BIOAVAILABILITY OF ORGANIC CONTAMINANTS IN RIVERS

A thesis submitted for the degree of Doctor of Philosophy

in

Environmental Sciences

by

Cyril Oziegbe Onogbosele

Department of Life Sciences
Brunel University London

April 2015

ABSTRACT

In rivers, association of organic contaminants with dissolved organic carbon may limit freely dissolved or bioavailable fractions and toxicity of organic contaminants. Consequently, assessment of toxicity of organic contaminants on the basis of their total chemical concentrations may lead to overestimation of risks to organic contaminants. Therefore, to achieve reliable and accurate risks assessment for organic contaminants, determination of bioavailability is important. The influence of humic acid on the bioavailability of organic contaminants in rivers was studied, using three chemicals with different properties as model contaminants, which at the start of the study were detected in wastewater effluents. It was hypothesized that in the presence of dissolved organic carbon, a fraction of the total concentration of an organic contaminant would not be bioavailable in river water. Therefore, the aim of the study was to determine bioavailability and its impact on toxicity. Bioavailability in the presence of humic acid was determined chemically and using a yeast estrogen screen assay. The chemical method comprised solid-phase extraction and liquid chromatography-mass spectrometry to determine freely dissolved and the fraction of the chemicals associated with dissolved organic carbon. The results indicated increased binding to dissolved organic carbon with the hydrophobicity of the test compounds except for perfluorooctane sulfonate. The dissolved organic carbon-water partition coefficient for ethinylestradiol was determined to be Log K_{DOC} 2.36. Log K_{DOC} values of 4.15 and 4.41 at 10 and 100 mg/L humic acid, respectively, were derived for hexabromocyclododecane indicating greater binding than ethinylestradiol due to the more hydrophobic character. The yeast estrogen screen was used as a biological method to measure the effect of humic acid on the bioavailability of ethinylestradiol and a more hydrophobic compound, dichlorodiphenyltrichloroethane. Results of the yeast estrogen screen indicated that the presence of humic acid had no effect on bioavailability of either of the chemicals.

ACKNOWLEDGEMENTS

I am grateful to my principal supervisor, Dr Mark Scrimshaw for the guidance, attention, support and encouragement extended to me during the PhD study. I also appreciate the effort he expended in reading and criticising the thesis manuscript constructively. I sincerely thank my second supervisor, Prof Susan Jobling for supporting me and sharing valuable ideas during the study.

I received assistance from Nicola Beresford in the Institute of Environment, Health and Societies, Brunel University London, during the use of yeast estrogen screen (YES) in the study. I thank her for sharing time and expertise with me. I wish to also express my thanks to Wuese Ahua for his contribution to the study on YES assay with DDT and humic acid. He expanded my original work on YES assay with DDT and humic acid. Some of the data on YES assay with DDT including measurements of the concentration of DDT with GC-MS were generated from his work.

I owe a debt of gratitude to my employer, Ambrose Alli University, Ekpoma, Nigeria, for approving my PhD study for financial sponsorship by Tertiary Education Trust Fund (TETFund), Abuja, Nigeria. I thankfully acknowledge funding from TETFund which supported the PhD study to a large extent.

I appreciate the love, care and support I received from my mother, Janet Onogbosele, which were sources of motivation for me during the study. With gratitude, I also place on record the moral and financial support I received from Mr and Mrs Peter Aikhuomobhogbe, Mr and Mrs Donatus Onogbosele, Rev Fr Jude Onogbosele, other members of Onogbosele's family and my special friend, Barr Emmanuella Idele, when I had a deficit in fund during the study. The support and encouragement extended to me by Mr Ayemhenre Esekheigbe and Prof Simeon Osuide also assisted me in completing the study. I thank them for their kind assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA-EQS	Annual average environmental quality standard
ANOVA	Analysis of variance
ASS	Activated sludge site
BCF	Bioconcentration factor
BFR	Brominated flame retardant
CAS	Chemical Abstracts Service
CF_2	Carbon-fluorine
CIP	Chemicals Investigation Programme
CPRG	Chlorophenol red-β-D-galactopyranoside
cps	Count per second
CUR	Curtain gas
d	Day
DCM	Dissolved and colloidal matter
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DP	Declustering potential
dph	Days post-hatch
E1	Estrone
E2	17β-estradiol
EC_{50}	Median effective concentration
EE2	17α-ethinylestradiol
EEQ	Estradiol equivalent
EI	Electron ionization

EP	Entrance potential
EPS	Expanded polystyrene
EQS	Environmental quality standard
EROD	Ethoxyresorufin-O-deethylase
ESI	Electrospray ionization
EU	European Union
EV	Electron Volts
FP	Focusing potential
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GSI	Gonadal somatic index
h	Hour
HA	Humic acid
HBCD	Hexabromocyclododecane
hER	Human estrogen receptor
HIP	High impact polystyrene
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HSI	Hepatosomatic index
IC_{50}	Median inhibitory concentration
IS	Ionspray voltage
K_{DOC}	Dissolved organic carbon-water partition coefficient
KHP	Potassium hydrogen phthalate
K_{OC}	Organic carbon-water partition coefficient
K_{OW}	Octanol-water partition coefficient

K_P	Partition coefficient
LC	Liquid chromatograph
LC_{50}	Median lethal concentration
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOEC	Lowest observed effect concentration
Log K_{ALB}	Logarithmic albumin-water partition coefficient
Log K_{DCM}	Logarithmic dissolved and colloidal matter-water partition coefficient
Log K_{DOC}	Logarithmic dissolved organic carbon-water partition coefficient
Log K_{HA}	Logarithmic humic acid-water partition coefficient
Log K_{OC}	Logarithmic organic carbon-water partition coefficient
Log K_{OW}	Logarithmic octanol-water partition coefficient
Log K_P	Logarithmic partition coefficient
LOQ	Limit of quantitation
LSI	Liver somatic index
Ltd	Limited
M	Mole
m/z	Mass-to-charge ratio
MAC-EQS	Maximum allowable concentration environmental quality standard
min	Minute
mM	Millimole
MS	Mass spectrometer
na	Not applicable
nd	Not detected
NEB	Nebulizer gas

nm	Nanometre
nM	Nanomole
NOEC	No observed effect concentration
NOM	Natural organic matter
NPOC	Non-purgeable organic carbon
ns	Not stated
p	Significance level at a given probability
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PBT	Persistent, bioaccumulative and toxic
PC	Personal computer
PFBS	Perfluorobutane sulfonate
PFOS	Perfluorooctane sulfonate
pKa	Dissociation constant
POPRC	Persistent Organic Pollutants Review Committee
POPs	Persistent organic pollutants
POSF	Perfluorooctanesulfonyl fluoride
PROD	Pentaoxyresorufin-O-depentylase
psi	Pound per square inch
R	Correlation coefficient
R^2	Regression coefficient
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
rpm	Revolution per minute
RSD	Relative standard deviation
SD	Standard deviation
SE	Standard error

SPE	Solid-phase extraction
SVHC	Substances of Very High Concern
t	t-test
TBBPA	Tetrabromobisphenol A
TBCDe	Tetrabromocyclododecene
TC	Total carbon
TFS	Trickling filter site
TIC	Total inorganic carbon
TOC	Total organic carbon
TSCA	Toxic Substances Control Act
UK	United Kingdom
UKWIR	UK Water Industry Research
US	United States
USA	United States of America
USEPA	United States Environmental Protection Agency
V	Volts
v/v	Volume/volume ratio
VTG	Vitellogenin
w/v	Weight/volume ratio
WFD	Water Framework Directive
WWTP	Wastewater treatment plant
XPS	Extruded polystyrene (XPS)
YES	Yeast estrogen screen

Chapter One: Introduction

1.1 Background information

Rivers are running surface waters potentially exposed to contamination with organic substances arising commonly from wastewater, runoff from land and atmospheric deposition, largely due to human activities (Loos *et al.*, 2009; Chari and Halden, 2012; Heeb *et al.*, 2012; Flores *et al.*, 2013; Zhang *et al.*, 2013). There is growing awareness that the total chemical concentration of an organic contaminant present in an environmental medium may not correlate directly with the fraction of the contaminant that is freely dissolved or bioavailable and hence its ecotoxicological effects are not related to total concentration (Dean and Scott, 2004; Akkanen *et al.*, 2012). In aquatic systems, including rivers, organic contaminants may be associated with dissolved organic matter (DOM) (mostly humic substances) and solids (particulates) or freely dissolved (Magnér *et al.*, 2009; Ruiz *et al.*, 2013).The freely dissolved fraction of organic contaminants is generally presumed to be the most potentially bioavailable fraction (Mézin and Hale, 2004; Bondarenko and Gan, 2009). Therefore, hazard ratings derived from the use of only total chemical concentration for organic contaminants in rivers may present unrealistic environmental scenarios for the organic contaminants. In addition, they may be unreliable and lead to overestimation of exposure and risks to target organisms and humans. This makes consideration of bioavailability of organic contaminants for accurate risk assessment a priority.

The concept of bioavailability is gaining recognition among environmental scientists with various definitions of the term expressed. Semple *et al*. (2004) reviewed different definitions of the term and remarked that there was seemingly no working definition for the term. However, from the authors' review, it can be deduced that bioavailability is a measure of the concentration of a contaminant in an environmental medium that is available to a target organism or has the potential to cause toxicity in a target organism. It is apparent that the freely dissolved fraction of an organic contaminant in an environmental medium and its potential for adverse effect in an organism is used as an indicator for bioavailability.

1.2 Model organic contaminants

In this present study, three model organic chemicals, 17α-ethinylestradiol (EE2), perfluorooctane sulfonate (PFOS) and hexabromocyclododecane (HBCD) were used to determine bioavailability of organic contaminants in rivers. These chemicals were selected because of their reported widespread occurrence in environmental samples including wildlife and humans (UNEP, 2006; EC, 2008; Nagpal and Meays, 2009). In addition they possess certain physical and chemical properties which may influence their environmental fate. These chemicals are of concern because of their persistent, bioaccumulative and toxic (PBT) potentials (Langston *et al.*, 2005; Al-Ansari *et al.*, 2010; Ahrens, 2011; Onogbosele and Scrimshaw, 2014). In addition, PFOS and HBCD in particular, have the potentials for long-range environmental transport (Remberger *et al.*, 2004; Dreyer *et al.*, 2009).

EE2 is a synthetic steroid oestrogen regarded as a derivative of the natural human oestrogen, 17β-estradiol (E2) (Aris *et al.*, 2014). EE2 is mostly used as a component of some oral contraceptives used by female humans (Khanal *et al.*, 2006). PFOS is a synthetic perfluorinated chemical widely used as a surfactant (surface-active agent) in a number of industrial applications and consumer products due to its hydrophobic (water-repellent) and lipophobic (oil-repellent) properties (Lindstrom *et al.*, 2011b). The brominated flame retardant (BFR), HBCD is used to resist, prevent or reduce flammability and hence damage in many applications or materials (Fromme *et al.*, 2014).

1.2.1 Chemical identities of model organic contaminants

1.2.1.1 Chemical identity of EE2

The Chemical Abstracts Service (CAS) number of EE2 is 57-63-6 (HSDB, 2012a). The CAS number is a unique numeric identifier for a chemical substance in scientific literature. As a final authority, CAS assigns CAS numbers to chemical substances to provide clear identities and links to their chemical information (ACS, 2014). Technical EE2 is a white to creamy white powder or crystals. It is soluble in ethanol, ether or acetone. It has a molecular formula, $C_{20}H_{24}O_2$ and a molecular weight of 296.4 g/mol (HSDB, 2012a). In its molecular structure (Figure1.1), there are two functional hydroxyl groups.

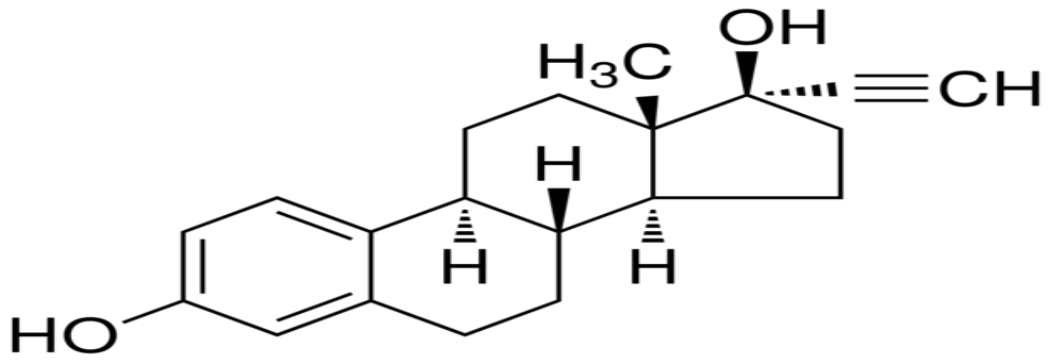


Figure 1.1 Molecular structure of EE2

1.2.1.2 Chemical identity of PFOS

Ionic PFOS does not have a specific CAS number, but the acid and salts have CAS numbers (OECD, 2002). The common forms of PFOS include perfluorooctane sulfonic acid (PFOS acid) (CAS number 1763-23-1), and salts such as potassium PFOS (CAS number 2795-39-3), ammonium PFOS (CAS number 29081-56-9), diethanolamine PFOS (CAS number 70225-14-8) and lithium PFOS (CAS number 29457-72-5) (OECD, 2002; Moermond *et al.*, 2010). PFOS has a molecular formula, $C_8F_{17}SO_3^-$ and a molecular weight of 499.1 g/mol (Moermond *et al.*, 2010). Characteristically, PFOS has a long chain of carbon atoms to which fluorine is attached (Figure 1.2). The strong carbon-fluorine covalent bond is responsible for the resistance of PFOS to degradation (Fernández-Sanjuan *et al.*, 2010). Potassium PFOS (used in this present study) is in the form of white powder.

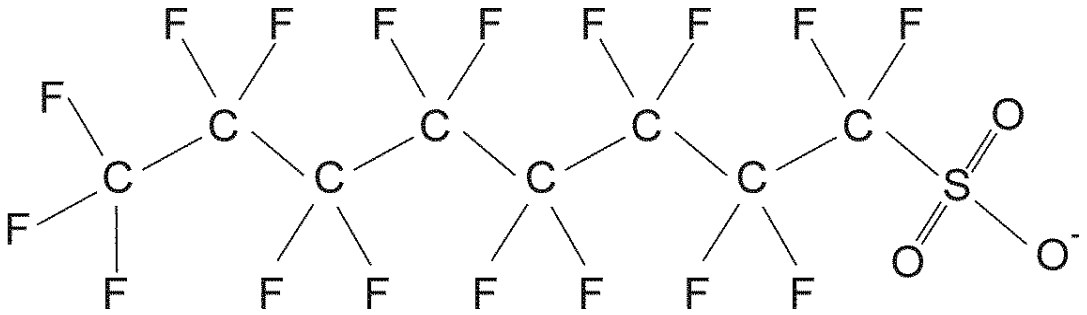


Figure 1.2 Molecular structure of PFOS

1.2.1.3 Chemical identity of HBCD

Technical HBCD is a white solid substance with two different names: hexabromocyclododecane (CAS number 25637-99-4) and 1,2,5,6,9,10-hexabromocyclododecane (CAS number 3194-55-6) (UNEP, 2010). Unlike the latter name, the former name refers to HBCD where the bromine substituents are not numbered (ECHA, 2008b; Environment Canada, 2011). The molecular formula of HBCD is $C_{12}H_{18}Br_6$ and its molecular weight is 641.7 g/mol (UNEP, 2010). The compound exhibits complex stereochemistry (Heeb *et al.*, 2005). Law *et al*. (2005) described 16 possible stereoisomers of HBCD comprising 6 pairs of enantiomers (chiral forms) and 4 mesoforms (achiral forms). However, the main stereoisomers (of environmental relevance) identified in the compound are alpha (α)-HBCD (CAS number 134237-50-6), beta (β)-HBCD (CAS number 134237-51-7) and gamma (γ)-HBCD (CAS number 134237-52-8) (ECHA, 2008b). α-HBCD accounts for 10-13% composition of the mixture; β-HBCD, 1-12% and γ-HBCD, 75-89% (Covaci *et al.*, 2006). Variation in the percentage composition of the different stereoisomers is due to technical differences in the production method for HBCD (UNEP, 2010). Structurally, HBCD is a cyclic aliphatic compound with bromine atoms attached (Figures 1.3).

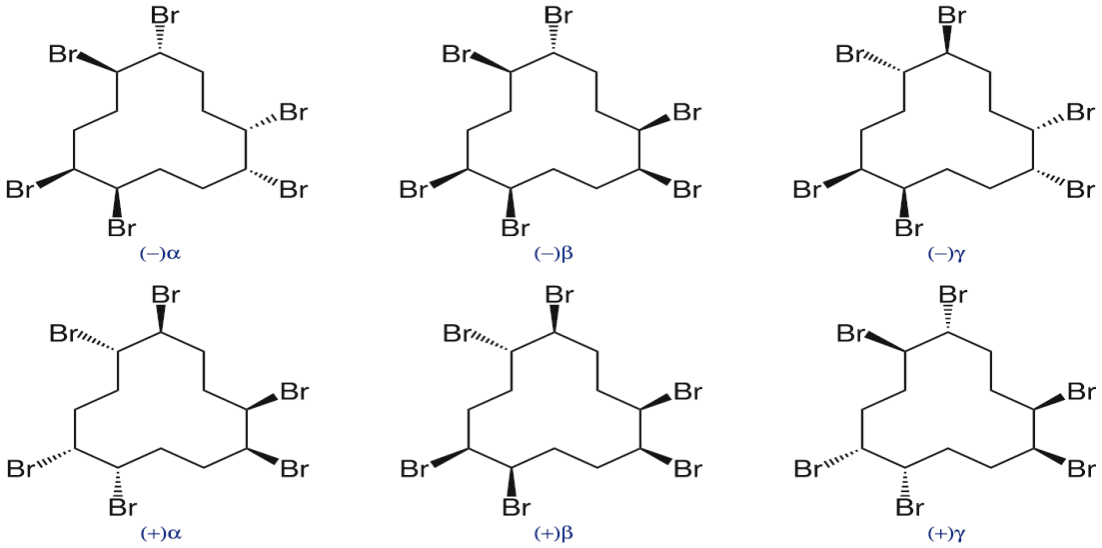


Figure 1.3 Molecular structures of α-, β- and γ-HBCD with their optical isomers

1.3 Importance of bioavailability

Laboratory-derived estimates of risks of aquatic contaminants are usually based on total concentrations of test chemicals to endpoints of concern in test organisms. Laboratory conditions under which test organisms are exposed to chemicals may not represent natural or real environmental conditions (Chapman *et al.*, 1998; Calow and Forbes, 2003; Vignati *et al.*, 2007). Commonly, laboratory toxicity tests are conducted under controlled conditions which may differ from real environmental conditions (Chapman, 2007), but in rivers or natural environments, the occurrence of a number of physical, chemical and biological factors may modulate the fate (including toxicity) of a contaminant (Akkanen *et al.*, 2012; Ruiz *et al.*, 2013). Therefore, bioavailability is relevant in environmental risks assessment.

Bioavailability data can inform accurate and reliable risks assessment of organic contaminants in rivers in relation to their adverse effects. In particular, knowledge and understanding of bioavailability will promote avoidance of overestimation or underestimation of exposure and risks of organic contaminants to river biota. To avoid faulty risks management decisions and actions, consideration of bioavailability is important in the determination of the appropriate scope and costs of risks management. Bioavailability is useful in regulatory controls. It is important for formulation of appropriate environmental quality standards (EQS) for contaminants in rivers. Bioavailability has been considered or used in update to the water quality

criteria for metals such as copper and lead in some jurisdictions (Reiley, 2007; EU, 2013; Han *et al.*, 2014).

1.4 Hypothesis of study

The overall hypothesis of this present study is that in the presence of dissolved organic carbon (DOC), a fraction of the total concentration of an organic contaminant would not be bioavailable in river water.

1.5 Aim and objectives of study

The overall aim of this study is to determine the bioavailability of organic contaminants in rivers and its influence on toxicity. The specific objectives of this study are as follows:

1) To determine the occurrence of organic contaminants in wastewater effluents.
2) To determine the impact of hydrophobicity of organic contaminants on their binding to DOM in rivers.
3) To determine the effect of binding of organic contaminants to DOM on their bioavailability in rivers.
4) To determine if there is a link between chemical measures of bioavailability and toxicity of organic contaminants.

Chapter Two: Literature Review

2.1 Environmentally relevant physical properties of EE2, PFOS and HBCD

The physical properties of EE2, PFOS and HBCD which are of environmental importance are presented in Table 2.1. Physical properties are useful in the prediction of the environmental fate of organic contaminants. The order of increasing molecular weight among the chemicals of concern is EE2, PFOS and HBCD. Molecular weight may influence the behaviour and environmental fate of chemicals. Generally, but not strictly, chemicals with higher molecular weights tend to be less mobile in the environment and their large size may restrict their transport through biological membranes and impact on their bioavailability (USEPA, 2014b).

