2 Protein expression and enzyme activity assays

The induction of several proteins or enzyme activities has been used to evaluate the estrogenic potency of possible toxicants. The induction of hormone receptor levels and the increased expression of secreted proteins such as HSP60, HP70[306]and Vtg[226] have been used as measurable end points. The expression of these proteins and enzyme activities give very precise indications about the estrogenic potency of a chemical but the results may be restricted to specific cell lines, thus it may be relevant only to the tissue and species studied.

The measurements of proteins generally involves laborious methodologies in particular:

- a step for cell lysis and protein extraction performed using mechanical and/or chemical treatments. Actually the detergent-based lysis methods are the most common[307].
- A step for protein separation.

  Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the method of choice to analyse complex protein mixtures. A single 2-D separation is a powerful and easy technique able to separate hundreds, if

not thousands, of proteins of different isoelectric points (pI) and different molecular weight values in only one run[308].

- A step for protein quantification.

  Protein quantification may be realized using a MS technique. This technique is robust, accurate and reproducible and achieves low limits of detection and may be coupled with 2D-PAGE[309,310] allowing not only protein identification but also quantitative analysis. However, sophisticated and expensive instruments and trained personnel are required.

  An alternative approach for the quantification of protein expression/modification in cell/tissue extracts is blotting. The blotting is always hyphenated with 2D-PAGE. It implies the transfer of the biological samples from the gel, used during the electrophoresis separation, to a membrane and their subsequent detection on the surface of the membrane[311]. Western blot, also known as immunoblotting, is a fast and easy routine technique based on chemiluminescence detection; this technique permits determination of the presence and the abundance of a protein or the amplitude of possible modifications in cell/tissue extracts or in nucleus/cytosol compartments[312].

1.6.3 Recombinant assays

Recombinant receptors/reporter gene assays identify receptor-specific ligands. These assays may be used to assess receptor-mediated toxicants classifying them upon their potential mechanism of action and their chemistry[303].

Recombinant receptors/reporter gene assays are broadly categorized into endogenous promoter-regulated reporter genes, response element-regulated genes, chimeric receptor/response element-regulated reporter genes and yeast-based assays.

1.6.3.1 Endogenous promoter-regulated reporter genes

This strategy implies the use of recombinant reporter genes consisting of endogenous promoters from estrogen-responsive genes linked to reporter genes encoding firefly luciferase[313], chloramphenicol acetyltransferase (CAT)[314], β-galactosidase (LacZ)[315], or alkaline phosphatase[316]. These reporters ensure sensitive, selective, stable and easily detectable enzyme activity.

1.6.3.2 Response element-regulated genes

This strategy overcomes pitfalls of the endogenous promoter-regulated reporter genes using reporter genes that are regulated by the 13 base pair (GGTCAcatTGACC) vitellogenin A2 estrogen response element (ERE). This strategy ensures that induction of the gene occurs only through the ERE. However ERE-regulated reporter genes exhibit poor responsiveness in the presence of serum-borne estrogens and are susceptible to induction, synergism or antagonism via receptors other than ER.

1.6.3.3 Chimeric receptor/response element-regulated reporter genes

This method, often referred to as the E2 bioassay, discriminates and assesses accurately the estrogenic potency of an alleged toxicant. Despite its complexity, it ensures a great selectivity, responsiveness and is less sensitive to the presence of serum-borne estrogens in media. The E2 bioassay exploits the receptor-mediated mechanism of action of estrogenic substances. It consists of 2 central components: the Gal4-HEGO chimeric receptor and the Gal4-regulated luciferase reporter gene. After treatment of transfected cells, the estrogenic substance binds to the ER ligand binding domain of the chimeric receptor and transforms the Gal4-HEGO construct into an activated, high-affinity DNA binding homodimer receptor complex. The Gal4 DNA binding domain then directs the activated chimeric receptor complex to the 17m5-G-Luc reporter gene where it binds to the Gal4 response elements (17mers). Binding of the activated complex to the 17mer response elements starts expression of the firefly luciferase cDNA which, in turn, results in the induction of luciferase activity. Thus, the determination of the luciferase activity is a measure of the estrogenic activity of a substance[303].

1.6.3.4 Yeast-based assays

Yeasts are known to be systems without endogenous receptors, with media devoid of steroids and a genetic disposition that facilitates the insertion of mammalian proteins and reporter genes. These advantages made yeasts convenient to investigate receptor structure and function as well as the activity of selected ligands such as alleged EDCs. There are two main yeast-based estrogenic assays available that exploit the yeast strain *Saccharomyces cerevisiae*.

The first uses the URA3 detectable marker as the reporter gene in an ER-mediated phenotypic transactivation assay[317]. The recombinant *S. cerevisiae* strain, PL3, expresses the human ER and contains the URA3 reporter gene that is regulated by three tandem EREs. Thus, expression of the URA3 gene and subsequent growth of PL3 on selective media lacking uracil is dependent on ER-mediated induction of OMPdecase activity, an enzyme involved in uracil synthesis. This assay can be used for qualitative screening, monitoring growth, or for quantitative assessment measuring the OMPdecase activity[317].

The other assay uses *S. cerevisiae* strains transformed with the human ER cDNA and an ERE-regulated LacZ reporter gene that encodes for the β-galactosidase enzyme[318]. This assay has been designed in a way that the β-galactosidase enzyme is secreted in to the media, thus facilitating its qualitative and quantitative assessments. Moreover it exhibits a low LOD (0.07 pM) due to its exquisite responsiveness.

The yeast estrogen screen (YES) assay exploits this type of modification. *S. cerevisiae* strains are in fact recombined in order to identify compounds that are human estrogen receptor (hER) active[319]. Normally these yeast cells do not contain an estrogen receptor, thus the DNA sequence of the hER is permanently integrated into the main chromosome of the yeast. Expression plasmids of the yeast cells carry the reporter gene *lac-Z* that encodes the enzyme β–galactosidase which is exploited to measure the receptors' activity. In the system thus designed (Figure 1.10), the hER is expressed in a form able to bind estrogen-responsive sequences (ERE). These sequences are situated within a strong promoter sequence on the expression plasmid. Once an active ligand is bound, the estrogen-occupied

receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. This process leads to the expression of the reporter gene *lac-Z* and the β-galactosidase enzyme produced is secreted into the medium, where it metabolizes the chlorophenol red-β-galactopyranoside (CPRG) that is a chromogenic substrate. This substrate is normally yellow while its product/metabolite is red and its absorbance can be colorimetrically measured at 570 nm.

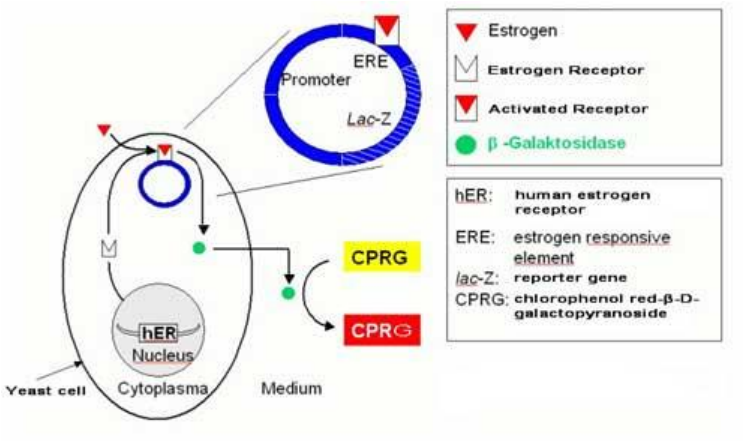


Figure 1.10 *Schematic representation of the estrogen-inducible expression system in yeast.*

Despite all the advantages already mentioned, factors such as permeability, variations in *S. cerevisiae* strains, differences in receptor levels and protein proteolysis mechanisms, non-receptor cell-specific factors, metabolic capabilities, multidrug resistance efflux pumps and endogenous yeast binding proteins[303] should be controlled and evaluated otherwise they could affect the value of estrogenic potency detected for a specific compound.

2 EXPANDING THE REPERTOIRE OF DIFFUSIVE GRADIENTS IN THIN FILMS IN SITU MEASUREMENTS OF ENDOCRINE DISRUPTING CHEMICALS IN AQUATIC SYSTEMS

2.1 Introduction

Environmental monitoring is essential to evaluate, protect and control the long term quality of natural resources such as water, sediments and air. Indeed, the deterioration of the quality of these primary resources is likely to be reflected in impacts on ecosystems.

Effective and efficient monitoring should be reliable, robust, fast and cheap, providing a representative picture of the *status* of the resource. Several approaches and methodologies are available to characterize analytically the presence of pollutants. In time, chromatographic techniques hyphenated with mass spectroscopy detection were chosen as the method for the environmental monitoring carried out herein[11,291]. The choice is due to the ability of these techniques to discriminate in a single analysis multiple classes of pollutants.

The sampling step is another crucial step that needs to be carried out carefully during monitoring. In fact an inappropriate sampling strategy would affect the quality and the meaning of the data collected. Routine methods for the detection of EDCs usually adopt an active sampling followed by a pre-concentration, generally SPE[263]. However this approach requires 2 separate steps prior the chromatographic analysis: one for the sampling and one for the concentration, becoming costly and time consuming, moreover with such an approach only instantaneous environmental concentrations can be evaluated.

The aim of this study was to develop and test an analytical method that exploits passive sampling to assess the presence of EDCs, in particular bisphenolic plastics and estrogens, in fresh water systems. Passive sampling combines sampling and concentration in only one step and allows the estimation of time-integrated concentrations.

Water quality was evaluated by DGT passive sampling coupled with HPLC/MS analysis that ensures a robust, automated and sensitive routine analysis. DGT is a promising sampling regime and it was chosen due to its ease of use and low cost.

The DGT approach has been extensively and successfully employed for the detection of inorganic micro-pollutants[237,238] in water, soil and sediments. However, organic species constitute the majority of pollutants in the environment and the potential and suitability of DGT has not been fully investigated yet. Only in recent years in fact has this sampling been deployed for the detection of some organic species[320]. Only one previous study focused on the quantification of plastic monomers[241] employing DGT as a sampling technique. The investigation conducted in this study would be beneficial in extending the application of DGTs for the simultaneously sampling of other types of plastic monomers and estrogens as well, known to be the most potent classes among the EDCs[4,22].

Several physical-chemical and chemical analyses have been employed to accurately characterize the DGT probe and to test its suitability for the monitoring of the selected disruptors as shown by the flow chart (Table 2.1). BPA (pKa = 9.6 at 25 °C)[321], BPAF (pKa = 9.2 at 25 °C)[322], E2 (pKa = 10.71 at 25 °C)[323] and EE2 (pKa = 10.25 at 25 °C)[324,325] had dissociation constants with high values thus, pH studies have not been carried out throughout the investigation. The ionic strength (IS) influence was not evaluated considering that the deployment sites included fresh waters low salinity, although application of this technique to more saline waters would require evaluation of the effect of ionic strength on DGT performance.

Table 2.1

Flow-chart of the physical-chemical and chemical analyses employed to characterize the DGT probe.

PHYSICAL-CHEMICAL ANALYSIS	CHEMICAL ANALYSIS
➢ Swelling kinetics and swelling ratio of the gels: Analytical scale, Caliper measurements.	➢ Adsorption of E2, EE2, BPA and BPAF by the material of the DGT probe: High-performance liquid chromatography (HPLC) mass spectrometry (MS).
➢ Thermal behaviour and homogeneity of the gels: Thermogravimetric analysis (TGA), Energy-dispersive X-ray (EDX) elemental analysis.	➢ Binding Kinetics of E2, EE2, BPA, BPAF onto Activated Charcoal gel: High-performance liquid chromatography (HPLC) mass spectrometry (MS):
➢ Approximated conformations and dimensions of E2, EE2, BPA and BPAF: Gaussian software simulations.	➢ Effective Diffusion coefficients measurements: High-performance liquid chromatography (HPLC) mass spectrometry (MS)
➢ Morphological characterization of the gels: Field emission scanning electron microscopy (FE-SEM).	

2.2 Materials and methods

2.2.1 Materials

All the glassware was silanized in order to prevent adsorption of solute to the glass surface and to increase the surface hydrophobicity. This is particularly important in the case of trace analyses like the ones conducted in this study. Organic solutes with different polarities, like proteins and hormones, in fact tend to stick easily to untreated glass walls because of the presence of silicates and silanol (SiOH) groups that act as ion-exchange and nucleophilic centres[326].

The short polymers of dichlorodimethylsilane react on contact with a silanol group (Si-OH) to give hydrochloric acid (HCl) and siloxane linkage (Si-O-Si). The polymerization of dichlorodimethylsilane is mediated by residual water absorbed to the glass surface. Chlorosilanes in fact react with the water and alcohols to give hydrochloric acid and silanol or alkoxylane, respectively. Therefore, in the polymerization reaction, the residual water reacts with the chloride end-group to give HCl and a silanol, which then reacts with more dichlorodimethylsilane and more water to give polymers[326].

Items were silanized by briefly soaking in a 5% solution of dichlorodimethylsilane, a silicon oil, using heptane as the volatile organic solvent. The solvent was removed by evaporation, depositing the dichloromethylsilane on the surface[326]. The dichlorodimethylsilane used to silanize the glassware was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) while n-heptane solvent was obtained from Merck Millipore (Burlington, Massachusetts, USA). Standard DGT mouldings of acetonitrile-butadiene-styrene (ABS), rigorously BPA free, were obtained from DGT Research Ltd, (Lancaster Lancashire, UK). Hydrophilic poly(tetrafluoroehtylene) (PTFE) filter membranes with diameters of 25 mm and pore sizes of 0.45 µm were obtained from Merck Millipore, (Burlington, Massachusetts, USA) and were soaked in deionised water for 24 h prior to use. Pyrex tempered glass sheets of 5 mm thickness and 10 cm x 10 cm dimension were employed as casting-moulds for the diffusive hydrogels while 17 cm x 6 cm and 5 mm thickness glass plates were employed to cast the binding hydrogels. Styrene sheet spacers of 0.5 mm thickness were purchased from Evergreen Scale Models (USA) and were used for both diffusive and binding hydrogels. Pyrex glass sheets, styrene spacers, DGT mouldings, PTFE filter membranes, hydrogels, glassware and vials were soaked or washed in SISTEMA® plastic containers (New Zealand) rigorously phthalate and BPA free.

Nalgene containers were adopted to store diffusive and resin hydrogels using the appropriate storage solutions.

DGT performance was tested using 1 L high containers, tweezers and syringes made up of hydrophobic perfluoroalkoxy alkane (PFA) purchased from Savillex (Long Beach, California, USA) to avoid adsorption of the targets on the surface of the containers during the measurement.

2.2.2 Chemicals and solutions

HCl fuming concentrated (37%) and HNO_3 concentrated (65%) were obtained from Merck Millipore (Burlington, Massachusetts, USA) and were used to prepare 10% acid washing solutions (100 mL in 1 L of deionized water). Agarose BioReagent LOW EEO (0.09-0.13) gel point 36°C (1.5% gel, ± 1.5 °C) was

purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Powdered activated charcoal (AC) (100-200 mesh) was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The sodium chloride salt was purchased from Riedel-de Haën (Wunstorfer, Germany) and solutions of 0.01 M (0.58 g in 1 L of deionized water) were prepared to store the diffusive gels. The analytical standards of BPA and BPAF (purity ≥ 99 ± %) were purchased from AccuStandards (New Haven, Connecticut, USA), while EE2 and β-E2 (purity ≥ 98 %) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock solutions of BPA, BPAF, EE2 and β-E2 were prepared at 1000 mg L^{-1} in methanol (HPLC grade) purchased from Scharlau Sentmenat (Barcelona, Spain) and stored in sealed volumetric flasks at 4°C. Deionised water (≥ 18.2 mΩ) was used to dilute stock solutions. Methanol and ethanol (HPLC grade) purchased from Scharlau Sentmenat (Barcelona, Spain) were employed for the elution of binding gels.

2.2.3 Instruments

Powders were weighed employing a Mettler AE200 analytical balance (d.p. 0.1 mg) Liquid reagents were dosed using automated pipettes (Finnpipette F1) of appropriate volumes (1-10 mL; 0.5-5 mL; 10-100 μL; 100-1000 μL) purchased form Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Gel solutions were homogenised using a Color Squid Number One stirrer or a MS1 minishaker both purchased from IKA (Staufen im Breisgau, Germany); stock solutions were homogenized using a Soltec (Milan, Italy) Sonica M S3 ultrasonic cleaners with sweep system.

Diffusive and binding gel sheet hydration, kinetics of swelling, adsorption studies, kinetics and elution efficiency were evaluated by shaking the soaked hydrogels/membranes/moulding in aqueous media/methanol/standard solution on a Ratek (Barkan, Israel) RPM5 medium rocking platform mixer – digital for the designated time.

Thermogravimetric analysis/differential thermal analysis (TGA/DTA) of the dry and swollen hydrogels were carried out using a SDT 2860 simultaneous DTA-TGA analyser purchased from TA instruments (New Castle, Delaware, USA).

A Labconco (Kansas City, Missouri, USA) FreeZone 6L Plus lyophilizer was adopted to freeze-dry the diffusive and resin hydrogels prior to morphological analysis.

An ion sputter Hitachi (Tokyo, Japan) E-1030 was used to cover the diffusive and resin gel with a thin homogeneous conductive layer of platinum to make the hydrogels conductive for the electron microscopy analysis.

A field emission scanning electron microscope (FE-SEM) Hitachi (Tokyo, Japan) S-4700 was employed for the morphological characterization and elemental analysis of the diffusive and resin gels.

The thicknesses of the swollen diffusive and binding hydrogels were evaluated using a J.B.S. vernier caliper (Beaverton, Oregon, USA) Digital M742 (200 mm).

A Thermo Fisher Scientific (Waltham, Massachusetts, USA) UltiMate 3000 standard (SD) HPLC unit hyphenated with a Bruker (Billerica, Massachusetts, USA) Amazon X ion trap mass spectrometer was used for the separation and quantification of the target analytes. A Phenomenex (Torrace, California, USA) Gemini NX-C18 110 Å (100 x 2 mm, 3 µm particle size) HPLC column, which delivers stability at pH 1-12, protected by a Gemini NX-C18 (4 X 2 mm ID) guard column were chosen to perform the chromatographic separations.

2.2.4 Diffusive gradient in thin-film sampling (DGT)

2.2.4.1 *DGTs diffusive gel preparation*

The diffusive hydrogel layer was made by aqueous gelation of agarose. 15 mL of diffusive gel solution (1.5%) was prepared by mixing 15 mL of deionized water with 0.225 g of agarose powder. The solution was heated to 90°C under stirring. Once the solution became colourless it was cooled down to 70°C. After that the

mixture was pipetted into pre-assembled and pre-heated (50°C) glass plates in a smooth and controlled way. Any air bubbles were removed by tilting the plates before continuing with pipetting.

Several steps were required to assemble the glass gel-casting moulds:

- glass plates of 10 cm x 10 cm and styrene sheet spacers (0.5 mm thick) were washed in HCl solution (10%), rinsed in deionized water, then washed in HNO_3 (10%) and rinsed again in deionized water. Plates and spacers were dried with clean tissue papers whilst avoiding touching the surfaces.
- Thickness spacers were kept around three edges and glass plates were clipped together to avoid displacement. A gap of some millimetres was left on the edge without the spacer of the plates to simplify the pipetting of the gel solution.

Once the mixture was all pipetted, the assembly was placed in a pre-heated oven at 36°C for 60 minutes to set the gel completely. Then the glass plates were opened by using Teflon coated razor blades and the gel was carefully transferred to 1 L of deionized water for hydration. Water was changed 3 times in 24 hours keeping the container on a rocker (rotational speed 20 rpm) for even mixing of water. The gels thus obtained were transferred to 0.01 M NaCl and stored at room temperature for up to 8 months.

The agarose hydrogels adopted in this study swell up to 1.08 times their original volume upon hydration (see section 2.2.4.6). Therefore, the 0.5 mm thick spacer adopted produces a diffusive gel of 0.54 mm.

2.2.4.2 *DGT binding gel preparation*

Activated charcoal was selected as sorbent because of its low cost and its efficiency in absorbing bisphenols as proven in previous studies[241]. The polymer-activated charcoal composite sorbent was prepared by gelation of agarose in the presence of uniformly dispersed powdered AC (100-200 mesh). To prepare the binding gels, 15 mL of diffusive gel solution, made as previously described, was gently stirred and kept at 90°C. A suspension of AC (100-200 mesh) was prepared

by mixing 3.75 mL of deionized water with 0.187 g of AC powder. The suspension was mixed through sonication. The suspension was added to the diffusive gel solution increasing the stirring to ensure a complete dispersion. After that, the mixture was quickly pipetted into pre-assembled glass plates in a smooth and controlled fashion. Any air bubbles were removed by tilting the plates before continuing with pipetting. The glass gel-casting moulds were assembled as described previously, but the assembly employed plates of dimensions 17 cm x 6 cm and styrene spacers of 0.5 mm of thickness. The assembly was kept in a pre-heated oven at 36°C for 90 minutes to set the gel completely. The casted resin gel was carefully transferred to 1 L of deionized water for hydration. Water was changed 3 times in 24 hours keeping the container on a rocker (rotational speed 20 rpm) to ensure mixing. The gels thus obtained were store in deionised water at room temperature up to 8 months[241]. The agarose hydrogels adopted in this study have an expansion factor of 1.02 thus the 0.5 mm thick spacer produces a resin gel of 0.51 mm (see section 2.3.3).

2.2.4.3 *DGT probe assembly*

Hydrophilic PTFE filter membranes of 0.45 µm pore size and 2.5 cm diameter were soaked in deionized water overnight. Some drops of deionised water were spread all over the surface of a Perspex cutting board to moisten it. The diffusive and resin gel sheets were stretched out on the cutting board using Teflon pliers avoiding unnecessary lifting that could cause damage. Both diffusive and resin gels were cut using a gel disc cutter into discs with diameters of 2.51 cm. The gel cutter was pressed and twisted firmly at the same time on the gels to produce discs with the correct shape and size. At this point it was possible to assemble the DGT probe. A resin gel disc was placed at the base of a clean piston, followed by one of diffusive gel and everything was protected with a disc of filter membrane. The outer sleeve with the window was put on top carefully avoiding air bubbles, it was pressed down until the bottom of the piston was reached (Figure 2.1). Another diffusive gel layer may be added under the resin disc if the appropriate height is not reached.

Figure 2.1
DGT probe finally assembled.
From the top: acetonitrile-butadiene-styrene (ABS) outer sleeve, poly(tetrafluoroethylene) (PTFE) filter membrane, agarose (1.5 %) diffusive gel disc, agarose (1.5 %) – activated charcoal (1%) resin gel disc, acetonitrile-butadiene-styrene (ABS) piston.

2.2.4.4 *DGT swelling ratio measurements*

The kinetics of swelling of agarose (1.5 %) and agarose (1.5 %) - AC with weight percentage 1.0% of activated charcoal were studied for 24 h. In the swelling measurements, the diffusive or resin gel was weighed (W_0) and immersed in aqueous media at room temperature and neutral pH. After the designated soaking time had elapsed, the wet samples were wiped dry with a tissue paper to remove excess liquid, and weighed (W_t). The swelling ratio (ΔW %) was calculated using the following equation (2.1).

$$(\Delta W\%) = \frac{(W_t - W_0) * 100}{W_0} \qquad 2.1$$

2.2.4.5 *DGT test of homogeneity and thermal behaviour*

The quality and homogeneity of the diffusive and resin gel layers are key factors in the performance of the DGT probes. The gel sheets need to be homogeneous and without air bubbles in the structure to ensure reproducible sampling. Thermogravimetric analysis was conducted on agarose (1.5%) and agarose (1.5%) - activated charcoal (1%) hydrogels to verify that the percentage of water and carbon was constant along the two-dimensions of the gel sheet. The samples were tested under 150 mL/min air flow, in the range 30 – 800°C employing a 40°C/min heating rate.

In the field emission scanning electron microscopy (FE-SEM) analysis, elemental analysis of freeze-dried, platinum-coated agarose (1.5%) and agarose (1.5%) -

activate charcoal (1%) was conducted using energy-dispersive x-ray spectroscopy analysis (EDX). This analysis enabled a chemical characterisation of the samples and verification of the different contents of carbon. A beam energy of 5 eV was adopted to produce sufficient x-rays from C K-α (0.27 eV), O K-α (0.53 eV), Si K-α (1.74 eV) while minimizing the electron beam penetration, avoiding biases in the elemental weight values by the presence of the carbon tape under the samples.

2.2.4.6 *DGT morphological analysis*

Agarose is a linear polymer made up of 2 basic units (Figure 2.2): D-galactose and 3,6-anhydro-L-galactopyranose linked by α-(1→3) and β-(1→4) glycosidic bonds[327].

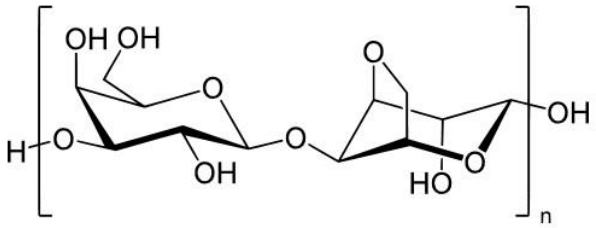


Figure 2.2
Basic agarose structure.

More agarose polymeric chains interact together creating helical fibres that aggregate into a supercoiled structure of 20 -30 nm in radius[328]. The helical fibers have a quasi-rigid structure and their lengths vary according to the agarose concentration[329]. The solidified fibres form a three-dimensional mesh of channels, held together with hydrogen bonds, of variable diameter (50 - 200 nm) depending on the concentration of agarose employed: higher agarose concentrations will correspond to lower average pore diameters. Indeed, hydrogel pore-sizes should be big enough to ensure the diffusion coefficient of the target analytes in the gels to be equal to their diffusion coefficient in the bulk solution.

Thus, the dimensions of the target molecules (BPA, BPAF, βE2 and EE2) and the pore size of both diffusive and binding gels were evaluated. The dimensions of the analytes were calculated through Gaussian simulation (DFT method B3LYP, basis set 6-31G). The surface appearance and the approximated pore-size of the swollen hydrogels made up using the water solution of agarose (1.5%) and water solution of agarose - activated charcoal (1%) were evaluated through morphological

characterization using a field emission scanning electron microscope (FE-SEM) while their thickness was evaluated through caliper measurements.

Prior to FE-SEM analysis, the samples were frozen using liquid nitrogen and then lyophilized in a freeze-drier (collector temperature -84°C; vacuum 0.002 mBar) for 24 h to minimise damage to the hydrogel structure prior the characterization of its three-dimensional structure. In fact, the hydrogel structure is peculiar and exists in relationship with the specific water content. Since the hydrogel is almost exclusively composed of water, it is not compatible with morphological analysis using scanning electron microscopy. Therefore, the freeze-drying technique was employed to subtract the water from the samples avoiding the collapse of the polymeric networks. Even if freeze-drying treatment does not allow the most accurate pore-size evaluation, it results in a valid economical option to evaluate their order of magnitude. Pore diameter measurements were performed using the acquired images and knowing the scaling. Hydrogels dried using just a tissue paper (air dried) were analysed as well, and their morphologies were compared with those of the freeze-dried samples. To realize the image acquisitions, the organic samples were made conductive by coating their surface with a thin layer of platinum sputtering at 80 s counts and current 20 mA. All the samples were fixed on aluminium stubs using graphite sticky-tape.

2.2.4.7 *DGT assessment of possible adsorption*

Filter membranes are commonly adopted to protect the DGT diffusive layer. Different types of membranes and moulding materials are commercially available and should be chosen to avoid adsorption of the target analyte during DGT deployment. According to previous studies[241] ABS DGT mouldings and hydrophilic PTFE filter membranes are the most suitable for bisphenol measurements due to their small adsorption of BPA (<3%), hence they were employed throughout this study.

ABS DGT mouldings, hydrophilic PTFE filter membranes and diffusive gels of 0.54 mm of thickness were tested by soaking them in 10 mL of 100 μg L^{-1} BPA, BPAF, EE2 and βE2 standards and then shaken horizontally for 6 h. The

concentration of the standard in the solutions before and after exposure was measured to evaluate the mass adsorbed.

2.2.4.8 *DGTs kinetics of adsorption and elution*

The elution factor allows an evaluation of the recovery of the target analyte from the binding gels. Each probe was immersed in 1 L of 1000 µg L^{-1} BPA, BPAF, βE2, EE2 with a matrix of 0.01 M NaCl and shaken for various times from 5 min to 3 h. Previous kinetic studies have demonstrated in fact that after 60 min almost 95% of the BPs in solution were absorbed[241] by the binding layer, thus it was chosen to monitor the kinetics for 3 h. AC gels were retrieved after the selected time and eluted. Several types of solvents, volumes and times of elution were tested to achieve a satisfactory recovery of the analytes from the binding gels. The immersion solutions and eluents were analysed to evaluate the kinetics of adsorption and the elution efficiencies, respectively. The elution factors f_e were calculated using equation 2.2 derived from the equation 1.3:

$$f_{\mathrm{e}} = \frac{C_{DGT}\,(V_g + V_e)}{M} \qquad 2.2$$

where C_{DGT} is the concentration of the analyte in the eluent, V_g is the volume of the gel of the binding layer, V_e is the volume of the eluting solution, and M is the mass of analyte accumulated in the binding layer.

The recovery of the trace analytes from the binding gel by batch elution[237] assessed how well DGT worked as a fully quantitative technique by comparing C_{DGT} (DGT measured concentration) and C_{soln} (concentration in solution measured by a conventional technique) (equation 2.3).

$$R\% = \frac{C_{DGT} * 100}{C_{soln}} \qquad 2.3$$

2.2.4.9 *DGT effective diffusion coefficient measurement*

Diffusion coefficients were measured by immersing DGTs assembled with AC resin gel and agarose diffusive gel layer and a hydrophilic PTFE filter membrane in 1 L of 1000, 750, 500, 250, 100, 50, 25, 10, 1 µg L^{-1} of BPA, BPAF, EE2, βE2 standard solutions for 24 h, under magnetic stirring, recording the temperature before and after the immersion (Figure 2.3). AC gels were then retrieved from the probes and the targets were eluted and analysed adopting the same protocols employed for the kinetics studies.

Figure 2.3
DGTs trial cell made up of silanized pyrex glass and hydrophobic perfluoroalkoxy alkane (PFA) container and screw cap.

The values of the effective diffusion coefficients *D* were calculated using equation 2.4 derived from the equation 1.4:

$$D = \frac{M\,\Delta_g}{C_{soln} A t} \qquad 2.4$$

where *M* is the mass of the target accumulated in the binding layer, Δ_g is the thickness of the filter membrane and diffusive gel layer, C_{soln} is the concentration of the target in solution measured by a conventional technique, *A* is the surface area and *t* is the time of deployment.