Table 2.1 Environmentally relevant physical properties of EE2, PFOS and HBCD

Property	EE2	PFOS	HBCD
Molecular weight	296.4g/mol	499.1 g/mol (Moermond *et al.*, 2010)	641.7 g/mol
Water solubility	4.8 mg/L (20 ^{o}C) 11.3 mg/L (27 ^{o}C) (HSDB, 2012a)	519-570 mg/L(24-25 ^{o}C)	0.0660 mg/L (20 ^{o}C) (technical HBCD) 0.0488 mg/L (α-HBCD) 0.0147 mg/L (β-HBCD) 0.0021 mg/L (γ-HBCD)
Log K_{OW}	3.6-4.15	2.57*	5.62
pKa	10.5-10.7	-3.27 (Brooke *et al.*, 2004)	Not applicable (NICNAS, 2012)
Vapour pressure	6.0x10^{-9} Pa (Ding *et al.*, 2014)	3.31x10^{-4} Pa (20 ^{o}C)	6.3X10^{-5} Pa (21 ^{o}C)
Henry's Law constant	7.94x10^{-12} atm m^{3}/mol	3.05x10^{-9} atm m^{3}/mol	6.08x10^{-4} atm m^{3}/mol (HSDB, 2011)

*Log K_{OC} (Higgins and Luthy, 2006)

EE2 (Banihashemi and Droste, 2014); PFOS (OECD, 2002); HBCD (EC, 2008) (unless otherwise stated)

The water solubility of a chemical is used as an indicator of its distribution in environmental media (USEPA, 2014b). The water solubility of PFOS is higher than that of EE2 or HBCD while HBCD has the lowest water solubility. The estimated water solubility of EE2 indicates that the chemical is moderately hydrophobic (Lai *et al.*, 2000), and therefore, has the potential for partitioning in the aquatic environment (Nagpal and Meays, 2009; Chen *et al.*, 2012). PFOS is moderately soluble in water (Beach *et al.*, 2006). If released to water, PFOS may remain in water unless adsorbed to particulate matter and sediments or bioaccumulated (OECD, 2002). The carbon-fluorine (CF_2) moiety of PFOS is both hydrophobic and lipophobic while the sulfonate group (SO_3^-) is hydrophilic (Higgins and Luthy, 2006). HBCD exhibits low water solubility (hydrophobicity) and tends to sorb to organic phases (for example, suspended solids and lipids) in the aquatic environment (Marvin *et al.*, 2011). The different solubilities of α, β- and γ-HBCD may result in distinctive rates of biological uptake and metabolism, and are possibly responsible for observed differences in their environmental behaviour (Janák *et al.*, 2005).

EE2, PFOS and HBCD have differing octanol-water partition coefficient (K_{OW}) values. K_{OW} (usually expressed in the logarithmic form, Log K_{OW}) is the ratio of the concentration of a chemical in octanol to its concentration in water when the two phases (octanol and water) are in equilibrium (Hayward *et al.*, 2006). Octanol is used as a surrogate phase for measuring the affinity of hydrophobic chemicals for lipid in organisms (Quinn *et al.*, 2014). K_{OW} or organic carbon-water partition coefficient (K_{OC}) (commonly expressed as Log K_{OC}) is useful in the evaluation of the potentials of a chemical for sorption to suspended solids and sediments (Holthaus *et al.*, 2002; Chen *et al.*, 2012), and for bioaccumulation (Petersen *et al.*, 2010) in the environment. K_{OW} is inversely related to water solubility; the lower the water solubility of a chemical, the greater the K_{OW}. The estimated value of the Log K_{OW} of EE2 indicates that it has the propensity to sorb to suspended solids and sediments (Chen *et al.*, 2012) or accumulate in biota (Lai *et al.*, 2002) in a water body. The Log K_{OW} of PFOS cannot be measured due to its surface-active properties (OECD, 2002). Alternatively, K_{OC} is used. Higgins and Luthy (2006) reported a Log K_{OC} of 2.57 for PFOS and observed that hydrophobicity was the dominant influence on the sorption of PFOS to sediments in water. PFOS does not partition to lipids, but rather binds to certain proteins in animals. Therefore, K_{OW} cannot be reliably used in the prediction

of the bioaccumulation of PFOS (OECD, 2002; Beach *et al.*, 2006). HBCD has a high Log K_{OW}. This suggests that HBCD has the potential for sorption to sediments and suspended solids in water (Environment Canada, 2011). The estimated Log K_{OW} also places HBCD in the optimum range (Log K_{OW} 5-7) for bioaccumulation (Wu *et al.*, 2011).

Dissociation constant (pKa) is used to evaluate the ionization potential of a chemical under environmental conditions. As the percentage of ionization increase, water solubility increase while vapour pressure, Henry's Law constant and K_{OW} decrease (USEPA, 2014b). The estimated pKa of PFOS indicates that PFOS can occur in the environment in the ionized form (Brooke *et al.*, 2004; Moermond *et al.*, 2010). HBCD does not ionize under environmental conditions (NICNAS, 2012).

Vapour pressure is used in the determination of the volatility of a chemical and its potential for long-range transport (USEPA, 2014b). EE2 has low vapour pressure (Ying *et al.*, 2002), and therefore, not prone to volatilisation from water. The estimated vapour pressure of potassium PFOS is 3.31×10^{-4} Pa. PFOS is not expected to significantly volatilise from water probably because of its ionic nature (OECD, 2002), and will mainly exist in the particulate phase rather than in a gaseous phase if released to air (UNEP, 2006). HBCD has fairly low vapour pressure (Abdallah *et al.*, 2008) and moderate volatility from water (USEPA, 2008). Based on the estimated vapour pressure, HBCD has the potential to exist in both gaseous and particulate phases in the atmosphere (HSDB, 2011). Existence in gaseous or particulate phase in the atmosphere enhances the potential of HBCD for long-range transport (ECHA, 2008b; USEPA, 2008).

Henry's Law constant which is also used to indicate the volatility of a chemical from water and partitioning in environmental matrices, is defined as the ratio of a chemical's concentration in the gas phase to that in the liquid phase at equilibrium (USEPA, 2014b). Based on the estimated Henry's Law constant of EE2, the chemical is not expected to volatilise from water surfaces (HSDB, 2012a). The Henry's Law constant of PFOS is higher than that of EE2, but lower than that of HBCD. PFOS is expected to exhibit very low or insignificant volatility from water based on the estimated Henry's Law constant (OECD, 2002). On the basis of the estimated Henry's constant, HBCD may volatilise from water surfaces. However,

volatilisation may be reduced by sorption of HBCD to suspended solids and sediments in water (HSDB, 2011).

2.2 The regulatory status of EE2, PFOS and HBCD

Globally, the production or use of EE2 has not been banned. In Annex II to the EU Water Framework Directive (WFD), EE2 was proposed for inclusion in the list of priority substances in 2012 (EC, 2012). Priority substances are contaminants which represent a significant risk to or via the aquatic environment (Coquery *et al.*, 2005). For inland surface waters including rivers, an annual average environmental quality standard (AA-EQS) of $3.5x10^{-5}$ µg/L EE2 was proposed (EC, 2012). However, in 2013, the chemical was included in the EU watch list of substances that require monitoring data for prioritisation exercise (EU, 2013). Starting from 2016, a final decision on the regulation of the chemical will be taken by the EU (Jobling and Owen, 2013). The WFD enacted in 2000 had as a primary objective, the achievement of 'good chemical status' for all surface waters by 2015 (Völker *et al.*, 2013). However, the deadline has been extended to 2021 for existing priority substances with revised EQS and to 2027 for newly identified priority substances (EU, 2013).

PFOS is among persistent organic pollutants (POPs) being restricted or banned globally. In the United States (US), PFOS is regulated. In the year 2000, the main producer of PFOS, 3M (based in the US) announced that it would stop production of the chemical in 2003 due to pressure from USEPA (United States Environmental Protection Agency) in relation to the environmental toxicity of PFOS (Zushi *et al.*, 2012). In the US, the Toxic Substances Control Act (TSCA) (with effect from 2003) requires companies to notify USEPA 90 days before commencement of the manufacture or import of PFOS for a significant new use. The notification is to allow time for the assessment of the intended use of the substance (Zushi *et al.*, 2012; USEPA, 2014a).

In May 2009, PFOS, its salts and derivatives were listed in Annex B (restriction of production and use) of the international treaty, Stockholm Convention on POPs (UNEP, 2009). Prior to this time, based on risks evaluation on the health and environmental effects of PFOS, an EU directive (Directive 2006/122/EC) in 2006

restricted the use of PFOS to a maximum content equal to or below 0.005% (50 mg/kg) by mass in PFOS-products, with some derogations (exemptions) (EU, 2006). In EU REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation, the chemical is derogated for use in some industrial applications such as photoresists or anti-reflective coatings for photolithography, photographic coatings (for films, paper or printing plates), mist suppressants for hard metal (chromium) plating and wetting agents for electroplating, and hydraulic fluids for aviation (EU, 2009). In 2010, the chemical was included in the EU regulation on POPs and the threshold in products was reduced to below or equal to 0.001% (10 mg/kg) by mass in products not yet in the market or in use (EU, 2010). In EU directive (Directive 2013/39/EU), PFOS is classified as a 'Priority Hazardous Substance' with AA-EQS of $6.5x10^{-4}$ µg/L and MAC-EQS (maximum allowable concentration EQS) of 36 µg/L for inland surface waters (EU, 2013).

The production and use of HBCD is being regulated globally. In the EU, under REACH framework, the European Chemicals Agency in October 2008, added HBCD to the list of 'Substances of Very High Concern (SVHC) due to its PBT potentials (ECHA, 2008a). In 2011, the substance was included in Annex XIV to REACH (list of substances subject to authorisation) and will be phased out with effect from 21 August 2015 (EC, 2013). In August 2013, HBCD was identified as a 'Priority Hazardous Substance' by the EU. By a directive (2013/39/EU) the AA-EQS and MAC-EQS for inland surface waters were set at 0.0016 and 0.5 µg/L, respectively (EU, 2013).

On 18 June 2008, Norway as a party to the Stockholm Convention proposed the listing of HBCD as a potential POP in Annex A (elimination of production and use) to the Convention (UNEP, 2010). Based on evaluation of the risk profile of HBCD, in October 2010, the Persistent Organic Pollutants Review Committee (POPRC) of the Convention at its sixth meeting concluded that HBCD has the potential for significant adverse human health and/or environmental effects to warrant global action (UNEP, 2010). The POPRC at its eighth meeting (POPRC-8/3) in October 2012 recommended that HBCD should be listed in Annex A to the Convention. However, it also recommended specific derogation for use of HBCD in expanded polystyrene (EPS) and extruded polystyrene (XPS) in building to allow transition time (at least 5

years) for the production and use of safer substitutes for HBCD (UNEP, 2012). In May 2013, the Conference of the Parties to the Convention considered and approved the recommendation by the POPRC to list HBCD in Annex A to the Convention with specific derogation (UNEP, 2013). The implication of the decision of the Conference is that the phase-out of HBCD must be implemented by countries that are parties to the Convention.

2.3 Production and uses of EE2, PFOS and HBCD

2.3.1 Production and uses of EE2

The synthesis of EE2 commenced in Berlin in 1938 (Jobling and Owen, 2010). It is difficult to obtain comprehensive data on the global production or use of EE2. However, for the European countries, UK, Germany, France, Italy, Netherlands and Belgium, Hannah *et al*. (2009) reported the estimated use of EE2 per year as 26.3, 51, 33.6, 19.9, 15.4 and 8 kg, respectively. The same authors reported the use of about 88 kg EE2 per year in USA. EE2 being the main component of many contraceptives is principally used by female humans for contraception (prevention of pregnancy). It inhibits ovulation by suppression of follicle stimulating hormone (FSH) and alters the structure of the endometrium (Hoffmann and Kloas, 2012).

2.3.2 Production and uses of PFOS

The manufacturing company, 3M in 1949 started the production of PFOS by electro-chemical fluorination of octanesulfonyl chloride ($C_8H_{17}SO_2Cl$) resulting in the product, perfluorooctanesulfonyl fluoride ($C_8F_{17}SO_2F$) (POSF). PFOS was then synthesized from POSF (UNEP, 2006; Paul *et al.*, 2008). Initially, PFOS was a chemical constituent of Scotchgard (stain repellent) manufactured by 3M for application to, and protection of consumer products (Renner, 2000). Following the phase-out of PFOS production in 2003, the company embarked on reformulation of Scotchgard and the replacement of PFOS with perfluorobutane sulfonate (PFBS) considered to be environmentally friendly (Renner, 2006).

Paul *et al*. (2008) reviewed the production of PFOS precursor, POSF, and reported a total global production of 122500 tonnes from 1970-2002. PFOS was mainly produced in the US until 2003 (Carloni, 2009). There are also indications of production of the chemical in Europe (Belgium, Bulgaria, Germany and Italy), Latin America (Brazil) and Asia (China and Japan) before its global ban or restriction (Carloni, 2009). Information on the production of PFOS or POSF in Africa and Oceania is lacking.

PFOS is used as a surfactant in industrial applications and consumer products. It is incorporated in fabric (clothing or textiles, carpets, leather products and upholstery), paper materials (food packaging, bags, cartons, paperboards and wraps), coatings for cookware, paints and adhesives (OECD, 2002; Pistocchi and Loos, 2009). Carloni (2009) also described the use of PFOS in chromium plating (mist suppressant), firefighting foams, photographic films, photolithography, aviation hydraulic fluids and pesticides.

2.3.3 Production and uses of HBCD

Production of HBCD started with the bromination of cyclododecatriene, resulting primarily in the formation of a mixture of three isomers (α-, β- and γ-HBCD) (Alaee *et al.*, 2003). The availability of HBCD in the global market was observed in the 1960s. However, production and use of the chemical in polystyrene flame retardancy commenced in the 1980s (EC, 2008). It is difficult to have comprehensive data on global production of HBCD from the start date to present. However, it is estimated that the global market demands for HBCD were 16,700 tonnes in 2001(Hale *et al.*, 2006; Morose, 2006) and 21,447 and 21,951 tonnes in 2002 and 2003, respectively (Morose, 2006; UNEP, 2010). Of the total estimate for 2001, Europe accounted for 9,500 tonnes (56.9%); Asia, 3,900 tonnes (23.3%); Americas, 2,800 (16.8%), and the rest of the world, 500 (3.0%) (Morose, 2006). In 2006, the demand and use of HBCD in Europe alone was 11,580 tonnes (UNEP, 2010).

HBCD is the third most used BFR after TBBPA and polybrominated diphenyl ethers (PBDEs) (Rani *et al.*, 2014). HBCD is principally used as a flame retardant in expanded polystyrene (EPS) and extruded polystyrene (XPS) used as thermal insulators by construction and building industries (Marvin *et al.*, 2011; Rani *et al.*,

2014). The chemical is also secondarily used for textile coatings, furniture, upholstered materials, transportation seating, bed mattress ticking, wall coverings and draperies susceptible to flammability (Stubbings and Harrad, 2014). Its use in high impact polystyrene (HIP) for electrical and electronic equipment such as electrical lines distribution boxes and electrical housings have also been reported (Stubbings and Harrad, 2014).

2.4 Sources of EE2, PFOS and HBCD in rivers

2.4.1 Sources of EE2 in rivers

Wastewater with EE2 from human use and excretion is the main source of EE2 in rivers (Jobling *et al.*, 2006; Hannah *et al.*, 2009; Wise *et al.*, 2010). There is incomplete removal of EE2 from wastewater during wastewater treatment processes, and when wastewater containing EE2 is discharged into rivers, it results in the contamination of rivers with EE2 (Silva *et al.*, 2012). Also, when animal wastes (from veterinary treatments) and sewage sludge containing EE2 are disposed to agricultural soils, they can contribute to the contamination of surface waters (including rivers) with EE2 through runoff (Lima *et al.*, 2012).

2.4.2 Sources of PFOS in rivers

The sources of release of PFOS to rivers are diverse. Hansen *et al*. (2002) recognized PFOS manufacturing site or process as one of the sources. River contamination with PFOS can also come from wastewater effluents (Becker *et al.*, 2008; Murakami *et al.*, 2008), the use and disposal of PFOS products (UNEP, 2006), landfill leachate, runoff from contaminated soils and atmospheric deposition (Ahrens, 2011). However, wastewater effluent is a significant source of PFOS in the aquatic environment (Bossi *et al.*, 2008). The study by Lein *et al*. (2008) on the sources of PFOS in rivers in Japan implicated wastewater effluent as a source of PFOS pollution of rivers. Loos *et al*. (2013) reported the occurrence of PFOS in effluents from a number of WWTPs in Europe. Like EE2, the application of sewage sludge contaminated with PFOS to agricultural soils as biosolids has been reported to cause

contamination or elevation of PFOS in rivers in the vicinity of contaminated soils (Lindstrom *et al.*, 2011a).

PFOS is incorporated into the products it protects, and therefore, vulnerable to release. The use and disposal of PFOS-products in landfills can lead to the release of PFOS into the environment, including rivers (Paul *et al.*, 2008). Busch *et al*. (2010) reported the occurrence of PFOS in landfill leachate in Germany.

2.4.3 Sources of HBCD in rivers

The contamination of rivers with HBCD can arise from the same pathways involved in the release of PFOS to rivers. HBCD can enter rivers from discharges of manufacturing sites (Covaci *et al.*, 2006; Guerra *et al.*, 2009), use and disposal of HBCD products (Wu *et al.*, 2011), effluents of WWTP, land runoff and atmospheric deposition (Zhang *et al.*, 2013). ECHA (2009) reported that in Europe a greater fraction of HBCD released to the environment is in wastewater followed by surface water and air. It is assumed that a considerable fraction of HBCD which reaches WWTP is adsorbed to sludge, and only about 20% is released to the environment (the receiving water) (Rüdel *et al.*, 2012). HBCD is susceptible to leaching and release from its products in use or after disposal to the environment because as an additive flame retardant it is not covalently bonded (chemically bound) to the materials it protects (Tomy *et al.*, 2005). Because HBCD is potentially volatile, atmospheric transport is a possible pathway for its release to the environment (Yu *et al.*, 2008).

2.5 Occurrence and levels of EE2, PFOS and HBCD in rivers

2.5.1 Occurrence and levels of EE2, PFOS and HBCD in river water

EE2, PFOS and HBCD have been detected in river water in various countries of the world. Worldwide, reported levels of EE2, PFOS and HBCD in river water range from 0.1-831 ng/L (Table 2.2a), <0.01-1371 ng/L (Table 2.2b) and 0.0095-2100 ng/L (Table 2.2c), respectively. However, the high concentrations of EE2 reported in the study by Kolpin *et al*. (2002) have been criticized. Ericson *et al*. (2002) argued that

interference by indistinguishable substance and the use of gas chromatography-mass spectrometry (GC-MS) analytical method accounted for the high concentrations of EE2 in US rivers reported by Kolpin *et al*. (2002). Further, they observed that based on analytical data, it is gas chromatography-tandem mass spectrometry (GC-MS/MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) that is more suitable for analysis of steroid estrogens to avoid overestimation of concentrations. Unlike EE2 and PFOS, measured concentrations of HBCD in river water are not common. This is probably due to the much higher hydrophobicity of HBCD than EE2 and PFOS, which results in it being associated with solids or organisms rather than in aqueous solution.

Generally, elevated concentrations of PFOS or HBCD in river water may be associated with closeness of rivers to PFOS or HBCD manufacturing sites or WWTPs which discharge effluents into rivers (Hansen *et al.*, 2002; EC, 2008; Konwick *et al.*, 2008). Though wastewater effluents may contain EE2, in-stream dilution of effluents can lower the concentrations of EE2 in river water (Ying *et al.*, 2009). The highest concentration (1520 ng/L) of HBCD reported in a survey of UK rivers in 2002 was measured in a river site close to a manufacturing site which ceased production of HBCD in 2003 (EC, 2008). Also, Oh *et al*. (2014) observed that the levels of HBCD in river water are reflective of the emission sources of HBCD for a given river. The authors reported low level (2.5-57 ng/L) of HBCD in the water of Tsurumi and Yodo Rivers, Japan, which receive domestic or municipal wastewater as possible source of HBCD compared to high level (180-2100 ng/L) of HBCD in Kuzuryu River, Japan, which receives industrial (textile) effluents.

Johnson *et al*. (2013) reported that the concentrations of EE2 in European rivers exceeded the initially proposed EU EQS for EE2. However, EE2 is currently on EU watch list of substances that may be regulated in the future. The levels of PFOS for European countries (Table 2.2b) are less than EU MAC-EQS of 36000 ng/L, but above (with the exception of Germany) EU AA-EQS of 0.65 ng/L for surface waters. HBCD levels in the European countries reported (Table 2.2c) also exceed EU AA-EQS of 1.6 ng/L for surface waters. When concentrations of EE2, PFOS and HBCD in rivers are above specific EQS, they are possibly of concern and may require further assessment.