2.2.5 High performance liquid chromatography (HPLC) separation

The chromatographic separation was performed by adapting protocols of previous studies[330,331]. The separation was carried out using a Phenomenex Gemini NX-C18 110 Å, HPLC column of 100 x 2 mm i.d. and 3 µm particle size protected by a Gemini NX-C18 guard column of 4 x 2 mm i.d.. A modified EPA method 539

was adopted as the elution program[263,332]. The column was kept at room temperature, a partial injection volume of 10 µL, a flow rate of 200 µL min^{-1} and a methanol (MeOH, NH_4OH 0.06 M)/water (H_2O, NH_4OH 0.06 M) fast gradient elution program were used (Figure 2.4): from 0 to 0.01 min 35% MeOH, NH_4OH 0.06 M; from 0.01 to 0.6 min a linear gradient elution up to 65% MeOH, NH_4OH 0.06 M; from 0.6 to 7.5 min an isocratic step at 65% MeOH, NH_4OH 0.06 M; from 7.5 to 8.5 min a linear gradient elution up to 85% MeOH, NH_4OH 0.06 M; from 8.5 to 13.0 min an isocratic step at 85% MeOH, NH_4OH 0.06 M; from 13.0 to 13.01 min a linear gradient elution down to 35% MeOH, NH_4OH 0.06 M; from 13.01 to 15.0 min an isocratic step at 35% MeOH, NH_4OH 0.06 M.

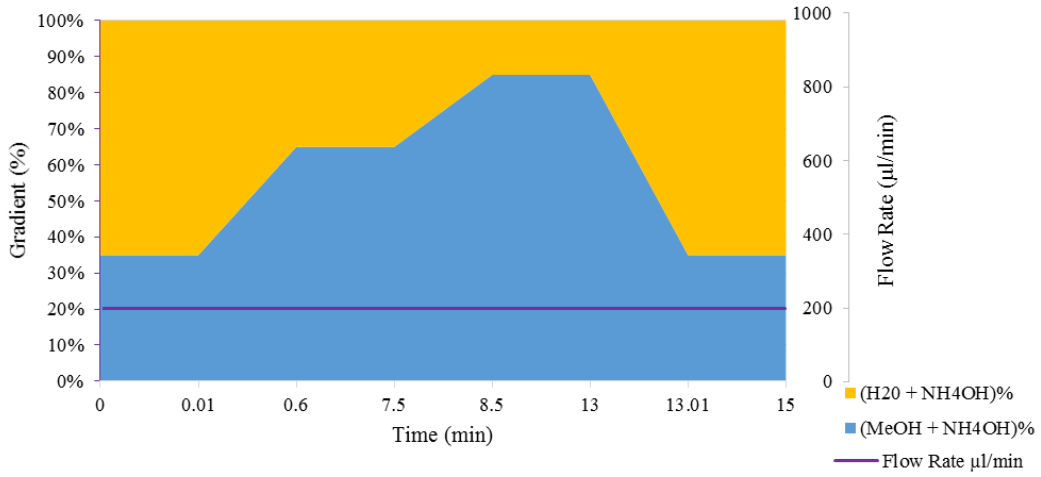


Figure 2.4

Schematic representation of the HPLC gradient and flow during the chromatographic runs.

After every analysis, a wash run of 15% MeOH/water (200 µL min^{-1}, 5 min) was used to avoid sample carry-over. Methanol and water were adopted as the mobile phase because results from previous works[333] demonstrated that the responses were higher than those using acetonitrile and water. Furthermore, water blank samples were injected between sample batches to control carry-over and background contamination. The Dionex Chromeleon Express Chromatography Management System was used as software to set the HPLC compartment.

2.2.6 Mass spectrometry (MS) detection

The liquid chromatography system was hyphenated with an ion trap mass spectrometer equipped with a heated electrospray ionisation source (ESI) operating in negative mode with optimized capillary energy (CE) for every analyte (spray voltage of -3000 V for BPA and BPAF, -4000 V for E2 and EE2) and nebulizer temperature of 220°C. Nitrogen (purity> 99.98%) was used as a sheath gas at 10 L/min flow rate and a pressure of 21.8 psi. The deprotonated molecule $[M-H]^-$ was used as precursor ion for tandem mass spectrometry and a transition was monitored for the identification of each target.

Tandem mass spectrometry (MS/MS) spectra were acquired between m/z 50 and 350 m/z using multi-reaction monitoring (MRM) and Ultrascan (32,500 m/z /s) as scan mode. A 1000 µg L^{-1} stock standard MeOH solution of each compound was infused at a flow-rate of 200 µL h^{-1} to optimize the source working conditions and to carry out the tandem mass spectrometry experiments. Bruker Trap Control software was used to set the mass spectrometer. Bruker Hystar 3.2 software was adopted to control the hyphenated HPLC/MS system and to program the timetables of the runs. Bruker Data Analysis 4.2 software was employed for the LC/MS data processing.

2.2.7 Standard linearity of response and detection limits of the method

DGT blank concentrations were calculated by measuring the mass of the targets in AC gels retrieved from DGT probes which were left assembled in the aqueous matrix for 24 h. LOD and LOQ were calculated as the minimum detectable amount of target with signal-to-noise ratios of 3 and 10 respectively[301,334] assuming 24 h as deployment time and temperature of 25°C with a 0.54 mm thick diffusive gel. LOD and LOQ were also calculated theoretically assuming 18 days of deployment. Quality assurance and quality control of all laboratory and field trials consisted of at least one distilled water blank, one triplicate sample, and one matrix recovery sample spike of 10 µg L^{-1} of the target analytes per 10 samples. The analysis results were expressed as the mean ± the standard deviation.

2.2.8 Environmental trials

The DGT-HPLC/MS methodology was tested under environmental conditions. It was chosen to deploy 6 DGT probes for 18 days at sampling sites along the Waikato River previously monitored (November 2013) adopting a validated and published SPE-HRGC/MS method[1]. Although very robust, the validated method was time consuming and laborious due to the very long sample preparation procedure. The comparison allowed to evaluate if the novel methodology was more convenient in terms of sample preparation and more suitable for the detection of the targets in the same concentration range of the previously validated technique.

2.2.9 Statistical analysis

All hydrogel characteristics, such as swelling kinetics, thermal behaviour and DGT laboratory and field deployments were performed in triplicate and the results were expressed as the mean ± standard deviation (SD). EXEL software was employed for the statistical analysis. The analysis of variance (ANOVA) and least significant differences (LSD) at the 5% significant level were adopted to identify statistically significant differences. Regression function and confidence level at 95% were adopted to calculate LOD and LOQ. LOD and LOQ were calculated analysing the DGT eluates of blanks and samples, assuming 24 h or 18 days as deployment time and 25°C temperature with a 0.54 mm thick diffusive gel. LOD and LOQ were calculated as minimum detectable amount of target with signal-to-noise ratios of 3 and 10 respectively[301,334]. Quality assurance and quality control of all laboratory and field trials consisted of at least one distilled water blank, one triplicate sample, and one matrix recovery sample spike of 10 µg L^{-1} of the target analytes per 10 samples. The analysis results were expressed as the mean ± the standard deviation.

2.3 Results and discussion

2.3.1 DGTs kinetics of swelling

Both agarose (1.5%) and the agarose (1.5%) – AC (1%) hydrogels were soaked and constantly shaken in water at room temperature and neutral pH. The kinetics of swelling were monitored for 24 hours (Table 2.2).

Table 2.2

Equilibrium swelling ratio (ΔW%) of the agarose (1.5%) and the composite agarose (1.5%) - AC(1%).

Time (h)	Agarose (1.5%) Average swelling ($\overline{\Delta W\%}$)	Agarose (1.5%)-AC (1%) Average swelling ($\overline{\Delta W\%}$)
0.25	38.56 ± 18.45	8.29 ± 22.26
0.5	94.67 ± 10.34	39.15 ± 13.04
1	71.82 ± 38.82	46.00 ± 4.12
2.5	66.20 ± 26.40	42.65 ± 28.90
24	92.33 ± 32.77	39.17 ± 11.14

The experimental data, shown in Table 2.2, were plotted in a graph (swelling versus time) and an appropriate trend line was added to evaluate in a more accurate way the maximum swelling ratio (ΔW%) of the pure and the composite agarose (Figure 2.5).

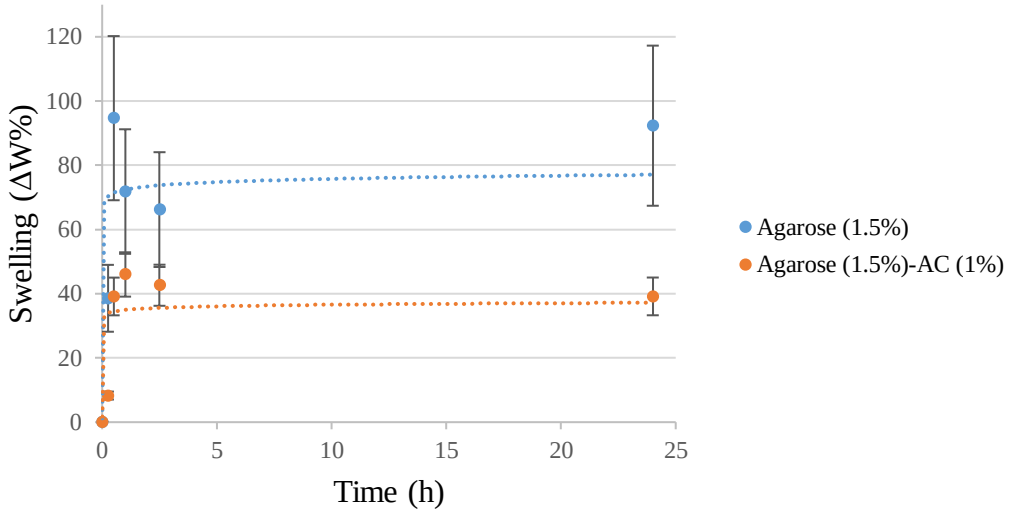


Figure 2.5

Trends of the agarose (1.5%) and agarose (1.5%) - AC (1%) swelling ratio as a function of time at room temperature and pH 7.

Both the diffusive and binding hydrogels reached the steady state, and so the maximum swelling ratio (ΔW%), after 1 h of immersion in the aqueous medium at room temperature and neutral pH. In particular the diffusive gel swelled approximately up to 70% while the resin gel swelled approximately up to 40%.

The activated charcoal decreases the space available for the water in the polymer mesh thus, diminishing the hydrogel final swelling ratio; more specifically its presence decreases the agarose functional groups that can create intermolecular hydrogen bonds with the water molecules[335] being the activated charcoal absorbed on the surface of the polymeric chains. The activated charcoal, however, does not interfere with the swelling kinetics because its presence does not affect the polymeric chains' mobility: the steady state in fact is reached at the same time for both agarose and agarose - AC.

2.3.2 DGT test of homogeneity and thermal behaviour

Agarose (1.5%) and the agarose (1.5%) – AC (1%) hydrogels homogeneity and thermal stability were evaluated after the synthesis through TGA/DTA characterizations (Figure 2.6). A linear temperature program was used in the range 30 – 800°C, with a heating rate of 40°C/min and 150 mL/min air flow. Both diffusive and binding gels were divided into a 3x3 grid and each sector was analysed in triplicate to verify that the ratio of water – carbon was constant along the two-dimensions of the gels.

The TGA curves of both agarose (1.5%) and agarose (1.5%) – AC (1%) show three stages of weight loss. The first weight losses happen in the range 30 – 170°C and are due to the loss of water, the second and third stage losses, connected to the pyrolysis of the organic part, happen respectively between 170 – 340°C and between 340 – 630°C. In Table 2.3 below, the average percentage losses for the three degradation stages of both agarose (1.5%) and agarose (1.5%) – AC (1%) are reported.

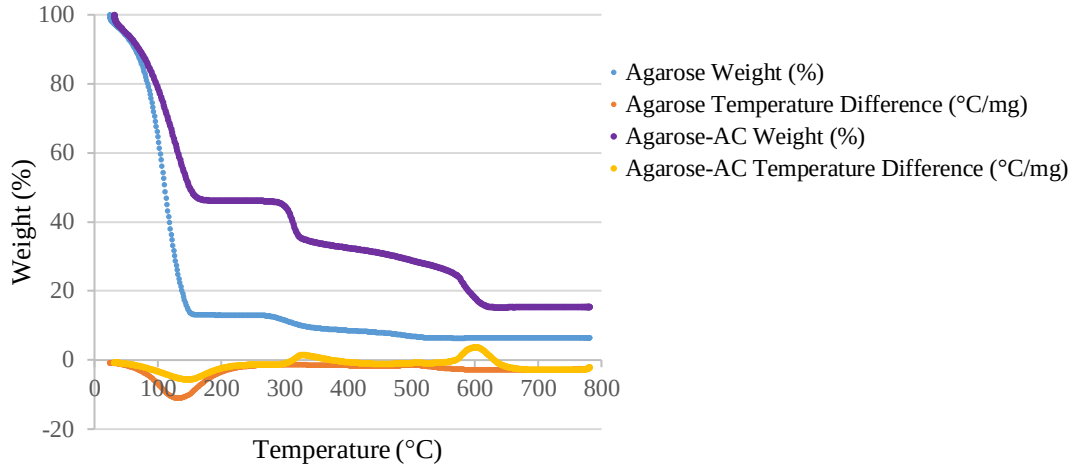


Figure 2.6

Thermograms (TGA) and differential thermograms (DTA) of agarose (1.5%) and agarose (1.5%) – AC (1%) in the temperature range 30 – 800 °C.

Table 2.3

Average percentage weight losses of the agarose (1.5%) and the composite agarose (1.5%) – AC (1%) in the temperature range 30 – 800°C.

Temperature range (°C)	Agarose (1.5%) weight loss (%)	Agarose (1.5%) – AC (1%) weight loss (%)
30 – 170	87	54
170 – 340	90	66
340 – 630	97	85

As expected, in the pure agarose hydrogel (87%) the biggest weight loss was represented by the water loss, while the agarose – AC composite showed a reduced water loss (54%) due to the presence of the carbon microparticles, that reduced the space available to the water in the polymer mesh, confirming the swelling measurement trends. Moreover the pure agarose degraded at lower temperatures (90% weight loss at the second stage) and showed a complete weight loss (97%), while the agarose – AC composite showed enhanced thermal stability of the product (66% weight loss at the second stage).

The weight loss percentages and the ratio of water - carbon were constant along the two-dimensions for both diffusive and binding gels; thus, it can be assumed that the diffusive properties of the pure agarose, and the binding ability of each resin hydrogel disc were constant. Moreover, the increased stability of the composite material used as the resin gel made it more suitable in environmental testing where the temperature could vary.

Despite possible deviations due to the presence of the conductive carbon tape, the EDX spectra of freeze-dried platinum-coated agarose (1.5%) and agarose (1.5%) – AC (1%) confirmed the higher content of carbon in the composite hydrogel (Figure 2.7).

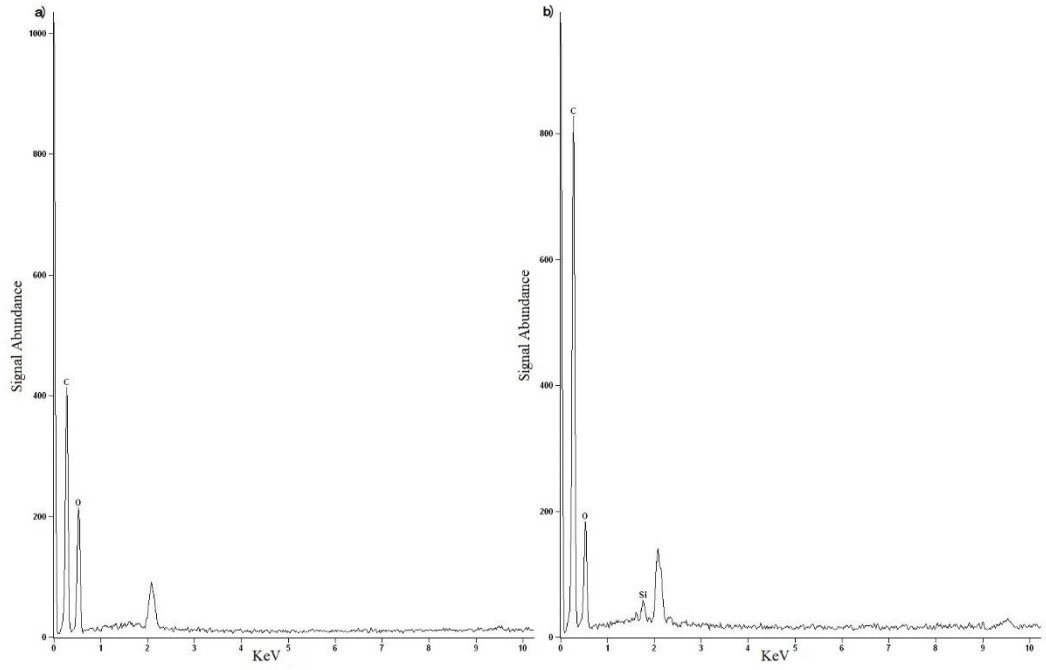


Figure 2.7

a) EDX spectrum of freeze-dried platinum-coated agarose (1.5%): x-rays peaks of carbon C K-α (0.27 eV), oxygen O K-α (0.53 eV) b) EDX spectrum of freeze-dried platinum-coated agarose (1.5 %) – activated charcoal (1 %): x-rays peaks of the carbon C K-α (0.27 eV), oxygen O K-α (0.53 eV), silicon Si K-α (1.74 eV).

It was possible to evaluate the atomic weight percentage of the carbon by examining the x-ray peak intensities. The results showed the composite hydrogel was enriched approximately by 10% more of carbon compared to the pure agarose hydrogel throughout the hydrogel sample (Table 2.4).

Table 2.4

Average content of carbon, oxygen and silicon in freeze-dried platinum-coated agarose (1.5%) and agarose (1.5 %) – activated charcoal (1 %) expressed in atomic weight percentage (wt%).

Element	Agarose (1.5%) (wt%)	Agarose (1.5 %) – activated charcoal (1 %) (wt%)
carbon	53.59 ± 0.96	61.82 ± 0.93
oxygen	46.41 ± 1.47	37.46 ± 1.48
silicon		0.72 ± 0.09

2.3.3 DGT morphological analysis

The optimised conformations of the target molecules (BPA, BPAF, βE2 and EE2), reported in Figure 2.8, Figure 2.9, Figure 2.10, Figure 2.11, were calculated through Gaussian simulation, using the DFT method B3LYP and as basis set 6-31G. The correlated approximated dimensions of the analytes are reported in Table 2.5.

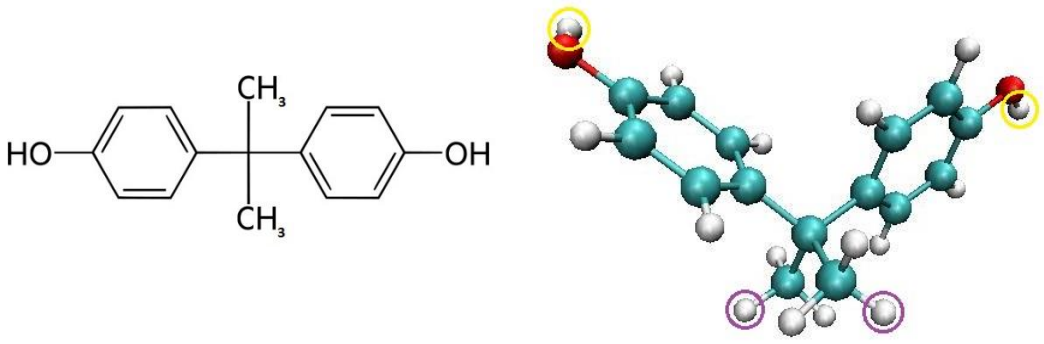


Figure 2.8

Bisphenol A (BPA), two-dimensional structural formula and three-dimensional optimised conformation (Gaussian simulation: method B3LYP, basis set 6-31G).

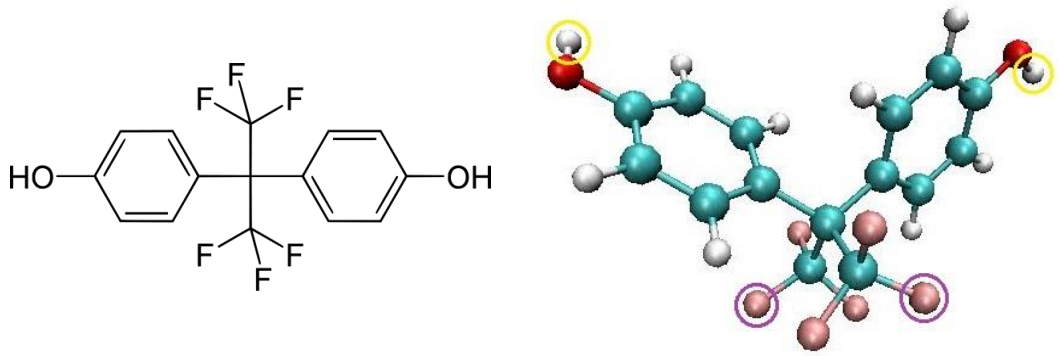


Figure 2.9

Bisphenol AF (BPAF), two-dimensional structural formula and three-dimensional optimised conformation (Gaussian simulation: method B3LYP, basis set 6-31G).

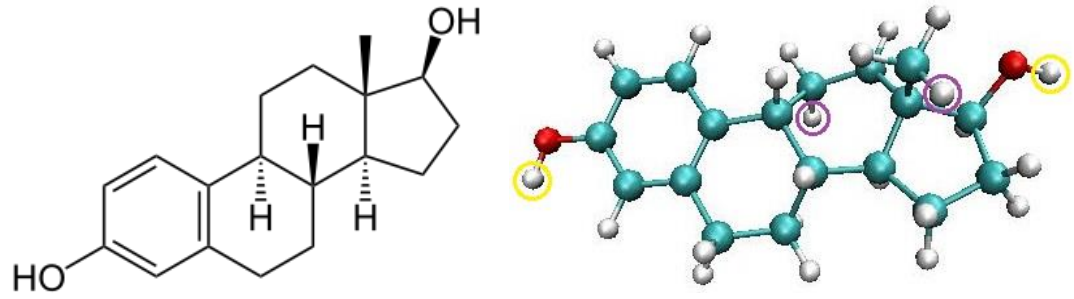


Figure 2.10

17β-Estradiol (E2) two-dimensional structural formula and three-dimensional optimised conformation (Gaussian simulation: method B3LYP, basis set 6-31G).

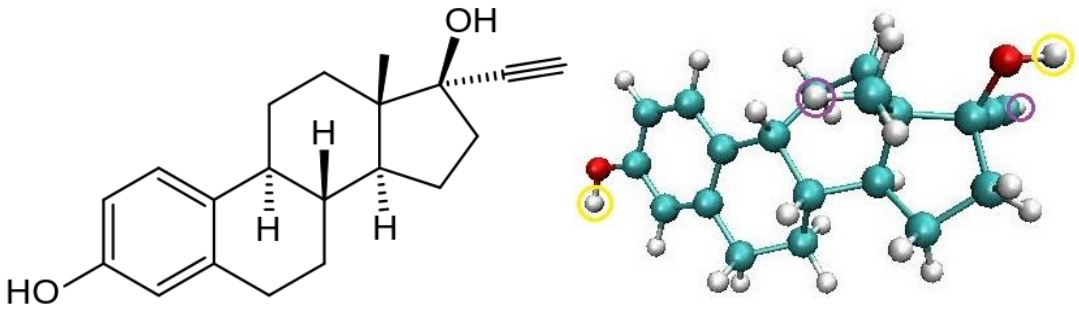


Figure 2.11

17α-Ethinyl estradiol (EE2) two-dimensional structural formula and three-dimensional optimised conformation (Gaussian simulation: method B3LYP, basis set 6-31G).

Table 2.5

Approximated BPA, BPAF, βE2 and EE2 dimensions measured using the respective Gaussian optimized conformations. Lengths were measured between the circled yellow atoms and widths between the circled purple atoms in the 3-D conformations shown in Figure 2.8, Figure 2.9, Figure 2.10, Figure 2.11.

Target Analyte	Approximated theoretical length (Å)	Approximated theoretical width (Å)
BPA	10.18	4.36
BPAF	10.23	4.83
βE2	12.32	5.11
EE2	12.32	6.77

The surface appearance of both diffusive and binding gels freeze-dried (Figure 2.12) and air-dried (Figure 2.13) was evaluated through FE-SEM morphological characterization using normal lens mode.

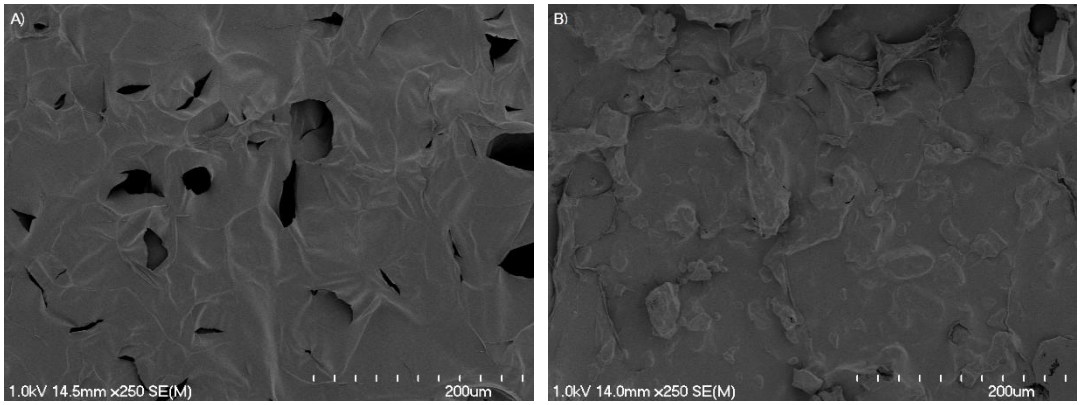


Figure 2.12

a) FE-SEM morphological characterization of freeze-dried, platinum-coated agarose (1.5%): magnification 250, accelerating voltage 1 kV, emission current 11000 nA, working distance 14500 μm; b) FE-SEM morphological characterization of freeze-dried, platinum-coated agarose (1.5%) – activated carbon (1%): magnification 250, accelerating voltage 1 kV, emission current 11000 nA, working distance 14000 μm.

The agarose hydrogel has a flatter surface compared to the one of the composite agarose-activated carbon hydrogel. This is due to the presence of visible and homogeneous powder aggregate along all the surface of the binding gel.

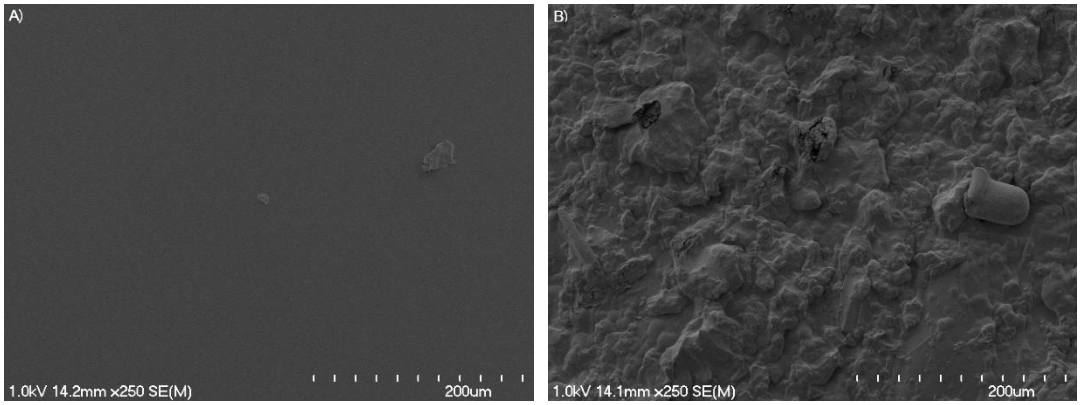


Figure 2.13

a) FE-SEM morphological characterization of air-dried, platinum-coated agarose (1.5%): magnification 250, accelerating voltage 1 kV, emission current 11000 nA, working distance 14200 μm; b) FE-SEM morphological characterization of air-dried, platinum-coated agarose (1.5%)- activated carbon (1 %): magnification 250, accelerating voltage 1 kV, emission current 11000 nA, working distance 14100 μm.

The air dried diffusive hydrogel shows a flat surface without particular features: this is due to the collapse of the three-dimensional structure and pore flattening. The collapse of the polymeric 3D-structure is also visible in the binding gel: the aggregates of activated charcoal powder are much more demarcated on the surface because they were not supported anymore by the three-dimensional polymeric network which collapsed around the encapsulated powder. Thus, further high resolution characterization were conducted only on the freeze-dried samples that showed a structure more representative of the real one, with less artifacts.

The pore size of the diffusive (Figure 2.14) and binding gels (Figure 2.15) required the use of ultra-high resolution lens mode. Hydrogels are soft materials that are very fragile: low working distance and low current were employed to avoid the alteration of the sample under the electron beam.

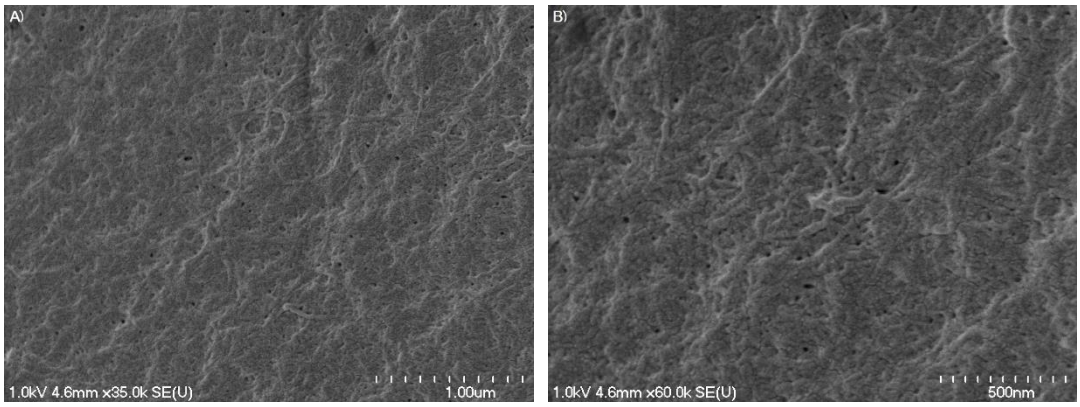


Figure 2.14

a) FE-SEM pore-size characterization of freeze-dried, platinum-coated agarose (1.5%): magnification 35.0 k, accelerating voltage 1 kV, emission current 10000 nA, working distance 4600 μm; b) FE-SEM pore-size characterization of freeze-dried, platinum-coated agarose (1.5%): magnification 60.0 k, accelerating voltage 1 kV, emission current 9500 nA, working distance 4600 μm.

The images acquired of the diffusive layer clearly show the presence of agarose polymeric fibres that form a 3-D mesh of well distributed channels. The diameter of the pore size depends on the ratio of agarose-water employed during the gelation process. Several random areas of 3.24 μm^2 (2081 nm x 1557 nm) of the agarose (1.5%) hydrogel were analysed to evaluate the average pore number and the weighted pore diameter. Measurements showed an average pore number of 22 ± 5 per μm^2 with a weighted pore diameter of Ø = 30 nm. Thus, it can be expected that the analytes of interest can freely diffuse.

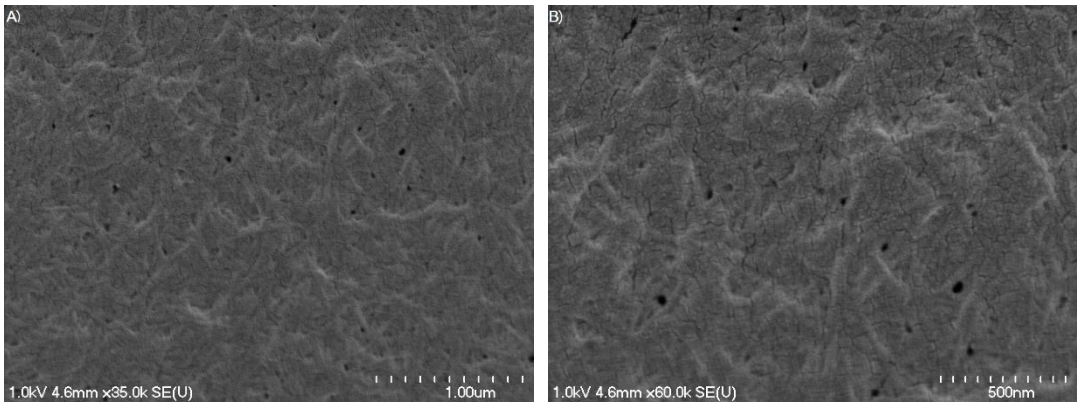


Figure 2.15

a) FE-SEM pore-size characterization of freeze-dried, platinum-coated agarose (1.5%) - activated carbon (1 %): magnification 35.0 k, accelerating voltage 1 kV, emission current 11000 nA, working distance 4600 μm; b) FE-SEM pore-size characterization of freeze-dried, platinum-coated agarose (1.5%) - activated carbon (1 %): magnification 60.0 k, accelerating voltage 1 kV, emission current 11000 nA, working distance 4600 μm .

The image captures of the binding layer show again the presence of agarose polymeric chains, but less defined due to the absorption of the activated charcoal powder around their surface. Several random areas of 3.24 μm^2 (2081 nm x 1557 nm) of the composite agarose (1.5%) – activate carbon (1 %) hydrogel were analysed to evaluate the average pore number and the weighted pore diameter. Measurements showed an average pore number of 13 ± 2 per μm^2 with a weighted pore diameter of Ø = 45 nm. The presence of the filler does not interfere negatively with the distribution of the pore channels or their size, in fact the pore number is similar to the one of the diffusive hydrogel and the average dimension of the pores is slightly bigger than 30 nm. The small difference in pore number between the binding and the diffusive layers may be due to the presence of the filler.

To house the materials on the stubs and evaluate their thicknesses using SEM imaging, it was necessary to cut the fragile freeze-dried hydrogels: this operation caused compression of the edges and so alteration of the thicknesses. Thus, caliper measurements (Table 2.6) of the more resistant fully hydrated hydrogels (gel strength $\geq$ 2500 g/cm^2 for 1.5% gel solution[336]) were preferred to SEM acquisition for the evaluation of the thicknesses.

Table 2.6

Calliper measurements of fully hydrated (24 h) agarose (1.5%) hydrogel and agarose (1.5%) - activate charcoal (1%) hydrogel thicknesses (mm) (mean ± standard deviation; n=3).

Material	Thickness (mm)
Agarose (1.5%)	0.54 ± 0.05
Agarose (1.5%) - activated carbon (1 %)	0.51 ± 0.04

The average thicknesses confirm again that the swelling is higher in the diffusive agarose gel (1.5 %) rather than the binding agarose (1.5 %) – activated carbon (1 %). The standard deviation of both measurements is consistent due to the difficulty in realizing precise measurements. Caliper measurements are more appropriate than FE-SEM acquisitions, but are anyway affected by errors due to the compression of the soft samples.

2.3.4 DGT adsorption assessment

Probe materials, diffusive gels and filter membranes should not adsorb the targets in order to ensure a good quality of measurements. ABS plastic probes, agarose diffusive gels of 2.51 cm of diameter and 0.54 mm of thickness, and hydrophilic PTFE membranes of 2.51 cm of diameter showed a relatively small adsorption < 3% (excluding the adsorption of BPAF by PTFE membrane < 7%) of BPA, BPAF, E2 and EE2 (Figure 2.16). Hence, they were employed throughout the study.

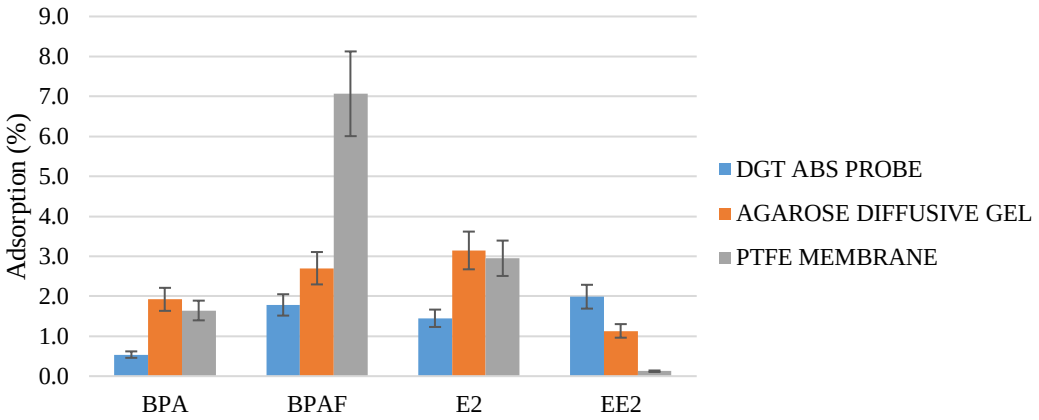


Figure 2.16

BPA, BPAF, E2, EE2 adsorption onto DGT ABS probes, 0.54 mm agarose diffusive gels and hydrophilic PTFE membranes. Error bars calculated from the standard deviation of the replicates (n=3).

2.3.5 DGT kinetics of adsorption and compound elution

Several solvents (10 mL MeOH; 9 mL MeOH + 1 mL NH_3 15 M; 9 ml MeOH + 1 mL buffer NH_3/HCl pH 10; 10 mL buffer NH_3/HCl pH 10; 9 mL MeOH + 1 ml EtOH; 10 mL EtOH) were tested to elute the targets from the loaded AC gels. The choice of the solvents to test was done according to previous BPs elution studies[241] and estrogen solubility studies[337,338]. It was chosen to elute BPA and BPAF in 10 mL of MeOH for 24 h and then to elute E2 and EE2 in 10 mL of EtOH for 24 h, as they proved to be the best solvents, volumes and times of elution among all the combinations tested. For all compounds the elution factor f_e adopted was equal to 0.8. The recovery efficiency of BPA, BPAF, E2, EE2 were investigated at various concentrations as shown in Figure 2.17.

The optimised protocols employed to recover the targets from the binding phase provided satisfactory recovery at all concentrations investigated and their average values are summarised in Table 2.7.

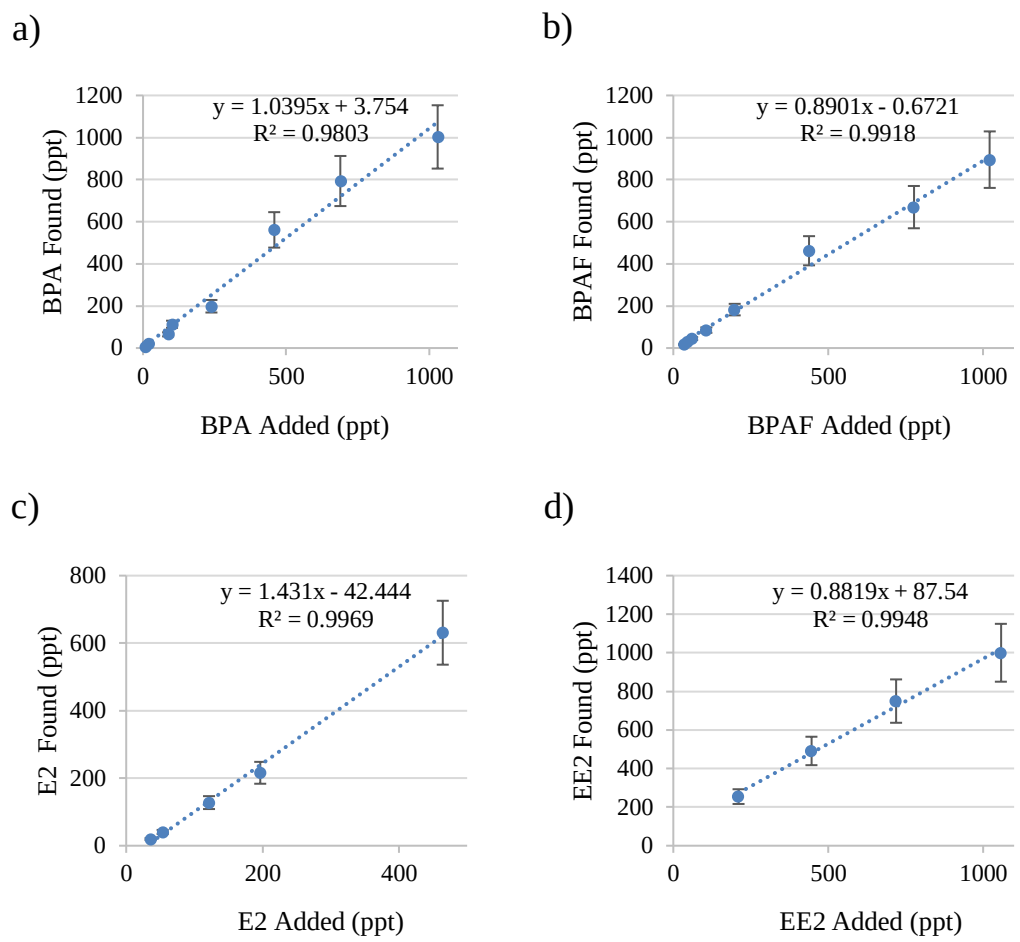


Figure 2.17

a) Recovery of different concentrations of BPA from AC binding gel in 10 mL MeOH batch experiments; b) recovery of different concentrations of BPAF from AC binding gel in 10 mL MeOH batch experiments; c) recovery of different concentrations of E2 from AC binding gel in 10 mL EtOH batch experiments; d) recovery of different concentrations of EE2 from AC binding gel in 10 mL EtOH batch experiments.

Table 2.7

Percentage recovery at 25°C of BPA, BPAF in 10 mL MeOH and E2, EE2 in 10 mL EtOH expressed as mean ± SD (n =3).

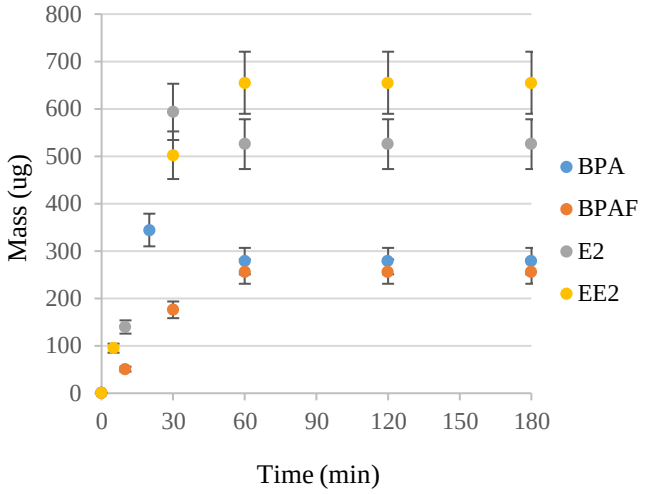
	BPA	BPAF	E2	EE2
Recovery %	103 ± 17	79 ±18	96 ± 31	107 ± 11

The adsorption of the standards into the AC gels were recorded at 25°C. The uptake of the standards by the binding gels was relatively fast: the adsorption increased linearly and reached plateau after 60 min (Figure 2.18).

BPA, BPAF, E2, EE2 were adsorbed by the binding phase with a percentage of 98%, 98%, 97%, 95% and an average binding rates of 2.05, 1.20, 2.48, 3.55 ng cm^{-2} min^{-1} over the first 60 min, respectively. The data were in accordance with the previous BPs adsorption study[241].

Figure 2.18

Kinetics of adsorption of BPA, BPAF, E2, EE2 by agarose (1.5%) - activated carbon (1 %) gels (25 °C, 1 L solution of 1000 µg/L and 0.01 M NaCl). Error bars calculated from the standard deviation of the replicates (n=3).



2.3.6 DGTs effective diffusion coefficients measurement

Gels based on agarose swell when immersed in water, to varying extents, according to the amount and type of agarose and the ratio of water-agarose chosen. The agarose hydrogels adopted in this study are 98% water when fully hydrated, thus all the water is effectively available for diffusion, there being no bound water. Moreover agarose gel has long been used in protein electrophoresis. Agarose gel has been adopted as a size-selective sieve to separate proteins in the range of 2000 – 200 000 Da which correspond to molecules with a radius not bigger than 20 nm[237].

A single gel composition of agarose (1.5%) as diffusive gel and agarose (1.5%) - activated carbon (1 %) as binding gel were used during all the work and their pore size, according to the morphological characterization, was estimated to be 30 nm and 45 nm, respectively. Hence, BPA, BPAF, βE2, EE2 can be expected to move

freely through the gels allowing the assumption that the diffusive coefficient (*D*) of the targets in the gels (Table 2.8) is equal to their diffusion coefficient in the bulk solution.

Table 2.8

Effective diffusion coefficients of BPA, BPAF, E2, EE2 in diffusive gels expressed as mean ± SD (n = 8) measured at 25°C using DGTs devices.

Diffusion coefficient (*D*) (10^{-6} cm^2 s^{-1}) (25°C)			
BPA	BPAF	E2	EE2
3.09 ± 0.61	3.55 ± 1.03	3.97 ± 0.98	3.18 ± 0.04

The effective diffusive coefficients, thus determined, will be used in the calculation of the concentrations. The values of their coefficients resulted in the same order of magnitude of the coefficients evaluated in other publications using the diffusive cell or alternative investigations[241,320].

2.3.7 Optimized mass spectrometry signals and high performance liquid chromatography retention times

Tandem MS parameters were optimized for each compound by directly infusing the standard at known concentration into the mass spectrometer (1000 µg L^{-1} in MeOH/water solution, 200 µL/h flow). It was chosen to operate in negative mode to perform the fragmentation in the mass spectrometer. The deprotonated molecules of the analytes $[M-H]^-$ were used as precursor ions and a transition ion was selected and monitored for the identification of each compound of interest. The cone voltage and the collision energy (CE) were optimized for every compound to obtain maximum intensity. The fragmentation schemes of all compounds are based on the observed mass/charge and compared with the literature[293,339].

A methanol (MeOH, NH_4OH 0.06 M)/water (H_2O, NH_4OH 0.06 M) gradient was used to elute the analytes. The column was kept at ambient temperature to carry the separation without degrading the targets. All the optimized retention times and MS/MS parameters are listed in Table 2.9.

Table 2.9

List of retention times and selected multiple reaction monitoring (MRM) transitions monitored for every target compound and relative optimized potentials[339,340,293].

Analyte	Retention Time (min)	Molecular Weight	Precurson Ion (m/z) $[M-H]^-$	Quantification Collision Energy (CE) (eV)	Product (m/z)
BPA	5.5	228.29	227	30	211
BPAF	4.3	336.23	335	30	265
E2	8.4	272.38	271	40	183
EE2	8.7	296.40	295	40	267

In the tandem mass (MS/MS) spectrum (Figure 2.19) of the $[M-H]^-$ ion of BPA, the characteristic product ion was observed at m/z 210.74 ($[M-H-CH_4]^-$, $C_{14}H_{11}O_2$), probably resulting from the loss of methane, from the precursor ion at m/z 227.

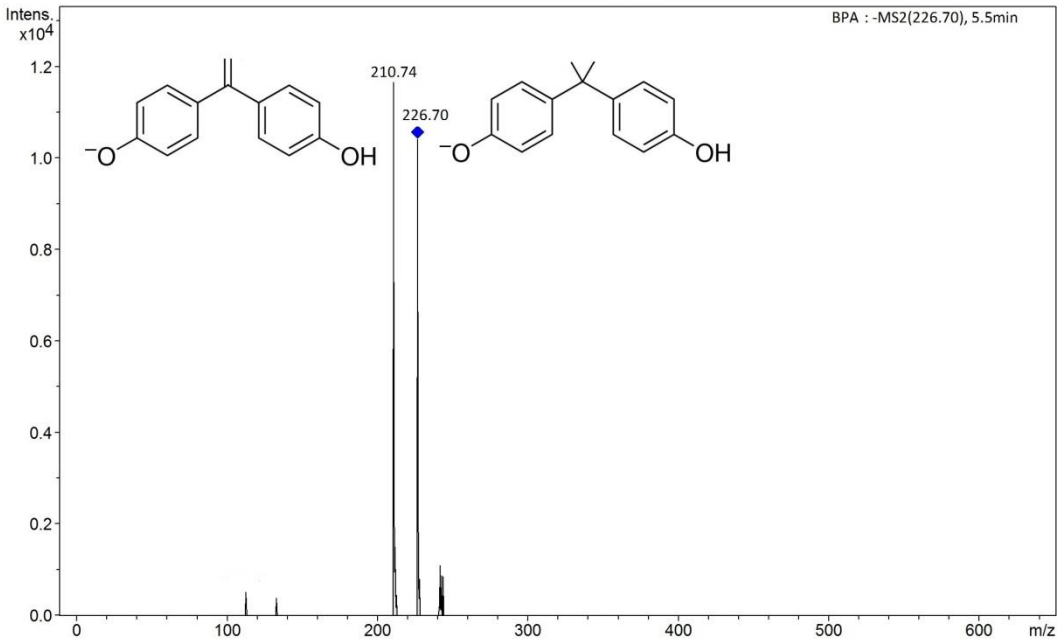


Figure 2.19

Tandem MS spectrum of BPA (1000 µg L^{-1} MeOH/water solution) directly infused in the mass spectrometer (200 µL/h flow) with relative ions intensities and structures; precursor ion marked by the blue tag.

The MS/MS fragmentation of deprotonated BPAF (Figure 2.20) was characterized by the ion at m/z 264.70 ($[M–H–CHF_3]^-$, $C_{14}H_9F_3O_2$) generated from cleavage of the C–C bond between the central carbon atom and the CF_3 group. The abundance of the $[M–H–C_{14}H_9F_3O_2]^-$ ion increased slightly increasing CE and no new peaks appeared, suggesting that the $[M–H–CHF_3]^-$ ion was stable.

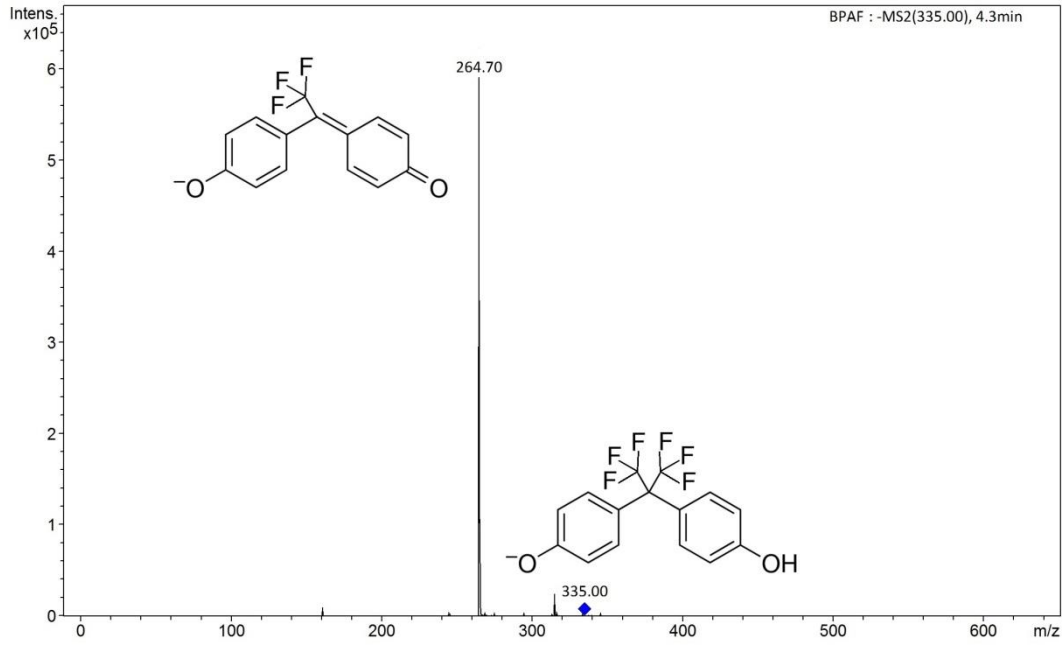


Figure 2.20

Tandem MS spectrum of BPAF (1000 µg L^{-1} MeOH/water solution) directly infused in the mass spectrometer (200 µL/h flow) with relative ions intensities and structures; precursor ion marked by the blue tag.

The MS/MS spectrum for estradiol (Figure 2.21) showed losses consistent with ring cleavages (i.e. losses of $C_5H_{12}O$, $C_8H_{14}O$) to give major product ions at m/z 183 ($[M–H–C_5H_{12}O]^-$, $C_{13}H_{11}O$) and 145 ($[M–H–C_8H_{14}O]^-$, $C_{10}H_9O$).

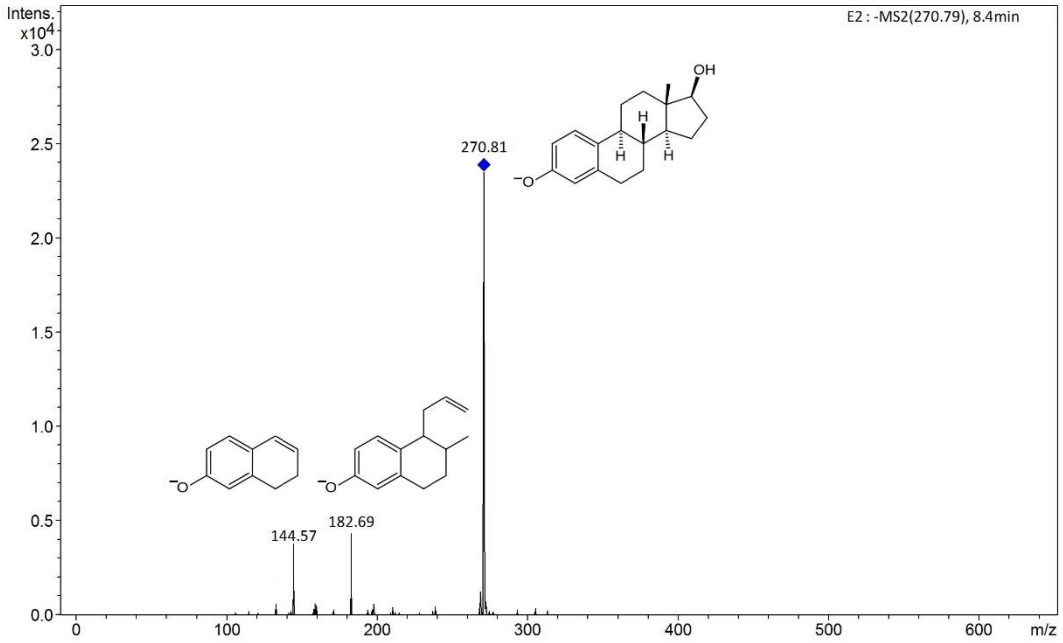


Figure 2.21

Tandem MS spectrum of E2 (1000 µg L^{-1} MeOH/water solution) directly infused in the mass spectrometer (200 µL/h flow) with relative ions intensities and structures; precursor ion marked by the blue tag.

The $[M–H]^-$ ion from ethynylestradiol gave major fragment ions in the MS/MS spectrum (Figure 2.22) at m/z 266.84 ($[M–H– C_2H_4]^-$, $C_{18}H_{20}O_2$), 182.65 ($[M–H–C_7H_{12}O]^-$, $C_{13}H_{12}O$), 144.71 ($[M–H–C_{10}H_{14}O]^-$, $C_{10}H_{10}O$) that are believed to be losses of C_2H_4, $C_7H_{12}O$ and $C_{10}H_{14}O$, respectively. The observation of m/z 145 is consistent with the stability of the phenol ring in the system and the observation of this ion in the daughter ion mass spectrum of each serves as a ready indicator of high specificity for these compounds.

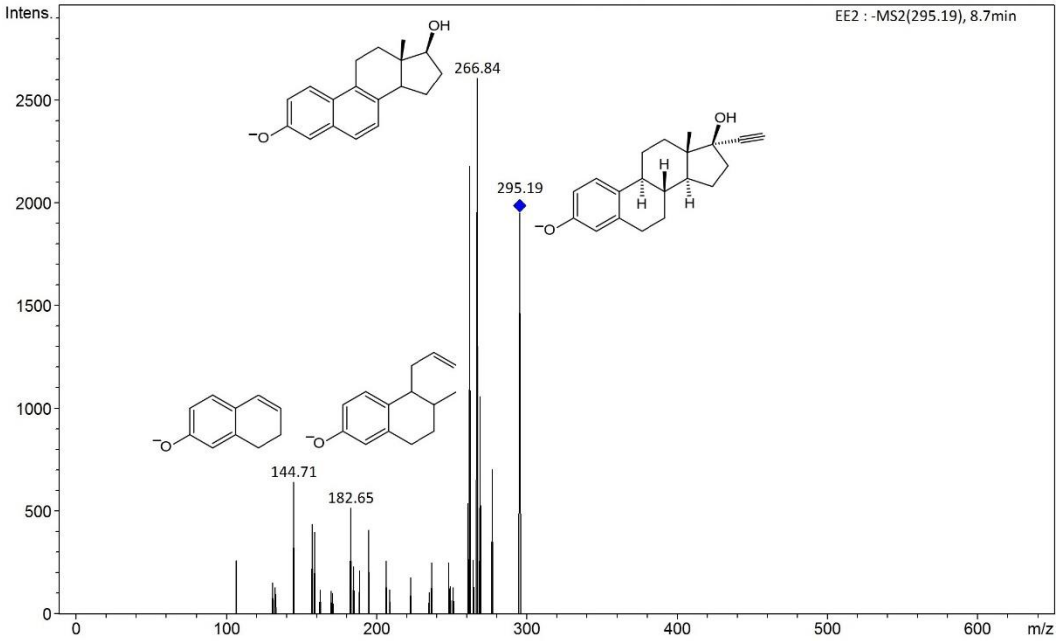


Figure 2.22

Tandem MS spectrum of EE2 (1000 µg L^{-1} MeOH/water solution) directly infused in the mass spectrometer (200 µL/h flow) with relative ions intensities and structures; precursor ion marked by the blue tag.