Table 2.2a Measured concentrations of EE2 in river water around the world

Location	Number of rivers sampled	Year of sampling	Concentration of EE2 (ng/L)	Reference
Brazil	1	2009	5.6-63.8	Moreira *et al.* (2011)
China	3	2007	nd-35.6	Lei *et al.* (2009)
Australia	5	2004-2005	0.0-0.52	Ying *et al.* (2009)
France	5	2001-2002	1.1-2.9	Cargouet *et al.* (2004)
UK	2	2000	nd-4.6	Williams *et al.* (2003)
Germany	4	2000	0.1-5.1	Kuch and Ballschmiter (2001)
USA	139	1999-2000	nd-831	Kolpin *et al.* (2002)

nd, not detected

Table 2.2b Measured concentrations of PFOS in river water around the world

Location	Number of rivers sampled	Year of sampling	Concentration of PFOS (ng/L)	Reference
South Africa	3	2011	<0.03-181.8	Mudumbi *et al.* (2014)
Canada	38	2001-2008	<0.02-34.6	Scott *et al.* (2009)
Japan	4	2004-2005	0.5-58	Takazawa *et al.* (2009)
China	2	2004	<0.01-99	So *et al.* (2007)
Australia	1	2001	0.2-34	Gallen *et al.* (2014)
USA	1	2000	16.8-140	Hansen *et al.* (2002)
Slovenia	1	ns	<1-1371	Loos *et al.* (2009)
UK	1	ns	<1-238	Loos *et al.* (2009)
France	1	ns	<1-97	Loos *et al.* (2009)
Germany	1	ns	<1-32	Loos *et al.* (2009)

ns, not stated

Table 2.2c Measured concentrations of HBCD in river water around the world

Location	Number of rivers sampled	Year of sampling	Concentration of HBCD (ng/L)	Reference
Denmark	ns	2012	0.096-2.9	Vorkamp *et al.* (2014)
Japan	3	2011	2.5-2100	Oh *et al.* (2014)
China	1	2010	0.0095-0.0824	He *et al.* (2013)
UK	4	2002	57-1520	EC (2008)

ns, not stated

2.5.2 Occurrence and levels of EE2, PFOS and HBCD in river sediment

EE2, PFOS and HBCD have been detected in river sediments in different countries around the world. In rivers, reported levels of EE2, PFOS and HBCD in sediments range from nd-12 ng/g dw (Table 2.3a), nd-83 ng/g dw (Table 2.3b) and nd-11310 ng/g dw (Table 2.3c), respectively. The reported levels of HBCD in sediments are higher than those of PFOS and EE2. This is probably due to much more hydrophobic character of HBCD than EE2 and PFOS. HBCD has been observed to exhibit high affinity for solid particles such as sediments and sewage sludge due to its hydrophobicity (Meng *et al.*, 2011). Though EE2 is more hydrophobic than PFOS, the reported level of PFOS in sediments is higher than that of EE2 probably due to the greater persistence of PFOS.

Sediments in the aquatic environment are considered as sinks or reservoirs for hydrophobic contaminants. Binding of hydrophobic contaminants to sediments can have impacts on the distribution, transport and adverse effects of hydrophobic contaminants in the aquatic environment. The mobility and bioavailability, and consequently, the toxicity of hydrophobic contaminants can be reduced due to binding to sediments (Ilyas *et al.*, 2011; Zhang *et al.*, 2013). However, hydrophobic contaminants bound to sediments may be available to sediments-dwelling biota (Zhang *et al.*, 2013).

Table 2.3a Measured concentrations of EE2 in river sediment around the world

Location	Number of rivers sampled	Year of sampling	Concentration of EE2 (ng/g dw)	Reference
China	1	2009	nd	Zhang *et al.* (2014a)
China	3	2007	0.81-9.26	Lei *et al.* (2009)
Germany	1	2006	nd	Grund *et al.* (2011)
UK	1	2005-2006	<0.04	Labadie and Hill (2007)
UK	2	2003	0.4-12	Liu *et al.* (2004)
Italy	1	2005	nd	Viganò *et al.* (2008)
France	5	2004	nd	Kinani *et al.* (2010)
Czech	2	ns	<1	Schmitt *et al.* (2010)
Spain	2	ns	<1	Schmitt *et al.* (2010)

dw, dry weight; ns, not stated; nd, not detected

Table 2.3b Measured concentrations of PFOS in river sediment around the world

Location	Number of rivers sampled	Year of sampling	Concentration of PFOS (ng/g dw)	Reference
South Africa	3	2011	0.0-83	Mudumbi *et al.* (2014)
China	2	2007	1.57-8.78	Li *et al.* (2010)
USA	1	2007	0.1-0.4	Senthil Kumar *et al.* (2009)
Germany	1	2006	<0.05-0.54	Becker *et al.* (2008)
Japan	5	2005	<0.33-6.4	Senthilkumar *et al.* (2007)
France	1	ns	4.3	Labadie and Chevreuil (2011)

dw, dry weight; ns, not stated

Table 2.3c Measured concentrations of HBCD in river sediments around the world

Location	Number of rivers sampled	Year of sampling	Concentration of HBCD (ng/g dw)	Reference
Japan	3	2011	5.7-7800	Oh *et al.* (2014)
Czech	9	2010	1.10-44.25	Hloušková *et al.* (2014)
Korea	3	2010	0.19-13	Jeong *et al.* (2014)
China	1	2010	1.35-26.4	Zhang *et al.* (2013)
China	7	2003-2004	0.026-206	Li *et al.* (2013)
Spain	1	2006	0.8-1850	Guerra *et al.* (2010)
Spain	1	2002	nd-514	Eljarrat *et al.* (2004)
UK	6	2000-2002	<2.4-1678	Morris *et al.* (2004)
UK	2	ns	<25-11310	EC (2008)
USA	1	2001	<0.075-3.7	Marvin *et al.* (2006)

dw, dry weight; ns, not stated; nd, not detected

2.5.3 Occurrence and levels of EE2, PFOS and HBCD in river biota

The occurrence of EE2, PFOS in river biota around the world has been reported. Reported measured concentrations of EE2, PFOS and HBCD in different river biota are presented in Table 2.4. Data on the levels of the contaminants in river biota are dominated by fish. However there is limited information on the levels of EE2 in river biota. EE2 accumulates in organisms because of its hydrophobic character. In fish, accumulation of EE2 in the bile has been observed (Al-Ansari *et al.*, 2013). EE2 concentrations in fish body could be regulated by metabolic biotransformation (Al-Ansari *et al.*, 2010)

Levels of PFOS in many species of river biota, particularly fish, have been reported. The range of measured concentrations of PFOS (0.10-842 ng/g ww) in fish reported by Hloušková *et al*. (2013) (Table 2.4) were observed in 48 samples of 10 fish species. The species were *Rutilus rutilus* (roach), *Abramis brama* (common bream), *Cyprinus carpio* (common carp), *Squalius cephalus* (European chub), *Carassius carassius* (Crucian carp), *Oncorhyncus mykiss* (rainbow trout), *Perca flaviatilis* (European perch), *Gobo gobio* (gudgeon), *Thymallus thymallus* (grayling) and *Scardinius erythrophthalmus* (rudd). The authors observed higher concentrations of PFOS in the fish samples collected from locations close to effluent discharge from a chemical factory. PFOS is known to commonly accumulate in the liver and blood of organisms due to its lipophobic and proteinophilic nature. However, in the study by Hloušková *et al*. (2013), accumulation of the chemical in fish muscles was reported.

.In Table 2.4, the concentrations (0.09-12.99 ng/g ww) of HBCD in fish reported by Hloušková *et al*. (2013) were detected in the same species of fish sampled for the determination of PFOS concentrations by the same authors. High concentrations of HBCD in fish were reported in the study by Eljarrat *et al.* (2004) (Table 2.4). In the study, the authors collected and analysed 23 samples of a single species of fish from locations in River Cinca, Spain, with drains from a highly industrialized town. HBCD being lipophilic is expected to accumulate in biota. In literature, most data on the levels of HBCD in aquatic organisms deal with accumulation of HBCD in marine organisms with fatty tissues. Lipophilic contaminants preferentially accumulate in liver and muscle of organisms because of their lipid content. In addition, the

accumulation of lipophilic contaminants in the liver may be due to the detoxifying function of the liver in the metabolism of xenobiotics (Xian *et al.*, 2008).

In the EU WFD, the biota EQSs relating to fish for PFOS and HBCD are 9.1 and 167 ng/g ww, respectively (EU, 2013). The levels of PFOS in fish in the EU countries reported are above the EQS. More recent data on levels of HBCD in river biota are needed to make comparison for HBCD. Regulation of concentrations of EE2, PFOS and HBCD in river water will possibly reduce the levels of the contaminants in river biota.

Table 2.4 Measured concentrations of EE2, PFOS and HBCD in river biota around the world

Contaminant	River biota	Concentration of contaminant (ng/g ww)	Location	Year of sampling	Number of rivers sampled	Reference
EE2	**Fish**					
	Moxostoma macrolepidotum (shorthead redhorse suckers)	0.48-2.30 (whole fish)	Canada	2002	1	Al-Ansari *et al.* (2010)
PFOS	**Fish**					
	10 species	0.10-842 (muscle)	Czech	2010	6	Hloušková *et al.* (2013)
	Eels and bream	4.2-80.7 (whole fish)	Germany	2007-2008	4	Schuetze *et al.* (2010)
	18 species	<10-1250 (whole fish)	USA	2005	3	Ye *et al.* (2008)
	Invertebrates					
	Hydropsyche exocellata (insect)	20-144	Spain	2006-2009	2	Fernández-Sanjuan *et al.* (2010)
	Procambarus clarkia (crab)	0.23-0.63	Spain	2006-2009	2	Fernández-Sanjuan *et al.* (2010)
HBCD	**Fish**					
	10 species	0.09-12.99 (muscle)	Czech	2010	6	Hloušková *et al.* (2013)
	Barbus graellsi (barbel)	nd-1172 (liver and muscle)	Spain	2002	1	Eljarrat *et al.* (2004)

ww, wet weight; nd, not detected

2.6 The fate of EE2, PFOS and HBCD in rivers

2.6.1 Persistence

EE2, PFOS and HBCD are potentially persistent (UNEP, 2006; Liu *et al.*, 2011; Marvin *et al.*, 2011). Half-lives of estrogenic steroids such as EE2 are estimated to be 2-6 d in water and sediment (Ying *et al.*, 2002). However, Jürgens *et al.* (2002) reported half-lives of EE2 in order of 10 d in English rivers. Aerobic and anaerobic conditions are critical for the persistence of EE2 in water. Under aerobic condition, degradation of EE2 may be rapid with shorter half-lives while under anaerobic condition, degradation may be slow with much longer half-lives (Langston *et al.*, 2005). Information on the half-lives of EE2 in river biota is lacking.

PFOS is extremely persistent (Wang *et al.*, 2009) with photolytic and hydrolytic half-lives in water estimated as 3.7 and 41 years, respectively (UNEP, 2006). There are no data on half-lives of PFOS in river sediments, but in river biota, half-lives of 49-152 d in the liver of *C. carpio*, and 12 d (in blood) and 14 d (in liver) of *O. mykiss* have been determined (Beach *et al.*, 2006).

There are limited experimental data on degradation half-lives of HBCD in rivers in particular. However, modelled estimates of half-lives of HBCD in water and aquatic sediments have been reported. Marvin *et al.* (2011) reported estimated half-lives of 60-130 d in water while EC (2008) reported estimated half-lives of 125-191 d in sediments. Data on half-lives of HBCD in biota seem to be limited to marine and terrestrial environments.

On the basis of reported values of degradation half-life, PFOS and HBCD are more persistent than EE2. Using half-lives as criteria for persistence in the aquatic environment, the regulatory thresholds based on Stockholm Convention on POPs are half-lives >60 d (in water) and >180 d (in sediments) (UNEP, 2001). PFOS and HBCD clearly meet the criteria, particularly for water.

2.6.2 Abiotic degradation

EE2 may not undergo degradation via hydrolysis since the chemical does not have functional groups that hydrolyze under environmental conditions (HSDB, 2012a). However, direct photolysis by sunlight is an important degradation process for EE2 since the chemical absorbs light at wavelength 281 nm (HSDB, 2012a). Lin and Reinhard (2005) reported photodegradation of EE2 in river water and air-saturated purified water exposed to irridiation with xenon arc lamp (765 W/m^2) with half-lives of 2 h and 341.7 h, respectively. Studies by Whidbey *et al.* (2012) indicated photodegradation of EE2 light intensity of 250 W/m^2 with half-life of 8 h. Jürgens *et al.* (2002) observed the susceptibility of EE2 to photodegradation in rivers.

PFOS is resistant to degradation by hydrolysis and photolysis in the environment (Giesy and Kannan, 2002; OECD, 2002; Beach *et al.*, 2006; UNEP, 2006; HSDB, 2012b). PFOS is resistant to hydrolysis and photolysis because of the stability conferred by the fluorine atoms and the absence of a chemical structure vulnerable to electrophilic or nucleophilic attack (HSDB, 2012b).

Like PFOS, HBCD is not expected to undergo degradation by hydrolysis or photolysis under environmental conditions (HSDB, 2011). Hydrolysis is not considered a significant degradation process for HBCD in the environment because of lack of hydrolyzable functional groups in its chemical structure (UNEP, 2010; HSDB, 2011). Photodegradation by sunlight is not expected due to lack of chromophores that absorb wavelenghths (HSDB, 2011).

2.6.3 Biodegradation

Liu *et al.* (2011) investigated biodegradation of EE2 in river water and concluded that the chemical was persistent. However, laboratory studies on microbial degradation of EE2 suggest that EE2 may undergo biodegradation in rivers in the presence of certain microbes. Kresinová *et al.* (2012) reported degradation of EE2 by the fungus, *Pleurotus ostreatus* (white rot fungus) under laboratory conditions. Larcher and Yargeau (2013) also reported the degradation of EE2 by seven species of heterotrophic bacteria. In the study, the percentage removal of EE2 by the bacteria after 300 h were, *Pseudomonas putida*, 21%; *Bacillus subtilis*, 27%; *P. aeruginosa*, 34%; *Rhodococcus zopfii,* 38%; *R. erythropolis*, 46%, and *R*. *equi*, 61%. With *R*.

rhodochrous, there was no detectable EE2 after 48 h. Metabolites were detected in the degradation of EE2 by the bacteria except in the degradation by *R*. *zoplii* and *R*. *rhodochrous*.

There are no data on biodegradation of PFOS in river water or sediments, but there are limited data on biodegradation of HBCD in rivers. BFRs such as HBCD that are considered persistent in abiotic media are not necessarily so in biotic media (Tomy *et al.*, 2011). Davis *et al.* (2005) studied biodegradation of HBCD in two river systems, using sediment microcosms. In the study, they reported biodegradation half-lives ranging from 11-32 d and 1.1-1.5 d under aerobic and anaerobic conditions, respectively. However, degradation products were not detected in the study. In a similar study, Davis *et al.* (2006) observed that microbes inhabiting aquatic sediments and anaerobic digester sludge mediate debromination and loss of HBCD, and the production of HBCD degradation products. In their study, the authors reported the decrease of total [^{14}C]HBCD from 195-109 nM and 192-74 nM in river sediment during 113 d under aerobic and anaerobic conditions, respectively. The degradation products identified in the study were tetrabromocyclododecene (TBCDe) ($C_{12}H_{18}Br_4$), 1,2-dibromocyclodedecane (DBCDi) ($C_{12}H_{18}Br_2$) and 1,5,9-cyclododecatriene (CDT) ($C_{12}H_{18}$).

2.6.4 Bioaccumulation

Studies have shown that EE2, PFOS and HBCD have the potential to accumulate in river biota (Lai *et al.*, 2002; Wu *et al.*, 2010; Ng and Hungerbühler, 2013). It is important to distinguish the terms, ‘bioaccumulation’ and ‘bioconcentration’. Arnot and Gobas (2006) described bioaccumulation or bioconcentration as the process by which a chemical substance is absorbed in an organism from the environment, but that bioaccumulation involves chemical uptake by an organism via all routes (dietary and ambient environmental sources) of exposure while bioconcentration involves chemical uptake only through the respiratory and dermal surfaces of the organism. Log K_{OW} and bioconcentration factor (BCF) are parameters commonly used in bioaccumulation assessment (Arnot and Gobas, 2006). BCF is the ratio of the concentration of a chemical in an organism to the total or freely dissolved concentration of the chemical in the environmental medium (usually water) (Mackay and Fraser, 2000). On the basis of BCF, bioaccumulation is designated as very high

(BCF>5000), high (BCF 5000-1000), moderate (BCF<1000-100) and low (BCF<100) (USEPA, 2014b). The different regulatory criteria for bioaccumulation assessment based on log K_{OW} and BCF include UNEP (Stockholm Convention on POPs), log K_{OW}≥5 and BCF≥5000; EU REACH, BCFs ≥2000 (bioaccumulative) and ≥5000 (very bioaccumulative); US (Toxic Substances Control Act and Toxic Release Inventory), BCFs of 1000-5000 (bioaccumulative) and ≥5000 (very bioaccumulative), and Environment Canada [Canadian Environment Protection Act (1999)], log K_{OW}≥5 and BCF≥5000 (Arnot and Gobas, 2006).

There are few studies on the bioaccumulation of EE2 in river biota. Available studies are commonly laboratory-based tests on fish modelled on river system. Länge *et al.* (2001) reported BCFs of 610 and 660 for the fish, *Pimephales promelas* (fathead minnow) exposed to 16 ng/L (245 days) and 64 ng/L (158 days) EE2, respectively, in a flow-through system. Al-Ansari *et al.* (2010) studied bioaccumulation of EE2 in fish in St. Clair River, Canada and concluded that EE2 accumulates in wild fish based on the detection of EE2 in samples of *M*. *macrolepidotum*. However, in the study, the BCF of EE2 in fish was not calculated. BCF of 337 in *Carassius auratus* (goldfish) exposed to 150 ng/L EE2 for 72 h in a flow-through system was reported by Al-Ansari *et al*. (2013). Yang *et al.* (2014) did not detect EE2 in algae and the bile of fish (*C*. *carpio*) in their investigation of bioaccumulation of endocrine disrupting chemicals in the Pearl River Delta, China. Blewett *et al.* (2014) observed that tissue-specific patterns of EE2 accumulation vary among species of fish probably due to differences in physiology, particularly rate of metabolism and excretion among species. In addition, the authors reported that <50% EE2 accumulated in the liver and gallbladder among six species of teleost fish while >50% of the chemical accumulated in the carcass.

Bioaccumulation of PFOS is unique. Because PFOS is both hydrophobic and lipophobic, it does not usually, accumulate in adipose tissues of biota unlike most POPs. Rather, it is proteinophilic; liver and blood rich in proteins are the preferential repositories of PFOS in biota. PFOS has been shown to bind to albumin and β-lipoproteins in blood plasma and fatty acids in liver of biota (Jones *et al.*, 2003; UNEP, 2006; Conder *et al.*, 2008; Ng and Hungerbühler, 2013). Martin *et al.* (2003) reported BCFs of 3100, 2900 and 690 in the blood, liver and carcass of *O*. *mykiss*,

respectively, in 12 d exposure to 0.35 µg/L PFOS in a flow-through system. BCF of 856 was observed in whole body of *Lepomis macrochirus* (bluegill sunfish) exposed to 86 µg/L PFOS for 62 d in a flow-through system (Beach *et al.*, 2006). Exposure of *C*. *carpio* to 2 and 20 µg/L PFOS for 58 d resulted in BCF of 1300 and 720, respectively (Inoue *et al.*, 2012).

Characteristically, HBCD bioaccumulates in adipose tissues (Haukås *et al.*, 2009). Veith *et al.* (1979) reported a BCF of 18,197 in *P*. *promelas* exposed to 6.2 µg/L HBCD for 32 d in a flow-through aquarium. In *O*. mykiss exposed to 3.4 µg/L HBCD for 35 d in a flow-through system, a BCF of 8,974 (whole fish) was estimated (Hardy, 2004). HBCD exhibits stereoisomer-specific bioaccumulation. In the environment, the chemical can undergo stereoselectivity such as bioisomerization, leading to selective enrichment of α-, β- and γ-HBCD. This has been observed in the dominance of α-HBCD in biota in relation to the much dominant γ-HBCD in the technical HBCD formulation and environmental sediments (Law *et al.*, 2006; Du *et al.*, 2012). Preferential bioaccumulation of α-HBCD was studied by Zhang *et al.* (2014b). In a 30-d exposure of *C*. *carpio morpha moblis* (mirror carp) to 1 µg/L of α-, β- and γ-HBCD each, the authors reported differing BCF values for the different isomers in the fish tissues (gill, viscera, muscle and skin). The BCF ranged from 30700-45200 (α-HBCD), 1030-1440 (β-HBCD) and 950-1730 (γ-HBCD). The BCF values for α-HBCD were much higher than the values for β- and γ-HBCD in the fish.

Variations in the magnitude of reported BCF values for EE2, PFOS and HBCD in different biota indicate differences in the potentials of the chemicals to accumulate in river biota. The bioaccumulation potential of EE2 is moderate while that of PFOS is high. HBCD has very high potential for bioaccumulation.

2.6.5 Long-range environmental transport

Data on long-range transport potential of EE2 in the environment are lacking. PFOS has the potential to undergo long-range atmospheric transport to remote regions of the world (UNEP, 2006; Dreyer *et al.*, 2009). However, in the ionic form, PFOS will mainly be distributed in water due to the magnitude of its water solubility (Zhao *et al.*, 2012). HBCD has the potential for long-range transport. It has been found in the air, water, sediments and biota in the Arctic and other remote regions of the world

without demonstrable existing sources of emission due to atmospheric deposition in gaseous or particulate state (Remberger *et al.*, 2004; Covaci *et al.*, 2006; Yu *et al.*, 2008; de Wit *et al.*, 2010).

2.6.6 Ecotoxicity

2.6.6.1 Ecotoxicity of EE2

2.6.6.1.1 Toxicity to algae

Generally, aquatic plants (including algae) seem to be relatively tolerant to EE2 (Nagpal and Meays, 2009). There are limited data on the toxicity of EE2 to algae. However, the few available data are based on laboratory toxicity tests on freshwater (including river) species of algae. Exposure of the green algae, *Pseudochirchneriella subcapitata* (= *Selenastrum capricornutum*) and *Chlorella* sp. to 200 µg/L EE2 for 7 d did not have any effects on the growth (based on cell count), chlorophyll fluorescence (indicator of photosynthesis), cell size and cell complexity of the organisms (Wang *et al.*, 2013). However, sensitivity of algae to EE2 at a higher concentration of EE2 has been observed. de Sá Salomão (2014) reported 96 h EC_{50} (median effective concentration) of 800 and 730 µg/L EE2 for *P. subcapitata* and *Desmodesmus subspicatus* (= *Scenedesmus subspicatus*), respectively, based on growth inhibition. In their study, the same authors estimated 96 h NOEC (no observed effect concentration) of <10 µg/L EE2 each for *P. subcapitata* and *D. subspicatus*, based on growth rate.

2.6.6.1.2 Toxicity to invertebrates

Studies have shown that EE2 is toxic to aquatic invertebrates such as crustaceans, insects, molluscs and rotifers (Hutchinson, 2002; Segner *et al.*, 2003; Caldwell *et al.*, 2008; Souza *et al.*, 2013). 21 d NOEC of ≥100000 ng/L EE2 for reproduction in *Daphnia magna* has been reported (Caldwell *et al.*, 2008).

2.6.6.1.3 Toxicity to fish

Evaluations of the toxicity of EE2 to fish or other vertebrates in rivers are commonly laboratory-based chronic toxicity tests. The toxic effects of EE2 on aquatic vertebrates, particularly fish, include endocrine disruption, reduction in fertility and

egg production, alteration of liver and gonadal indices, feminization of males and alteration of sex ratio (Rose *et al.*, 2002; Nagpal and Meays, 2009; Caldwell *et al.*, 2012; Pérez *et al.*, 2012). Exposure of fish to 0.1-10 ng/L EE2 has been reported to cause vitellogenin induction and histological alterations such as occurrence of testicular oocytes and feminization of males in fish (Nagpal and Meays, 2009; Al-Ansari *et al.*, 2010; Pérez *et al.*, 2012).

Induction of plasma vitellogenin (VTG) in fish due to exposure to EE2 in rivers has been observed to cause incidence of intersex fish with feminized reproductive organs (Jobling *et al.*, 1998; Jobling *et al.*, 2006). Intersexuality in fish populations may lead to reduction in the fertility of affected fish (Jobling *et al.*, 2002). Pawlowski *et al.* (2004) reported 21 d (LOEC) lowest observed effect concentration of 1 ng/L EE2 for induction of VTG in *P*. *promelas*. Xu *et al.* (2008) studied the effect of EE2 on reproduction in *D*. *rerio* exposed to 0.4, 2 and 10 ng/L concentrations of EE2 for 3 months. The authors reported induction of VTG at 21 days post-hatch (dph) fish at 10 ng/L EE2. In fish exposed to 2 and 10 ng/L EE2, at 21 dph, increased mortality and sex ratio were observed. At 180 dph, malformation of sperm duct and reduction in sperm number were observed in males at 2 and 10 ng/L EE2 exposure.

2.6.6.2 Ecotoxicity of PFOS

2.6.6.2.1 Toxicity to algae

The toxicity of PFOS to algae has been studied in few species of algae in laboratory-based tests. PFOS has been reported to cause growth inhibition (reduced cell density) in the freshwater algae, *P*. *subcapitata*, *Scenedesmus obliquus* (green alga), *Chlorella vulgaris* (green alga), *Anabaena flos-aque* (blue-green alga) and *Navicula pelliculosa* (diatom). The 96 h EC_{50} and 96 h NOEC for growth inhibition in *P*. *subcapitata* by PFOS were observed to be 71 and 44 mg/L, respectively, in the study by OECD (2002). However, Boudreau *et al.* (2003) reported 96 h IC_{50} (median inhibitory concentration) of 48.2 mg/L and 96 h NOEC of 5.3 mg/L PFOS for growth inhibition in *P*. *subcapitata*. Boltes *et al.* (2012) reported 72 h EC_{50} of 35 mg/L based on growth inhibition in *P*. *subcapitata* while Liu *et al.* (2008) observed 72 h IC_{50} of 77.8 mg/L for growth inhibition in *S*. *obliquus*. In *C*. *vulgaris*, 96h IC_{50} of 81.6 mg/L and 96 h NOEC of 8.2 mg/L PFOS for growth inhibition was reported by Boudreau *et*

al. (2003). OECD (2002) also reported 96 h EC_{50} of 176 and 305 mg/L PFOS for growth inhibition in *A*. *flos-aque* and *N*. *pelliculosa*, respectively. The same authors also reported 96 h NOEC of 94 and 206 mg/L PFOS for growth inhibition in *A*. *flos-aque* and *N*. *pelliculosa*, respectively.

The reported 96 h EC_{50} values of PFOS for growth inhibition in freshwater algae range from 71-305 mg/L. Also, the 96 h NOEC values determined for growth inhibition range from 5.3-206 mg/L PFOS. Among freshwater algae, *P. subcapitata* appears to be the most sensitive to PFOS exposure while *N*. *pelliculosa* is probably the least sensitive, based on reported NOEC values for growth inhibition. Variations in toxicity endpoint values of PFOS for the same species of alga may be due to differences in algal growth media, test protocols and endpoint estimation methods used (OECD, 2002; Boudreau *et al.*, 2003).

2.6.6.2.2 Toxicity to invertebrates

Laboratory-based studies on acute (short-term) and chronic (long-term) exposures of daphnids to PFOS have indicated adverse effects of PFOS on the mobility, survival, growth and reproduction of daphnids. The toxicity of PFOS has been investigated in two sensitive species of daphnids, *D. magna* and *D*. *pulicaria*. In the toxicity, the most sensitive endpoint appears to be immobility with acute (48 h) NOEC of 0.8 mg/L determined for *D*. magna by Boudreau *et al.* (2003). The same authors determined 48 h LC_{50} (median lethal concentration) of 112 mg/L for immobility of *D*. *magna*. In static toxicity test, the lowest 48 h EC_{50} value of 27 mg/L, based on immobility was determined for *D*. *magna* (OECD, 2002). Boudreau *et al.* (2003) also reported 48 h LC_{50} of 130 mg/L for inhibition of survival in *D*. *magna* exposed to PFOS. Li (2009) observed significant mortality (50%) in *D*. *magna* at 63 mg/L PFOS in a 48 h exposure. The author also reported 48 h NOEC of 20 mg/L for the same endpoint. 48 h EC_{50} values of 37.36 mg/L (Ji *et al.*, 2008) and 210 mg/L (OECD, 2002) for mortality of *D*. *magna* have also been determined.

Sanderson *et al.* (2004) reported chronic (21 d) LOEC value of 50 mg/L for inhibition of survival of *D*. magna exposed to PFOS. For survival of *D*. *magna*, 21 d NOEC values ranging from 5-25 mg/L PFOS have been determined (OECD, 2002; Sanderson *et al.*, 2004; Li, 2010). OECD (2002) determined a 21 d NOEC of 12

mg/L for inhibition of growth or reproduction in *D*. *magna* and concluded that PFOS had no effects on reproduction at the highest concentration that had no effect on survival. However, lower 21 d NOEC values of 1.25 mg/L (Ji *et al.*, 2008) and 10 mg/L (Li, 2010) for inhibition of reproduction in *D*. *magna* have been observed. Though there may be uncertainties about the reliability of some endpoint values, probably due to discrepancies in test protocols and other parameters, there is need for further investigation on the relative sensitivity of reproduction, survival and mortality of *D*. *magna* to PFOS exposure.

In the evaluation of the toxicity of PFOS to *D*. *pulicaria*, Boudreau *et al.* (2003) reported 48 h NOEC value of 13.6 mg/L, based on immobility. In the same study, a 48 h LC_{50} of 169 mg/L for inhibition of survival was determined. Sanderson *et al.* (2004) reported a 21 d LOEC of 13 mg/L and 21 d NOEC of 6 mg/L for survival of *D*. *pulicaria* exposed to PFOS. On the basis of higher NOEC value for mobility in *D*. *pulicaria* compared to *D*. *magna*, it is most likely that *D*. *magna* is more sensitive than *D*. *pulicaria* to PFOS exposure. Data on the effects of PFOS on growth and reproduction in *D*. *pulicaria* are lacking.

2.6.6.2.3 Toxicity to fish

A number of studies on the acute and chronic toxicity of PFOS to fish have been carried out. PFOS exhibits moderate acute toxicity to fish (UNEP, 2006) with an estimated lowest observed 96 h LC_{50} of 2.5 mg/L in a study of exposure of adult *D*. *rerio* to PFOS (Sharpe *et al.*, 2010). Sharpe *et al.* (2010) also reported maternal transfer of PFOS to embryos in the *D*. *rerio*. In studies of 96 h acute toxicity of PFOS to *P*. *promelas*, *L*. *macrochirus* and *O*. *mykiss* in freshwater media, the LC_{50s} determined for mortality were 4.7, 7.8 and 22 mg/L PFOS, respectively (OECD, 2002). In addition, 96 h NOEC of 4.5 mg/L for survival was determined for *L. macrochirus* by the same author.

In chronic toxicity studies, several effects of PFOS in different species of fish have been reported. In *P*. *promelas* early life-stages, 42 d NOEC of 0.30 mg/L was determined, based on survival and growth inhibition (OECD, 2002). The low value of

the concentration endpoint suggests that the early life-stages of *P*. *promelas* are highly sensitive to PFOS exposure. Ankley *et al.* (2005) studied the effect of exposure of sexually mature *P*. *promelas* to 0.03, 0.1, 0.3 and 1.0 mg/L of PFOS. The 21 d EC_{50} for fecundity was determined as 0.23 (0.19-0.25) mg/L PFOS. There were histopathological alterations in the ovaries of the adult female fish. For insignificant mortality of *L*. *macrochirus* in PFOS exposure, a 62 d NOEC of 0.086 mg/L was determined by OECD (2002). Inhibition of growth, impairment of reproductive organs, the disruption of the endocrine and alteration of sex ratio in chronic exposure of *D*. *rerio* to PFOS have been studied. Wang *et al.* (2011b) reported the suppression of growth of 8 h post-fertilization *D*. *rerio* at 0.25 mg/L PFOS on exposure to 0.005, 0.05 and 0.25 mg/L PFOS for 5 months. They also reported that male gonad development was impaired in a dose-dependent pattern, and that the sex ratio was altered with predominance of female in the group with high dose of PFOS. In *D*. *rerio* fry (14 d post-fertilization) exposed to 0.01, 0.05 and 0.25 mg/L PFOS via water for 70 d followed by 30 d recovery in clean water, hepatic VTG gene expression was significantly up-regulated in both sexes at 0.01-0.25 mg/L without mortality and alteration of sex ratio (Du *et al.*, 2009). In contrast to the findings by Wang *et al.* (2011b), it seems that alteration of sex ratio in *D*. *rerio* exposed to PFOS depends on the duration of exposure and age of the fish. Apparently, the longer the duration of exposure and the younger the developmental stage of the fish, the greater the sensitivity to PFOS exposure in relation to alteration of sex ratio. Shi *et al.* (2008) investigated developmental toxicity and alteration of gene expression in *D*. *rerio* embryos (4 h post-fertilization) exposed to 0.1, 0.5, 1.0, 3.0 and 5.0 mg/L PFOS for 132 h (5.5 d). Growth (body length) was reduced at 3.0 and 5.0 mg/L while the frys exhibited developmental abnormalities (epiboly deformities, hypopigmentation, yolk sac edema, tail and heart malformations, swim bladder inflation and curved spines) at ≥1.0 mg/L PFOS. The study also showed disruption of thyroid development at 0.1-1.0 mg/L PFOS. Shi *et al.* (2009) also reported disruption of thyroid hormone status in *D*. *rerio* larvae (2 h post-fertilization) at 0.2 and 0.4 mg/L PFOS exposures for 15 d. The triiodothyronine levels were significantly increased following exposure to PFOS.

Xiphophorus helleri (swordtail fish) males exposed to PFOS (0.1, 0.5 and 2.5 mg/L) for 21 d, and then transferred to clean water for recovery in 7 d, showed that PFOS

has estrogenic effect, evidenced by inhibition of and up-regulation of VTG mRNA expression in the fish (Han and Fang, 2010). In female *X*. *helleri* exposed to the same range of concentrations of PFOS, but for a longer duration of 42 d, elevation of gonadal somatic index (GSI) (gonad weight as a percentage of whole body weight) was observed in the 0.5 mg/L PFOS. In the juveniles (20-30 d old) exposed to 0.1 mg/L PFOS for 90 d, there was increased hepatosomatic index (HSI) or liver somatic index (LSI) (liver weight as a percentage of whole body weight) and impairment of growth (Han and Fang, 2010). Hagenaars *et al.* (2008) studied the toxicity of PFOS to *C. carpio*) exposed to 0.1, 0.5 and 1.0 mg/L PFOS for 14 d and reported lowered HSI and disruption of liver metabolism in the fish. In similar studies by Oakes *et al.* (2005) in which juveniles (16.4 cm body length, 22.7 g body weight) and adult females (34.8 cm body length, 511 g body weight) of *O*. *mykiss* were subjected to 12 d 3.0 mg/L and 14 d 1.0 mg/L PFOS exposures, respectively, the LSI was lowered in both juveniles and the adult females. In addition, testosterone and VTG levels were observed to be significantly elevated in the juveniles (both males and females), but were unaffected in the adult females. Induction of VTG in fish in response to xenobiotics exposure varies with maturity and species (Nicolas, 1999). Increased LSI observed in fish exposed to contaminants is often caused by hyperplasia (increase in cell number) or hypertrophy (increase in cell size) as an adaptive response of the liver to detoxification of xenobiotics (Oakes *et al.*, 2005).

2.6.6.3 Ecotoxicity of HBCD

2.6.6.3.1 Toxicity to algae

There are limited data on the toxicity of HBCD to algae. Roberts and Swigert (1997) reported 72 h EC_{50} >0.0025 mg/L and 72 h LOEC >0.0025 mg/L for growth inhibition in *P*. *subcapitata*. This species of algae appears to be more sensitive to exposure to HBCD than PFOS. Apparently, inhibition of algal growth by HBCD, PFOS or EE2 implies impairment of autotrophic production in the aquatic environment. Algae form mostly the autochthonous food base in aquatic communities (Doi, 2009).

2.6.6.3.2 Toxicity to invertebrates

The toxicity of HBCD to daphnids has not been extensively studied. Acute flow-through toxicity of HBCD to *D*. *magna* was studied by Graves and Swigert (1997a) who measured immobility in the daphnid following 48 h exposure to HBCD. The EC_{50} was estimated as >0.0032 mg/L. In a 21 d flow-through toxicity test, LOEC of 0.0056 mg/L and NOEC of 0.0031 mg/L for reduced growth and survival or reproduction, respectively, in *D*. *magna* were determined by Drottar and Krueger (1998).The same authors reported significantly reduced body length, dry weight and number of young of *D*. *magna* in 21 d exposure to 0.11 mg/L HBCD. However the concentration endpoint was not determined. There are no data on the relative toxicity of α-, β- and γ-HBCD to daphnids. The reported toxicity endpoints data indicate that HBCD is more toxic to *D*. *magna* than PFOS and EE2.

2.6.6.3.3 Toxicity to fish

Acute toxicity of HBCD has been studied in few species of freshwater fish. Graves and Swigert (1997b) studied 96 h exposure of *O*. *mykiss to* 0.0015, 0.0022, 0.0032, 0.0046 and 0.0068 mg/L HBCD in a flow-through test. Mortalities or other effects were not observed in the exposed fish. However, Deng *et al.* (2009) reported oxidative stress and apoptosis, reduced survival and malformations in embryos of *D*. *rerio* exposed to 0.05, 0.1, 0.5 and 1.0 mg/L HBCD for 96 h. At ≥0.1 mg/L HBCD, growth of the larvae was significantly reduced. In a similar study, 96 h exposure of *D*. *rerio* embryos to 0.002, 0.01, 0.5 and 10 mg/L HBCD resulted in induction of oxidative stress with increased concentration of HBCD, using lipid peroxidation as biomarker (Hu *et al.*, 2009).

Studies on the chronic toxicity of HBCD in fish have shown both adverse effects and no effects of the chemical on fish species. Drottar *et al.* (2001) studied the effect of HBCD on the early life-stage of *O*. *mykiss*. Newly-fertilized eggs of the fish were exposed to 0.00043, 0.00085, 0.0017, 0.0034 and 0.0068 mg/L HBCD in 88 d flow-through test. The total exposure period included 27 d hatching period and 61 dph period. No significant effects on hatching success, swim-up by larvae, survival of larvae and fry, and growth were observed. Based on each of these, NOEC ≥0.0037

mg/L HBCD was determined. Similarly, in 940 day old *Platichthys flesus* (European flounder) exposed to HBCD via food and sediment, no effects on behaviour, survival, growth rate, relative weights of liver and gonad, the endocrine and the general health of the fish were observed. The nominal HBCD exposures were 0.0003-3.0 mg/g lipid (food) and 0.00008-8.0 mg/g TOC (sediment) (Kuiper *et al.*, 2007).

To assess the effects of HBCD on liver enzyme activity in *O*. *mykiss* using ethoxyresorufin-O-deethylase (EROD) activity and other liver activities as biomarkers, Ronisz *et al.* (2004) injected intraperitoneally, juveniles of *O*. *mykiss* with HBCD dissolved in fish peanut oil. The fish were given treatments of 50 mg HBCD/ kg body weight for 5 d and <500 mg HBCD/kg body weight for 28 d. The result indicated significant increase in LSI (~40%) and inhibition of EROD activity by HBCD in the 28 d exposure. 5 d exposure did not show significant changes in LSI, but indicated increased catalase activity. Increased LSI and induction of catalase suggested that HBCD may induce peroxisome, a negative hormonal response. However, the possibility of peroxisome proliferating activity of HBCD was further investigated by the authors without conclusions. Induction of hepatic enzymes based on increased activity of the biomarkers, EROD and pentaoxyresorufin-O-depentylase (PROD), and oxidative stress in *Gobiocypris rarus* (Chinese rare minnow) exposed to 0.1-0.5 mg/L waterborne HBCD for 42 d have also been reported (Zhang *et al.*, 2008). Like in PFOS, maternal transfer of HBCD to offspring in *D*. *rerio* exposed to HBCD via food for 42 d was reported by Nyholm *et al.* (2008). Legler (2008) reviewed the endocrine-disrupting effects of HBCD and other BFRs in fish and concluded that HBCD can potentially impact, particularly the thyroid system in fish. Palace *et al.* (2008) reported altered thyroid hormone status and changes in liver enzyme activity in juveniles of *O*. *mykiss* exposed (via food) to lipid-corrected concentrations of approximately 0.029, 0.012 and 0.023 mg/kg (29.14, 11.84 and 22.84 µg/kg) body weight α-, β- and γ-HBCD, respectively, for 56 d followed by a depuration (clearance) period of 112 d. There was significant increase in epithelial cell heights of the thyroids, indicating thyroid gland hypertrophy. In addition, detoxification processes of the liver were inhibited. In studies of exposure of juvenile *O*. *mykiss* to 0.005 mg/kg HBCD via food for 32 d, followed by oral dosing with thyroid hormone to examine tissue disposition over 14 d period, Palace *et al.* (2010) also reported alteration of thyroid hormone (thyroxine) metabolism in the fish. The

authors concluded that HBCD may interfere with the thyroid of fish by lowering iodine uptake by the thyroid or by increasing elimination rates of the thyroid hormone.

Generally, the lower concentration endpoints in the toxicity of HBCD to fish compared to PFOS, indicate that HBCD is more toxic to fish than PFOS. However, data on the toxicity of both chemicals to fish show that their toxicity may be influenced by concentration of chemical, the duration of exposure to fish, the species of fish, age and gender of fish. However, the studies lack consideration of DOC and other natural environmental factors which may impact on bioavailability and toxicity of the chemicals.

2.7 Natural organic matter in rivers

In rivers, natural organic matter (NOM) occurs as structurally complex substances derived from the decomposition and biotransformation of plant and animal matter from allochthonous (outside the water body) and autochthonous (within the water body) sources. NOM may be derived from the leaves of plants and runoff from soils in the terrestrial environment, and from the decomposition of dead bodies of organisms such as algae, macrophytes, microbes and animals within the water body (Fan *et al.*, 2001; Croue, 2004). DOM is the dissolved fraction of NOM (McDonald *et al.*, 2004). Sometimes, the terms, 'DOM' and 'DOC' are used interchangeably. However, DOC is operationally defined as the organic carbon component of DOM which can pass through 0.45 or 0.7 µm filter (Ritson *et al.*, 2014). NOM comprises humic substances and non-humic substances (for example, carbohydrates, proteins and transphilic acids). Non-humic substances which account for 20-40% of DOC are less hydrophobic than humic substances (Fan *et al.*, 2001). Humic substances (humic acid, fulvic acid and humins) comprise 50-90% of DOC (Janoš, 2003). Elementally, humic substances contain about 50% carbon, 4-5% hydrogen, 35-40% oxygen, 1-2% nitrogen and <1% sulphur and phosphorus (Linnik *et al.*, 2013). In natural aquatic environments, concentrations of DOC vary from <1 to >50 mg/L (Evans *et al.*, 2005). Humic substances may exhibit binding to organic contaminants with covalent bonding, hydrogen bonding, hydrophobic or van der Waals interaction

(Landrum *et al.*, 1984; Neale *et al.*, 2008) and influence their transport and bioavailability (Akkanen *et al.*, 2004).