2.3.8 Standard linearity of response and limits of detection

The calibration curves of BPA (Figure 2.23), BPAF (Figure 2.24), E2 (Figure 2.25), EE2 (Figure 2.26) were based on at least five concentration points ranging from 1000 to 1 µg L^{-1} for each target. The curves were built both analysing the standard water solutions and the DGT eluents in the instrument employing a 0.54 mm diffusive agarose (1.5%) gel layer, assuming 24 h of deployment in 1 L of standard solutions at 25°C. All the curves showed satisfactory correlation coefficients greater than 0.99.

a) b)

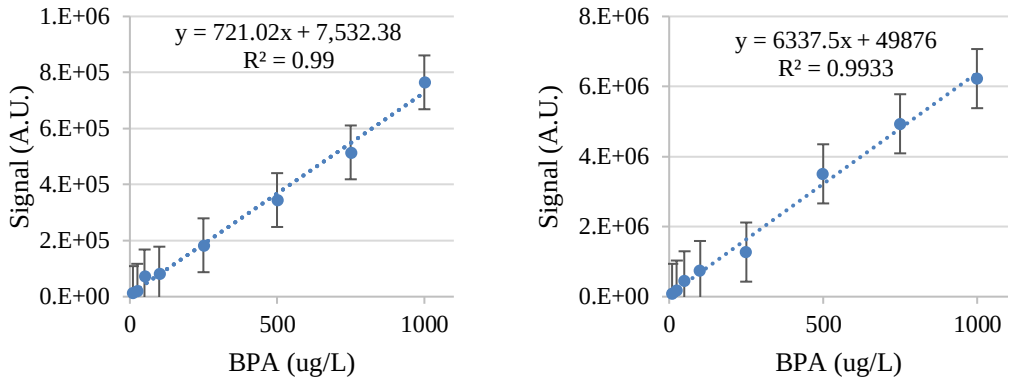


Figure 2.23

a) calibration curve for BPA standard water solutions (n = 3); b) calibration curve for BPA from DGTs eluents (n = 3).

a) b)

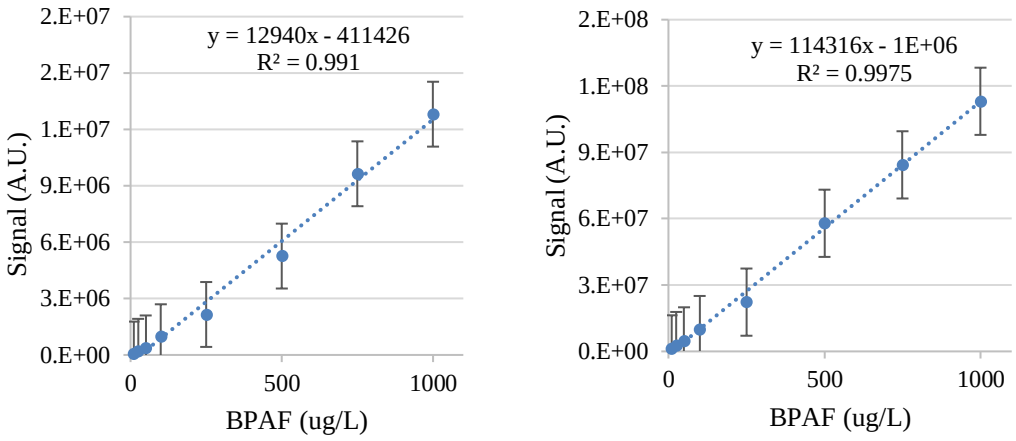


Figure 2.24

a) calibration curve for BPAF standard water solutions (n = 3); b) calibration curve for BPAF from DGTs eluents (n = 3).

a) b)

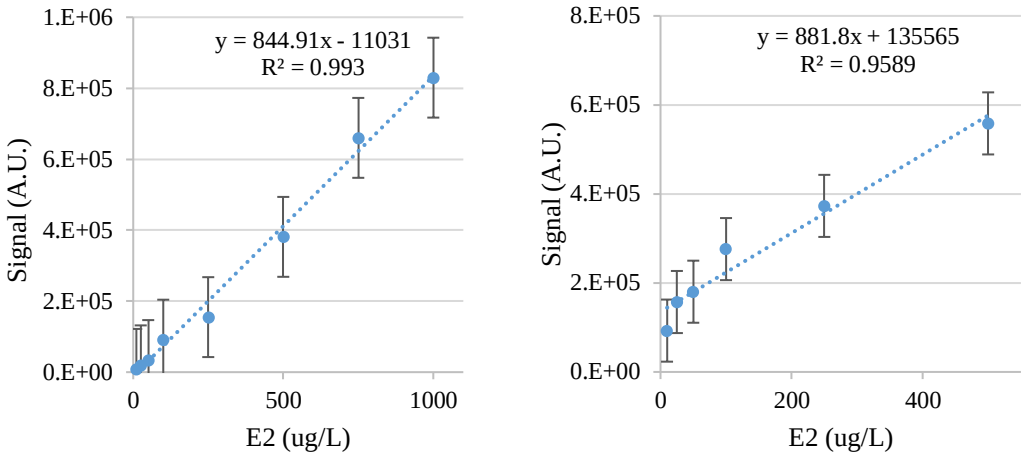


Figure 2.25

a) calibration curve for E2 standard water solutions (n = 3); b) calibration curve for E2 from DGTs eluents (n = 3).

a) b)

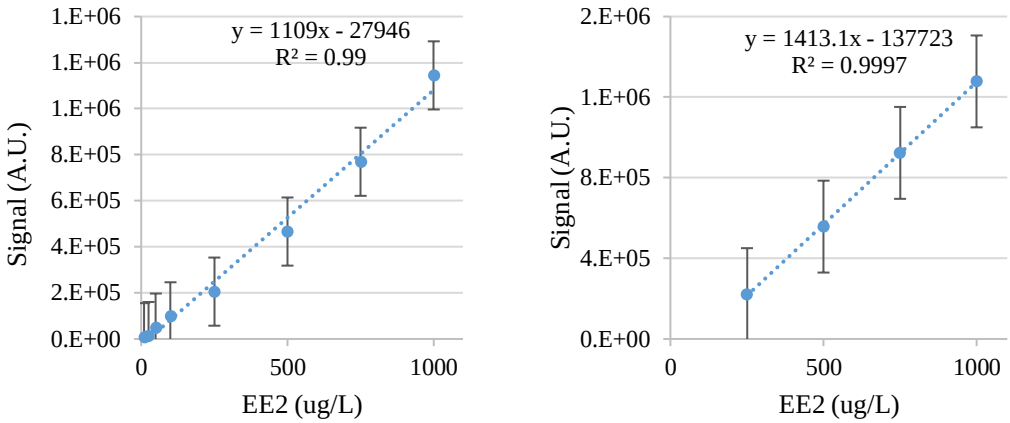


Figure 2.26

a) calibration curve for EE2 standard water solutions (n = 3); b) calibration curve for EE2 from DGTs eluents (n = 3).

In Table 2.10 and Table 2.11 are summarized the limits of detection and quantification of the DGT-HPLC/MS method developed for the BPA, BPAF, E2, EE2 assuming 1 day (lab trial) and 18 days (environmental exposure) of deployment. During both deployment times, blank concentrations analysis showed that no targets could be detected, indicating no measurable release of analytes from DGT mouldings.

Table 2.10

Blank concentrations, limits of detections (LODs) and quantifications (LOQs) expressed in µg/L of the DGT-HPLC/MS method calculated employing a 0.54 mm diffusive agarose (1.5%) gel layer, assuming 24 h of deployment in 1 L of standard solutions at 25°C.

	Gel blank conc.	LOD (µg L^{-1})	LOQ (µg L^{-1})
BPA	N.D.[a]	0.0220	0.0734
BPAF	N.D.	0.0007	0.0024
E2	N.D.	0.1647	0.5491
EE2	N.D.	0.0309	0.1031

[a] not detected

Table 2.11

Blank concentrations, limits of detections (LODs) and quantifications (LOQs) expressed in µg/L of the DGT-HPLC/MS method calculated employing a 0.54 mm diffusive agarose (1.5%) gel layer, assuming 18 days (432 h) of deployment in 1 L of standard solutions at 25°C.

	Gel blank conc.	LOD (µg L^{-1})	LOQ (µg L^{-1})
BPA	N.D.[a]	0.00100	0.00300
BPAF	N.D.	0.00004	0.00014
E2	N.D.	0.00006	0.00019
EE2	N.D.	0.00171	0.00573

[a] not detected

2.3.9 Trial deployments

6 DGT probes were exposed for 18 days at selected sampling sites along the Waikato River. The DGTs were eluted and analysed according to the methodology described in the previous sections. The data were compared with those from a previous monitoring of the same sites adopting active sampling andthe SPE-HRGC/MS method[1]. In Table 2.12 and Figure 2.27 are reported only the concentrations of BPA, because E2 and EE2 concentrations were <LOD in both investigations.

Table 2.12

Environmental concentrations of BPA evaluated via SPE-HRGC/MS and DGT-HPLC/MS at selected sites along the Waikato River, New Zealand. Concentrations expressed in ng L^{-1}.

	[BPA] ng/L SPE Monitoring 2013	[BPA] ng/L DGT Monitoring 2018
Lake Taupo Upstream	0,83	1,12
Hamilton Waikato Upstream	0,96	2,19
Huntly Waikato Upstream	0,90	3,47
Tuakau	0,87	1,48

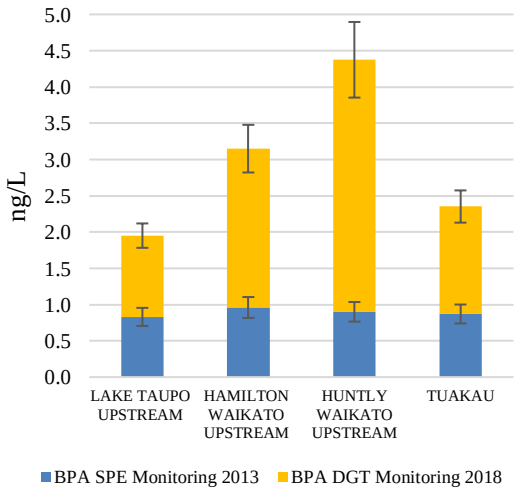


Figure 2.27

Comparison of BPA concentrations evaluated via SPE-HRGC/MS and DGT-HPLC/MS at selected sites along the Waikato River, New Zealand; concentrations expressed in ng L^{-1}.

The results showed that the DGT technique was suitable for the detection of EDC trace concentrations in the range of ng L^{-1}. This outcome is in accordance with a similar study in the literature[241]. The concentration values of the SPE and DGT investigations were very close despite the DGT analysis occurring several years later from the SPE analysis. The novel DGT approach was very convenient in terms of sample preparation, ease of deployment and low cost resulting in a competitive and effective alternative to the traditional active sampling approaches for the monitoring of the EDCs.

2.4 Summary chapter II

This study of the performance characteristics of the agarose (1.5%) – AC (1%) DGT-HPLC/MS method applied here to EDC measurements shows good agreement with the only previous study[241].

In particular, agarose (1.5%) and agarose (1.5%) – AC (1%) gels resulted in constant water-carbon ratios along the two-dimensions ensuring a consistent diffusive property and binding ability.
This consistency was confirmed by EDX spectra of the freeze-dried platinum-coated hydrogels. The agarose (1.5%) – AC (1%) gel was enriched approximately by 10% more carbon compared to the agarose (1.5%) throughout the sample.

BPA, BPAF, βE2 and EE2 optimised conformations and approximated dimensions were calculated through Gaussian simulation (DFT method B3LYP; basis set 6-31G). Lengths and widths: BPA (10.18 Å; 4.36 Å), BPAF (10.23 Å; 4.83 Å), βE2 (12.32 Å; 5.11 Å) and EE2 (12.32 Å; 6.77 Å). Freeze-dried, platinum-coated hydrogel samples were morphologically characterized by FE-SEM. Agarose (1.5%) was found to have an average pore number of 22 ± 5 per μm^2 with a weighted pore diameter of Ø = 30 nm while the agarose (1.5%) – AC (1%) had an average pore number of 13 ± 2 per μm^2 with a weighted pore diameter of Ø = 45 nm. Both gels ensured free diffusion of the target analytes.

ABS plastic probes, agarose diffusive gels of diameter Ø =2.51 cm and 0.54 mm thickness, and hydrophilic PTFE membranes of diameter Ø = 2.51 cm were tested to ensure they avoided target adsorption. All materials showed a relatively small adsorption < 3% (excluded the adsorption of BPAF by PTFE membrane of < 7%).

Kinetic studies of absorption of the agarose (1.5%) – AC (1%) showed relatively fast BPA, BPAF, E2, EE2 uptake of 98%, 98%, 97%, 95% and average binding rates of 2.05, 1.20, 2.48, 3.55 ng cm^{-2} min^{-1} over the first 60 min, respectively.

MeOH (10 mL; 24 h) was the be best solvent for the recovery of BPA (R% = 103 ± 17; 25°C), and BPAF (R% = 79 ±18; 25°C) while EtOH (10 mL; 24 h) was more suitable for recovery of E2 (R% = 96 ± 31; 25°C) and EE2 (R% = 107 ± 11; 25°C) from the binding hydrogel.

A HPLC-MS method was optimised to detect and quantify BPA, BPAF, E2, EE2 adopting a MeOH, NH_4OH 0.06 M/ H_2O, NH_4OH 0.06 M fast gradient elution program (15 min; ambient t). Tandem MS parameters were optimized for each compound. The negative mode was chosen to fragment the targets, and the deprotonated molecules [M-H]- were used as precursor ions and a transition ion was selected and monitored for the identification of each compound of interest.

DGT blank concentrations, LOD (1.00, 0.61, 1.31, 0.20 µg/L for BPA, BPAF, E2, EE2 respectively) and LOQ (3.34, 2.03, 4.39, 0.68 µg/L for BPA, BPAF, E2, EE2 respectively) were calculated for each compound (24 h as deployment time; 25°C; 0.54 mm thick diffusive gel).

The DGT-HPLC/MS methodology was tested with environmental samples from sampling sites along the Waikato River in November 2013 and compared to a validated and published SPE-HRGC/MS method[1]. The comparison of the quantifications demonstrated the novel DGT technique is suitable for the environmental detection of EDC trace concentrations (ng L^{-1}). DGT-HPLC/MS was convenient in terms of sample preparation, ease of deployment and low cost compared to the traditional active sampling approaches for the monitoring of the EDCs.

3 *IN SITU* MONITORING OF ESTROGENIC CONCENTRATION IN FRESH WATER AND THROUGH DRINKING AND WASTEWATER TREATMENT PROCESSES

3.1 Introduction

3.1.1 Drinkable water treatment plant (HWTP) in Hamilton

Hamilton City Council's Water Treatment Plant (HWTP) was built in 1971 but a series of improvements and upgrades have been applied to provide a peak production capacity of 106 ML/day[341]. However, a Resource Consent from the Waikato Regional Council sets the maximum amount of water, accordingly with city population growth, that can be taken daily from the river to be treated in the plant[341].

The water supply system is designed to ensure that all urban areas of the city have access to enough water at the right flow and pressure. A series of "lines of defence" along the treatment, distribution and management systems guarantee that the water is safe to drink and use after reaching the households[341]:

- Screening. Coarse grills and then narrower screens are used to take out large and small pieces of debris before water from the Waikato River enters the Water Treatment Plant. The river water is then pumped by large pumps up to the plant.
- Coagulation/sedimentation. This treatment removes the particulate material such as sediments, micro-organisms, organic and inorganic material and other small particles. Aluminium sulphate (alum) and polymer are added to assist the sedimentation. Alum breaks weak bonds that hold small particles in suspension. This, along with the addition of polymer, helps the suspended material to coagulate to form "floc" that are bigger, heavier particles. This floc separates from the water to create a sludge layer that is continually removed and discharged to the city's wastewater system. The clearer water flows out of the top of the sedimentation tank for further treatment.

- Filtration. Fine sand is used to filter and remove any leftover floc that have not clumped together or settled in the sedimentation tanks. The sand gravity-feeds the water, leaving remaining floc trapped on top and within the sand. These filters are cleaned automatically every 50-100 hours by backwashing with a mixture of water and air. Coagulation and Filtration represent the primary defence against impurities and larger micro-organisms (in particular *Cyrptosporidium* and *Giardia*) found in river water. The sand-filtered water is lifted up by large pumps to the Granular Activated Carbon (GAC) filters.
- Carbon filtration. Granular Activated Carbon (GAC) filters are made up of activated carbon. Each grain of carbon has a huge surface area because it is covered with tiny gaps and holes. This allows the carbon to absorb any organic dissolved material not able to be removed in earlier treatment processes. This can include organic compounds produced by algae (such as *Cyanobacteria* or Blue Green Algae) naturally found in river water that can be toxic or can give water an unpleasant taste and odour.
- UV disinfection. The disinfection using ultraviolet (UV) light deactivates protozoa (single-celled microscopic organisms) such as *Giardia* and *Cryptosporidium*, highly resistant to chlorine treatment, by permanently altering their DNA structure so they are unable to infect or reproduce.
- Chlorine disinfection. The addition of chlorine makes safe the treated water killing any remaining bacteria and viruses left at this stage. Chlorine-dosed water is sent to a large water reservoir and left there for over an hour to maximise its effect.
- Residual disinfection. A small amount of chlorine is left in the water leaving the treatment plant to provide "residual disinfection" so the water remains safe between leaving the plant and coming out of the tap.
- Reservoir storage. The treated water flows through a bulk water main that transports water to the distribution network and eight reservoirs around the city. Additional pumps push the water into or out of specific reservoirs. The pumps ensure that agreed levels of service for pressure and flow can be achieved in both elevated and low lying areas of the city.

- Backflow prevention. Back flow preventers are installed at various high risk supply points throughout the Council's water supply network in conjunction with the protection provided by the low chlorine residual in the water after treatment. These fittings are designed to stop any affected water flowing back into the system in case a contamination incident does occur.

Water leaving the plant, stored in reservoirs and within the distribution network is continuously tested through advanced "online" technology to monitor that the water is maintained safe for drinking. Hamilton City Council's Water Treatment Plant owns an "Aa" grade according to the A1 to E grading system set by the Ministry of Health.

The Ministry of Health is responsible for setting drinking water standards in New Zealand. The standards specify the maximum acceptable values (MAV) of micro-organisms, organic and inorganic chemicals that are of health significance. However, the Ministry of Health does not include MAV for the EDCs, nor for bisphenols and estrogens more specifically, in the organic chemical deteminands datasheets[342]. According to the Ministry there are insufficient data to derive a MAV for bisphenol A in drinking-water. However, the Ministry of Health reports that, according to the World Health Organization (WHO), BPA is frequently found in drinking-water and its concentration may vary from 0.005 µg L^{-1} up to 1 µg L^{-1} maximum[342]. No comprehensive monitoring of bisphenols and estrogens in the drinkable water of New Zealand is undertaken, since the EDCs are not to have possible health or aesthetic significance. According to the New Zealand Herald[343], the only data available date back to 2017 where 16 bores were tested in Lower Hutt and it appeared that the highest concentration of BPA found was 0.28 µg L^{-1}.

3.1.2 Wastewater treatment plant (WWTP) in Hamilton

Domestic wastewater includes everything that is flushed down toilets/sinks/showers from the residential properties, while trade waste may contain different types of chemicals/pollutants which need to be treated before being discharged to the Hamilton City Council (HCC) wastewater network.

The Pukete Wastewater Treatment Plant (WWTP) was built in 1975 and represents Hamilton's only wastewater treatment facility. Complex processes ensure the efficient removal of solids, oils, greases, bacteria, chemicals and heavy metals from wastewater before discharging the treated wastewater (effluent) to the Waikato River. Thus, the end products of wastewater treatment result in fully treated effluent and biosolids.

The Pukete WWTP includes primary, secondary and tertiary treatment stages[344,345]:

- Primary treatment. It removes large objects such as rags, food scraps, twigs and small heavy particles like sand and gravel. This material is taken to landfill. The wastewater flows to large primary sedimentation tanks. Heavy solids, scum and grease are separated from the wastewater and pumped into "digesters" that consist of large heated tanks. The naturally occurring bacteria in this sludge help to break down some of the solids. This digested "sludge" becomes less smelly and many disease causing organisms are destroyed. The sludge is then dewatered and taken to landfill. The methane produced during the digestion is used to generate electrical energy. The remaining wastewater (primary effluent) flows to aeration tanks for secondary treatment.
- Secondary treatment. It removes most of the nutrient nitrogen found in wastewater, reducing the amount of nitrogen entering the river. Excess nitrogen in the river stimulates the growth of nuisance plants and algae, reducing water quality. The activated sludge is then settled out in "clarifiers" that consist in large open-air tanks and is returned to the aeration tanks to repopulate them with bacteria. The clear "secondary effluent" flows on for tertiary treatment.
- Tertiary treatment. During this treatment, the effluent is exposed to high doses of ultraviolet radiation to disinfect the water and to stop the spread of diseases. The final effluent consists in a high quality clear liquid, low in solids and bacteria, that can be discharged back into the Waikato River.

To date, no data are available regarding the EDCs concentration of the WWTP treated wastewater (effluent). However, steroid estrogens, their conjugates and their total estrogenic activity have been analysed by Gadd at 18 New Zealand dairy farms as reported by Tremblay et al. (2011)[346]. Steroid estrogen concentrations were elevated in dairy shed effluents with potential to cause environmental effects if discharged directly to the aquatic environment with minimal dilution. The study suggested as well that when disposed of on land, there is a possibility for water-soluble conjugated steroids to leach through soils to the aquatic environment and pose a problem if hydrolysed at a later time. Also assessed was the reduction of estrogens from dairy effluent using a two pond system and advanced pond system treatment options. The results suggested a 50-100 percent decrease in total steroid concentrations and 62-100 percent decrease in estrogenic activity. Estrogenic steroid hormones have been detected in groundwater and stream waters of intensively farmed dairy catchments. It was noted that estrogenic activity of the effluent at times exceeded suggested guideline values for protection of freshwater fish.

3.1.3 Waikato River: water assessment for selected endocrine disrupting chemicals and hormonal activity

The Waikato River is the longest river in New Zealand. This river starts in the volcanic zone of the central North Island, 2797 m above sea level. It flows into Lake Taupo and 425 km from exiting the lake, finally reaches the Tasman Sea at Port Waikato[347].

In the 1950s the Waikato River was one of the dirtiest in New Zealand. Measurements taken downstream of Hamilton City revealed that the concentrations of contaminants increased ten times between the 1950s and the early 1970s due to the growth of the city[348]. Hamilton's wastewater in fact was discharged to the river after holding in 14 septic tanks which were emptied into the river three times a year. Moreover sewage from other towns, effluent from the Horotiu freezing works, Kinleith paper mill, power projects and dairy farms discharged directly into the Waikato River[348].

The water quality began to improve in the 1970s thanks to major improvements both in the urban wastewater treatment plant (WWTP) and to the quality of the industrial effluents. The levels of contamination in fact decreased ten times during the late 1970s and the early 1980s. In 1987, the Waikato Regional Council started a comprehensive monitoring program to assess the water quality of the river. Since then the water is periodically sampled at 10 monitoring sites along the length of the river[348].

The Waikato Regional Council compiled a first technical report at the end of the 2013 to evaluate the presence of EDCs and hormonal activity in the Waikato River[1]. Potential sources of EDCs to the Waikato River are represented by wastewater discharge from a number of WWTPs and dairy waste along its length and the prevalence of intensive dairy farming in the region.

The analysis was carried out using a combined approach of biological assays and trace chemical analysis, in order to measure the total endocrine disrupting activity but also the concentration of the selected EDCs in concentrated sample extracts (water samples were concentrated 20000x for bioassays and 30000x for chemical analysis).

More specifically the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay[349] was chosen to monitor the total estrogenic activity and the PC-3-androgen receptor-luciferase-MMTV cell line (PALM) assay[350] was deployed to assess the androgenic activity, while high resolution gas chromatography-mass spectrometry (HRGC-MS) was selected for the analytical analysis.

The total hormonal activity and the target EDCs were evaluated in eight water samples collected between Taupo and Tuakau (Table 3.1).

Table 3.1

MELN and PALM bioassay results for estrogenic and androgenic activities in eight samples from the Waikato River catchment[1]. Re-edited from "Waikato Regional Council" (2013).

Site	Estrogenic activity		Androgenic activity	
	Agonist (17β-estradiol equivalent, ng/L)	Antagonist (ICI 182 780 equivalent, ng/L)	Agonist (R1881 equivalent, ng/L)	Antagonist (bicalutamide equivalent, µg/L)
Taupo Gates	0.13	BDL (< 17.4)	BDL (< 7.1)	BDL (< 2.3)
Ohaaki Bridge	0.17	BDL (< 17.4)	BDL (< 7.1)	BDL (< 2.3)
Waipapa	BDL (< 0.072)	176.2	BDL (< 6.9)	10.1
Hamilton Narrows	BDL (< 0.068)	40.1	BDL (< 7.1)	10.8
Horotui Bridge	BDL (< 0.072)	16.1	BDL (< 6.9)	BDL (< 1.5)
Huntly Tainui	BDL (< 0.072)	30.2	BDL (< 6.9)	4.6
Mercer Bridge	BDL (<0.068)	31.5	BDL (< 7.1)	15.4
Tuakau Bridge	BDL (< 0.072)	28.5	BDL (< 6.9)	2.4

None of the sites monitored showed detectable androgenic activities. Some sites showed estrogen and androgen antagonistic activities and only at the Taupo Gates and Ohaaki Bridge sites was estrogenic activity at a level close to the detection limit of the technique. A reasonable explanation of the loss of estrogenic and androgenic activities in treated effluents is caused by the degradation of agonistic compounds and by the presence and production of antagonist compounds[351]. This assumption may explain the presence of estrogenic activity at Taupo Gates and Ohaaki Bridge, known to be the more pristine sites, where the presence of antagonistic chemicals is less likely and estrogenic activity may come from agricultural activities. Several classes of EDCs were investigated during the analytical analysis but BPA was the only EDC detected in all samples of the Waikato River. The detection in the upper waters of the Waikato River can be considered background concentrations within Lake Taupo. The maximum concentration of BPA detected was 4.26 ng/L at the Horotiu Bridge sample site downstream of Hamilton city.

Estrogenic activity was detected at levels close to the detection limit of the bioassay both in the Taupo Gates and Ohaaki Bridge samples. Other studies proposed that the loss of estrogenic/androgenic activity in treated effluents is caused by the degradation of agonistic compounds and by the presence and/or

production of antagonist compounds[351]. This assumption may explain the presence of estrogenic activity at the more pristine sites of Taupo Gates and Ohaaki Bridge where it is more difficult to find the presence of antagonistic chemicals and the estrogenicity may be from agricultural activities.

However, previous investigations[346] demonstrated that the Waikato River receives relevant input of steroid hormones from the WWTP effluent points, animal waste effluents and diffuse agricultural sources. The daily absolute mass of steroid hormone residues that comes from the sources mentioned and enters the greater Waikato catchment is relevant compared to the other sources of pollution, thus aquatic organisms that live close to the discharge of WWTPs effluent may be impacted by residues of hormones and EDCs continuously released into the river.

In conclusion these analyses do not represent a comprehensive study: the water quality was assessed but not the quality of the sediments, in which the EDCs may potentially accumulate. Moreover the analysis were not time-integrated: the samples were obtained from a one-off sampling and the concentrated samples analysed, thus the results need to be interpreted with caution.

Future investigation focused on this class of pollutants within the Waikato River would benefit from the deployment of integrative passive sampling and biomarker-based surveys.

3.1.4 Study location

The Ministry of Health in New Zealand does not set maximum acceptable values (MAVs) in drinking-water for estrogens such as E2 and EE2 and bisphenols such as BPA and BPAF[342], because there are insufficient data to derive MAVs. Thus, no comprehensive monitoring of bisphenols and estrogens in drinkable water is available currently in New Zealand. However, according to the World Health Organization (WHO), BPA is frequently found in drinking-water and its concentration may vary from 0.005 µg L^{-1} up to 1 µg L^{-1} [342].

Steroid estrogens, their conjugates and their total estrogenic activity have been analysed by Gaddat from 18 New Zealand dairy farms, as reported by Tremblay et al. (2011)[346]. Their concentrations appeared elevated in dairy shed effluents with potential to cause environmental effects if discharged directly to the aquatic

environment with minimal dilution. Moreover, there was a possibility for water-soluble conjugated steroids to leach through soils to the aquatic environment when disposed of on land and posed a problem if hydrolysed at a later time. Estrogenic steroid hormones have been detected in groundwater and stream waters of intensively farmed dairy catchments. It was noted that estrogenic activity of the effluent at times exceeded suggested guideline values for protection of freshwater fish.

Therefore, it appeared necessary to evaluate in a more comprehensive way the actual level of the selected EDCs during the drinking and wastewater treatment processes and along the Waikato River. The *in situ* integrated monitoring seemed the most practical, least invasive and cost effective approach to evaluate human and environmental exposure to the unregulated E2, EE2,BPA and BPAF compounds. Thus, this investigation consisted of:

- test the DGT samplers under environmental conditions and broaden their application to new organic species. The DGT deployment would provide the first EDC survey in New Zealand adopting this technique. The study would enrich and support other case studies in the literature where plastic monomers were quantified[241];
- characterize the efficiency of removal of the selected ECs throughout the drinkable water treatment processes (river-to-tap) in Hamilton. The DGT deployment represents the first EDC survey at the Waiora Water Treatment Plant (HWTP) adopting this technique; routine analysis at the HWTP does not involve the survey of EDCs such as estrogens due to the lack of threshold limits in the water supply set by the Ministry of Health;
- characterize the efficiency of removal of the selected EDCs during the wastewater treatment processes (effluent-to-treated wastewater) in Hamilton. The DGT deployment represents the first EDCs survey at the Pukete Wastewater Treatment Plant (WWTP) adopting this approach, routine analysis does not involve the survey of EDCs because this is not required by the Council, in fact EDCs are assessed only on a five yearly

basis as required by the resource consent for the WWTP by the Waikato Regional Council;

- monitor the water quality of the Waikato River from source-to-outfall, at particular interest point locations used as water sources by different councils.

The DGTs were placed in specific study sites, in particular:

- they were deployed in the Waikato River (upstream) and at critical stages of the water treatment in the HWTP such as after sedimentation, after UV disinfection, in the distribution storage reservoir at Pukete and from domestic tap water (Figure 3.1);

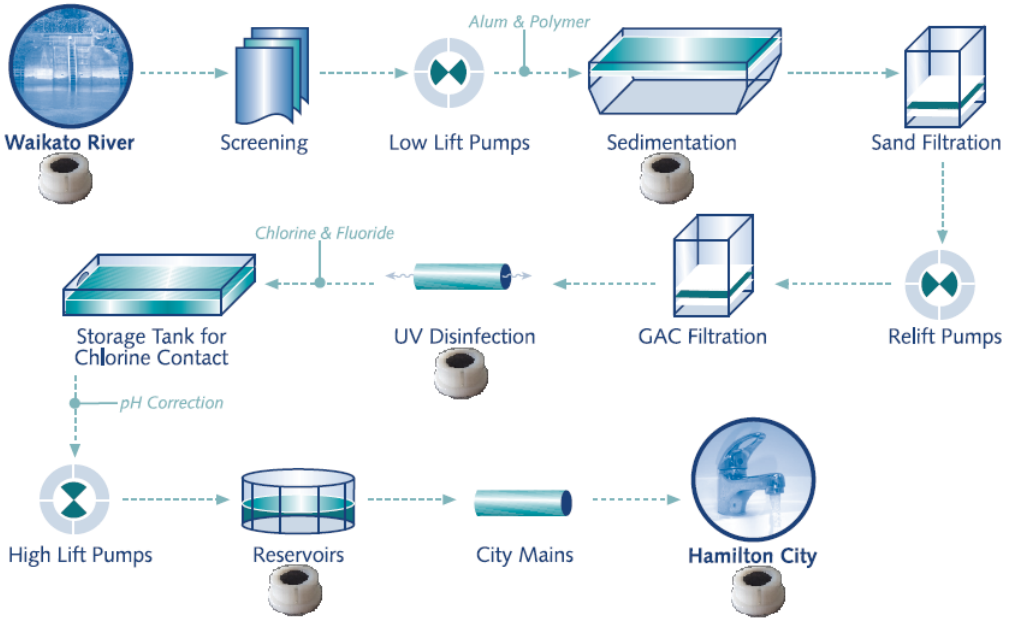


Figure 3.1

The treatment process of Hamilton's tap water and proposed DGT deployment locations[352]*. Re-edited from the "Hamilton City Council" (2010).*

- they were deployed at the PWTP at crucial stages such as after primary sedimentation, after secondary clarifiers, after UV disinfection and in the River (downstream) (Figure 3.2);

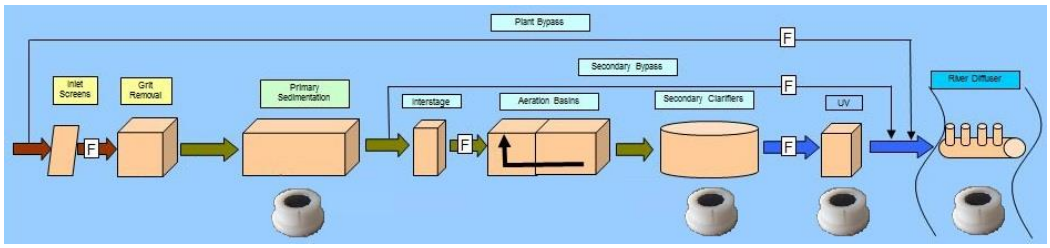


Figure 3.2

The treatment process of Hamilton's wastewater and proposed DGT deploymentlocations[353]. Re-edited from the "Hamilton City Council" (2013).