Chapter Three: Materials and Methods

3.1 Materials used

3.1.1 Chemicals

3.1.1.1 Analytical standards

The analytical standards used were EE2 (≥98% purity; Sigma-Aldrich, UK), potassium PFOS (98% purity; Sigma-Aldrich, UK), HBCD (95% purity; Sigma-Aldrich, UK), potassium hydrogen phthalate (KHP) (≥99.95% purity; Sigma-Aldrich, UK) and dichlorodiphenyltrichloroethane (DDT - o,p'-DDT) (Sigma-Aldrich, UK).

3.1.1.2 Solvents

The following solvents were used: deionized water (Milli-Q, UK), double-distilled water (Brunel University London laboratory), methanol, acetonitrile and hexane (HPLC grade; Rathburn Chemicals Ltd, UK) and ethanol (absolute; Hayman Ltd, UK).

3.1.1.3 Reagents

The reagents used were purchased from Sigma-Aldrich, UK, unless otherwise stated. They were ammonium hydroxide solution (≥25% ammonia in water), formic acid (≥98% purity), humic acid (sodium salt, technical grade), hydrochloric acid (HCl) (2 M; Bernd Kraft GmBH), potassium dihydrogen phosphate (≥99% purity), ammonium sulphate (≥99% purity), potassium hydroxide (pellets, ≥85% purity), magnesium sulphate (anhydrous), iron (III) sulfate (hydrate, 97% purity), L-leucine (≥98.5% purity), L-histidine, adenine (≥99% purity), L-arginine (hydrochloride, ≥98.5% purity), L-methionine (≥99% purity), L-tyrosine (≥99% purity), L-isoleucine (≥98.5% purity), L-lysine (hydrochloride, ≥98.5% purity), L-phenylalanine (≥98.5% purity), L-glutamic acid (≥98.5% purity), L-valine (≥98.5% purity), L-serine (≥98.5% purity), L-aspartic acid (≥98% purity), L-threonine (≥99% purity), copper (II) sulfate (anhydrous, ≥99% purity), chlorophenol red-β-D-galactopyranoside (CPRG) (>96% purity; Roche, USA), thiamine (hydrochloride), pyridoxine (≥98% purity), D-pantothenic acid (hemicalcium salt), inositol, biotin (≥96% purity) and D-(+)-glucose (anhydrous, ≥99.5% purity).

3.2 Liquid chromatography-mass spectrometry analytical method

3.2.1 Preparation of stock solutions and serial dilutions of analytical standards

High purity EE2, PFOS and HBCD were used as standards in the chemical analysis of samples by liquid chromatography-mass spectrometry (LC-MS). Stock solutions of 1000 mg/L each for EE2, PFOS and HBCD were prepared. The stock solution of EE2 was prepared by weighing 10 mg EE2 in a weighing foil on a microbalance (0.1 mg sensitivity; Presica, UK) and dissolving it in 10 ml methanol in a 20 ml volumetric flask. The flask was covered with the lid, shaken manually to dissolve the chemical before transferring the content to a clean glass bottle which was covered and stored in the refrigerator at 4 ^{o}C. Stock solutions of PFOS and HBCD were prepared by dissolving 100 mg PFOS or HBCD in 100 ml methanol in a 100 ml volumetric flask, using the same procedure for EE2. The chemicals were also stored in the refrigerator at 4 ^{o}C. For each chemical, the stock solution was serially diluted in methanol with volumetric flasks to achieve the concentration range used in LC-MS analyses. All the glass bottles used to store the three chemicals in varying concentrations were labelled to reflect name and concentration of chemical, solvent used, date of preparation and name of researcher.

3.2.2 LC-MS

The LC-MS system comprised of a triple quadruple MS with Turbo Ionspray interface (API 365, Sciex, Canada), coupled to a LC system. The LC consisted of solvent degasser, pump and column oven (Series 1050, Hewlett Parkard) and an autosampler (Series 200, Perkin Elmer). Two chromatography columns were used during the work. For the multi-residue method initially used, an Ascentis C18 column (10 cm × 2.1 mm, 2.7 µm particle size) (Sigma-Aldrich, UK) was used. For single compunds an ACE C18 column (10 cm x 2.1mm, 3.0 µm particle size (Hichrom Ltd, UK) was used. Methanol, acetonitrile and deionized water with ammonium hydroxide solution (30 mM) and formic acid (20 mM) at a pH of 7.4, constituted the mobile phase used. This is similar to mobile phases used by others to enhance ionization (Gros *et al.*, 2012; Petrović *et al.*, 2014).

Calibration standards (EE2, PFOS and HBCD) and samples were prepared in appropriately labelled vials (2 ml, Chromacol Ltd, UK) with caps sealed by means of crimper. Vials were serially arranged in the rack of the autosampler before analysis and the first run was always repeated. The MS was equilibrated for a minimum of 20 min to stabilize it. Samples were analysed with injection volume of 50 µl each at a flow rate of 0.2 µl/min and varying gradients of the mobile phase (Table 3.2). The MS was operated in electrospray ionization (ESI) negative mode due to the nature of the ionization of EE2, PFOS and HBCD. The ionization parameters, nebulizer gas (NEB), curtain gas (CUR), ionspray voltage (IS) and temperature were optimized at 12 psi, 8 psi, -4200 V and 400 ^{o}C, respectively. Air was used as the NEB while nitrogen was used as the CUR. The parent ions of the analytes with the respective values of their optimized declustering potential (DP), focusing potential (FP) and entrance potential (EP) are shown in Table 3.1. Identification of analytes was done by matching ionic masses of parent compound with retention time. Quantitation of anaytes was done with the use of Analyst Software 1.42 (MDS Sciex, Applied Biosystems). It involved calibration for linearity.

Table 3.1 Instrument settings for LC-MS

Analyte	Parent ion (*m/z*)	DP (V)	FP (V)	EP (V)
EE2	295.4	-45	-260	-10
PFOS	498.9	-30	-150	-3.5
HBCD	640.8	-3	-130	-10

m/z, mass-to-charge ratio; V, Volts

3.3 Determination of the occurrence of EE2, PFOS and HBCD in wastewater effluent

Wastewater effluent samples were collected from eight sites of WWTPs in the UK from 20-30 June 2011 to determine the concentrations of EE2, PFOS and HBCD. The sampling sites comprised of four trickling filter sites (TFS1, TFS2, TFS3 and TFS4) and four activated sludge sites (ASS1, ASS2, ASS3 and ASS4), so code-named because of confidentiality requirement. Samples were collected with a steel bucket connected to a long pole and then transferred by means of a glass funnel into

2.5 L dark brown Winchester glass bottles pre-rinsed with methanol and deionized water. Capped bottles containing the samples were transported to the laboratory at Brunel University London on the same day of collection for filtration and extraction.

The samples were filtered with glass microfiber filters (1.2 µm pore size; Whatman GF/C) on the day of collection to avoid possible modification of samples. Solid-phase extraction (SPE) involving the use of Oasis HLB cartridges (6 ml, 200 mg sorbent; Waters Ltd, UK) was carried out on the same day. Appropriately labelled cartridges were mounted on a vacuum manifold (Agilent Technologies, USA) with the capacity for 20 cartridges simultaneously. Each cartridge was sequentially pre-conditioned with 5 ml methanol followed by 5 ml deionized water. At a flow rate of 20 ml/min, 1 L of each effluent sample in pre-cleaned, labelled, 1 L measuring glass cylinder was passed through the column of the cartridge by means of pre-cleaned (with methanol and deionized water), labelled, transparent plastic tubes connected to the cartridges. After passing the samples through the cartridge completely, the cartridges were dried for 2 h each, wrapped in foil and put in re-sealable plastic bags. They were stored in a freezer at -18 ^{o}C until analysed in May 2012 following full validation of LC-MS analytical methods. There is evidence of stability of EE2 when extracted onto SPE cartridges and stored under these conditions (Koh *et al.*, 2009).

The analytes were eluted from each cartridge with 5 ml of a mixture of acetonitrile and methanol (1:1, v/v) into appropriately labelled polypropylene tubes placed in a rack inside the manifold. The extract in uncapped polypropylene tube was evaporated in a rotary evaporator (miVac Quattro) for 50 min and reconstituted in 1 ml of acetonitrile (for HBCD) or a mixture of acetonitrile and water (1:1, v/v) (for PFOS) before analysis by LC-MS method. Chemical analysis of EE2 was done as part of the Chemicals Investigation Programme (CIP) by UK Water Industry Research (UKWIR) because the sensitivity of the LC-MS used at Brunel University London was not sufficient to detect EE2 at environmentally relevant concentrations. PFOS and HBCD were analysed at Brunel University London.

3.4 Chemical determination of bioavailability

3.4.1 Sorption Study

3.4.1.1 Determination of TOC content of Aldrich humic acid

3.4.1.1.1 Analytical method used

A personal computer (PC)-controlled total organic carbon (TOC) analyzer (TOC-V_{CPN}, Shimadzu Corporation, Japan) was used in the measurement of TOC of humic acid samples. The TOC analyzer used a platinum 680 ^{o}C combustion catalytic oxidation method with detection range of 0.004-25000 mgC/L. The instrument automatically created a calibration curve from a range of concentrations of standard solution of KHP used. Sample acidification with 2M HCl to lower the pH to 2 prior to analysis, and sparging were also automatically done by the instrument. Acidified samples were sparged with oxygen (compressed air) connected to the instrument to remove total inorganic carbon (TIC) as carbon dioxide. Since total carbon (TC) is the aggregate of TOC and TIC, the instrument measured TOC of samples as non-purgeable organic carbon (NPOC) after the purging (stripping) of TIC.

3.4.1.1.2 Preparation and analysis of samples

To ensure humic acid was not retained on SPE cartridges following Landrum *et al*. (1984) method, the TOC content of Aldrich humic acid was measured before and after SPE. A stock solution of 1000 mgC/L KHP standard was prepared from 2125 mg KHP in 1 L deionized water, using a volumetric flask. The solution was stored in capped 250 ml glass bottle kept in a dark cupboard in the laboratory. For sample analysis, the stock solution of the standard was serially diluted to give a range of concentrations: 0.0 1.0, 5.0 and 10.0 mgC/L, using cleaned volumetric flasks. 20 ml of each concentration was transferred to cleaned, appropriately labelled 40 ml vials (borosilicate glass; Fisher Scientific, UK). A stock solution of 10000 mg/L humic acid was prepared from 5000 mg humic acid in 500 ml deionized water. After thorough mixing, the solution was transferred into a 1 L volumetric flask, sealed and stored a dark cupboard in the laboratory. 100 ml of each humic acid sample was prepared from serial dilutions of the stock solution to give concentrations of 5.0, 10.0 and 20.0 mg/L humic acid in deionized water, using cleaned volumetric flasks. 20 ml of each

sample was put in appropriately labelled 40 ml vials for TOC analysis. Before TOC analysis, the pH of the humic acid samples was measured using a pH meter (Delta 350, Mettler). Determination of the TOC of the humic acid samples was done before and after SPE to check for possible differences in TOC values. The samples for SPE were passed through the columns of cartridges (3 ml, 500 mg sorbent; C18, Sep-Pak), using SPE procedure. The cartridges were preconditioned with 120 ml of deionized water each to prevent addition of the carbon content of sorbents to the samples. The samples were passed to the column with glass funnel and there was no elution or evaporation of samples since humic acid was used alone. The filtrates were simply analysed for TOC after SPE. Blanks (deionized water) were analysed for TOC along with samples for quality control.

To start the analysis, a calibration curve was first generated automatically by the instrument, using the calibration points selected and the concentrations of standard solutions prepared. NPOC analysis was manually selected. The standard solutions in sealed vials were serially arranged in a rack and connected to the 8-port sampler of the instrument by means of flow lines passing through their lids. After manually creating a method for the analysis, the calibration curve file was incorporated in the method. The injection parameter, sparge time was optimized at 3 min in order to purge all the inorganic carbon. Analysis of each sample in triplicates was also selected. The humic acid samples in sealed vials were serially arranged in a rack and anaysed the same way as the standard solutions. The instrument automatically generated the results of the TOC analysis, indicating NPOC values with mean and standard deviation. Nitrogen was used as the carrier gas at 130 psi.

3.4.1.2 Determination of the impact of humic acid on bound and freely dissolved fractions of EE2, PFOS and HBCD in deionized water

3.4.1.2.1 Experimental design

Two sets of experiments were designed in the study (Appendix 1-7). The first set comprised range-finding experiments used to determine the concentrations of EE2, PFOS or HBCD needed to measure sorption. This involved the determination of bound and freely dissolved fractions of constant concentrations of EE2, PFOS or

HBCD at varying concentrations of humic acid. The second set involved the measurement of bound and freely dissolved fractions of varying concentrations of EE2 or HBCD at constant concentration of humic acid to determine the partition constant of EE2 or HBCD. PFOS was not included in the second set of experiments due to its poor retention in the SPE cartridge column. In both sets of experiments, Landrum *et al.* (1984) method was used.

Each experimental unit was in triplicates. Constant concentrations of 5 mg/L EE2, 5 mg/L PFOS and 50 µg/L HBCD each at varying concentrations (0, 1, 10 and 100 mg/L) of humic acid were used in the first set of experiments. In this set of experiments, the controls had only EE2, PFOS or HBCD treatments. In the second set of experiments, constant concentrations of 10 and 100 mg/L humic at varying concentrations of 0.1-0.5 mg/L and 1.0-5.0 mg/L EE2, respectively, were used. In addition, 10 and 100 mg/L humic each at 5-50 µg/L HBCD were used. In the controls of the second set of experiments, the factors tested were varied. Water solubility and the limit of the analytical method used were considered in the selection of the concentrations of EE2, PFOS and HBCD used in the experiments.

3.4.1.2.2 Preparation and chemical analysis of samples

A mixture of EE2, PFOS or HBCD and humic acid in 100 ml of deionized water in pre-cleaned 250 ml conical flask was prepared to reflect concentrations of the chemicals specified in the experimental designs. The control experiments only had specific concentrations of EE2, PFOS, HBCD or humic acid in 100 ml deionized water. Each conical flask containing prepared sample was sealed with foil, gently shaken manually for 10 sec and equilibrated for 18 h at 4 ^{o}C in the refrigerator to allow binding of the analytes to humic acid. After equilibration, the pH of each sample was measured with a pH meter. Before extraction, each SPE cartridge (3 ml, 500 mg sorbent; C18, Sep-Pak) was preconditioned with 120 ml deionized water. 10 or 20 ml of each sample was extracted on the cartridges at a flow rate of 10 ml/0.5 min. Glass funnels were used to pass the sample to the cartridge column. Drying of cartridges, elution, evaporation and reconstitution of sample extracts were done using the procedure previously described in section 3.3. Sample extracts for EE2 or PFOS were reconstituted in I ml of a mixture of acetonitrile and water (1:1, v/v) while 1 ml of acetonitrile was used for HBCD because of the relatively low water solubility.

Chemical analysis of samples and quantitation of analytes involved the use of the LC-MS method previously described. The type of samples analysed, the column and the mobile phase used with gradients are presented in Table 3.2. The ion chromatograms for the analytes are shown in Figure 3.1-3.3.

The partition coefficient, K_{DOC} (dissolved organic carbon-water partition coefficient) for EE2 and HBCD was calculated using the following equation (Wang *et al.*, 2011):

$K_{DOC} = C_{DOC}/C_{free}$. [DOC]

Where C_{DOC}, concentration of chemical bound to DOC

C_{free}, concentration of chemical freely dissolved

DOC, dissolved organic carbon

The K_{DOC} values were expressed in L/kg.

Table 3.2 Mixtures of EE2, PFOS or HBCD and humic acid with LC-MS column, mobile phase and gradient used in their analyses

LC-MS sample	Column	Mobile phase	Gradient
EE2 5.0 mg/L or PFOS 5.0 mg/L or HBCD 50.0 µg/L and humic acid 0-100 mg/L	Ascentis C18	A (aqueous) B (methanol) C (acetonitrile)	0-2 min (60% A, 20% B, 20% C), 2-15 min (80% A and 40% B) and 15-20 min (5% A and 95% B)
EE2 0.1-0.5 mg/L and humic acid 10.0 mg/L	ACE 18	A (aqueous) B (methanol)	Isocratic (25% A and 75% B) 8 min run
EE2 1.0-5.0 mg/L and humic acid 100.0 mg/L	ACE 18	A (aqueous) B (methanol)	Isocratic (25% A and 75% B) 8 min run
HBCD 5.0-50.0 µg/L and humic acid 10.0 or 100.0 mg/L	ACE 18	A (aqueous) B (methanol) C (acetonitrile)	Isocratic (5% A, 5% B and 90% C) 5 min run

Isocratic = no gradient

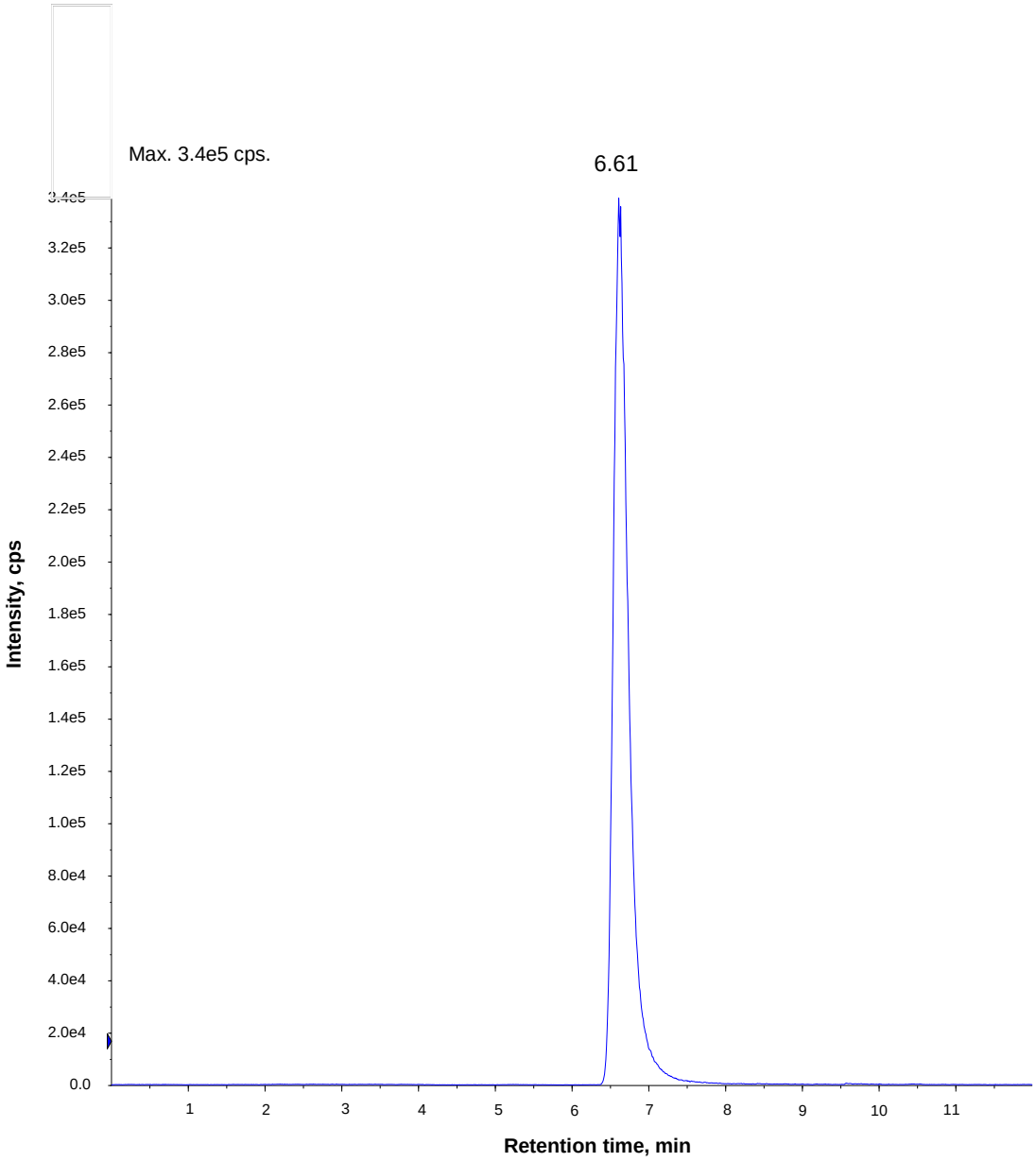


Figure 3.1 Ion chromatogram for EE2

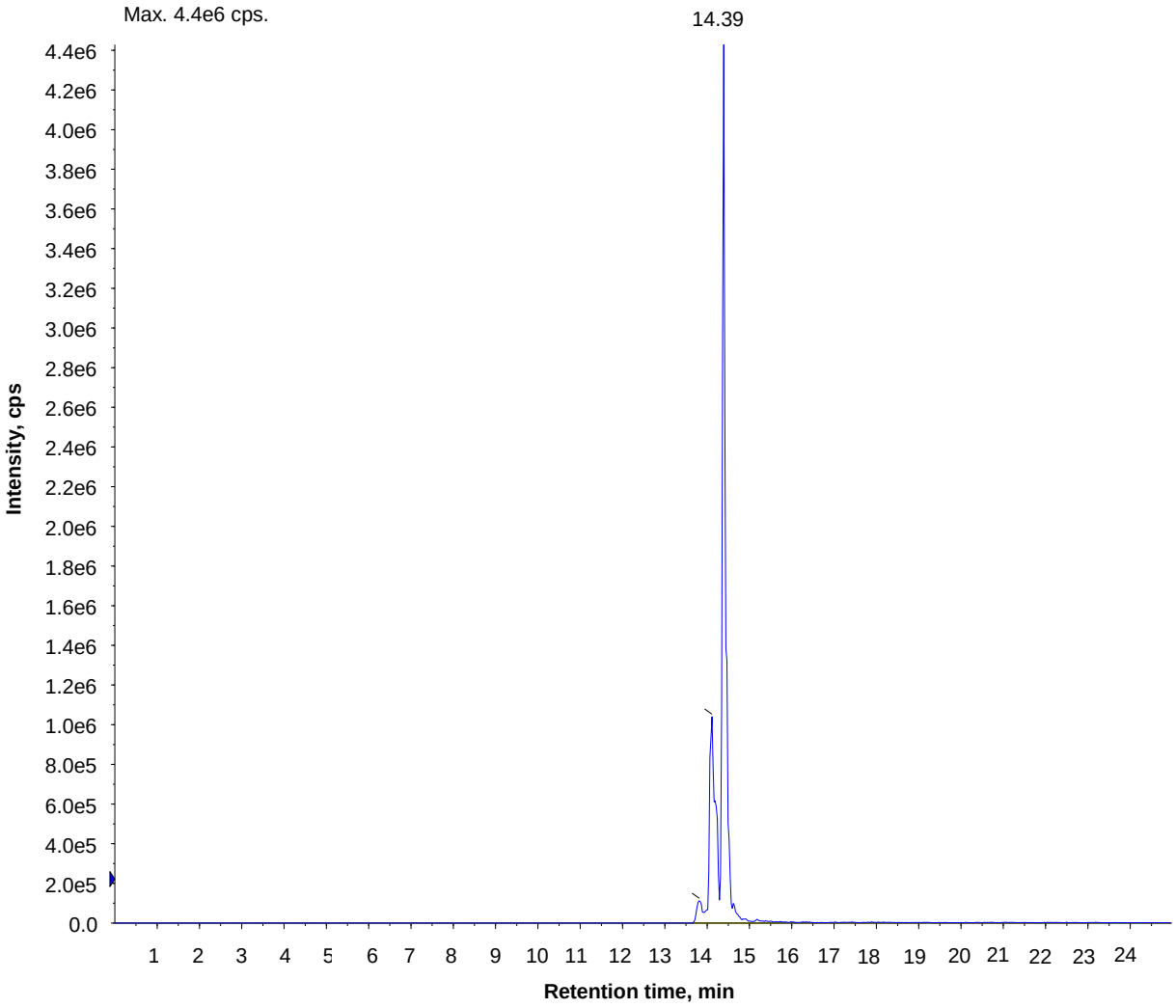


Figure 3.2 Ion chromatogram for PFOS

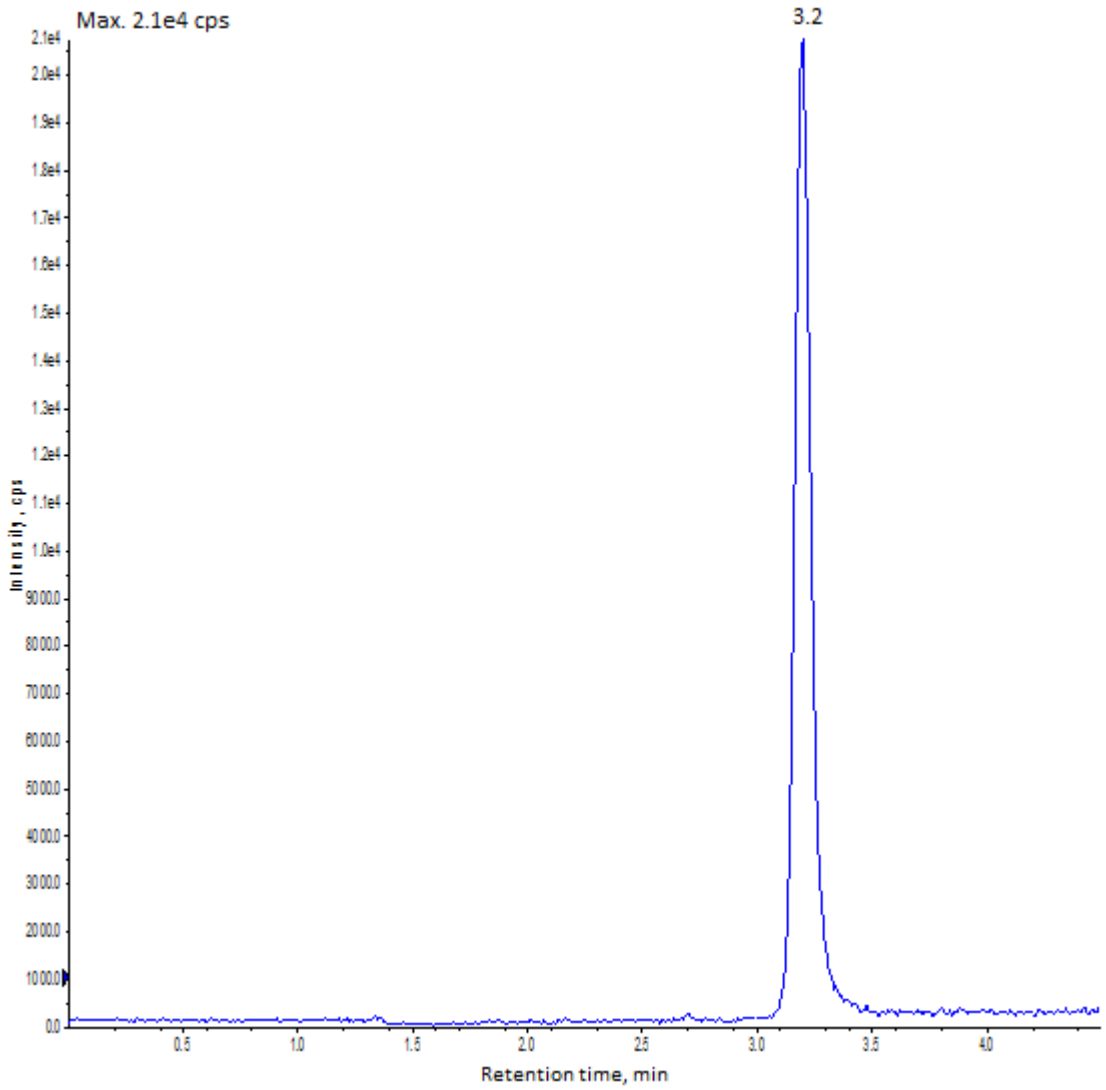


Figure 3.3 Ion chromatogram for HBCD

3.5 Biological determination of bioavailability

3.5.1 Yeast estrogen screen assay

3.5.1.1 Assay method

The yeast estrogen screen (YES) assay method described by Routledge and Sumpter (1996) was modified and used as an endpoint to determine the chemical that was bioavailable. In the method, a genetically modified strain of yeast, *Saccharomyces cerevisiae* with the deoxyribonucleic acid (DNA) sequence of human estrogen receptor (hER) incorporated into its genome was used as the test organism. The modifications to the original method were the inclusion of humic acid solution in the assay medium and the equilibration of mixtures of EE2 or DDT and humic acid in assay plates for 18 h before yeast exposure.

3.5.1.2 Preparation of components of medium

The reagents, minimal medium (pH 7.1), glucose solution, L-aspartic acid solution, vitamin solution, L-threonine solution, copper (II) sulfate solution and CPRG used in the assay medium were prepared using the procedure used and described by Routledge and Sumpter (1996). Preparation of the minimal medium was done by adding 13.61 g potassium dihydrogen phosphate (KH_2PO_4), 1.98 g ammonium sulphate [$(NH_4)_2SO_4$], 4.2 g potassium hydroxide (KOH), 0.2 g magnesium sulphate ($MgSO_4$), 1 ml iron (III) sulphate [$Fe_2(S0_4)_3$] solution (40 mg/50 ml double-distilled water), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine and 375 mg L-serine to 1 L double-distilled water in a sterilized glass beaker. The beaker was placed on a stirrer to dissolve the components of the medium. 45 ml aliquots of the minimal medium were dispensed into 150 ml glass bottles each, sterilized at 121 ^oC for 10 min and stored at room temperature.

A 20% w/v solution of D-(+)-glucose was prepared. 20 ml aliquots of the glucose solution in glass bottles were sterilized at 121 ^oC for 10 min and stored at room temperature. A stock solution of L-aspartic acid was prepared at 4 mg/ml in double-distilled water. 20 ml aliquots of the solution in glass bottles each were sterilized at

121 ^{o}C for 10 min and stored at room temperature. Preparation of vitamin solution was done by adding 8 mg thiamine hydrochloride, 8 mg pyridoxine, 8 mg D-pantothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml double-distilled water) to 180 ml double-distilled water in a sterile 250 ml conical flask. To achieve further sterilization, the solution was passed through a 0.2 µm pore size filter (Whatman) in a laminar air flow cabinet, and 10 ml aliquots were dispensed into sterile glass bottles and stored in the refrigerator at 4 ^{o}C. A stock solution of L-threonine was prepared at 24 mg/ml double-distilled water, sterilized at 121 ^{o}C for 10 min in 10 ml aliquots in glass bottles each, and stored at 4 ^{o}C in the refrigerator. A solution of 20 mM (3.19 mg/ml) copper (II) sulfate in double-distilled water was prepared. The solution was sterilized by filtering through a 0.2 µm pore size filter (Whatman) in a laminar flow cabinent, and 5 ml aliquots were dispensed into sterile glass bottles each and stored at room temperature. A stock solution of CPRG was prepared at 10 mg/ml in double-distilled water, put in sterile glass bottles and stored at 4 ^{o}C in the refrigerator.

3.5.1.3 Preparation of humic acid, EE2 and DDT

Prior to the assay, stock solutions of humic acid, EE2 and DDT were prepared. A stock solution of 4000 mg/L of humic acid (400 mg humic acid/100 ml deionized water) was prepared using sterile 250 ml conical flask, and serially diluted to 2000, 400 and 40 mg/L and stored in 150 ml sterile bottles away from light at room temperature. Before serial dilution, the stock solution of humic acid in sealed conical flask was shaken for 30 min at 200 rpm in an orbital shaker (PSU-10i, Grant-bio) to ensure thorough mixing. A stock solution of 500 µg/L of EE2 or $1x10^{7}$ µg/L DDT in absolute ethanol was prepared and stored in a sterile bottle. A volume of 120 µl of 500 µg/L EE2 or $1x10^{7}$ µg/L DDT was serially diluted in a 96-well microplate (Sarstedt, Germany) with 12 wells each in 8 rows (Figure 3.4), to give concentrations ranging from 500-0.244 µg/L EE2 or $1x10^{7}$-$4.88x10^{3}$ µg/L DDT from the 1st well to the 12th well as a result of 1:2 dilutions. The serial dilution was done by pipetting 120 µl of absolute ethanol to each well (from the 2nd to the 12th) in a row of the microplate inside a laminar air flow cabinet to reduce the formation of aerosol and prevent contamination. 120 µl of 500 µg/L EE2 or $1x10^{7}$ µg/L DDT was pipetted to the 1st well up to the 12th well in the same row containing absolute ethanol, and serially diluted

by mixing with the pipette five times in each well before transferring 120 µl to the next well sequentially, starting from the 2nd to the 12th well. During the serial dilution, the microplate was intermittently covered with the lid to prevent evaporation of the ethanol.

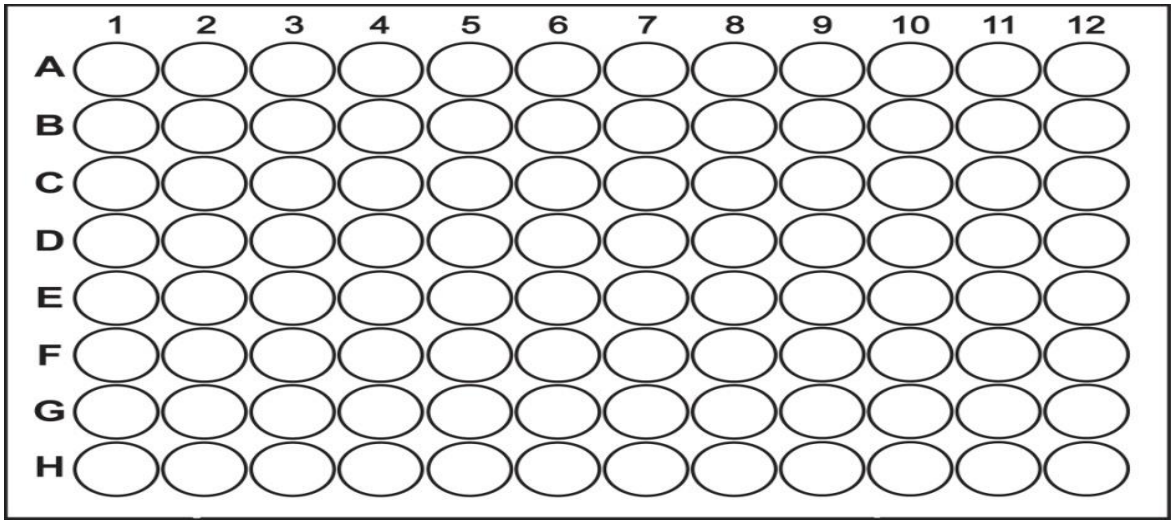


Figure 3.4 Design of 96-well microplate

Microplates were prepared for the equilibration of varying concentrations of humic acid with EE2 or DDT. The experimental units which were in triplicates each comprised treatments for a mixture of EE2 and deionized water or DDT and deionized water, and EE2 or DDT with I, 10, 50 or 100 mg/L humic acid each. However, DDT was not mixed with 1 mg/L humic acid. Deionized water and negative controls for humic acid at all concentrations used were also included in the experimental units. In addition to these, ethanol was used as a negative control for DDT.

Using a multi-channel pipette (Labsystems Titertek), 10 µl each of serially diluted EE2 or DDT was transferred to each well in the various units for EE2 or DDT and mixtures of EE2 or DDT and humic acid in the appropriately labelled equilibration microplates, and allowed to evaporate completely for 30 min. After evaporation, using a multi-channel pipette, 100 µl of deionized water in a sterile trough was transferred to each well in the units for deionized water, and deionized water with EE2 or DDT. Similarly, 100 µl of 40 mg/L humic acid was transferred to each well in

the units for 1 mg/L humic acid and a mixture of EE2 and 1 mg/L humic acid; 100 µl of 400 mg/L humic acid to each well for 10 mg/L humic acid and a mixiture of EE2 or DDT and 10 mg/L humic acid; 100 µl of 2000 mg/L humic acid to each well for 50 mg/L humic acid and a mixture of EE2 or DDT and 50 mg/L humic acid, and 100 µl of 4000 mg/L humic acid to each well for 100 mg/L humic acid and a mixture of EE2 or DDT and 100 mg/L humic acid. Each microplate was covered with the lid, sealed with autoclave tape, shaken for 2 min by means of titer plate shaker (Lab-Line Instruments) to ensure mixing, and equilibrated for 18 h at 4 ^{o}C in the refrigerator.

3.5.1.4 Yeast culture

A growth medium for a genetically modified yeast strain, *S. cerevisiae* (hER; Department of Genetics, Glaxo, UK), was prepared by pipetting 5 ml glucose solution, 1.25 ml aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution and 125 µl copper (II) sulfate solution each to 45 ml minimal medium in a 150 ml sterile glass bottle. The bottle was covered, shaken thoroughly to enhance mixing and then poured into a sterile 250 ml conical flask. 125 ml of thawed yeast previously stored in a cryogenic vial at -20 ^{o}C in the freezer was pipetted into the growth medium. The flask was then sealed with a white foam bung wrapped with foil and incubated for 24 h in an orbital shaker at 28 ^{o}C and 200 rpm until turbid.

3.5.1.5 *In vitro* assay procedure

Microplates were used for the assay. They were appropriately labelled to correspond to the design used in the equilibration experiment. Using a multi-channel pipette, 10 µl of absolute ethanol poured in a sterile trough was transferred into each well in the units for negative controls (deionized water, ethanol, 1, 10, 50 or 100 mg/L humic acid) and evaporated completely for 30 min. After 24 h of incubation, a medium for the cultured yeast was prepared by pipetting 10 ml glucose solution, 2.5 ml aspartic acid solution, 1 ml vitamin solution, 0.8 ml L-threonine solution, 250 µl copper (II) sulfate solution and 1 ml CPRG into 90 ml minimal medium in a 150 ml sterile glass bottle. The bottle was covered and shaken thoroughly to enhance mixing of the contents. To determine the amount of yeast to be added to the medium, turbidity test was carried out. 100 µl each of the cultured yeast was pipetted into two wells in a microplate and their absorbance was determined at 620 nm using the software,

Softmax Prov5 with absorbance reader (Spectra Max). The amount of yeast that was added was estimated from the ratio of standard number of yeast cells to graphical estimate of number of yeast cells (derived from absorbance readings). For example, the amount of yeast that was added in the YES for EE2 was estimated as follows:

Absorbance reading (mean) = 0.201 (0.200-0.202) nm
Standard number of yeast cells = $4x10^7$
Graphical estimate of number of yeast cells = $3x10^7$
Amount of yeast added: $4x10^7/3x10^7$ = 1.33 ml yeast per 45 ml minimal medium

For EE2 or DDT, the specific volume of cultured yeast estimated was added to 90 ml of the medium, using a pipette. The bottle was covered and then shaken manually to ensure mixing. The yellow yeast medium was put in a sterile trough. By means of a multi-channel pipette, 195 µl of the medium was transferred into each well in all the rows of the assay microplates, including the gap rows between experimental units. Each equilibrated microplate was shaken for 2 min with titer plate shaker, and 5 µl of the medium in each well was transferred by means of a multi-channel pipette to the corresponding well in each assay microplate containing the yeast medium. This resulted in 1: 40 dilutions each of the initial concentrations of humic acid in the respective experimental units of the equilibration plates to 1, 10, 50 or 100 mg/L humic acid. With the same factor, EE2 was diluted to a concentration range of 12.5-0.0061 µg/L while DDT was diluted to the range of 250000-122.07 µg/L (1^{st} to 12^{th} well). DDT positive control was not equilibrated or dried, but directly added to the yeast medium. The microplates were covered, sealed with autoclave tape and shaken for 2 min each with a titer plate shaker to ensure mixing before incubation. They were placed in the incubator (Memmert, Fisher Scientific) at 32 ^oC for 4 days.

After 48 h of incubation, the first absorbance evaluation was done when gradual colour change from light yellow to red was first observed in some wells. Wells containing EE2 or mixtures of EE2 and humic acid showed colour change from yellow to red. In DDT assay microplates, only the positive control wells showed colour change from yellow to red. Each assay microplate was brought out of the incubator and shaken with titer plate shaker for 2 min 1 h prior to the determination of absorbance. Each microplate plate was unsealed and absorbance was measured at two wavelengths of 540 (because of optimum absorbance for CPRG which is

~575) and 620 nm (for turbidity) at room temperature. Absorbance was determined up to the 4th day of incubation to obtain data. All the negative control wells had light yellow colour at the end of incubation. Red colour indicated estrogenicity while yellow colour indicated lack of estrogenicity.

Correction for turbidity was done, using the following equation:
Corrected value = chemical absorbance (540 nm) – [chemical absorbance (620 nm) – blank absorbance (620 nm)].

The magnitude of expression or suppression of biological response (estrogenicity) in yeast to the test chemical at different concentrations of humic acid was used as a measure of bioavailability.

3.5.2 Further YES assay with DDT

Further YES assays with DDT were conducted with experimental units in duplicates. With some modications, the YES procedure previously described was used. A stock solution of 1 g/L DDT in absolute ethanol was prepared and used. The same concentrations of humic acid stock solutions previously used were prepared and diluted to achieve the 1, 10 and 100 mg/L humic acid concentrations used with DDT. Different approaches were adopted in the method of exposing the yeast to DDT or DDT and humic acid. These were drying of DDT in ethanol (evaporation of ethanol), direct addition of DDT in ethanol to the yeast medium and the dissolving of DDT in deionized water before exposure to the yeast. Nominal concentrations of DDT were compared with GC-MS measured concentrations of DDT.

3.5.2.1 Equilibration of DDT

Where an equilibration step was required, the necessary volume of the DDT (10 µl) was transferred to a separate microplate and labelled. The DDT was allowed to dry in a laminar air flow cabinet in the equilibration microplate for 30 min. 100 µl of deionized water was then added to the wells that previously contained the DDT and the microplate was sealed with autoclave tape and put in a refrigerator at 4 ^{o}C 18 h. 90 µl of deionized water was also added to different rows of the equilibration microplate to which 10 µl of DDT was added without allowing the rows to dry. The different conditions required for the equilibration step were necessary to understand

how the assay would perform when DDT was allowed to dry and when it was not dried. The different types of equilibration were performed on separate equilibration microplates to allow covering, and minimise evaporation of deionized water and DDT where it was not allowed to dry.

3.5.2.2 Gas chromatography- mass spectrometry

The samples determined by GC-MS, which used a Clarus 500 (Perkin Elmer, UK) were taken from the equilibration microplates and diluted 10 or 100 times with hexane into which they were extracted to fit the linear range of the instrument. Either 50 µl was then taken and added to a chromatography vial or 500 µl of hexane was added to achieve a 10 times dilution. For the more concentrated solutions, 10 µl was taken from the well and added to a chromatography vial with 990 µl of hexane to obtain a 100 times dilution. This extraction was done for all the wells on the assay microplate with DDT in them.