- they were deployed all along the Waikato River (Figure 3.3), in order: Lake Taupo (upstream), Taupo Waikato River (downstream), Cambridge Waikato (upstream), Cambridge Waikato (downstream), Hamilton Waikato River (upstream), Hamilton Waikato (downstream), Huntly Waikato River (upstream), Huntly Waikato (downstream), Tuakau. The locations were chosen due to the lack of a comprehensive survey of the effluents of the main urban areas such as Taupo, Cambridge, Hamilton and Huntly and their impact on the water quality. Moreover, the sampling points were chosen to compare the data of this investigation with those arising from the Regional Council technical report in 2013[1] and to evaluate longitudinal changes in the water quality.

Figure 3.3
Monitoring sites along the Waikato River[354]*. Re-edited from the "Waikato Regional Council" (2016).*

3.2 Materials and methods

3.2.1 Materials and field deployments

Standard DGT mouldings of acetonitrile-butadiene-styrene (ABS), rigorously BPA free, were obtained from DGT research Ltd (Lancaster, Lancashire, UK), and were employed for the field study. Final probes were assembled following the procedure reported in paragraph 2.2.4.3.

Hydrophilic poly(tetrafluoroehtylene) (PTFE) filter membranes with diameters of 25 mm and pore sizes of 0.45 µm were obtained from Merck Millipore (Burlington, Massachusetts, USA) and were soaked in deionised water for 24 h prior use.

Diffusive agarose (1.5%) hydrogels of 0.54 mm of thickness and binding agarose (1.5%) activated charcoal (1%) hydrogels of 0.51 mm of thickness were synthetized in the laboratory following the procedure explained in sections 2.2.4.1.and 2.2.4.2 respectively.

HOBO TidbiT v2 Water Temperature Data Logger (max depth 1,000 ft, accuracy ± 0.2 °C), purchased from ONSET (Bourne, Massachusetts, USA) were employed as temperature recorders for river, treatment plant and tap water sampling.

Probe holders in SISTEMA® plastics (New Zealand) and polylactic acid (PLA), rigorously phthalates and BPA free, were specially designed in the laboratory for the sampling of the tap water in the domestic environment (Figure 3.4).

Figure 3.4
a) tap water probes holder b) tap water probe holder with DGTs and temperature recorder c) tap water probe holder completely assembled d) tap water probe holder assembled to store DGTs.

Galvanised steel chains, galvanised steel cages, stainless steel connectors and nichrome wires (NCrA: 80% Ni, 20% Cr) were chosen to secure the probes and the loggers during the sampling exposure (Figure 3.5).

Figure 3.5
a) DGTs secured on a stainless steel bar b) DGTs secured on a galvanised steel chain c) DGTs secured in a galvanised steel cage.

DGT mouldings were washed in HCl solution (10%), rinsed in deionized water, then washed in HNO_3 (10%) and rinsed again in deionized water. Galvanised steel

chains, metal connectors, and nichrome wires, were washed in NaClO. Temperature loggers were cleaned with EtOH (70% v/v) sanitizer for the deployments at the drinkable water treatment plant.

The DGT probes were retrieved from the environment, and transported in deionized water using SISTEMA® (New Zealand) plastic boxes (BPA free). The binding gels were recovered in the laboratory and were eluted in methanol/ethanol for 24 h on a Ratek (Barkan, Israel) RPM5 medium rocking platform mixer – digital.

A Thermo Fisher Scientific UltiMate 3000 standard (SD) HPLC unit (Waltham, Massachusetts, USA) hyphenated with a Bruker Amazon X ion trap mass spectrometer (Billerica, Massachusetts, USA) were used for the separation and quantification of the target analytes. A Phenomenex (Torrance, California, USA) Gemini NX-C18 110 Å (100 x 2 mm, 3 µm particle size) HPLC column, which delivers stability at a pH 1-12, protected by a Gemini NX-C18 (4 X 2 mm ID) guard column were chosen to perform the chromatographic separations.

3.2.2 Analytical Methods

HPLC-MS samples preparation and analysis

At least 6 DGT probes were employed for each monitored site. DGTs were retrieved from the environment and the exposure time and the water temperature were recorded. During the transportation to the laboratory the probes were stored in deionized H_2O. Only the DGTs where significant biofouling (accumulation of microorganisms, plants, algae, or small animals on wetted surfaces) did not occur on the protective filter membrane were analysed, to avoid bias in the target quantifications. DGTs were disassembled and the loaded AC hydrogels were recovered. Two combined AC hydrogels were eluted in 10 mL of MeOH for 24 h to extract BPA and BPAF and were sequentially eluted in 10 mL of EtOH for 24 h to extract E2 and EE2. The combined elution was adopted to increase the S/N during the chromatographic analysis and to decrease the variance values as well.

The MeOH and EtOH DGTs eluted were then analysed using HPLC-MS by adopting the HPLC elution program described in sections 2.2.5 and 2.2.6. Calibration curves, like those shown in section 2.3.8, were analysed with each batch of samples and were employed to ascend to the mass of target absorbed by the binding layer. This mass was used to calculate the environmental concentration of the target adopting Equation 1.4 from section 1.5.1.2 taking into account the number of probes eluted together and the exposure time.

3.2.3 Statistical Analysis

All the environmental locations were monitored using 6 probes in order to calculate a mean value and the relative standard deviation both during the analytical analysis and the bioassay evaluations. The statistical analysis was performed adopting EXEL software. QA and QC followed the standards written in section 2.2.9.

3.3 Results and Discussion

At least 6 DGT probes per site were exposed in the environment for a variable time (15 to 18 days) to allow a sufficient accumulation of the selected analytes. The combined elution of 2 probes per time, following the developed protocol, proved sufficient to obtain clear HPLC signals with a high S/N ratio.

3.3.1 Drinkable Water Treatment Plant (HWTP) river-to-tap water monitoring in Hamilton

The monitoring of the HWTP from river-tap water took place between October and November 2017. During the exposure time no significant rainstorms took place. It was possible to retrieve all probes without significant damage taking place. Table 3.2 and Figure 3.6 report the DGT-HPLC/MS analytical quantification and the trends of the BPA, BPAF, E2, EE2 concentrations at the HWTP monitoring.

Table 3.2

Waiora Treatment Plant (HWTP) river-to-tap water monitoring (October-November 2017, Hamilton). BPA, BPAF, E2 and EE2 concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

	BPA	BPAF	E2	EE2
Waikato River Upstream	5.19 ± 0.78	0.69 ± 0.10	<LOD	16.82 ± 2.52
Waiora Sedimentation	4.25 ± 0.64	0.74 ± 0.11	0.60 ± 0.09	19.08 ± 2.86
Waiora After UV	4.89 ± 0.73	0.86 ± 0.13	2.70 ± 0.41	18.25 ± 2.74
Pukete Reservoir	5.05 ± 0.76	0.84 ± 0.13	1.21 ± 0.18	16.08 ± 2.41
Tap Water	5.84 ± 0.88	1.01 ± 0.15	2.89 ± 0.43	16.55 ± 2.55

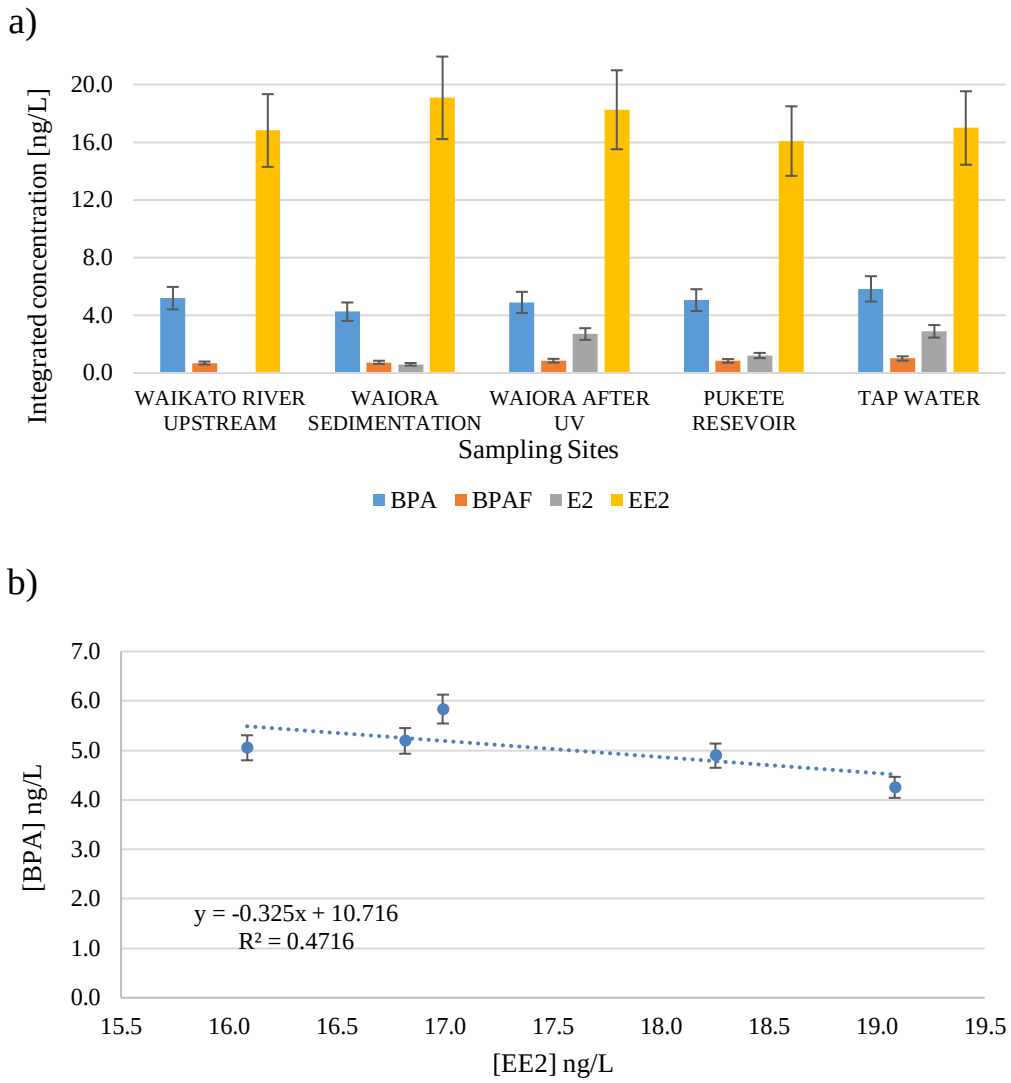


Figure 3.6

a) Waiora Treatment Plant (HWTP) river-to-tap water monitoring (October-November 2017, Hamilton). BPA, BPAF, EE2 and E2 trends along the treatment process. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method; b) Waiora Treatment Plant (HWTP) river-to-tap water monitoring (October-November 2017, Hamilton). EE2-BPA concentration relationship. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

The concentrations determined by DGT-HPLC/MS are comparable with those of other published studies that were monitoring surface freshwaters that serve urban areas around the world such as in China[19], Harbin Songhua River (BPA in river water 29-64 ng L^{-1}; BPA in tap water 15 – 63 ng L^{-1}), USA[19] (BPA in tap water max 420 ng L^{-1}), Turkey[355], Buyukcekme watershed (EE2 in watershed 11.70-14.00 ng L^{-1}), Italy[356], Venice (BPA in the lagoon water 16 ng L^{-1} and EE2 18 ng L^{-1}), Portugal[357], Ave Right River (BPA in river water 29.8 ng L^{-1}) and previous Waikato River monitoring[1] (BPA in river water max 4.26 ng L^{-1}). The concentration of all targets seemed to fluctuate around the same value, thus it was not possible to identify a specific trend or to appreciate a consistent drop in concentrations after a particular type of treatment. One obvious finding is inefficacy of the primary sedimentation. This step in fact appeared to make these pollutants more available in the water phase rather than promoting their removal. EE2 appeared slightly depleted during the treatments, but contributed the most to the estrogenic activity due to its higher potency and higher concentration compared to the other target compounds. The analysis showed that EE2 and BPA are the main pollutants, their trend was similar to the trends of other published monitoring of surface waters[358] and could depend on the different chemistry and partitioning of the these compounds[359]. EE2 shows, in fact, a relatively high octanol/water partition coefficient ($K_{OW} \approx 4$)[360] compared to BPA ($2.2 \leq K_{OW} \leq 3.4$)[361] making it more likely to bind to organic matter. This process of adsorption happens quite quickly and can explain why EE2 concentration fluctuates in a greater degree compared to BPA concentration that on the contrary appears stable along the different treatments (horizontal asymptote Figure 3.6 b). Another explanation could be given by the fact that EE2 conjugates are often converted back into their free forms during water treatment processes regaining potency as shown in other treatment plants monitoring[8,9,10].

3.3.2 Wastewater Treatment Plant (WWTP) effluent-to-treated wastewater water monitoring in Hamilton

The monitoring of the PWTP from effluent-to-treated wastewater took place between October and November 2017. It was possible to retrieve all probes without significant damage taking place because during the exposure time no significant rainstorms took place. In Table 3.3 and Figure 3.7 are reported the DGT-HPLC/MS analytical quantification and the trends of the BPA, BPAF, E2, EE2 concentrations determined from the PWTP monitoring.

Table 3.3

Pukete Wastewater Treatment Plant (PWTP) effluent-to-treated wastewater monitoring (October-November 2017, Hamilton). BPA, BPAF, E2 and E2 concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

	BPA	BPAF	E2	EE2
Pukete Sedimentation	6.67 ± 1.00	0.96 ± 0.14	6.69 ± 1.00	17.71 ± 1.76
Pukete Secondary Clarifiers	6.08 ± 0.91	1.01 ± 0.15	8.61 ± 1.29	24.57 ± 2.46
Pukete After UV	4.85 ± 0.73	0.93 ± 0.14	<LOD	15.57 ± 1.56
Waikato Downstream	5.02 ± 0.75	1.06 ± 0.16	2.82 ± 0.42	16.68 ± 1.67

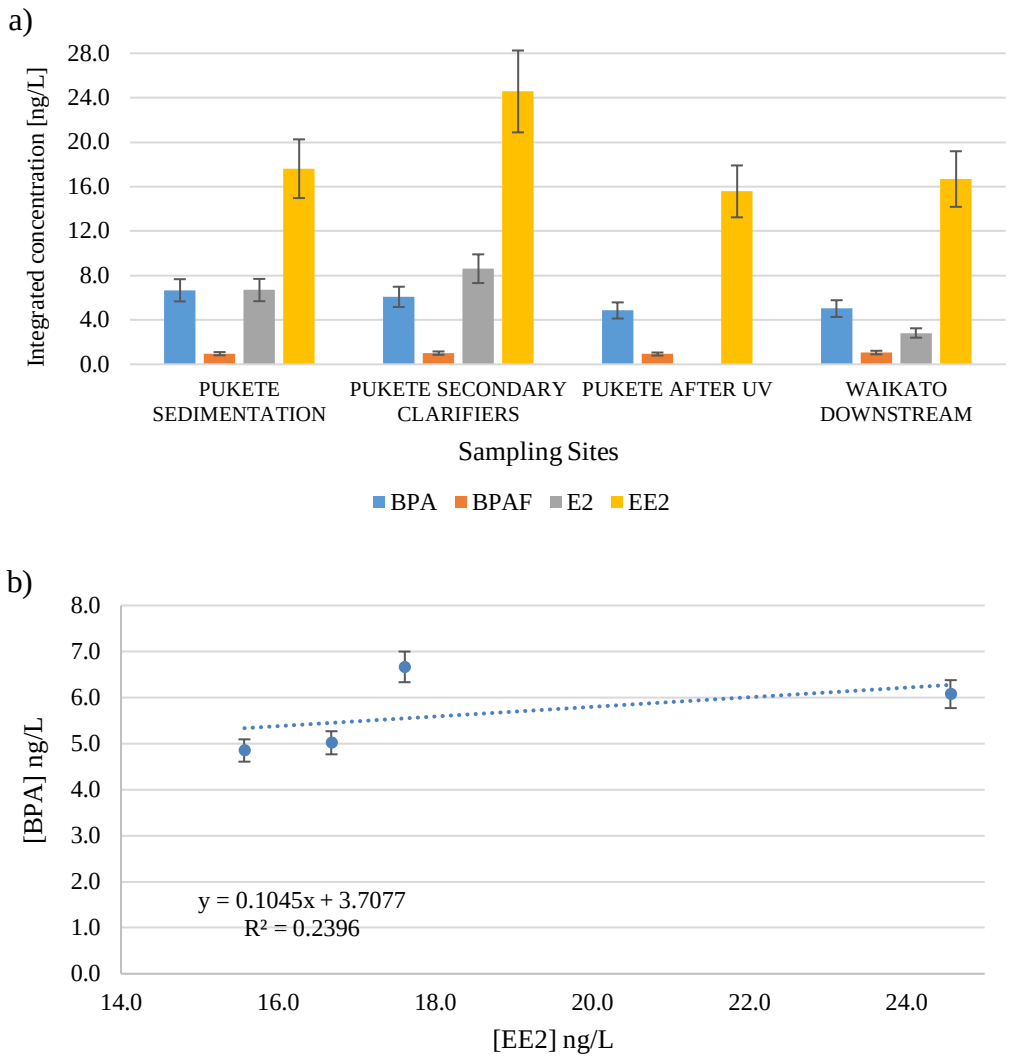


Figure 3.7

a) Pukete Wastewater Treatment Plant (PWTP) effluent-to-treated wastewater monitoring (October-November 2017, Hamilton). BPA, BPAF, E2 and EE2 trends along the treatment process. Concentrations expressed in ng L^{-1}as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method; b) Pukete Wastewater Treatment Plant (HWTP) effluent-to-treated wastewater monitoring (October-November 2017, Hamilton). EE2-BPA concentration relationship. Concentrations expressed in ng L^{-1}as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

EC concentrations determined from DGT deployments at the PWTP were within the range of those published in other monitoring studies of wastewaters such as USA[19], Cape Cod (BPA in treated wastewaters 20-55 ng L^{-1}), Canada[19], Southern Ontario (BPA effluent wastewaters 31-233 ng L^{-1}) and surface freshwaters that serve urban areas[355,356,357] and previous Waikato River monitoring[1]. As expected, the inflow concentrations were higher from the outflow ones. In this case as well, the primary treatment appeared ineffective in the removal of the selected pollutants, the water quality in fact appeared to worsen after the treatment rather than improve with respect to ECs, and it is likely that the treatment promotes the availability of the pollutants in the water phase. EE2 contributed the most to the estrogenic activity and the BPA-EE2 ratio was similar to the one evaluated during the HWTP monitoring. EE2 concentration fluctuated in a greater degree compared to BPA concentrations that were constant along the different treatments (horizontal asymptote Figure 3.7 b). These fluctuations could depend on the high octanol/water partition coefficient of EE2[360] allowing it to bind rapidly to the organic matter. Moreover, estrogen conjugates may be often converted back into their free forms during water treatment processes regaining potency as shown in other treatment plant monitoring[8,9,10].

A systematic study of removal efficiency of EDCs from fresh water and waste water (Table 3.4) was conducted by the European Community during the POSEIDON Project[362].

Table 3.4

EDCs removal efficiency from different types of treatments[362]. Legend = Ibu: Ibuprofen; Dicl: Diclofenac; Bezf: Bezafibrate; Clof: clofibric acid; E1: estrone ; E2: 17β-Estradiol; EE2: 17α-Ethynylestradiol; SMX: Sulfamethoxazole; Rox: Roxithromycin ; Carb: Carbamazepine; Diaz: Diazepam; Iopr: Iopromide; Diatr: Diatrizoate; Iopam: Iopamidol. - - : < 10%; + : from 10 to 50%; + + : from 50 to 90 %; + + + : > 90%; n.d.: no data. The values in brackets are predicted.

	Pharmaceuticals				**Estrogens**			**Antibiots**		**Neutral Drugs**		**Iodinated contrast media**		
	Ibu	Dicl	Bezf	Clof	E1	E2	EE2	SMX	Rox	Carb	Diaz	Iopr	Diatr	Iopam
Waste Water Treatments														
Primary Treatment	--	--	--	--	+	+	+	--	--	--	--	--	--	--
Nitrification	+++	+	+++	--	+++	+++	+++	+++	+	--	--	++	--	--
Active Sludge	+++	+	+++	--	+++	+++	+++	++	++	--	--	++	--	--
Bioreactor membranes	+++	+	+++	--	+++	+++	+++	n.d.	++	--	n.d.	++	--	--
Biofilters	n.d.	+	n.d.	--	+++	+++	++	n.d.	+	--	n.d.	++	--	--
Ozonization	+/++	+++	++	n.d.	+++	+++	+++	+++	+++	+++	+	+	--	+
Tap Water Treatments														
Bank Filtration	+++	+++	++	(--)	+++	+++	+++	++	+++	--	n.d.	++	--	--
Flocculation	--	--	--	--	n.d.	n.d.	n.d.	--	--	--	--	--	--	--
Ozonization	+	+++	++	+	+++	+++	+++	+++	+++	+++	+	+	--	--
AOPs	++	++	++	++	++	++	++	++	++	++	++	++	+	++
GAC	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	++	+	++
Ultrafiltration/PAC	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	++	(+)	(++)
Nanofiltration	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	(+++)	(+++)
Chlorination	--	++	--	--	(++)	(++)	(++)	(+++)	(++)	--	--	(--)	(--)	(--)
ClO_2	--	+++	--	--	+++	+++	+++	+++	++	--	--	--	--	--

Both the experimental data arising from monitoring of the water treatment plants in this study and the POSEIDON study highlighted that primary treatments are not suitable for the removal of estrogens and pharmaceuticals in general.

According to the POSEIDON project, active sludge and GAC filtration is effective in the removal of the pollutants with estrogenic activity. However, this finding did not seem to agree with the results of the DGT monitoring. It was not possible to evaluate consistent breakdown of the compounds after these types of treatments.

Several studies highlighted how sewage treatment processes can be responsible for the deconjugation into biologically active forms of most estrogens[8,9]. The deconjugation can be catalysed as well by the presence of microorganisms in the treatment plant such as *Escherichia coli*.

According to POSEIDON, reverse osmosis appeared very effective for the removal of this class of pollutants despite the onerous cost. Ozonisation proved the most robust, efficient removal methodology among all techniques, allowing 100% removal of BPA, E2 and EE2 with consequent reductions in estrogenic activity[362]. Ozone has been employed successfully as an oxidant for the treatment of the drinkable water and has been proposed for the removal of plastics, estrogens and pharmaceuticals as well[363,364].

The outcomes of the monitoring suggested that the actual technologies available at the treatment plants do not ensure efficient removal of the compounds monitored. In case of upgrades to the treatments plants, ozonisation could be proposed as a possible technology for the breakdown of persistent EDCs, however it appears necessary that a deeper and systematic monitoring adopting both active and passive analytical approaches is warranted to evaluate discrepancies in quantification.

3.3.3 Waikato River source-to-outfall water monitoring

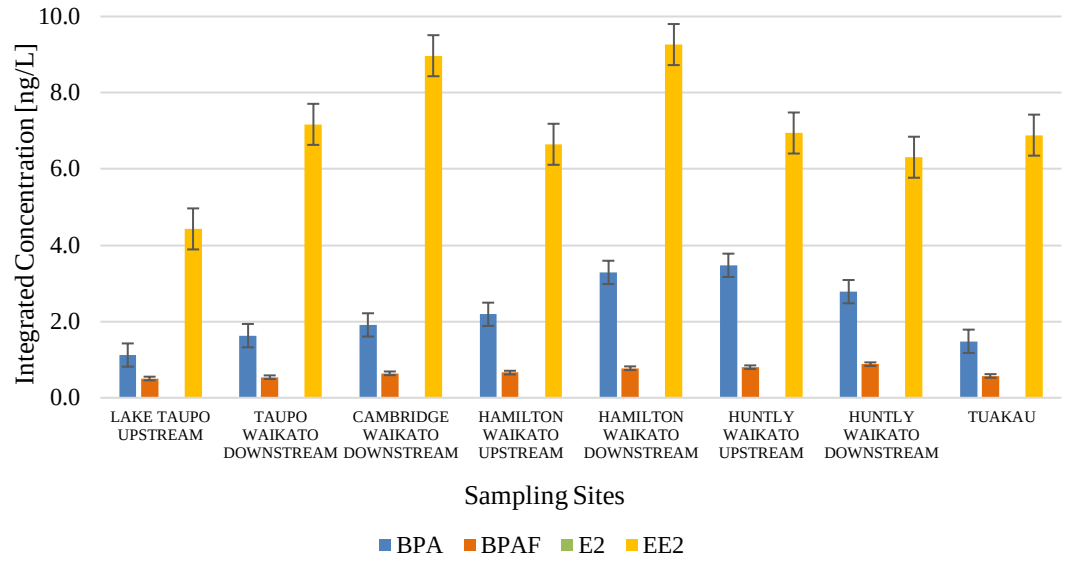
The monitoring of water quality of the Waikato River from source-to-outfall took place between January and February 2018. Rainstorms, prior to the retrieval time, caused significant flooding, diluting the pollutants of interest in the environment and damaging the DGT probes as well. All probes deployed at Cambridge Upstream were lost. However, the consistent number of probes deployed ensured replicated analysis at all other sites.

In Table 3.5 and Figure 3.8 are reported the DGT-HPLC/MS analytical quantification and the trends of the BPA, BPAF, E2 and EE2 concentrations arising from the Waikato River source-to-outfall monitoring.

Table 3.5

Waikato River source-to-outfall monitoring (January-February 2018, New Zealand). BPA, BPAF, E2 and E2 concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

	BPA	BPAF	E2	EE2
Lake Taupo Upstream	1.12 ± 0.17	0.51 ± 0.08	<LOD	4.43 ± 0.66
Taupo Waikato Downstream	1.63 ± 0.24	0.54 ± 0.08	<LOD	7.17 ± 1.08
Cambridge Waikato Downstream	1.91 ± 0.29	0.64 ± 0.10	<LOD	8.97 ± 1.35
Hamilton Waikato Upstream	2.19 ± 0.33	0.66 ± 0.10	<LOD	6.65 ± 1.00
Hamilton Waikato Downstream	3.29 ± 0.49	0.78 ± 0.12	<LOD	9.26 ± 1.39
Huntly Waikato Upstream	3.47 ± 0.52	0.80 ± 0.12	<LOD	6.94 ± 1.04
Huntly Waikato Downstream	2.78 ± 0.42	0.88 ± 0.13	<LOD	6.31 ± 0.95
Tuakau	1.48 ± 0.22	0.57 ± 0.09	<LOD	6.89 ± 1.03



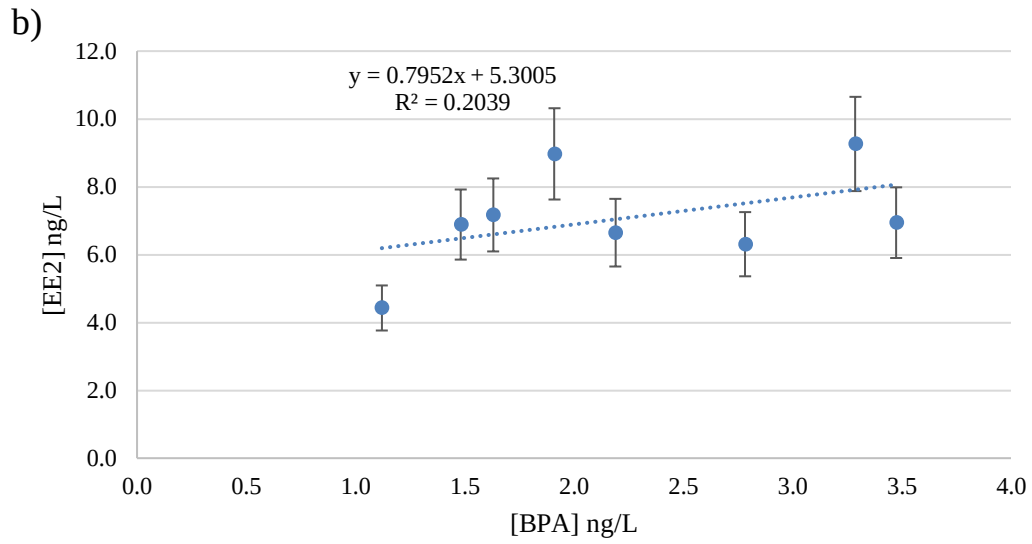


Figure 3.8

a) Waikato River source-to-outfall monitoring (January-February 2018, New Zealand). BPA, BPAF, E2 and E2 trends along the treatment process. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method; b) Waikato River source-to-outfall monitoring (January-February 2018, New Zealand). EE2-BPA concentration relationship. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

EC concentrations were in accordance with those of other studies of surface freshwaters that serve urban areas[19,355,356,357] and previous Waikato River monitoring[1]. The concentration of all targets appeared to be higher downstream at all sites monitored. EE2 proved to be always the compound that contributed most to the estrogenic activity. It was possible to appreciate a moderate worsening of water quality moving from Taupo to Tuakau with EC concentrations peaking at Hamilton downstream. It can be reasonably assumed that the high concentration of estrogens in the Waikato region may be connected to the intensively farmed dairy catchments. Previous studies proved in fact the estrogenic concentration of groundwater and stream waters of the dairy shed effluents at times exceeded suggested guideline values for protection of freshwater fish with potential to cause environmental effects if discharged directly to the aquatic environment with minimal dilution[346]. Like at the HWTP and WWTP, EE2 and BPA contributed most to the pollution. EE2 values tended to vary in a greater degree compared to BPA values that appear quite similar at the different sampling sites. EE2 fluctuations could depend on its high octanol/water partition coefficient that allows EE2 to exchange rapidly from the water to the organic matter and vice versa[360].

In Table 3.6 and Figure 3.9 are compared the values of the ECs recorded in October-November 2017 and January-February 2018 during the monitoring of the Waikato River at Hamilton upstream and downstream sites.

Table 3.6

Seasonal monitoring (October-November 2017, January-February 2018) of the Waikato River Upstream and Downstream in Hamilton, New Zealand. BPA, BPAF, E2 and E2 concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

	Hamilton Upstream November 2017	Hamilton Upstream February 2018	Hamilton Downstream November 2017	Hamilton Downstream February 2018
BPA	5.19 ± 0.78	2.19 ± 0.33	5.02 ± 0.75	3.29 ± 0.49
BPAF	0.69 ± 0.10	0.66 ± 0.10	1.06 ± 0.16	0.78 ± 0.12
E2	<LOD	<LOD	2.82 ± 0.42	<LOD
EE2	16.82 ± 2.52	6.65 ± 1.00	16.68 ± 1.67	9.26 ± 1.39

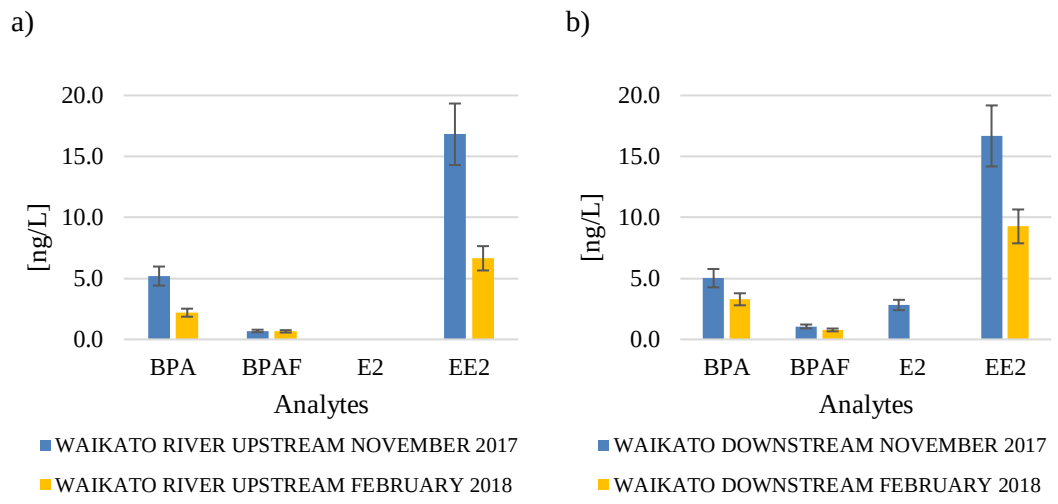
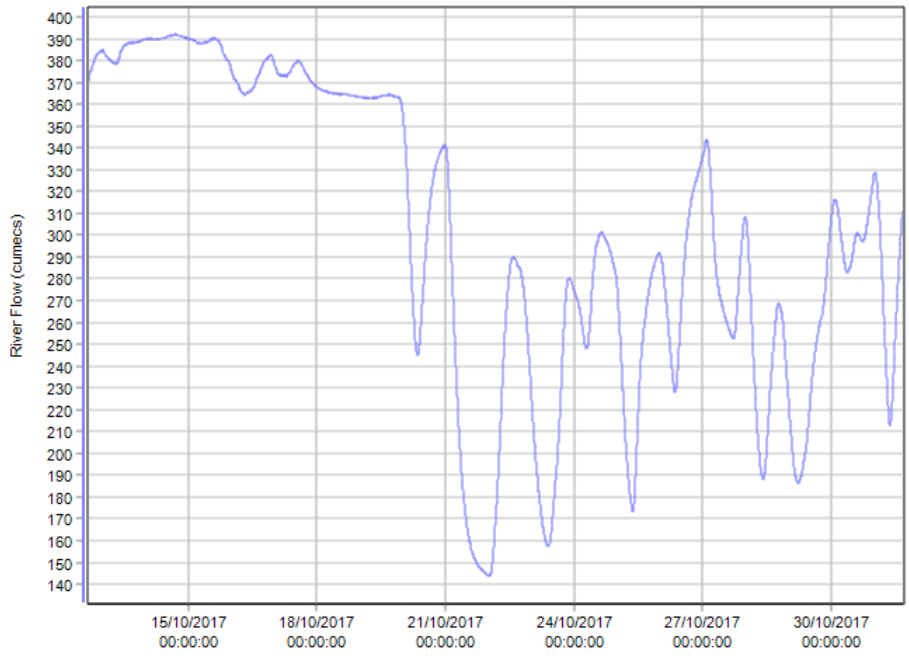


Figure 3.9

a) Seasonal comparison of BPAF, E2 and E2 concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Upstream in Hamilton, New Zealand;
b) seasonal comparison of BPAF, E2 and E2 concentrations expressed in ng L-1 as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Downstream in Hamilton, New Zealand.

From October 2017 to February 2018, there was a notable dilution in concentrations of all ECs at both Upstream and Downstream sites. As stated earlier, prior to the retrieval time, consistent rainstorms caused significant flooding affecting the concentrations of the pollutants investigated. The Waikato Regional Council[365] recorded at Victoria Bridge in Hamilton city an average flow of 257 m^3 s^{-1} in October 2017 compared with an average flow of 303 m^3 s^{-1} in February 2018 and their trends are reported in Figure 3.10.

a)



b)

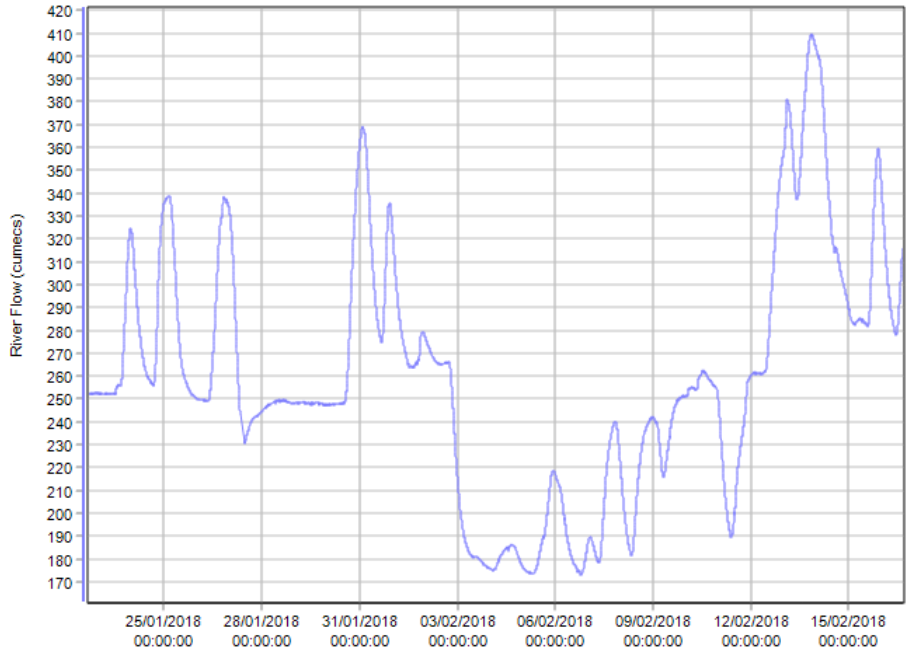


Figure 3.10

a) Waikato River flow trend recorded in October 2017 at Victoria Bridge in Hamilton city. Flows expressed in cumecs (m^3 s^{-1}); b) Waikato River flow trend recorded in February 2018 at Victoria Bridge in Hamilton city. Flows expressed in cumecs (m^3 s^{-1}).

To evaluate the hypothesis that the change in concentrations was due to the weather conditions and not caused by other factors, the data have been correlated in Figure 3.11 and Table 3.7.

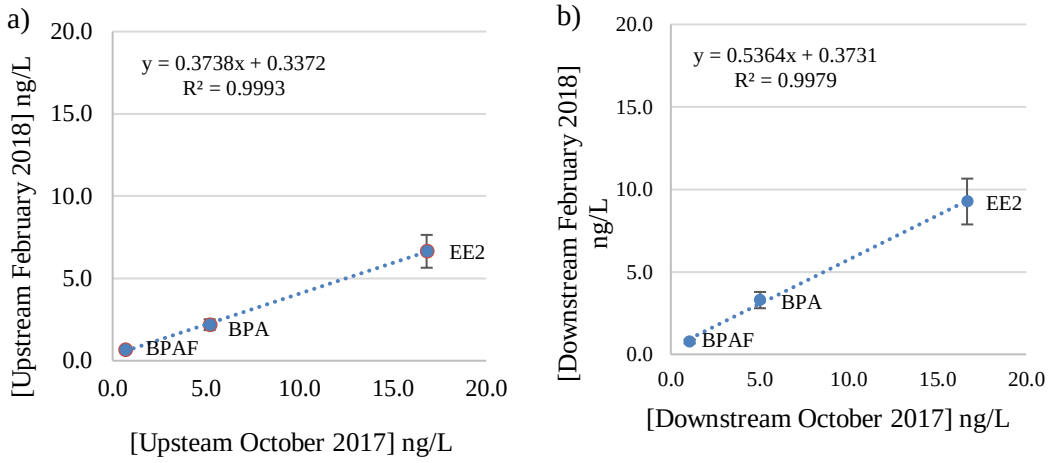


Figure 3.11

a) Correlation between BPA, BPAF, E2 and EE2 concentrations collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Upstream in Hamilton, New Zealand. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method; b) Correlation between BPA, BPAF, E2 and EE2 concentrations collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Downstream in Hamilton, New Zealand. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

Table 3.7

Dilution factors of BPA, BPAF, E2 and EE2 determined from the ratio of January-February 2018 and October-November 2017 concentrations during the monitoring of the Waikato River Upstream and Downstream in Hamilton, New Zealand.

[February 2018]/ [October 2017]	Waikato River Upstream Hamilton	Waikato River Downstream Hamilton
BPA	0.4	0.7
BPAF	1.0	0.7
EE2	0.4	0.6

The correlations clearly show the variation in concentrations are due to the dilution caused by the weather conditions at both sites ($R^2 > 0.9$). All ECs showed similar dilution factors both Upstream and Downstream.

BPA and EE2 are the EDCs that have the highest concentrations and contribute the most to estrogenic disruption in the Waikato River. In Figure 3.12 and Table 3.8 are reported their seasonal ratios. BPA end EE2 concentrations were highly correlated at these two sampling sites ($R^2 > 0.9$), with a ratio ≈ 0.3.

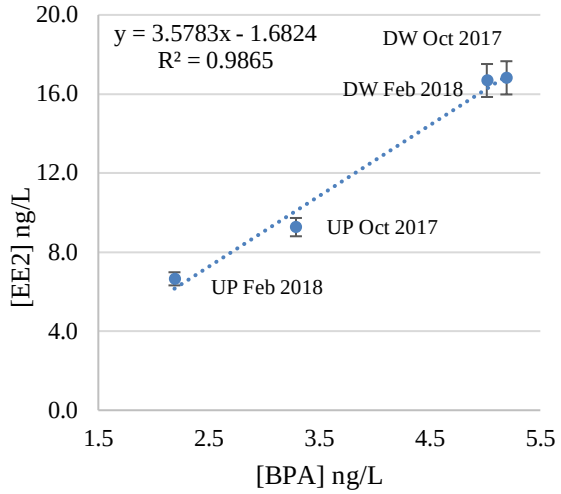


Figure 3.12

Correlation between BPA and EE2 concentrations collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Upstream and Downstream in Hamilton, New Zealand. Concentrations expressed in ng L^{-1} as mean ± standard deviation (n=6) evaluated via DGT-HPLC/MS method.

Table 3.8

BPA-EE2 ratios calculated using the values collected in October-November 2017 and January-February 2018 during the monitoring of the Waikato River Upstream and Downstream in Hamilton, New Zealand.

	[BPA] /[EE2] Waikato River Upstream Hamilton	[BPA] /[EE2] Waikato River Downstream Hamilton
October 2017	0.3	0.3
February 2018	0.3	0.4

The data collected from the monitoring of the Waikato River are compared with those from a previous monitoring of the same sites adopting an SPE-HRGC/MS method[1] in Table 3.10 and Figure 3.13. In Table 3.9 are compared the LODs of both techniques.

Table 3.9

Comparison of the limits of detection (LODs) of the SPE-HRGC/MS and DGT-HPLC/MS methods employed for the survey of BPA, E2 and EE2 in the Waikato River.

	SPE-HRGC/MS LOD[a] g/L	DGT-HPLC/MS LOD g/L
Bisphenol A	5.0E-11	1.0E-09
17β-Estradiol	1.0E-11	5.6E-11
17α-Ehynylestradiol	5.0E-11	1.7E-09

[a] limit of detection determined for 10 L of SPE extracted aqueous sample

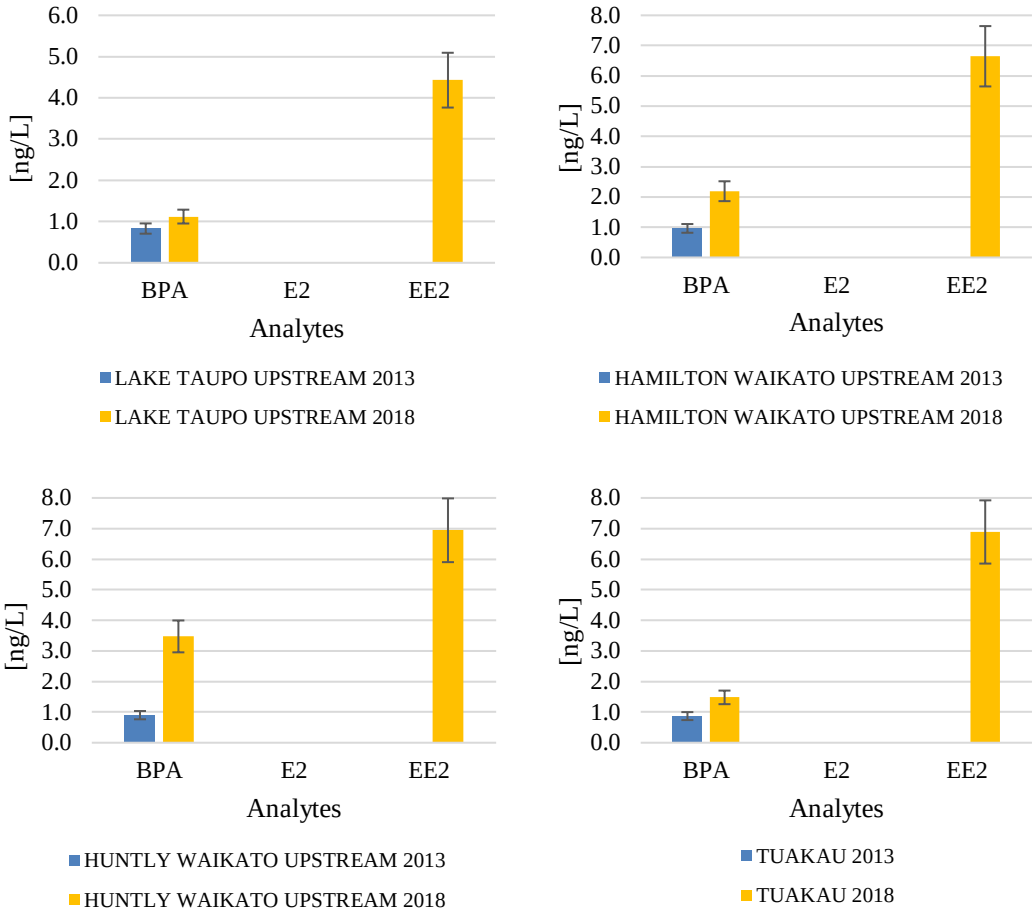
Table 3.10

BPA, E2 and EE2 concentrations in the Waikato River evaluated by SPE-HRGC/MS[1] in November 2013 and by DGT-HPLC/MS in February 2018. Concentrations expressed in ng L^{-1}.

	Lake Taupo Upstream 2013	Lake Taupo Upstrea m 2018	Hamilton Waikato Upstrea m 2013	Hamilton Waikato Upstrea m 2018	Huntly Waikato Upstrea m 2013	Huntly Waikato Upstrea m 2018	Tuakau 2013	Tuakau 2018
BPA	0.83	1.12	0.96	2.19	0.9	3.47	0.87	1.48
E2	<LOD[a]	<LOD[b]	<LOD[a]	<LOD[b]	<LOD[a]	<LOD[b]	<LOD[a]	<LOD[b]
EE2	<LOD[a]	4.43	<LOD[a]	6.65	<LOD[a]	6.94	<LOD[a]	6.89

Figure 3.13

Comparison of the BPA, E2 and EE2 concentrations at the different sampling points along the Waikato River evaluated by SPE-HRGC/MS[1] in November 2013 and by DGT-HPLC/MS in February 2018. Concentrations expressed in ng L^{-1}.



At all sites E2 concentration resulted in <LOD during both studies. It appears the water quality worsened slightly at all sites from 2013 to 2018 due to increased concentrations of BPA and EE2. However, a more comprehensive investigation is required because the limited set of analysis is not sufficient to confirm the outcomes.

3.4 Summary chapter III

DGT samplers were tested under environmental conditions. The environmental monitoring aimed to assess the efficiency of removal of the selected ECs during the water treatment processes from river-to-tap and from effluent-to-treated

wastewater in Hamilton as well as the water quality of the Waikato River source-to-outfall, at particular locations of interest.

6 DGT probes were employed for each site monitored for a long time (15 to 18 days) to enhance the accumulation of the selected analytes; combined elution of 2 probes per time successfully increased the high S/N ratio during the HPLC runs.

HWTP monitoring took place between October and November 2017. The concentrations determined by DGT-HPLC/MS were comparable with those of other published studies that were monitoring surface freshwaters that serve urban areas around the world[19,355,356,357]. EC concentrations fluctuated around the same value, thus it was not possible to identify a specific trend or to appreciate a consistent drop in concentrations after a particular type of treatment. Primary sedimentation was obviously inefficient in the removal of the targets, making them more available in the water phase and worsening the water quality. No consistent breakdown of the compounds happened even after GAC filtration. EE2 and BPA resulted in the most concentrated pollutants and their trend was similar to that of other published monitoring of surface waters[358]. EE2 concentration fluctuated to a greater degree compared to BPA concentration that appears stable along the different treatments.

PWTP monitoring happened between October and November 2017. EC concentrations were within the range of those published in other monitoring studies of wastewaters[363]. Inflow concentrations were higher than the outflow ones. The primary treatment appeared ineffective in the removal of the selected pollutants that appeared more available in the water phase. BPA-EE2 ratio was similar to the one evaluated during the HWTP monitoring. EE2 concentration fluctuated to a greater degree compared to BPA concentrations that were constant along the different treatments. The outcomes suggested that the actual technologies available at the treatment plants do not ensure efficient removal of the compounds monitored.

The Waikato River monitoring took place between January and February 2018. EC concentrations were in accordance with those of other studies of surface freshwaters that serve urban areas[19,355,356,357] and previous Waikato River monitoring[1]. All targets were more concentrated downstream at all sites monitored. EE2 proved to be always the compound that contributed most to the estrogenic activity. A moderate worsening of water quality was appreciated moving from Taupo to Tuakau. The most polluted sites were Hamilton and Huntley after the outlets of WWTPs that serve the main councils in the area, moreover effluents from extensive dairy farming activities distributed along the whole length of the river flow together making the lower sites more polluted than the upper sites. Like at the HWTP and WWTP, EE2 and BPA contributed most to the pollution and EE2 values varied to a greater degree compared to PBA.

The values of the ECs recorded in November 2017 and February 2018 during the monitoring of the Waikato River at Hamilton upstream and downstream sites were compared. All EC concentrations were diluted from November 2017 to February 2018. The correlation of the data showed this variation was attributable to the weather conditions at both sites ($R^2 > 0.9$). Prior to the retrieval time, consistent rainstorms caused significant flooding affecting the concentrations of the pollutants investigated. BPA and EE2 had the highest concentrations and were highly correlated at these two sampling sites ($R^2 > 0.9$), with a ratio ≈ 0.3.

The data collected from the monitoring of the Waikato River via DGT-HPLC/MS were compared with those from a previous monitoring of the same sites adopting an SPE-HRGC/MS method[1]. At all sites, E2 concentrations were <LOD during both studies. The water quality appeared slightly worse at all sites from 2013 to the 2018 due to increased concentrations of BPA and EE2. However, a more comprehensive investigation is required because the limited set of analysis is not sufficient to confirm the outcomes.

It appeared from the investigation that a further systematic monitoring, adopting both active and passive analytical approaches, is required to evaluate eventual discrepancy in quantification and to a draw a clear water quality assurance.

4 *IN VITRO* BIOASSAY ANALYSIS OF ENDOCRINE DISRUPTOR POTENCY IN FRESH WATER AND THROUGH DRINKING AND WASTEWATER TREATMENT PROCESSES

4.1 Introduction

The development of the DGT-HPLC/MS method focused on the analytical quantification of bisphenolic plastic and estrogens in the Waikato River and Hamilton treatment plants. These data give specific information about the exposure to ECs in the aquatic ecosystem to the selected ECs but are not sufficient to give a complete picture of the effects due to this exposure.

The interaction of these pollutants can be in fact unpredictable due not only to additive but also synergistic effects[55]. Moreover, the eventual presence of other ECs not taken into account may contribute to the final total disruption.

Bioassays are powerful and well-validated tools used in routine analysis due to their specificity, selectivity, speed and relatively low cost[366]. They allow correlation of the exposure, arising from the analytical quantification, with the dose and thus, the effect on the fresh water ecosystem[367,368].

YES bioassay has been employed in previous studies to assess the estrogenic potency of particular classes of ECs, such as surfactants,[319] or to evaluate the total estrogenic potency of environmental extracts[368,369].

Therefore, it was decided to combine the analytical measurements (exposure) with tandem deployment of a biomarker-based measurement (dose) to quantify the total estrogenic potency of the matrices analysed[370].

Among all the approaches available, it was chosen to implement the YES (Yeast Estrogen Screen) assay to evaluate the estrogenic potency of the xenoestrogens present in the samples. The choice is driven by the fact that this bioassay has been employed for more than two decades[318], thus it has been extensively implemented in order to increase its sensitivity (sub-nanomolar range), robustness, ease of use, responsiveness (only 18 h to get the results) and the relatively low cost[371].

The contribution that this study makes is to extend the application of the YES assay to eluate derived from passive sampling rather than eluate derived from active sampling. This type of hyphenation would allow correlation of the *in situ* integrated ECs concentrations with the relative integrated estradiol equivalency quantity (EEQ). Moreover, it will provide the first case-study of YES assay deployment at the drinkable and waste water treatment plants in Hamilton and along the Waikato River in New Zealand.

In the study commissioned by the Waikato Regional Council to assess EDCs and their hormonal activity[1] it was chosen to deploy the MELN assay, that consists of reporter cell lines[372], rather than the YES assay, that exploits a genetically modified yeast[318], to quantify the total estrogenic potency. MELN assay appeared slightly more sensitive compared to YES assay in detecting the ECs of interest, however its implementation was time consuming and more laborious compared to the YES assay that was implemented in a way to reduce bias due the operator steps and to reduce the time required for the assay results.

4.2 Materials and methods

4.2.1 Materials

96-well plates, culture flasks and plate sealer were purchased from Xenometrix AG (Allschwil, Switzerland).

4.2.2 Chemicals

XenoScreen XL YES accelerated high-sensitivity agonist assay kit for the detection of compounds with estrogenic activity was purchased from Xenometrix AG (Allschwil, Switzerland). The kit includes lyticase, Streptomycin/Ampicillin (to store at -20°C), CPRG substrate solution, basal medium, vitamin solution, 2 mercaptoethanol, 10x DO medium, 10x SD medium (to store between 2°C – 8°C), L-aspartic acid solution, Cu(II)-sulfate solution, lacZ lysis buffer (to store at room temperature between 20 – 25°C, protecting the liquids from the light)

Saccharomyces cerevisiae strains with human estrogen (hERα) receptor on filter discs (to store at -20°C) were purchased from Xenometrix AG (Allschwil, Switzerland).

High purity EE2 and E2 (≥ 98%) were purchased form Sigma-Aldrich (St. Louis, Missouri, USA) while BPA and BPAF (≥ 99+%) were obtained from AccuStandards (New Haven, Connecticut, USA). Sterile deionized water and sterile dimethyl sulfoxide (DMSO) purchased from Merck Millipore (Burlington, Massachusetts, USA) were employed to make up the standard solutions.

4.2.3 Instruments

The yeast cells were handled in a microbiological safety cabinet (class II, type A2) Heraeus (Hanau, Germany) model HERAsafe to avoid bacteria contamination.

The yeast cells were grown using an orbital shaker-incubator (31°C, 100 rpm) purchased from Ratek (Barkan, Israel) model OM11.

The yeast cultures were inspected with an inverted microscope Olympus (Tokyo, Japan) model IMT-2.

The colour development of the bioassay was evaluated colorimetrically at 570 nm and 690 nm using a microplate spectrophotometer Thermo Scientific (Waltham, Massachusetts, USA) model Multiskan GO 60.

MeOH and EtOH DGT eluates were dried and dissolved in sterile water and DMSO 1% (drying rate at ambient temperature) using a rotary concentrator Savant SpeedVac model SC110 purchased from Thermo Scientific (Waltham, Massachusetts, USA).

4.2.4 Yeast Estrogen Screen (YES) assay

4.2.4.1 Yeast-Based Assay preparation

The XenoScreen XL YES assay[371], employed throughout the study, is able to identify agonistic activities of test compounds both in water samples and samples dissolved in organic solvents. This assay employs lyticase and a detergent to facilitate the secretion of the intracellular β-galactosidase, leading to a reduced incubation time of 18 h, compared to the 48 h of the standard YES assay, and to an enhanced sensitivity (Figure 4.1).

Yeast culture preparation

To prepare the yeast culture it is necessary to prepare first the growth medium by mixing the complete content of the vitamins solution, L-threonine solution, L-aspartic acid solution, and 300 µL of the Cu(II) sulfate solution in a flask with the basal medium. The growth medium can be stored up to 6 months at -20°C.

To start the culture, 5 mL of growth medium was added in a tissue culture flask and then the filter disc containing the yeast strains was added using sterile forceps. The vented filter cap was tightened. The flask was placed on a wide surface and the yeast cells were incubated on an orbital shaker set at 31°C ensuring a moderate shaking of 100 rpm to supply adequate oxygen necessary for the optimal growth of the yeast cells. After one week the culture growth was stopped once it appeared clearly turbid.

The dense cultures of the yeast strains were diluted 1:10 one day before starting the assay and were stored at 4°C. The culture resumes growth once diluted in fresh medium and incubated at 31°C. It is possible to store the grown cells by freezing them (glycerol 15% w/w and growth medium, - 80°C) or storing them on porous glass beads (glycerol 15% w/w and growth medium, - 80°C). Healthy cells will take 2-3 days to grow to dense cultures again prior to dilution in fresh medium for the assay.

After the growing days, the culture appearance was investigated using an inverted microscope to determine that plenty of cell clusters were present. Using a serological pipette the yeast clumps were broken and the cells were well dispersed. At this point, 200 µL of the yeast culture were added in a flat bottom 96-well plate and it was checked that OD_{690} value was at least 0.2. If the criterion was not met the incubation of the yeast was continued.

Preparation of Controls

Stock solutions (E2 1 µM, EE2 1 µM, BPA 10 mM, BPAF 1 mM) were prepared in DMSO. The volumes of the stock solutions to be added to the sterile water were calculated for half-logarithmic dilution steps with the final volume of sample dilution of 80 µL. Solvent controls were always run along with the standards. All target compounds were tested at 8 different concentrations in two independent experiments in duplicate to build the dose-response curves for the calculation of estrogenic activities. In particular the concentrations investigated were in the range 2.1 pM - 6.7 nM for the E2 and EE2, and 21 nM - 6.7 µM for the BPA and 2.1 nM - 6.7 µM for the BPAF.

YES assay samples preparation and analysis

0.5 mL of MeOH DGT eluate and 0.5 mL of EtOH DGT eluate of each environmental sample were combined and dried using a rotor-concentrator (room temperature, 6 hours). They were then dissolved in 1 mL of sterile H_2O-DMSO (1%), and 80 µL were transferred to the 96-well plate for the single-dose tests in duplicate to estimate the relative activities. The E2 standard (stock solution 1 µM) was employed as positive control and was assayed in the range 2.1 pM - 6.7 nM. The E2 standard along with the environmental samples and the solvent were tested in duplicate.

Preparation of Test Medium

To prepare the test medium for one plate, 1.56 mL 10x SD medium, 1.56 mL 10x DO medium, 1.88 mL water, 25 µL Cu-sulphate 20 mM, and 133 µL

ampicillin/streptomycin were combined and mixed. The medium was stored at room temperature prior to the addition of the cells.

Yeast Cells Dilution

900 µL of growth medium was added to 100 µL of YES cells and 300 µL of growth medium to 2 wells in the 96-well plate. Into 2 wells, 2 x 300 µL of the 1:10 diluted YES cells was added to 2 wells and the OD_{690} was measured. The means of the measured duplicate values were then calculated and the growth medium value was subtracted from the YES cell value to calculate the OD_{690} net. The necessary volume of cells to be added to the test medium V_{cells} was calculated using the formula (3.7).

$$V_{cells} = \frac{25 * 5\ \text{mL}}{OD_{net} * 10000} \qquad 4.1$$

Transfer of YES Yeast Cells to the assay plate

The calculated amount of the original YES culture was added to 5 mL of the test medium, this was then mixed and distributed in 40 µL aliquots into all wells of the YES assay plate.

Incubation of the assay plate

A gas-permeable foil was used to seal the plate and transfer it to a plastic container provided with wet paper towels. The lid was closed to ensure a humid environment that avoids evaporation from the wells. The wells were incubated for 18 h at 31°C under gentle agitation (100 rpm). After 18 h the cells were investigated using the inverted microscope: if the cells in the solvent control wells covered less than 50% of the surface, the incubation was continued for several hours to avoid weak colour development.

Addition of lysis buffer and substrate

The lacZ reaction mixture was freshly prepared by adding 5 mL lacZ lysis buffer, 13.6 µL 2ME, 200 µL CPRG substrate solution and 50 µL of lyticase solution. 50 µL of this mixture was added to each well of a fresh 96-well plate. The assay plate was removed from the incubator and mixed using a vortex (30-60 s; 800 rpm) or a multichannel pipette avoiding the formation of bubbles. Eventually, the bubbles were removed with a gas flame to ensure the yeast cells were evenly distributed in the wells. The data obtained reading the plate at 690 nm was used to calculate the growth factor. 30 µL was added to the fresh 96-well plate then covered with a lid and incubated (31°C; 100 rpm). In case of a rapid strong colour development ($OD_{570} \geq 2.0$ at the highest concentration of E2 positive control) the experiment was stopped after 30 min, otherwise incubated for 1 h.

Reading the assay plates

The plate was tilted and rotated to distribute the developed colour, eventual bubbles among the yeast cells were removed at the flame. The OD_{570} was read again and the OD_{690} was used to correct the OD_{570} for non-specific adsorption and diffraction effects due to cell debris after lysis.