Extraction was carried out in the GC vial, by holding the capped vial on a Whirlimixer for 1 min, and then allowing the aqueous layer to settle. Injection was done into the GC directly from the vial, as the sample needle did not reach the aqueous layer. The recoveries for DDT were measured for a range of dilutions of DDT in 50 mg/L humic acid, and are presented in Table 3.3. The method gave good recoveries, which were acceptable for identifying differences in the DDT concentrations serially diluted in the wells.

From the two stock solutions of 10 and 0.1 mg/L DDT in hexane, standard solutions were made by pipetting 0.1ml of the stock solution and adding 0.9 ml of hexane to GC vials for each one of the solutions to make 1 mg/L and 0.01 mg/L standard solutions. The stock solutions were then put in chromatography vials without diluting to bring the total number of standard solutions to four (10, 1, 0.1 and 0.01 mg/L).

Table 3.3 Recovery of DDT from 50 mg/L humic acid into hexane by the extraction method

DDT in well (mg/L)	Measured DDT (mg/L)	% recovery
125.0	154	123
62.5	76	122
31.3	32	102
15.6	13	83
7.8	7.5	96

DDT, dichlorodiphenyltrichloroethane

The GC-MS method used a BPX5 30 m by 0.25 mm column (0.1µm film thickness) from SGE (Milton Keynes, UK.). The operating parameters for the instrument are given in Table 3.4. Due to the relatively high concentrations of DDT, the instrument was run in a scan from 50-370 m/z, and DDT was quantified with the m/z 235.

Table 3.4 Gas Chromatography-mass spectrometry parameters used in the measurement of DDT

Carrier Gas	Helium at 1ml/min
Initial Temperature	70 ^{o}C
Initial Hold Time	1 min
Temperature Programme	20 ^{o}C/min up to 220 ^{o}C for 15 min Injector 250 ^{o}C Transfer line at 250 ^{o}C Source 150 ^{o}C
Ionization Mode	
Ionization	EI + 70 EV

EI, electron ionization; EV, electron Volts

3.6 Quality assurance

To enhance quality assurance, replication of samples and the use of analytical standards and blanks in chemical analysis of samples were employed. Relative standard deviation (RSD) was computed and used to determine the reproducibility and precision or accuracy of data. Limits of quantitation (LOQ) and limit of detection (LOD) were used to evaluate uncertainty about measured concentrations of analytes in LC-MS analyses. LOD and LOQ were calculated from calibration curves derived from regression analysis (Motwani *et al.*, 2007; Shrivastava and Gupta, 2011). For example, LOD and LOQ values in Table 4.3 were calculated as follows:

Y = a + bX
Where a, intercept of regression line
b, slope of regression line
Y = 638.55 + 1.58X
LOD = 3.3(SD of intercept/slope of calibration curve)
LOQ = 10(SD of intercept/slope of calibration curve)
SD of intercept = SE of intercept x $\sqrt{n}$
SE = SD/$\sqrt{n}$
Where SD, standard deviation; SE, standard error; n, sample size
n = 11
SE of intercept = 3.52
SD of intercept = 3.52 x $\sqrt{11}$
= 3.52 x 3.32
= 11.69
Therefore, LOD = 3.3(11.69/1.58)
= 3.3(7.40)
= 24.42
LOD = 24.42 ng/ml (0.02 mg/L)
LOQ = 10(11.69/1.58)
=10(7.40)
LOQ = 74 ng/ml (0.07 mg/L)

3.7 Statistical analysis of data

Microsoft Excel was used to conduct all statistical analyses. F-test was used in the comparison of variances of samples while t-test was used to compare the means of samples with a 95% confidence level. Pearson's correlation, regression and one-way analysis of variance (ANOVA) were also used in the statistical analyses.

Chapter Four: Results

4.1 The occurrence of EE2, PFOS and HBCD in final effluent

It was considered important to determine the concentrations of the compounds in UK effluents, as although relevant from the EU legislative perspective there was little data on PFOS and HBCD for the UK. The EE2 analysis was undertaken as part of the CIP, and all results are reported in Table 4.1. Concentrations of the analytes in the eight sites ranged from 0.05-0.67 ng/L EE2, 2-19 ng/L PFOS and 1-33 ng/L HBCD, with mean values of 0.28, 10.88 and 10.63 ng/L, respectively.

The concentrations varied over each of the four TFS and ASS (Figure 4.1). EE2 concentrations ranged from 0.18-0.67 ng/L (TFS) and 0.05-0.37 ng/L (ASS). PFOS concentrations ranged from 6-19 ng/L (TFS) and 2-18 ng/L (ASS) while HBCD concentrations ranged from 7-33 ng/L (TFS) and 1-12 ng/L (ASS). EE2, PFOS and HBCD lowest concentrations of 0.05, 2 and 1 ng/L, respectively, were detected in the ASS while their highest concentrations of 0.67, 19 and 33 ng/L, respectively, were detected in the TFS. However, the variations in concentrations of the chemicals in the TFS and ASS were not uniform.

The site with the highest concentrations was a trickling filter works, and the lowest concentrations were at an activated sludge site, however, there was no clear indication that an activated sludge works would discharge lower concentrations than trickling filter sites. A detailed analysis of why concentrations in effluents did not show a consistent pattern by work type is not intended here. The concentrations in the effluents exceed existing and proposed EQS and should dilution not be sufficient, the receiving waters will fail quality standards. Therefore, the role of binding to DOC and possible effect on bioavailability may be important in determining future assessment of compliance with EQS, as is at present the case with metals.

Table 4.1 Concentrations of EE2, PFOS and HBCD in wastewater effluent samples in the UK

Effluent site	Sampling date	Concentrations of chemicals in effluent (ng/L)		
		EE2	PFOS	HBCD
TFS1	June 2011	0.51	10	9
TFS2	June 2011	0.18	6	7
TFS3	June 2011	0.67	7	9
TFS4	June 2011	0.18	19	33
ASS1	June 2011	0.05	18	12
ASS2	June 2011	0.19	10	9
ASS3	June 2011	0.37	2	1
ASS4	June 2011	0.06	15	5

TFS, trickling filter site; ASS, activated sludge site

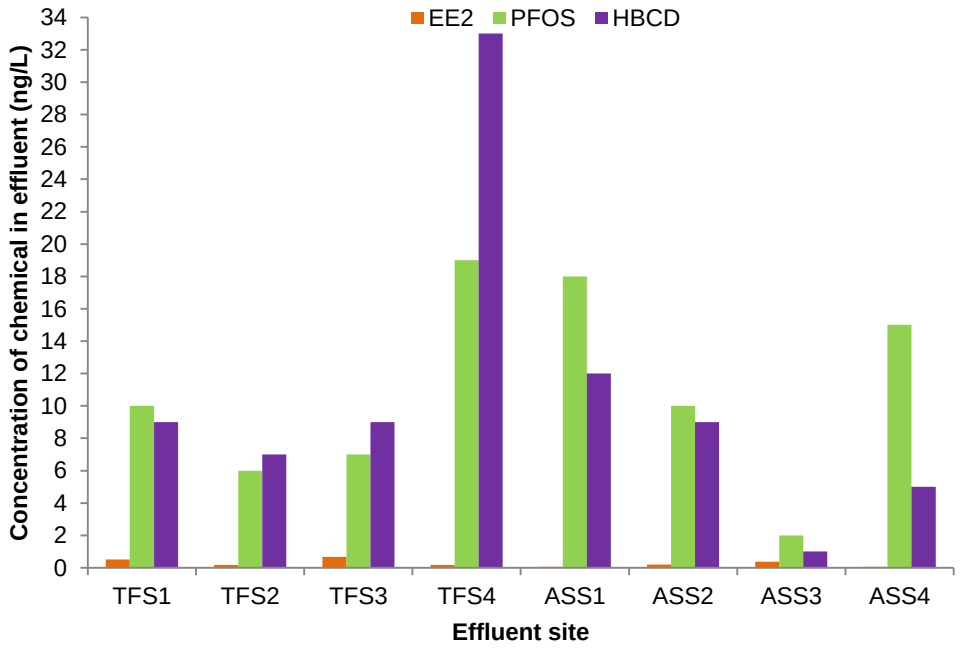


Figure 4.1 Comparison of EE2, PFOS and HBCD concentrations in wastewater effluents from selected sites in the UK (TFS, trickling filter site; ASS, activated sludge site)

4.2 Chemical determination of bioavailability

4.2.1 Determination of adsorption to DOC using SPE

4.2.1.1 The elution of Aldrich humic acid through SPE cartridges

Before undertaking studies assessing binding of the chemicals to the humic acid, which were dependent on the humic material not being retained by the C18 SPE cartridge, the retention of humic on the cartridge was assessed. The results of the analysis of Aldrich humic acid samples for TOC determination are presented in Table 4.2. The mean pH values for 5, 10 and 20 mg/L humic acid were 7.5, 7.5 and 7.7, respectively, which were above those recorded in subsequent work, and may indicate an issue with the pH probe, or an effect of the addition of the contaminants in later work. The mean TOC values for 10 mgC/L KHP standard solutions ranged from 9.98-10.02 mgC/L while the blank (deionized water) had 0.03-0.06 mgC/L, indicating that the TOC analysis was well calibrated.

Mean TOC values of 1.39 mgC/L (2.16% RSD) and 1.52 mgC/L (1.97% RSD) were measured for 5 mg/L humic acid before and after SPE, respectively. 2.50 mgC/L (3.20% RSD) and 2.48 mgC/L (2.82% RSD) mean TOC were measured for 10 mg/L humic acid before and after SPE, respectively. The mean TOC values measured for 20 mg/L humic acid before and after SPE were 4.68 mgC/L (3.85% RSD) and 4.42 mgC/L (3.17% RSD), respectively. Before SPE, 5, 10 and 20 mg/L humic acid were composed of 27.8, 25.0 and 23.4% TOC (mean) respectively. After SPE, the same concentrations of humic acid were composed of 30.4, 24.8 and 22.1% TOC (mean), respectively. Statistical analysis with t-test did not indicate significant difference in TOC before and after SPE for 5 mg/L ($t = 0.99$, $p>0.05$), 10 mg/L ($t = 0.39$, $p>0.05$) and 20 mg/L ($t = 2.04$, $p = 0.05$) humic acid. This implied that SPE did not exert any significant effect on the amount of TOC of the various concentrations of humic acid used.

Table 4.2 Measured TOC of Aldrich humic acid in deionized water before and after SPE

Nominal KHP (mgC/L)	**TOC of KHP (mgC/L)**		**TOC of blank (mgC/L)**		**Nominal humic acid (mg/L)**	**pH of humic acid**		**TOC of humic acid before SPE (mgC/L)**		**TOC of humic acid after SPE (mgC/L)**	
	Replicates	Mean (+SD)	Replicates	Mean (+SD)		Replicates	Mean (+SD)	Replicates	Mean (+SD)	Replicates	Mean (+SD)
10	9.91	9.98±0.06	0.04	0.06±0.03	20	7.7	7.7±0.0	4.78	4.68±0.18	4.27	4.42±0.14
	10.02		0.06			7.7		4.79		4.45	
	10.01		0.09			7.7		4.48		4.54	
10	9.96	10.01±0.04	0.07	0.04±0.04	10	7.5	7.5±0.0	2.41	2.50±0.08	2.54	2.48±0.07
	10.03		0.05			7.5		2.57		2.48	
	10.03		0.00			7.5		2.52		2.41	
10	9.93	10.02±0.08	0.08	0.03±0.04	5	7.5	7.5±0.0	1.37	1.39±0.03	1.49	1.52±0.03
	10.09		0.00			7.5		1.39		1.52	
	10.04		0.00			7.5		1.42		1.54	

KHP, potassium hydrogen phthalate; TOC, total organic carbon; blank, deionized water; SD, standard deviation; SPE, solid-phase extraction

4.2.2 The binding of EE2 to DOC

4.2.2.1 Range-finding with constant concentration of EE2 at varying concentrations of humic acid

Before undertaking the work to determine the partition coefficient, K_{DOC}, that is, humic acid or DOC-water partition coefficient, it was necessary to check what concentrations of EE2 and DOC would be suitable for the exercise. From an analytical perspective, enough EE2 needed to be bound to the DOC passing through the SPE cartridge to allow for measurement of the difference in the amount of EE2 retained on the cartridges. Results of humic acid-bound and freely dissolved fractions of constant concentration of EE2 with increasing concentration of humic acid are presented in Table 4.3. The mean pH values of the analysed samples ranged from 5.9-6.9, lower that previously observed for humic acid alone.

The nominal concentrations of EE2 added (EE2 before SPE) were determined by LC-MS. It appeared that measured values before SPE were higher (4.73 and 4.79 mg/L EE2) at lower concentrations (0 and 1 mg/L) of humic acid. However, the sum of the EE2 determined in the two fractions following SPE was in all cases within 20% of the measured starting concentration. Humic acid-bound fractions of EE2 increased with increasing humic acid concentration. However, at 0 and 1 mg/L humic acid, humic acid-bound EE2 was not detected indicating the importance of the range-finding exercise. The impact of the increasing concentration of humic acid on humic acid-bound and freely dissolved fractions of EE2 is illustrated in Figure 4.2.

The range-finding exercise indicated that it would be possible to observe binding of EE2 to humic acid at a concentration of 10 mg/L humic acid. This concentration was preferred to the higher options as it was environmentally relevant, reflecting concentrations in many rivers, although the characteristics of DOC do vary. The concentrations of EE2 selected were based on the limits of the analytical method, and ranged from 0.1 to 5.0 mg/L.

Table 4.3 Bound and freely dissolved fractions of constant concentration of EE2 at varying concentrations of humic acid

Nominal EE2 (mg/L)	HA (mg/L)	pH			EE2 before SPE (mg/L)			EE2 bound to HA (mg/L)			EE2 freely dissolved (mg/L)			Total EE2 (mg/L)
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	
5.0	100.0	6.9	6.9±0.00	0.00	2.46	2.98±0.45	15.10	0.81	0.81±0.02	2.47	1.98	2.07±0.21	10.14	2.88
		6.9			3.24			0.79			1.94			
		6.9			3.23			0.83			2.31			
	10.0	6.7	6.7±0.00	0.00	3.77	3.37±0.64	18.99	0.67	0.66±0.02	3.03	2.19	2.36±0.15	6.36	3.02
		6.7			2.63			nd			2.45			
		6.7			3.70			0.64			2.44			
	1.0	6.2	6.2±0.06	0.97	4.77	4.79±0.02	0.42	nd	nd	nd	3.94	4.83±0.77	15.94	4.83
		6.2			4.80			nd			5.35			
		6.1			4.79			nd			5.20			
	0.0	5.8	5.9±0.06	1.02	4.48	4.73±0.20	4.23	nd	nd	nd	3.48	4.19±0.64	15.27	4.19
		5.9			4.84			nd			4.71			
		5.9			4.86			nd			4.39			

EE2, ethinylestradiol; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; nd, not detected

LOD = 0.02 mg/L; LOQ = 0.07 mg/L.

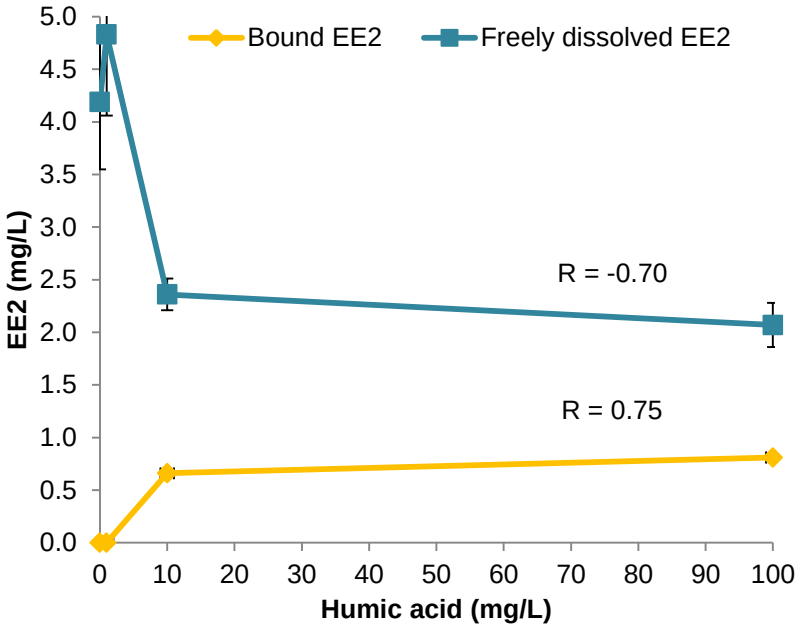


Figure 4.2 The impact of increasing concentration of humic acid on bound and freely dissolved fractions of 5 mg/L EE2 in deionized water

4.2.2.2 Determination of the partition constant of EE2 to DOC

The derivation of partition coefficient for EE2 to the Aldrich humic acid involved determining the humic acid-bound and freely dissolved fractions of EE2 at a constant concentration (10 mg/L) of humic acid (Table 4.4). The mean pH values of analysed samples ranged from 5.7 to 6.8. Before SPE, the LC-MS measurements of the nominal concentrations (0.1-0.5 mg/L) of EE2 ranged from 96-100% at 10 mg/L humic acid. In controls with 0.1 and 0.5 mg/L EE2 without humic acid, the measurements were 80.0 and 99.8%, respectively.

Humic acid-bound EE2 was not detected at any concentration of EE2. There was some indication that at the higher concentrations of EE2 added (0.4 and 0.5 mg/L) the freely dissolved fraction had decreased following the SPE (Figure 4.3). The hypothesis was that the difference in EE2 concentrations should be measurable in the eluate, that is, the EE2 associated with the humic acid which passed through the SPE cartridge. However, nothing was detected here, and the control sample with no humic acid, and a nominal concentration of EE2 at 0.5 mg/L also indicated some experimental error. This was determined to be associated with the concentrations of EE2 used in the experiment, which were challenging for the LC-MS, which throughout the work, had difficulty in ionizing the EE2.

Table 4.4 Bound and freely dissolved fractions of varying concentrations of EE2 at 10 mg/L humic acid

HA (mg/L)	Nominal EE2 (mg/L)	pH			EE2 before SPE (mg/L)			EE2 bound to HA (mg/L)			EE2 freely dissolved (mg/L)			Total EE2 (mg/L)	Log K_P
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)		
10.0	0.5	6.6	6.7±0.06	0.90	0.499	0.500±0.001	0.20	nd	nd	nd	0.295	0.291±0.005	1.72	0.291	0.0
		6.7			0.501			nd			0.286				
		6.7			0.500			nd			0.292				
	0.4	6.8	6.8±0.06	0.88	0.371	0.384±0.014	3.65	nd	nd	nd	0.263	0.264±0.002	0.76	0.264	0.0
		6.7			0.383			nd			0.263				
		6.7			0.398			nd			0.267				
	0.3	6.3	6.3±0.06	0.95	0.298	0.298±0.001	0.34	nd	nd	nd	0.300	0.299±0.001	0.33	0.299	0.0
		6.2			0.299			nd			0.298				
		6.3			0.297			nd			0.298				
	0.2	6.3	6.3±0.00	0.00	0.201	0.200±0.002	1.00	nd	nd	nd	0.200	0.199±0.002	1.01	0.199	0.0
		6.3			0.198			nd			0.200				
		6.3			0.200			nd			0.197				
	0.1	6.6	6.5±0.12	1.85	0.099	0.100±0.001	1.00	nd	nd	nd	0.100	0.100±0.002	2.00	0.100	0.0
		6.4			0.100			nd			0.098				
		6.4			0.100			nd			0.101				
0.0	0.5	5.6	5.7±0.06	1.05	0.509	0.499±0.010	2.00	nd	nd	nd	0.368	0.262±0.101	38.55	0.262	na
		5.7			0.499			nd			0.252				
		5.7			0.489			nd			0.167				
0.0	0.1	5.9	5.8±0.12	1.88	0.088	0.080±0.007	8.75	nd	nd	nd	0.081	0.089±0.008	8.99	0.089	na
		5.7			0.076			nd			0.097				
		5.7			0.076			nd			0.088				
10.0	0.0	6.5	6.4±0.12	1.88	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na
		6.5			nd			nd			nd				
		6.3			nd			nd			nd				

EE2, ethinylestradiol; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; Log K_p, Logarithmic partition coefficient; nd, not detected; na, not applicabe.

LOD = 0.01 mg/L; LOQ = 0.02 mg/L.