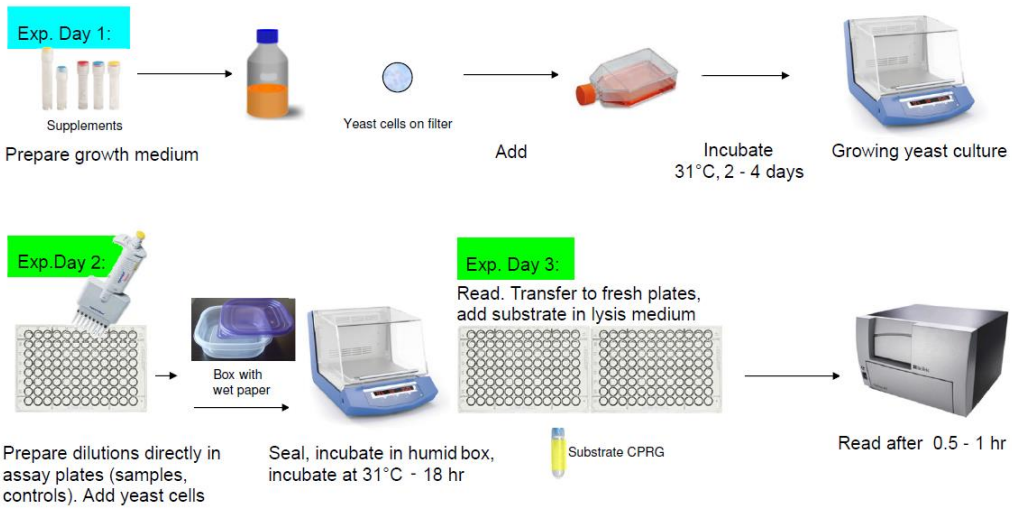


Figure 4.1

Schematic representation of the YES assay steps; Re-edited from the Xenometrix protocol[371].

4.2.4.2 Calculation, data evaluation and statistical analysis

The calculations were carried out following the protocol of the producer and the Xenometrix Excel workbook[371]. The data evaluation required first the calculation of:

- enrichment factor (*EF*)

$$EF = \frac{V_{water}}{V_{extract}} = \frac{C_{HPLC}}{C_{DGT}} \qquad 4.2$$

where V_{water} is the volume of water and $V_{extract}$ is the volume of the extract. *EF* can be evaluated only if the YES assay is coupled with an active sampling method such as SPE. This investigation on the contrary has the innovation of hyphenating DGT passive sampling with the YES assay. The enrichment of the target analytes with such sampling cannot be evaluated adopting the traditional *EF* definition due to the difficulty of ascertaining the volume of water sampled. Thus, *EF* will be calculated adopting the ratio between the concentration of the analyte computed from the HPLC (C_{HPLC}) calibration curves and the concentration computed from the DGT equation (1.4) (C_{DGT}).

- Dilution factor (*DF*)

$$DF = \frac{V_{sample}}{V_{assay}} \qquad 4.3$$

where V_{sample} is the volume of sample added and V_{assay} is the total assay volume.

- relative enrichment factor (*REF*)

$$REF = \frac{EF}{DF} \qquad 4.4$$

defined as the ratio of the previously described enrichment and dilution factors.

- the growth factor (*G*)

$$G = \frac{A_{690,S}}{A_{690,N}} \qquad 4.5$$

where $A_{690,S}$ is the absorbance of the sample at 690 nm before lysis and $A_{690,N}$ is the absorbance of the solvent control at 690 nm before lysis.

- β-galactosidase activity (relative units) (U_S)

$$U_S = \frac{A_{570,S}}{A_{690,S}} \qquad 4.6$$

where $A_{570,S}$ is the absorbance of the sample at 570 nm minus the absorbance of the sample at 690 nm after lysis.

- Induction ratio (I_R)

$$I_R = \frac{I}{G} * \frac{A_{570,S}}{A_{570,N}} \qquad 4.7$$

where $A_{570,N}$ is the absorbance of the solvent control at 570 nm minus the absorbance of the solvent control at 690 nm after lysis. The induction was corrected according to the growth factor for each well, considering growth factors with values < 0.5 as cyto-toxic effects, thus the activity of the β-galactosidase was not influenced by inhibited growth of yeast cells. I_R 10 is defined as the I_R which is 10% of (I_R max - I_R solvent) above the I_R solvent. I_R sample ≥ I_R 10 indicates an agonistic effect.

EC_{50} values indicate the concentration of agonist needed to reach 50% of the maximum signal. They were calculated with 95% confidence intervals in case the compounds exhibited a complete dose-response curve. The estradiol equivalency factor (*EEF*), or relative potency, allows the comparison of a certain chemical with the E2 standard and it is defined as the ratio between E2 EC_{50} and the chemical EC_{50}.

$$EEF_{chemical} = \frac{EC50_{E2}}{EC50_{chemical}} \qquad 4.8$$

The estradiol equivalency quantity (*EEQ*) or estrogenic activity of a sample corresponds to the concentration of E2 that would give the same activity as the sample. EEQs were evaluated from the horizontal displacement of the test sample dose-response curve relative to the standard curve ($EC50_{E2}/EC50_{sample}$) considering the different overall dilutions (*DF*)[373].

$$EEQ = \frac{EC50_{E2}}{EC50_{sample}} * DF \qquad 4.9$$

The calculated *EEQ* (*cEEQ*) can be estimated by multiplying the individual estradiol equivalency factor *EEF* by the observed analyte concentrations[373] evaluated via HPLC-MS analysis.

$$cEEQ = \sum (EEF_{chemical} * C_{chemical}) \qquad 4.10$$

4.2.4.3 Statistical Analysis

The limit of detection (LOD) was calculated as the mean of the solvent control plus 3 standard deviations, while the limit of quantification (LOQ) was calculated as mean of solvent control plus 9 standard deviations[302,371].

The estrogenic activity of the target compounds was evaluated by building calibration curves of eight points. Every trial was performed in duplicate and the results were expressed as the mean ± standard deviation (SD). Dose-response curves were fitted using a sigmoid dose-response function. The statistical analysis was carried out employing EXEL software. The analysis of variance (ANOVA) and least significant differences (LSD) at the 5% significant level were adopted to identify statistically significant differences.

4.3 Results and Discussion

4.3.1 Dose-response curves, active concentration intervals and EC_{50} values

The yeast culture was started by adding the filter disc containing the yeast strains to the growth medium in a tissue culture flask that was incubated on an orbital shaker (31°C; 100 rpm). The culture appearance was investigated on an inverted microscope after the growing days (

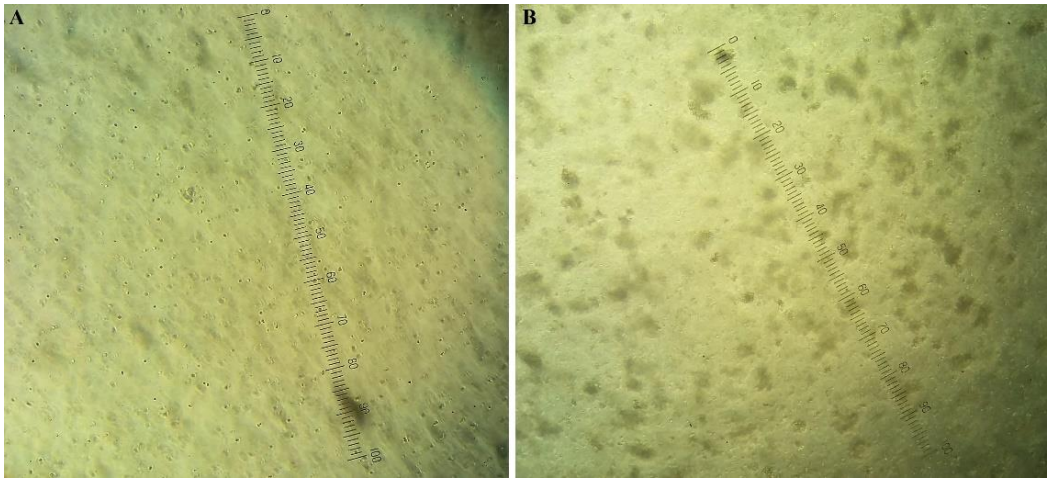
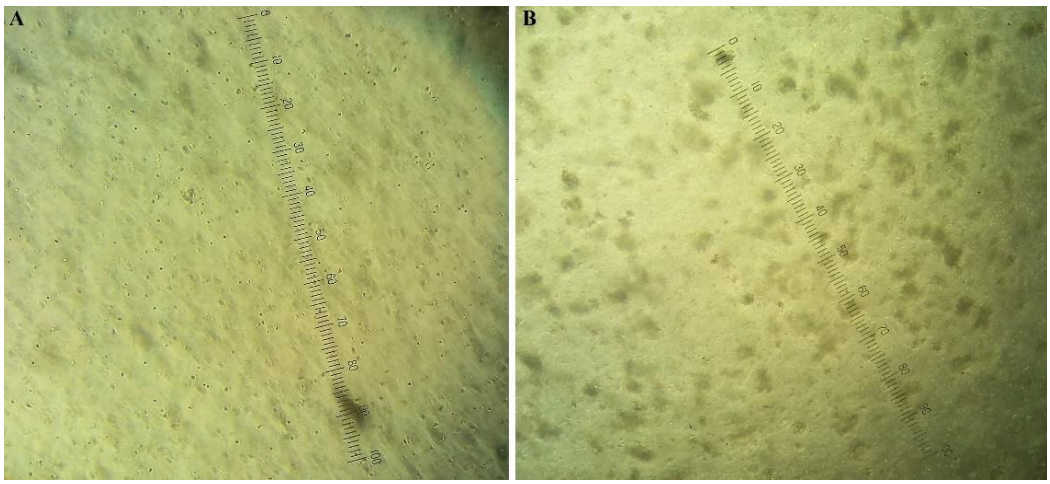


Figure 4.2) to check the health and the density of the yeast cells. The yeast was



ready for the assay once plenty of cell clusters were present.

Figure 4.2

a) yeast culture after 1 day of incubation (growth medium; 31 °C; 100 rpm), appearance of the first cells ; b) yeast culture after 3 days of incubation (growth medium; 31 °C; 100 rpm), evident cell clusters.

An eight-point standard E2 curve was built in the range 6.67 E-09 M to 2.11 E-12 M according to the concentrations recommended by the producer protocol[371]. In Figure 4.3 are shown the values and the trends of the cell density necessary to evaluate the growth factor (G) and the β-gal expression necessary to estimate the β-galactosidase activity (relative units) (U_S), respectively. The growth factor (G) and the β-galactosidase activity (U_S) evaluation allows the calculation of the induction ratio (I_R) as reported in Table 4.1. The evaluation of G and U_S will be systematic for the calculation of I_R in all YES assay trials. The induction ratio (I_R) was normalised and plotted in Figure 4.4.

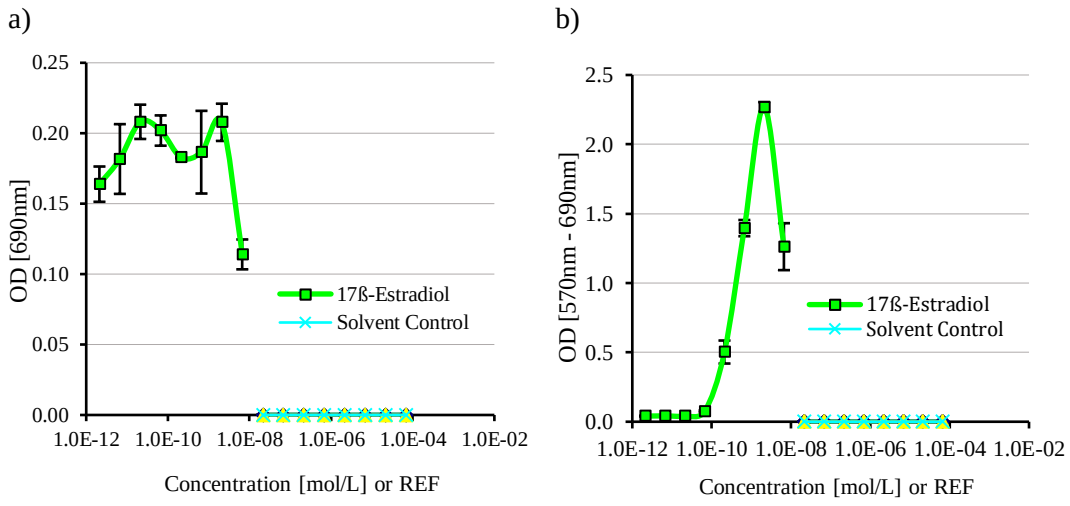


Figure 4.3

a) Cell density during the YES assay 17β-Estradiol calibration (range 6.67 E-09 M - 2.11 E-12 M); b) β-gal expression during the YES assay 17β-Estradiol calibration (range 6.67 E-09 M - 2.11 E-12 M).

Table 4.1

Calculated growth factor (G), β-galactosidase activity (U_S) and induction ratio (I_R) during the 17β-Estradiol calibration (range 6.67 E-09 M - 2.11 E-12 M).

Growth factor (G)			**β-galactosidase activity (U_S)**			**Induction Ratio (I_R)**		
REF or Conc.	17ß-Estradiol	Solvent Control	REF or Conc.	17ß-Estradiol	Solvent Control	REF or Conc.	17ß-Estradiol	Solvent Control
2.11E-12	0.82820	0.29752	2.11E-12	0.24696	0.29752	2.11E-12	1.15717	1.39407
6.67E-12	0.91843	0.24833	6.67E-12	0.23159	0.24833	6.67E-12	1.08513	1.16360
2.11E-11	1.05187	0.20721	2.11E-11	0.19529	0.20721	2.11E-11	0.91505	0.97094
6.67E-11	1.02053	0.20358	6.67E-11	0.37201	0.20358	6.67E-11	1.74311	0.95389
2.11E-10	0.92373	0.15110	2.11E-10	2.74979	0.15110	2.11E-10	12.8844	0.70802
6.67E-10	0.94294	0.19396	6.67E-10	7.48322	0.19396	6.67E-10	35.0633	0.90882
2.11E-09	1.05010	0.19813	2.11E-09	10.9202	0.19813	2.11E-09	51.1677	0.92837
6.67E-09	0.57623	0.23039	6.67E-09	11.0719	0.23039	6.67E-09	51.8785	1.07955
	$G \leq 0.5$						**$I_R \geq I_R 10$**	(≥ 6.217)

a) b)

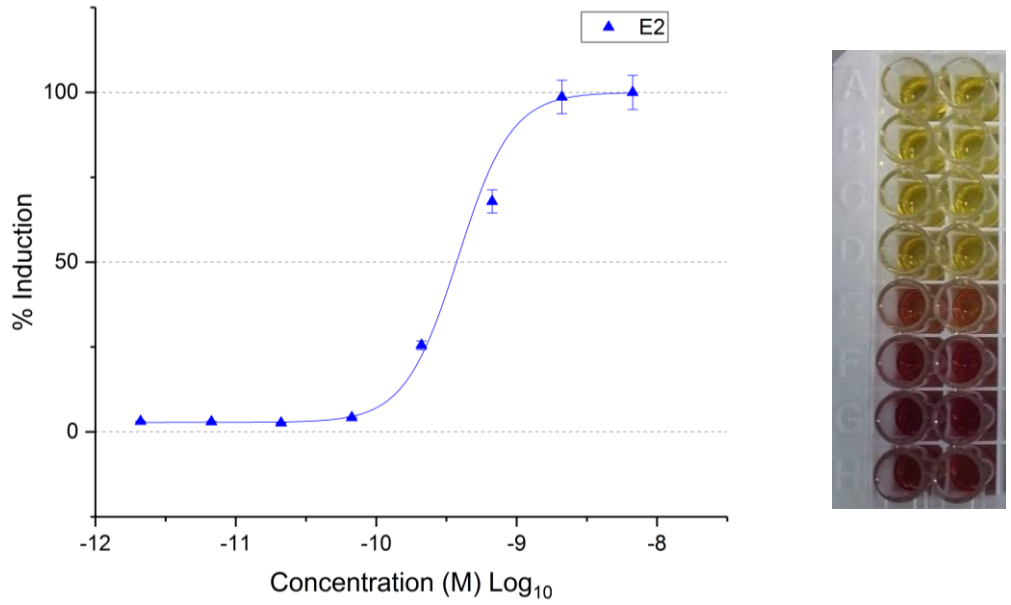


Figure 4.4

a) Experimental E2 data normalized vs dose-response curve (n=2; error bars expressed as percentage error 5%); b) plate showing the response of the YES screen to E2.

The experimental EC_{50} value of the 17-β estradiol was revealed to be in the sub-nanomolar range (EC_{50} = 4.47 E-10 M) and in the same order of magnitude to literature values adopting the same assay[370]. The limit of detection (LOD) was 4.01 E-11 M while the limit of quantification (LOQ) was 7.67 E-11 M.

The concentration intervals tested for each target in the assay were chosen according to the active concentration range of each compound evaluated in previous investigations[370]. The E2 equivalency factors (EEF) of the target analytes were calculated in the YES assay according to an eight-point standard E2 curve. EE2, BPA and BPAF proved to have agonistic endocrine activity showing all $I_R \geqslant 10\%$ of the difference between the maximum E2 and the solvent control in the agonist assay. Their dose-response curves (Figure 4.5) showed satisfactory coefficients of regression R^2 ($\geqslant 0.9$). All the compounds investigated dose-dependently increased the secretion of β-galactosidase in the yeast assay.

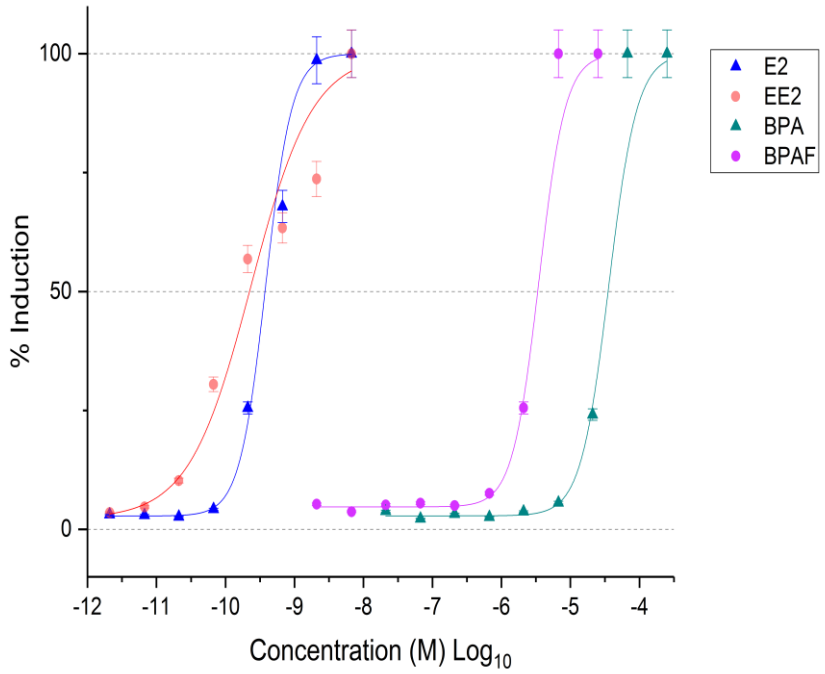


Figure 4.5

EE2, BPAF and BPA experimental data normalized vs fitted dose-response curves compared with the control E2 experimental data vs fitted dose-response curve (n=2; error bars expressed as percentage error 5%).

EC_{50} values of EE2, BPAF and BPA were compared with the EC_{50} value of E2: EE2 proved to be more potent (EC_{50} = 4.10 E-10 M) while BPAF (EC_{50} = 1.04 E-05 M) and BPA (EC_{50} = 2.02 E-05 M) were less potent. EC_{50} values followed the trends and the values found in the literature[369,370,373]. Experimental EC_{50} values are summarized and compared in Table 4.2. *EEFs* evaluated experimentally (Table 4.3) were comparable with those of other published studies[368,369 ,373,374].

Table 4.2

Summary of the experimental EC_{50} for E2, EE2, BPAF and BPA compared with some from the literature.

	E2	EE2	BPAF	BPA
EC_{50}	4.47 E-10 M	4.10 E-10 M	1.04 E-05 M	2.02 E-05 M
EC_{50}^{369}	2.10 E-10 M	1.80 E-10 M	-	2.00 E-05 M
EC_{50}^{373}	4.40 E-10 M	-	6.08 E-07 M	4.10 E-06 M

Table 4.3

Summary of the experimental estradiol equivalency factors (EEFs) for E2, EE2, BPAF and BPA compared with some from the literature.

	E2	EE2	BPAF	BPA
EEF	1	1.09	3.2 E-05	2.00 E-05
EEF[368]	1	1.20	-	1.00 E-05
EFF[373]	1	-	7.23 E-04	1.07 E-04

4.3.2 Monitoring of EC potency through drinkable water treatment processes (river-to-tap) in Hamilton

In Table 4.4 and Figure 4.6, the calculated estradiol equivalency quantities (cEEQ) are reported from the DGT-HPLC/MS analysis and the estradiol equivalency quantities (EEQ) from the YES assay during the river-to-tap water monitoring at the Waiora Treatment Plant that took place during October and November 2017.

Table 4.4

The calculated estradiol equivalency quantities (cEEQ) arose from the DGT-HPLC/MS analysis, the estradiol equivalency quantities (EEQ) and arose from the YES assay and their ratio (cEEQ/EEQ) during the river-to-tap water monitoring (October-November 2017) at the Waiora Treatment Plant (HWTP, Hamilton). Values expressed in g L^{-1}.

	Analytical cEEQ	**Bioassay EEQ**	**cEEQ/EEQ**
Waikato River Upstream	1.8E-09	1.8E-09	0.993
Waiora Sedimentation	2.1E-08	1.4E-07	0.144
Waiora After UV	2.2E-08	8.5E-08	0.263
Pukete Resevoir	1.8E-08	5.6E-08	0.331
Tap Water	4.7E-09	<LOD	-

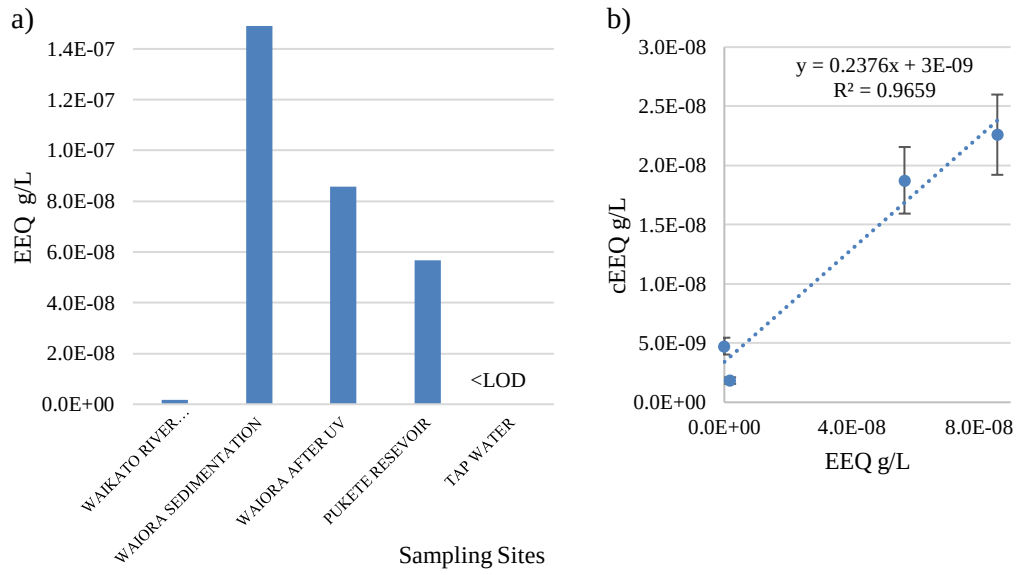


Figure 4.6

a) Waiora Treatment Plant (HWTP) river-to-tap water monitoring (October-November 2017, Hamilton). YES assay Estradiol equivalency quantities (EEQ) trend along the treatment process. Concentrations expressed in g L^{-1} (n=2);
b) Waiora Treatment Plant (HWTP) observed EEQs via YES assay vs calculated cEEQs via DGT-HPLC/MS expressed in in g L^{-1} (n=2).

The EEQs and the DGT-HPLC/MS agreed on the inefficacy of the primary sedimentation treatment that appeared to worsen the water quality, making the xenoestrogens more available to the water phase. The depletion of EE2 concentration during the treatment process corresponded to a decrease in estrogenic activity, until not detectable EEQ (<LOD) in the tap water. The divergence between cEEQ and EEQ in the tap water could be explained by the fact that the tap water was monitored with only 2 DGT probes for practicality, while all the other sampling sites were monitored with 6 DGTs. Thus, in the absence of a consistent number of replicate analyses the values are not reliable and can be used only as an indication.

Both cEEQs and EEQs evaluated during the water monitoring at the HWTP appeared to be in strong accordance (R^2 > 0.9). The regression factor demonstrates a small matrix effect, and high selectivity of the DGT with eluates that are enriched essentially by the analytes of interest. This assumption is reasonable because the HWTP has the highest water quality among all the sites analysed, with low interference by organic matter and particulates.

4.3.3 Monitoring of EC potency through the wastewater treatment processes (effluent-to-treated wastewater) in Hamilton

In Table 4.5 and Figure 4.7 are reported the calculated estradiol equivalency quantities (cEEQ) arising from the DGT-HPLC/MS analysis and the estradiol equivalency quantities (EEQ) determined from the YES assay during the effluent-to-treated wastewater monitoring at the Pukete Wastewater Treatment Plant that took place during October and November 2017.

Table 4.5

The calculated estradiol equivalency quantities (cEEQ) arose from the DGT-HPLC/MS analysis, the estradiol equivalency quantities (EEQ) and arose from the YES assay and their ratio (cEEQ/EEQ) during the effluent-to-treated wastewater monitoring (October-November 2017, Hamilton) at Pukete Wastewater Treatment Plant (PWTP). Values expressed in g L^{-1}.

	Analytical cEEQ	**Bioassay EEQ**	**cEEQ/EEQ**
Pukete Sedimentation	2.5E-08	5.4E-07	0.048
Pukete Secondary Clarifiers	3.5E-08	8.1E-06	0.004
Pukete After UV	1.7E-09	1.4E-09	1.171
Waikato Downstream	2.1E-08	4.2E-08	0.501

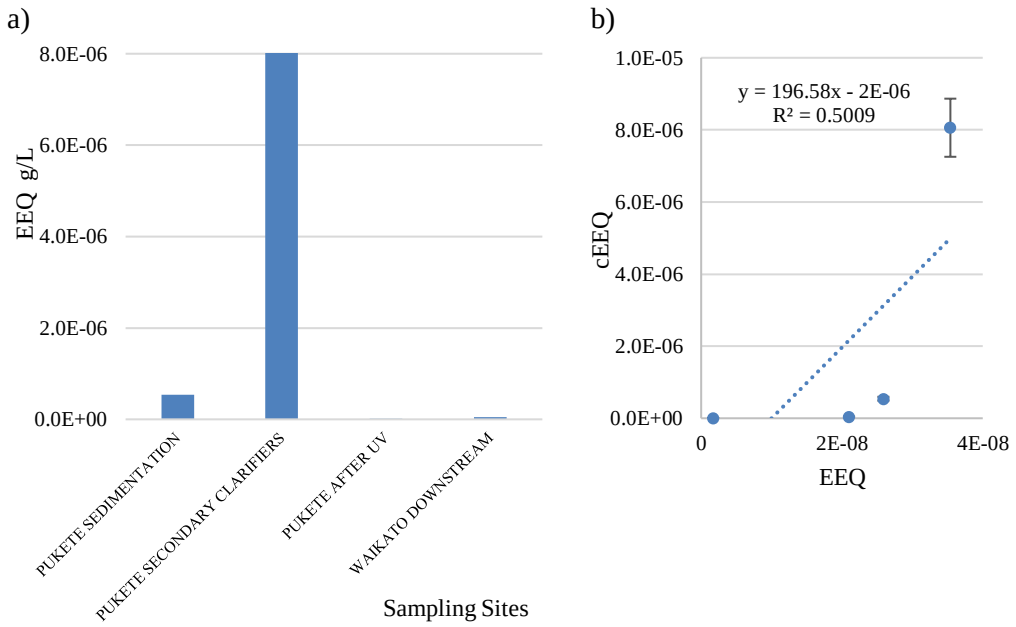


Figure 4.7

a) Pukete Wastewater Treatment Plant (PWTP) effluent-to-treated wastewater monitoring (October-November 2017, Hamilton). YES assay Estradiol equivalency quantities (EEQ) trend along the treatment process. Concentrations expressed in g L^{-1} (n=2). b) Pukete Wastewater Treatment Plant (PWTP) observed EEQs via YES assay vs calculated cEEQs via DGT-HPLC/MS expressed in in g L^{-1} (n=2).

cEEQs and EEQs values determined during the water monitoring at the WWTP appeared to be higher than those from the water monitoring at the HWTP, moreover upstream values were depleted compared to downstream. The EEQs agreed with the DGT-HPLC/MS dosages and confirmed the inefficacy of the primary sedimentation treatment that worsen the water quality making the estrogenic compounds more available in the water phase. The depletion of the concentration of all the analytes during the treatment processes corresponded in a progressively decreased estrogenic activity.

cEEQs and EEQs values determined during the wastewater monitoring at the WWTP appeared not to be in strong accordance (R^2 = 0.5) as at the HWTP. The low regression factor demonstrates a bigger matrix effect and low selectivity of the DGT. It is reasonable to assume the DGT eluates are enriched by other compounds with estrogenic activity due to the low water quality and the high

organic matter and particulates. The difference between EEQ and cEEQ values could also be enhanced by synergistic coaction of the target compounds[55] with estrogenic compounds not investigated during the HPLC/MS analysis. The highest divergence between cEEQ and EEQ values is notable at the beginning treatment stages (sedimentation and secondary clarification) that have the lowest water quality. Fine sediments and particulates are particularly present at these stages. They not only work as sinks, accumulating pollutants on their surface[13], but can interfere with the efficiency of sampling of the DGT settling on its protective membrane.

4.3.4 Monitoring of EC potency along the Waikato River (source-to-outfall)

In Table 4.6 and Figure 4.8 are reported the calculated estradiol equivalency quantities (cEEQ) calculated from the DGT-HPLC/MS analysis and the estradiol equivalency quantities (EEQ) calculated from the YES assay during the Waikato River source-to-outfall monitoring that took place during January and February 2018.

Table 4.6

The calculated estradiol equivalency quantities (cEEQ) from the DGT-HPLC/MS analysis and the estradiol equivalency quantities (EEQ) from the YES assay during the source-to-outfall monitoring (January-February 2018, New Zealand) along the Waikato River. Values expressed in g L^{-1}.

	Analytical cEEQ	**Bioassay EEQ**	**cEEQ/EEQ**
Lake Taupo Upstream	4.8E-09	1.1E-09	4.583
Taupo Waikato Downstream	7.8E-09	8.8E-08	0.089
Cambridge Waikato Downstream	9.7E-09	2.6E-08	0.374
Hamilton Waikato Upstream	7.2E-09	N.D.[a]	-
Hamilton Waikato Downstream	1.0E-08	2.8E-06	0.004
Huntly Waikato Upstream	7.5E-09	N.D.[a]	-
Huntly Waikato Downstream	6.8E-09	3.1E-06	0.002
Tuakau	7.5E-09	1.3E-08	0.008

[a]N.D. not reliable data

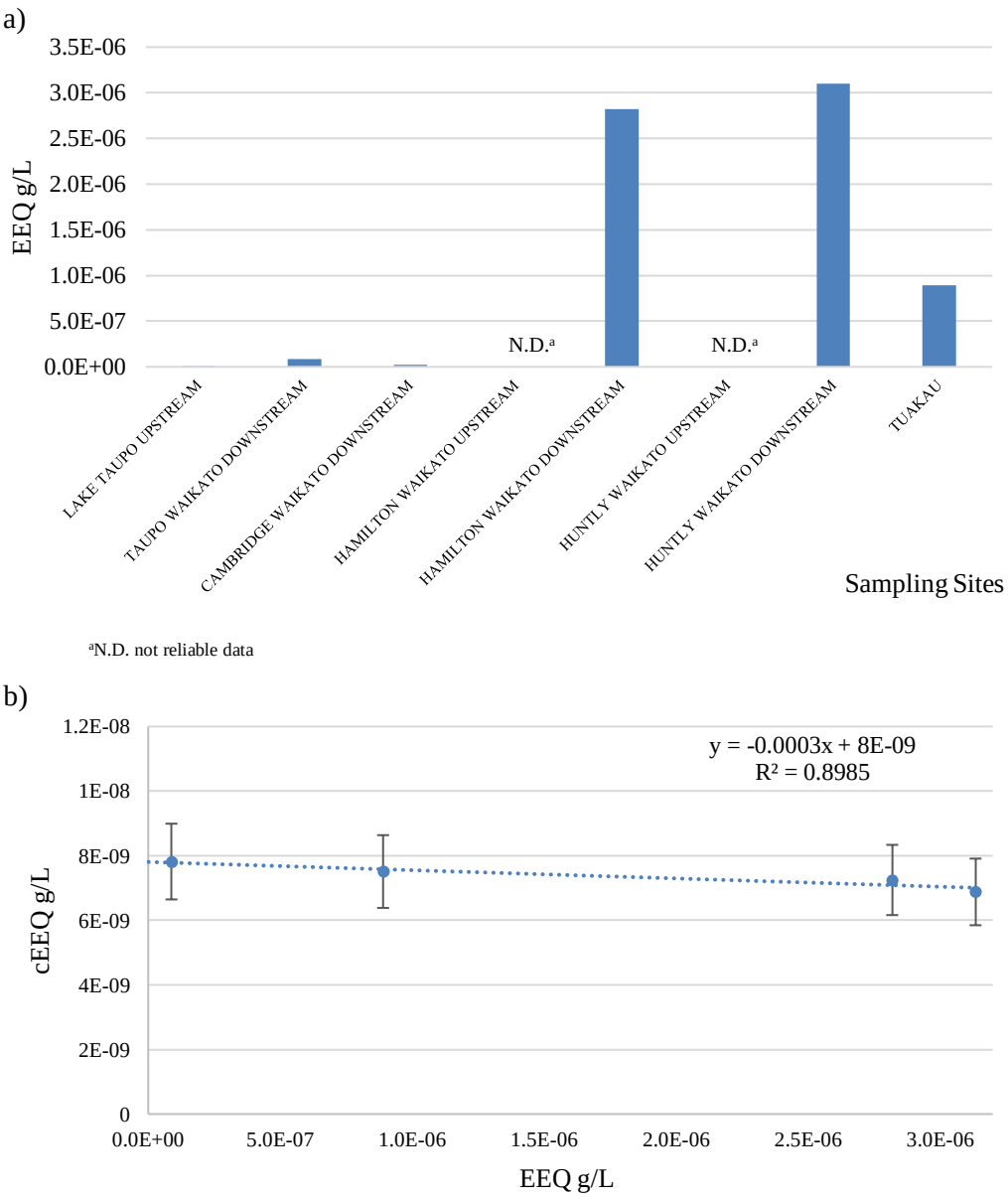


Figure 4.8

a) Waikato River source-to-outfall monitoring (January-February 2018, New Zealand). YES assay Estradiol equivalency quantities (EEQ) trend along the sampling sites. Concentrations expressed in g L^{-1} (n=2); b) Waikato River source-to-outfall monitoring observed EEQs via YES assay vs calculated cEEQs via DGT-HPLC/MS expressed in in g L^{-1} (n=2).

Higher estrogenic potencies were detected downstream at all sites monitored along the Waikato River. EEQs increased from the source to the outfall and the highest values were detected in Hamilton and Huntley after the outlets of WWTPs that serve the main councils in the area. Tuakau still showed a consistent estrogenic activity but there was a notable dilution. The peak activity recorded downstream in Hamilton could also be connected to the extensive farming of the area. As reported by Tremblay et al. (2011)[346], estrogenic steroid hormones have been detected in groundwater and stream waters of intensively farmed dairy catchments. Their total estrogenic activity was elevated in dairy shed effluents that at times exceeded suggested guideline values for protection of freshwater fish. The theoretical predicted EEQs (cEEQs) differed from the experimental EEQs at some sites, however this divergence of values was smaller than the one at the WWTP ($R^2 = 0.89$). The regression factor was intermediate between the R^2 of the HWTP and WWTP ($R^2_{WWTP} < R^2_{Waikato\ water} < R^2_{HWTP}$); the difference of the values could depend again on the presence of other ECs in the DGT eluate that could act synergistically with the species of interest[55]. Organic matter and fine sediments may be again the reason of the low DGT elution selectivity. They can accumulate other EC species[13] with similar affinity for the DGT elution solvents compromising the total estradiol equivalency quantities (EEQ) evaluated with the YES assay. However, surface waters are well mixed, due to the reasonable and constant water flow, that avoids the accumulation of material on the DGT membrane. Thus, EEQs and cEEQs do not differ to the same degree like in the WWTP.

The estradiol equivalency quantities (EEQs) of selected ECs in the Waikato River[1] were evaluated by the Regional Council in the technical Report 2013/55 drafted in November 2013 as well. The monitoring was carried out adopting an active sampling, extracting 10 L of aqueous sample with SPE cartridges to determine the enrichment factor (EF) and LOD prior to the MELN bioassay, that exploits reporter cell lines[372].

On the contrary, in this study the estradiol equivalency quantities (EEQs) of selected EDCs in the Waikato River was carried out adopting DGT passive sampling, assuming 18 days of deployment to determine the enrichment factor (EF) and LOD prior to the YES assay, that exploits a recombinant brew yeast[371]. The limits of detection (LODs) of the different assays are compared in Table 4.7. The MELN assay appears to be more sensitive compared to the YES assay, however both assays ensure quantification in the ng L^{-1} range. T

he EEQs detected by the Regional Council during November 2013 are compared with those evaluated by this investigation in the same locations during January and February 2018. The values are displayed both in Table 4.8 and Figure 4.9.

Table 4.7

Comparison of MELN assay LODs and YES assay LOD adopted to evaluate Estradiol equivalency quantities (EEQs) of the Waikato River in November 2013 and January-February 2018 respectively.

	MELN LOD[a] g/L	YES LOD g/L
17β-Estradiol	7.20E-11	1.04E-09

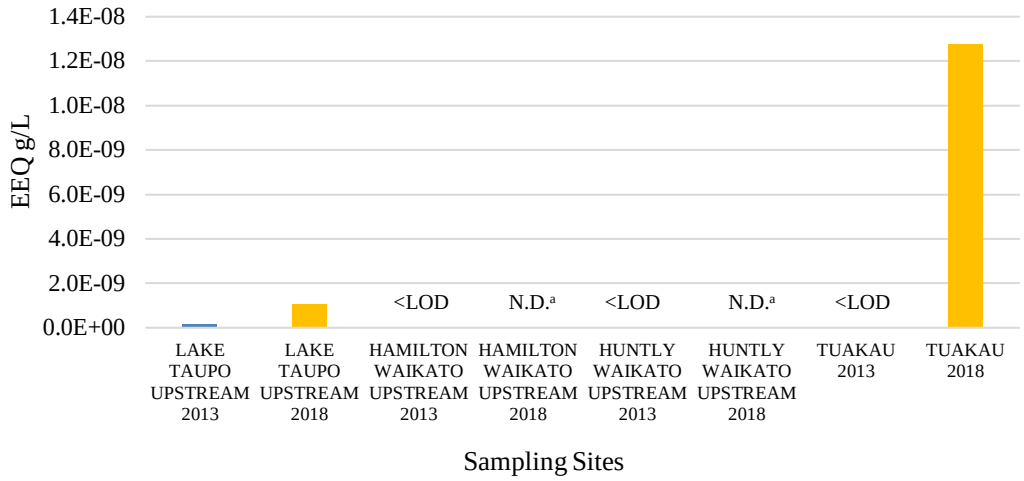
[a] limit of detection determined adopting the enrichment factor (EF) arose from 10 L of SPE extracted aqueous sample

Table 4.8

Estradiol equivalency quantities (EEQs) evaluated at the same sampling points along the Waikato River in in November 2013 and January-February 2018 respectively.

	Lake Taupo Upstream 2013	Lake Taupo Upstream 2018	Hamilton Waikato Upstream 2013	Hamilton Waikato Upstream 2018	Huntly Waikato Upstream 2013	Huntly Waikato Upstream 2018	Tuakau 2013	Tuakau 2018
Bioassay Total EEQ g/L	1.3E-10	1.0E-09	<LOD	N.D.[a]	<LOD	N.D.[a]	<LOD	1.3E-08

[a]N.D. not reliable data



[a]N.D. not reliable data

Figure 4.9

Comparison of the Estradiol equivalency quantities (EEQs) recorded at the same sampling points along the Waikato River in in November 2013 and January-February 2018 respectively.

EEQs values from the monitoring of Hamilton Upstream and Huntly Upstream sites cannot be compared due to not reliable data obtained from the YES assay, while Taupo upstream and at Tuakau showed increased EEQs from 2013 to 2018. However, more investigation is required to ascribe these differences to the different measurement methodologies adopted, to a seasonal effect or to a real deterioration of the water quality.

4.4 Summary chapter IV

DGTs-HPLC/MS dosages were coupled with a biomarker-based assay to quantify the total estrogenic potency of the matrices analysed and thus, evaluate their impact on the aquatic ecosystem. YES (Yeast Estrogen Screen) assay was implemented among all the assays available due to its robustness, test speed and relatively low cost. This test exploits *Saccharomyces cerevisiae* yeast strains recombined in order to identify compounds that are human estrogen receptor (hER) active.

DGT eluates were treated in order to be compatible with the YES assay. To suit the YES assay requirements, 0.5 mL of MeOH DGT eluate and 0.5 mL of EtOH DGT eluate of each couple of AC hydrogels were combined, dried (room temperature, 6 hours) and dissolved in 1 mL of sterile H_2O-DMSO (1%). The enrichment factor EF necessary for the data analysis of the assay was calculated adopting the ratio between the concentration of the analyte computed from the HPLC (cHPLC) calibration curves and the concentration computed from the DGT equation assuming 18 days of DGT deployment.

Single-dose tests in duplicate of control, solvent and samples were conducted to estimate the relative estrogenic activities. E2 was employed as control. An eight-point standard E2 curve was built in the range 6.67 E-09 M to 2.11 E-12 M. The experimental EC_{50} value of the 17-β estradiol revealed to be in the sub-nanomolar range (EC_{50} = 4.47 E-10 M) in accordance with the literature[370]. The limit of detection (LOD) was 4.01 E-11 M while the limit of quantification (LOQ) was 7.67 E-11 M.

EE2, BPA and BPAF were assayed in concentration ranges suggested by the literature[370]. All EDCs proved to have agonistic endocrine activity ($I_R \geqslant 10\%$). EE2 proved to be more potent (EC_{50} = 4.10 E-10 M) then E2, while BPAF (EC_{50} = 1.04 E-05 M) and BPA (EC_{50} = 2.02 E-05 M) less potent in agreement with the literature[370,373]. The experimental *EEFs* (EEF_{EE2}= 1.09; EEF_{BPAF} =3.2 E-05; EEF_{BPA} = 2.0 E-05) were comparable with those published[368,374,373].

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the river-to-tap water monitoring at the HWTP that took place in October and November 2017 confirmed the inefficacy of the primary sedimentation treatment that again appeared to worsen the water quality. The depletion of EE2 concentration during the treatment process corresponded to a decrease in estrogenic activity, until not detectable EEQ (<LOD) in the tap water.

cEEQs and EEQs values of the water monitoring at the HWTP appeared in strong accordance ($R^2 > 0.9$) due to the high quality of the water of the HWTP. The limited presence of organic matter and particulates corresponded in a low small matrix effect and to a high selectivity of the DGT with eluates enriched by the analytes of interest.

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the WWTP that took place in October and November 2017 appeared to be higher than those from the water monitoring at the HWTP, moreover upstream values were depleted compared to downstream. The EEQs confirmed the inefficacy of the primary sedimentation treatment. The depletion of the concentration of all the analytes during the treatment processes corresponded in a progressively decreased estrogenic activity.

cEEQs and EEQs values of the WWTP monitoring appeared not to be in strong accordance ($R^2 < 0.9$) as at the HWTP. DGT eluates were enriched by other EC compounds due to the low water quality and the bigger matrix effect caused by the high organic matter and particulates. The divergence of cEEQs and EEQs values was higher at the beginning treatment stages (sedimentation and secondary clarification) that have the lowest water quality.

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the Waikato River source-to-outfall monitoring that took place in January and February 2018 showed higher estrogenic potencies downstream at all sites monitored along the Waikato River. EEQs increased from the source to the outfall and the highest values were detected in Hamilton and Huntley after the outlets of WWTPs. The peak activity downstream in Hamilton could also be connected to the effluents from the intense farming of the area.

Tuakau showed as well a consistent estrogenic activity. cEEQs differed from EEQs at some sites, however this divergence was smaller than the one at the WWTP ($R^2 = 0.89$).

Comparison of EEQ river values with a previous study in 2013 showed increased EEQs from 2013 to 2018 at Taupo upstream and at Tuakau. However, more analysis is required to attest a deterioration of the water quality.

Overall, the YES assay hyphenation to the DGT-HPLT/MS proved to be a reliable total estrogenic potency evaluation with excellent potential for use in time integrated passive sampling. The assay attested the presence of other compounds with estrogenic activity in the DGT eluates suggesting this type of DGT could be suitable for the sampling and accumulation of other analytes.

5 CONCLUSION AND DISCUSSION

5.1 Expanding the repertoire of diffusive gradients in thin films in situ measurements of endocrine disruptors chemicals in aquatic systems

DGTs with agarose diffusive gel and agarose-activated charcoal (AC) as a binding gel were chosen for the sampling of the BPA, BPAF, βE2 and EE2 known to be potent and persistent ECs. Several physical-chemical and chemical analysis approaches were conducted to attest the suitability of the hydrogels selected.

Diffusive and binding gels reached their maximum swelling ratio of 70% and 40%, respectively, after 1h of immersion.

EDX spectra of freeze-dried platinum-coated agarose (1.5%) and agarose (1.5%) – AC (1%) confirmed the consistency of the composite hydrogel that was enriched approximately by 10% in carbon compared to the pure agarose hydrogel throughout the sample.

The ratio water-carbon proved to be constant along the two-dimensions for both diffusive and binding gel. Thus, the diffusive property of the pure agarose, and the binding ability of each resin hydrogel disc were consistent.

The binding gel showed enhanced thermal stability (66% weight loss compared to the 90% weight loss of the diffusive gel, in the range 170-340ºC), making it particularly suitable in the case of long environmental deployments where the temperature conditions could vary dynamically over short timescales.

Optimised conformations of the target molecules (BPA, BPAF, βE2 and EE2) were calculated through Gaussian simulation (DFT method B3LYP, basis set 6-31G). The approximated lengths and width were respectively 10.18 Å and 4.36 Å for the BPA; 10.23 Å and 4.83 Å BPAF; 12.32 Å and 5.11 Å βE2; 12.32 Å and 6.77 Å and EE2.

FE-SEM morphological characterisations of freeze-dried, platinum-coated samples proved that of the diffusive gel has an average pore number of 22 ± 5 per μm^2 with a weighted pore diameter of 30 nm, while the binding gel had an average pore number of 13 ± 2 per μm^2 with a weighted pore diameter of 45 nm. These values confirmed these gels are suitable for the detection of organic molecules with a bigger steric hindrance such as polycarbonatic monomers (BPA, BPAF) and estrogens (E2, EE2).

Adsorption of the targets by the materials employed should be avoided during DGT deployment. ABS plastic probes, agarose diffusive gels of 2.51 cm of diameter and 0.54 mm of thickness and hydrophilic PTFE membranes of 2.51 cm of diameter showed a relatively small adsorption < 3% (excluding the adsorption of BPAF by PTFE membrane < 7%) of BPA, BPAF, E2 and EE2 proving suitable for this study.

Kinetic studies showed the uptake of BPA, BPAF, E2, EE2 by the binding phase was relatively fast with a percentage of 98%, 98%, 97%, 95% of sorption and average binding rates of 2.05, 1.20, 2.48, 3.55 ng cm^{-2} min^{-1} over the first 60 min, respectively.

BPA and BPAF were recovered from the binding gel in 10 mL of MeOH for 24 h while E2 and EE2 were recovered in 10 mL of EtOH for 24 h, as they proved to be the best solvents, volumes and times of elution with percentage recoveries at 25°C of 103 ± 17, 79 ±18, 96 ± 31, 107 ± 11, respectively.

The effective diffusion coefficients of the E2, EE2, BPA and BPAF evaluated from the DGT devices were 3.97×10^{-6}, 3.19×10^{-6}, 3.09×10^{-6}, 3.55×10^{-6} at 25 °C with average binding rates of 2.05, 1.20, 2.48, 3.55 ng cm^{-2} min^{-1} over the first 60 min.

An HPLC-MS method was successfully optimised to detect and quantify the compounds of interest. A methanol (MeOH, NH_4OH 0.06 M)/water (H_2O, NH_4OH 0.06 M) fast gradient elution program of 15 min was chosen to elute the analytes carrying the separation at ambient temperature to avoid their degradation. Tandem MS parameters were optimized for each compound. The negative mode

was chosen to perform the fragmentation in the mass spectrometer. The deprotonated molecules of the analytes $[M-H]^-$ were used as precursor ions and a transition ion was selected and monitored for the identification of each compound of interest.

DGT blank concentrations along with LOD (1.00, 0.61, 1.31, 0.20 µg/L for BPA, BPAF, E2, EE2 respectively) and LOQ (3.34, 2.03, 4.39, 0.68 µg/L for BPA, BPAF, E2, EE2 respectively) were calculated for each compound assuming 24 h as deployment time and 25 °C of temperature with a 0.54 mm thick diffusive gel.
The investigation demonstrated DGT passive sampling is a valid alternative to active sampling for the monitoring of organic pollutants. DGT was successfully deployed for the trace detection of emergent EDCs such as bisphenol A (BPA) and its analogue bisphenol AF (BPAF), the natural estrogen estradiol (E2) and its synthetic analogue ethinylestradiol (EE2).

Preliminary environmental trials of the DGT-HPLC/MS methodology were conducted along the Waikato River at the same sampling sites monitored in November 2013 by a validated and published SPE-HRGC/MS method[1]. The comparison of the results demonstrated the novel DGT technique is suitable for the environmental detection of EDCs trace concentrations (ng L^{-1}). DGT-HPLC/MS resulted to have a convenient sample preparation, was easy to deploy and had low cost compared to the validated active sampling technique adopted for the monitoring of the EDCs.

However, other investigations are still required to fully understand the potential of the DGT, thus developed. This approach should be tested to assess if it is suitable for deployment in sediments and for the detection of other BPA analogues or other estrogens and their metabolites. Other solvents or mixtures and other times of elution should be tested to recover simultaneously all the targets absorbed by the agarose (1.5%) – AC (1%) binding phase, reducing in this way the sample preparation and the analysis time. DGT was shown to be a very robust sampling methodology despite changes in temperature, pH, ionic strength (IS) and flow[241].
The ionic strength (IS) influence was not assessed considering that fresh waters were investigated. However, IS influence should be evaluated adopting the DTG

probes in other type of environments such as salty waters where salting in/out phenomena can occur. DGT approach had the advantage of being cheap compared to other passive sampling such as POCIS thanks to the quantity and type of binding material required[375], moreover the reduced probe size allowed an easy deployment and retrieval even in places not very accessible.

5.2 In situ monitoring of endocrine disruptors concentration in fresh water and through drinking and wastewater treatment

The environmental monitoring aimed to assess the environmental exposure to the most potent EDCs among all the classes, in particular hormones[4] and plastics[22]. The efficiency of removal of the selected ECDs was monitored during the drinkable water treatment processes (river-to-tap) and wastewater water treatment processes (effluent-to-treated) in Hamilton as well the water quality of the Waikato River at particular interest locations throughout its length (source-to-outfall).

Multiple DGT probes were deployed simultaneously at each site monitored. DGT sampling rates were reduced compared to other passive sampling like POCIS, thus longer exposure times (15 – 18 days) were required to enhance the accumulation of the targets and so increase the sensitivity of the method. Combined elution of 2 probes per analysis successfully increased the S/N ratio and decreased the variance during the HPLC runs.

The monitoring of the HWTP river-to-tap water and of the PWTP effluent-to-treated wastewater took place between October and November 2017. During the monitoring no significant rainstorms took place. The monitoring of water quality of the Waikato River source-to-outfall took place between January and February 2018. However, rainstorms, prior the retrieval time, caused significant flood, and diluted the pollutants of interest in the environment.

DGT-HPLC/MS quantification collected during the HWTP monitoring resulted in line with those of other monitoring of surface freshwaters that serve urban areas around the world[19,355,356,357]. EDCs concentrations fluctuated around the same

value, along the different treatment steps. The trends highlighted that primary treatments are not suitable for the removal of bisphenolic plastics and estrogens because they appear to worsen the water quality making these pollutants more available. It was not possible to appreciate a consistent breakdown of the compounds even after GAC filtration. EE2 and BPA resulted the most concentrated pollutants and their trend was similar to that of other published monitoring of surface waters[358]. EE2 concentration fluctuated in a greater degree compared to BPA concentration that appears stable along the different treatments.

EDCs concentrations recorded during the PWTP monitoring were comparable with those published in other monitoring studies of wastewaters[363]. Inflow concentrations resulted higher from the outflow ones. Similarly to the HWTP, the primary treatment resulted ineffective in the removal of the selected pollutants. BPA-EE2 trend followed the one evaluated during the HWTP monitoring. EE2 concentration changed in a greater degree while BPA concentration resulted constant along the different treatments.

The outcomes suggested that the actual technologies available at the treatment plants do not ensure efficient removal of the compounds monitored.

The Waikato River monitoring was carried out between January and February 2018. EDCs concentrations resulted in accordance with those of other studies of surface freshwaters that serve urban areas[19,355,356,357] and previous Waikato River monitoring[1]. All targets resulted more concentrated downstream at all sites monitored. EE2 proved to be the most concentrated contributing more to the estrogenic activity. Moving from Taupo to Tuakau the water quality worsened in a moderate degree. The most polluted sites resulted Hamilton downstream and Huntley downstream. The high estrogenic concentration downstream in Hamilton could also be connected to the effluents produced by the intense farming activity. Like at the HWTP and WWTP, EE2 and BPA contributed most to the pollution and EE2 values varied in a greater degree compared to PBA.

The EDCs recorded in November 2017 and February 2018 during the monitoring of the Waikato River at Hamilton upstream and downstream sites were compared. All EDCs resulted diluted from November 2017 to February 2018 both Upstream

and in particular Downstream. The correlation of the data showed this dilution was attributable to the weather conditions at both sites ($R^2 > 0.9$), in fact during the DTGs exposure consistent rainstorms caused significant flooding. BPA and EE2 had the highest concentrations and resulted highly correlated at both sampling sites ($R^2 > 0.9$), with a ratio ≈ 0.3.

The data from the monitoring of the Waikato River via DGT-HPLC/MS were compared with those from a previous monitoring of the same sites adopting and SPE-HRGC/MS method[1]. E2 concentration resulted <LOD at all sites in both monitoring. Increased concentrations of BPA and EE2 at all sites from the 2013 to the 2018 appeared to slightly worsen the water quality.

It appeared from the investigation that DGT technique was not sufficiently evaluated to recommend it for regular EC monitoring. To validate the technique a direct comparison of DGT passive sampling and active sampling should be done in a further systematic monitoring. The tandem quantifications would allow to evaluate eventual discrepancy in quantification at the different stages of the water treatment and would provide a clear water quality assurance for the Waikato River and local communities. Moreover, DGTs should be deployed for different time frames, in waters with different qualities (clean-river-wastewater effluents) along with a validated active method to attest the consistency of the time integrated sampling despite the matrix effect.

5.3 In vitro bioassay analysis of endocrine disruptors potency in fresh water and through drinking and wastewater treatment processes

The DGTs-HPLC/MS analytical dosages were coupled with biomarker-based surveys to quantify the total estrogenic potency of the matrices analysed and thus, evaluate their impact on the aquatic ecosystem.

The YES (Yeast Estrogen Screen) was implemented among all the assays available due to its robustness, responsiveness and relatively low cost. This test exploits *Saccharomyces cerevisiae* yeast strains recombined in order to identify compounds that are human estrogen receptor (hER) active[371].

E2 was adopted as control and an eight-point standard E2 curve was built in the range 6.67 E-09 M to 2.11 E-12 M. The experimental EC_{50} value of the control resulted to be in the sub-nanomolar range (EC_{50} = 4.47 E-10 M) and in the same order of magnitude to literature values[370]. The limit of detection (LOD) of the method was 4.01 E-11 M while the limit of quantification (LOQ) 7.67 E-11 M.

EE2, BPA and BPAF were tested in the same concentration intervals investigated in a previous study[370]. All targets demonstrated agonistic endocrine activity ($I_R \geqslant 10\%$). The EE2 EC_{50} was more potent (EC_{50} = 4.10 E-10 M) than E2 while BPAF (EC_{50} = 1.04 E-05 M) and BPA (EC_{50} = 2.02 E-05 M) were less potent. EC_{50} agreed with those of the literature[370,373]. The experimental EEFs (EEF_{EE2}= 1.09; EEF_{BPAF} =3.2 E-05; EEF_{BPA} = 2.0 E-05) were comparable with other published [368,374,373]. All dose-response showed satisfactory coefficients of regression (R^2> 0.9).

DGT eluents were treated in order to be compatible with the YES assay. In particular, 0.5 mL of MeOH DGT eluted and 0.5 mL of EtOH DGT eluted of each couple of AC hydrogels were combined, dried (room temperature, 6 hours) and dissolved in 1 mL of sterile H_2O-DMSO (1%). The investigation had the innovation of hyphenating a passive sampling with this bioassay. To allowed the the data analysis of the YES assay it was assumed as enrichment factor (EF) the ratio between the concentration of the analyte computed from the HPLC (C_{HPLC}) calibration curves and the concentration computed from the DGT equation, assuming 18 days of deployment. Single-dose tests in duplicate of control, solvent and samples were conducted to estimate the relative estrogenic activities.

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the monitoring of the HWTP done between October and November 2017 confirmed the inefficacy of the primary sedimentation treatment that worsened the water quality. The decreased estrogenic activity during the treatment process was influenced by the depletion of EE2 concentration, until not detectable EEQ (<LOD) in the tap water. cEEQs and EEQs values resulted in strong accordance (R^2 > 0.9) due to the high quality of the water. The limited

organic matter and particulate corresponded in a small matrix effect and to a high selectivity of the DGT with eluted enriched essentially by the analytes of interest.

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the monitoring of the WWTP done in October and November 2017 appeared to be higher than those from the monitoring of the HWTP, moreover downstream values were depleted compared to upstream. EEQs confirmed the inefficacy of the primary sedimentation treatment. The depletion of all EDCs concentration during the treatment processes corresponded in a progressively decreased estrogenic activity. cEEQs and EEQs values of the WWTP monitoring appeared not to be in strong accordance ($R^2 < 0.9$) as at the HWTP. cEEQ-EEQ discrepancy was due to the low water quality, the high organic matter and particular that caused a big matrix effect, and DGT eluted enriched by other EDCs compounds. This divergence resulted higher at the beginning treatment stages (sedimentation and secondary clarification) that had the lowest water quality.

cEEQ calculated from DGT-HPLC/MS analysis and EEQ calculated from the YES assay during the monitoring of the Waikato River in January and February 2018 showed higher estrogenic potencies downstream at all sites along the river. EEQs increased from the source to the outfall with pick activities in Hamilton and Huntley after the outlets of WWTPs. The pick estrogenic activity downstream in Hamilton could also be connected to the effluents produced by the extensive farming of the area. Tuakau showed as well a consistent estrogenic activity. cEEQs differed from EEQs at some sites, however the divergence was smaller than the one at the WWTP ($R^2 = 0.89$).

EEQs of selected EDCs in the Waikato River were evaluated by the Regional Council during a survey in November 2013[1]. The method employed consisted in an active sampling and SPE extraction of 10 L of aqueous sample, prior MELN assay, that exploits reporter cell lines[372]. EEQs values of both monitoring resulted in strong accordance at all sites investigated, Taupo upstream and at Tuakau showed increased EEQs from the 2013 to the 2018. However, more analysis are required to attest a deterioration of the water quality.

YES assay hyphenation to the DGT-HPLT/MS was convenient because it provided a correct total estrogenic potency evaluation.

However, more trials are required to validate the DGT-YES hyphenation. DGT eluates and samples concentrated though routine SPE should be assayed simultaneously to attest the assumption employed during the DGT-YES hyphenation are correct and that the value of estrogenic potency evaluated via DGT-YES are consistent with those evaluated with the SPE-YES.

The bioassay revealed the presence of other compounds that contributed to the estrogenic activity in the DGT eluates, especially at the most polluted sampling sites. This outcome suggested this type of DGT could be suitable for the sampling and accumulation of other analytes that could be investigated through HPLC-MS analysis in future studies.

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