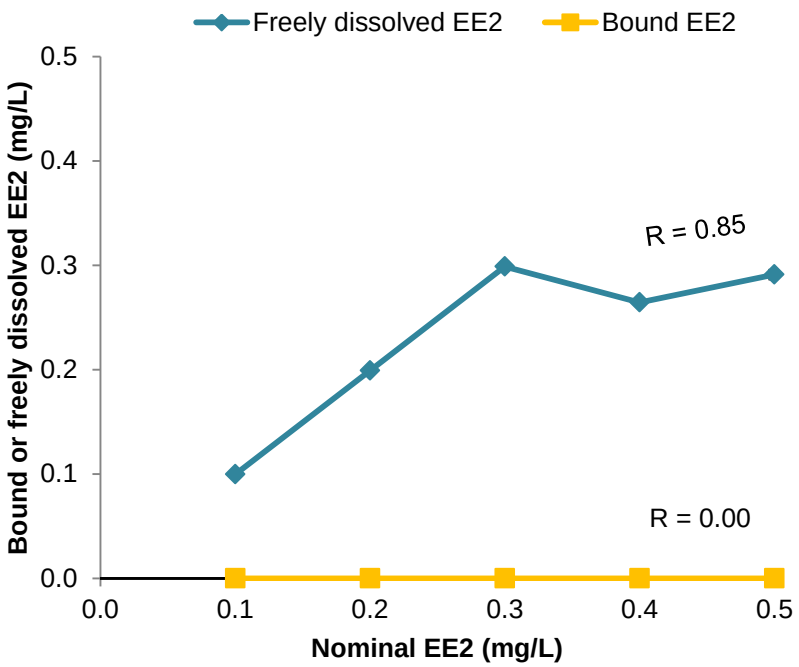


Figure 4.3 The impact of constant concentration (10 mg/L) of humic acid on bound and freely dissolved fractions of increasing concentration of EE2 in deionized water

Therefore the experiment was repeated, this time using concentrations of EE2 an order of magnitude greater than in the initial test, ranging from 1 to 5 mg/L. To ensure that the ratio of humic acid to EE2 was unchanged, the humic acid concentration was increased to 100 mg/L. Table 4.5 shows the concentrations of humic acid-bound EE2 and the freely dissolved fractions. The mean pH values of the analysed samples ranged from 5.6 to 7.2. The experimental design was less challenging for the LC-MS, and the measured concentrations of nominal concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L EE2 before SPE were all within 20% of expected values. In controls without humic acid, at 1.0 and 5.0 mg/L EE2, the measurements of nominal EE2 were 78.4 and 91.1%, respectively. The measurement of the freely dissolved and humic acid-bound fractions by LC-MS indicated that EE2 was measurable in both, and that concentrations of EE2 were increasing at increasing nominal concentrations (Figure 4.4). However, there was no significant difference between the concentrations of humic acid-bound EE2 and freely dissolved EE2 ($t = -3.12$, $p<0.05$). Being able to detect the EE2 in both fractions was required to determine the partition coefficient. The sorption isotherm (Figure 4.5) shows that EE2 was binding to the humic acid, although the fit of the regression was influenced again by variation in the experimental data ($R^2 = 0.47$,

$p<0.05$). The partition coefficient was derived from the slope of the line in Figure 4.5 which gave a K_{DOC} of 231 and log K_{DOC} 2.36. From Table 4.5 the partition coefficients calculated at individual concentrations seem to show a decrease with increasing EE2 indicating that at higher concentrations, binding sites may be limited.

Table 4.5 Bound and freely dissolved fractions of varying concentrations of EE2 at 100 mg/L humic acid

HA (mg/L)	Nominal EE2 (mg/L)	pH			EE2 before SPE (mg/L)			EE2 bound to HA (mg/L)			EE2 freely dissolved (mg/L)			Total EE2 (mg/L)	Log K_p
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)		
100.0	5.0	6.9	6.9±0.06	0.87	4.970	4.967±0.010	0.20	0.232	0.266±0.060	22.56	4.710	4.693±0.050	1.07	4.959	2.75
		7.0			4.960			0.335			4.640				
		6.9			4.970			0.231			4.730				
	4.0	7.1	7.2±0.12	1.67	3.990	3.950±0.070	1.77	0.258	0.246±0.040	16.26	3.670	3.707±0.030	0.81	3.953	2.82
		7.1			3.990			0.274			3.720				
		7.3			3.870			0.205			3.730				
	3.0	7.0	6.9±0.06	0.87	3.000	3.000±0.010	0.33	0.177	0.177±0.003	1.69	2.810	2.813±0.010	0.36	2.990	2.80
		6.9			3.000			0.175			2.820				
		6.9			2.990			0.180			2.810				
	2.0	7.2	7.1±0.17	2.39	1.630	1.637±0.010	0.61	0.220	0.214±0.010	4.67	1.540	1.527±0.020	1.31	1.741	3.15
		7.2			1.640			0.218			1.510				
		6.9			1.640			0.205			1.530				
	1.0	7.0	7.2±0.15	2.08	1.000	1.000±0.002	0.20	0.161	0.161±0.002	1.24	0.815	0.818±0.003	0.37	0.979	3.29
		7.2			0.997			0.159			0.820				
		7.3			1.000			0.162			0.818				
0.0	5.0	5.7	5.6±0.12	2.14	4.450	4.553±0.090	1.98	0.041	0.073±0.030	41.10	4.940	4.890±0.060	1.23	4.963	na
		5.7			4.630			0.090			4.830				
		5.5			4.580			0.087			4.900				
0.0	1.0	6.3	6.2±0.15	2.42	0.704	0.784±0.100	12.76	nd	nd	nd	0.951	0.976±0.020	2.06	0.976	na
		6.2			0.900			nd			0.979				
		6.0			0.748			nd			0.997				
100.0	0.0	6.8	6.9±0.06	0.87	nd	nd	nd	nd	nd	nd	nd	nd		nd	na
		6.9			nd			nd			nd				
		6.9			nd			nd			nd				

EE2, ethinylestradiol; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; Log K_p, logarithmic partition coefficient; nd, not detected; na, not applicable.

LOD = 0.004 mg/L; LOQ = 0.012 mg/L.

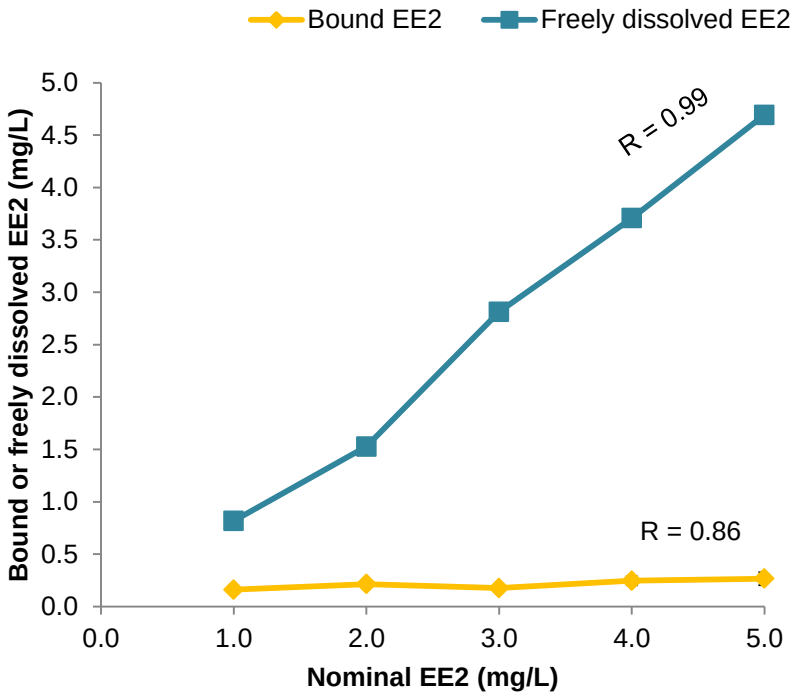


Figure 4.4 The impact of constant concentration (100 mg/L) humic acid on bound and freely dissolved fractions of varying concentrations of EE2 in deionized water

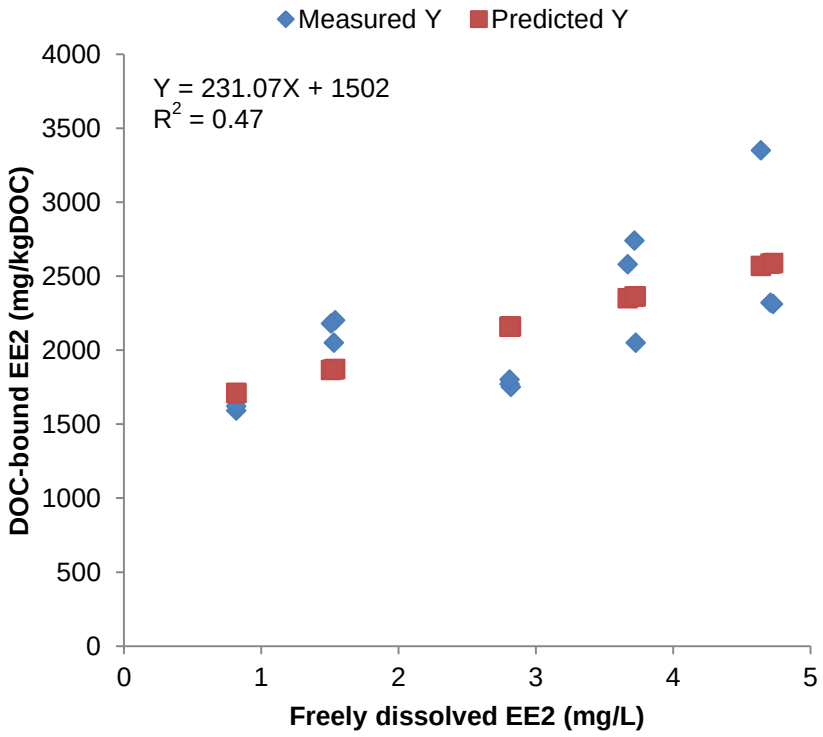


Figure 4.5 Sorption isotherm of increasing concentration of EE2 at 100 mg/L humic acid in deionized water (DOC, dissolved organic carbon; measured Y, measured DOC-bound EE2; predicted Y, predicted DOC-bound EE2)

4.2.3 The binding of PFOS to DOC

4.2.3.1 Range-finding with constant concentration of PFOS at varying concentrations of humic acid

Like in the analysis of EE2, range-finding exercise was done to evaluate concentrations of PFOS and humic acid that would be appropriate for the determination of partition coefficient for PFOS. The relatively low hydrophobicity of PFOS made it more imperative to undertake the exercise. Unlike contaminants with greater hydrophobicity, less hydrophobic ones may not exhibit much binding to DOC, and therefore, may have less retention by SPE cartridge. Results of humic acid-bound and freely dissolved fractions of constant concentration of PFOS with increasing concentration of humic acid are presented in Table 4.6. The mean pH values of the analysed samples ranged from 5.8-6.9 which was also lower than values previously reported for humic acid alone.

Before SPE, the mean nominal concentrations of PFOS determined by LC-MS at 0-100 mg/L humic acid ranged from 104.0-119% of the initial concentration of 5 mg/L PFOS. This indicated some experimental error. Because PFOS is much less hydrophobic than EE2 and HBCD, it did not exhibit the characteristic of a classical hydrophobic chemical in terms of DOC-bound and freely dissolved fractions in the presence of humic acid. The high fractions (5.30-5.77 mg/L) of PFOS detected in the eluate from SPE cartridge were not probably due to much binding to DOC, but due to low retention by the cartridge. At 0.0 mg/L humic acid (control), all the PFOS passed through the cartridge. Low concentrations of PFOS considered as freely dissolved, ranging from and 0.50-0.65 mg/L were retained by the cartridge. Increasing concentration of humic acid did not appear to have any positive impact on DOC-bound or freely dissolved fractions of 5 mg/L PFOS (Figure 4.6). The assumption that PFOS in eluate is the fraction of PFOS bound to humic acid may be unreliable.

The range-finding exercise indicated that it would be unlikely to achieve binding of PFOS to humic acid. Therefore, further work was not carried out to determine the partition constant of PFOS to DOC at varying concentrations of PFOS or humic acid. The results on PFOS shows limitations in the modified Landrum *et al's* reverse-phase separation method used in the determination of DOC-bound and freely

dissolved fractions of PFOS. The method seems more suitable for use when dealing with contaminants with greater hydrophobicities.

Table 4.6 Bound and freely dissolved fractions of constant concentration of PFOS at varying concentrations of humic acid

Nominal PFOS (mg/L)	HA (mg/L)	pH			PFOS before SPE (mg/L)			PFOS bound to HA (mg/L)			PFOS freely dissolved (mg/L)			Total PFOS (mg/L)
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	
5.0	100.0	6.8	6.9±0.06	0.87	5.89	5.84±0.09	1.54	3.85	5.30±1.43	26.98	0.48	0.50±0.03	6.00	5.80
		6.9			5.74			6.71			0.54			
		6.9			5.90			5.34			0.49			
	10.0	6.1	6.1±0.06	0.98	5.28	5.63±0.41	7.28	5.52	5.77±1.17	20.28	0.48	0.51±0.03	5.88	6.28
		6.1			6.08			7.04			0.54			
		6.2			5.54			4.74			0.51			
	1.0	5.7	5.8±0.06	1.03	6.32	5.20±1.05	20.19	5.35	5.75±0.42	7.30	0.42	0.63±0.18	28.57	6.38
		5.8			5.05			5.71			0.68			
		5.8			4.24			6.18			0.78			
	0.0	6.2	6.2±0.00	0.00	5.14	5.98±0.76	12.71	6.03	6.07±0.56	9.23	0.55	0.65±0.09	13.85	6.72
		6.2			6.18			6.65			0.72			
		6.2			6.63			5.54			0.69			

PFOS, perfluorooctane sulfonate; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction.

LOD = 0.04 mg/L; LOQ = 0.11 mg/L.

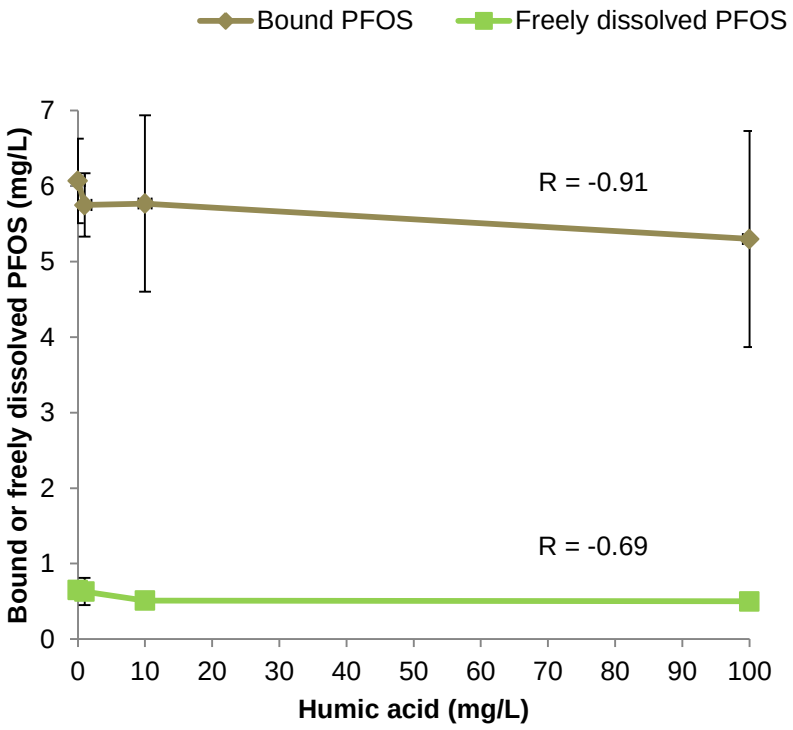


Figure 4.6 The impact of increasing concentration of humic acid on bound and freely dissolved fractions of 5 mg/L PFOS in deionized water

4.2.4 The binding of HBCD to DOC

4.2.4.1 Range-finding with constant concentration of HBCD at varying concentrations of humic acid

Prior to determination of the partition coefficient for HBCD, range-finding exercise was also undertaken to evaluate concentrations of HBCD and humic acid that would be suitable. Unlike the relatively high concentrations of EE2 and PFOS used in the determination of binding to DOC, low concentrations of HBCD were used due to the much lower water solubility of HBCD. The experiment was designed to ensure that the nominal concentrations of HBCD used were below its water solubility. Table 4.7 shows results of humic acid-bound and freely dissolved fractions of constant HBCD with increasing concentration of humic acid. The mean pH of analysed samples ranged from 5.7-6.9, also lower than values previously determined for humic acid alone.

Before SPE, the percentage of the nominal concentration of 50 µg/L HBCD at 0-100 mg/L humic acid measured by LC-MS ranged from 7.72-55.46%. The higher measured values were at higher (10 and 100 mg/L) humic acid while the lower values were at lower (0 and 1 mg/L) humic acid, probably due to adsorption of HBCD to glass during sample preparation. When DOC is lacking or low in concentration, chemicals with high K_{OW} may adsorb to glass during chemical analysis in the laboratory. Humic acid-bound fractions of HBCD increased with increasing concentration of humic acid. However, humic acid-bound HBCD was not detected at both 0 and 1 mg/L humic acid. Figure 4.7 illustrates the impact of increasing concentration of humic acid on humic acid-bound and freely dissolved fractions of HBCD. There was significant, positive, linear correlation ($R = 0.85$, $p>0.05$) between increasing concentration of humic acid and humic acid-bound HBCD, but significant, negative, linear correlation ($R = -0.91$, $p>0.05$) for freely dissolved HBCD.

From the results of the range-finding exercise, it was observed that HBCD would bind to humic acid at a concentration of 10 mg/L because of its relatively high hydrophobicity. The concentrations (5-50 µg/L) of HBCD finally selected were informed by the limits of the analytical method.

Table 4.7 Bound and freely dissolved fractions of constant concentration of HBCD at varying concentrations of humic acid

Nominal HBCD (µg/L)	HA (mg/L)	pH			HBCD before SPE (µg/L)			HBCD bound to HA (µg/L)			HBCD freely dissolved (µg/L)			Total HBCD (µg/L)
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	
50.0	100.0	6.9	6.9±0.00	0.00	30.60	27.73±3.38	13.81	25.00	26.93±2.39	8.87	8.20	4.86±2.89	59.47	31.79
		6.9			28.60			29.60			3.18			
		6.9			24.00			26.20			3.20			
	10.0	6.4	6.4±0.00	0.00	22.10	19.33±4.04	20.90	19.00	17.27±3.53	20.44	7.40	10.87±5.29	48.67	28.14
		6.4			21.10			19.60			16.95			
		6.4			14.70			13.20			8.25			
	1.0	5.8	5.7±0.06	1.05	5.05	8.78±3.28	37.36	nd	nd	nd	18.15	16.15±3.04	18.82	16.15
		5.7			11.20			nd			12.65			
		5.7			10.10			nd			17.65			
	0.0	5.6	5.7±0.06	1.05	3.40	3.86±0.99	25.65	nd	nd	nd	22.85	17.53±4.61	26.30	17.53
		5.7			5.00			nd			14.75			
		5.7			3.19			nd			15.00			

HBCD, hexabromocyclododecane; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; nd, not detected.
LOD = 0.13 µg/L; LOQ = 0.40 µg/L.

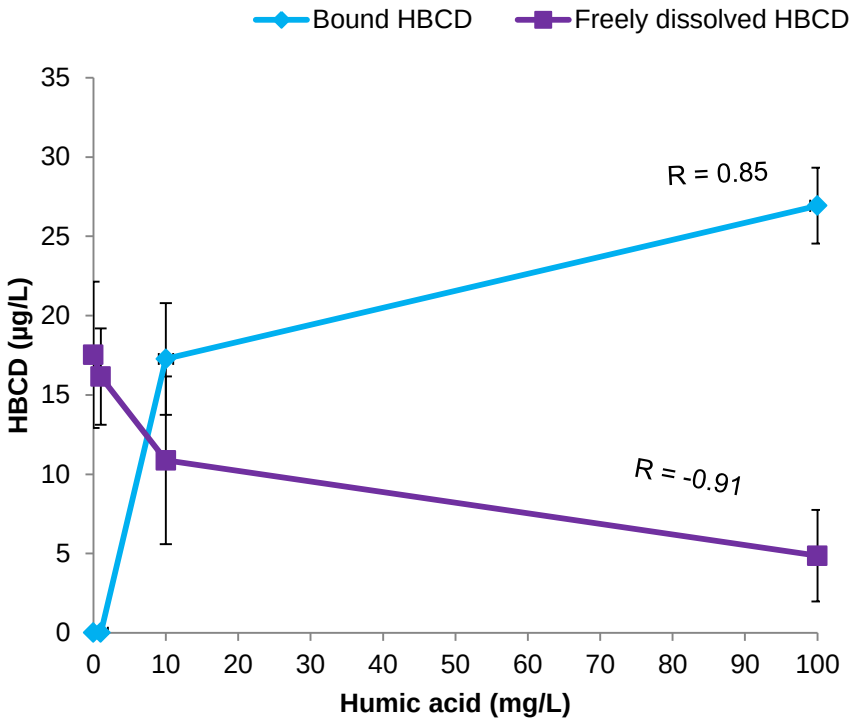


Figure 4.7 The impact of increasing concentration of humic acid on bound and freely dissolved fractions of 50 µg/L HBCD in deionized water

4.2.4.2 Determination of the partition constant of HBCD to DOC

Results of the partition coefficient for HBCD derived from the determination of humic acid-bound and freely dissolved fractions of HBCD at constant concentration (10 mg/L) of humic acid are presented in Table 4.8. The mean pH values of the analysed samples ranged from 5.7-6.4. Before SPE, 39.5-56.8% of the nominal concentrations (5-50 µg/L) of HBCD at 10 mg/L humic acid were measured by LC-MS. However, in controls of 5 and 50 µg/L HBCD without humic acid, lower measurements of 16.00 and 1.98% of the nominal concentrations, respectively, were observed before SPE. Negligible fractions of 10.8 and 4.0% of HBCD controls of 5 and 50 µg/L, respectively, were detected in the eluate following SPE. Poor ionization of HBCD was observed during the LC-MS analysis.

The lower values of concentrations of HBCD controls measured were probably due to adsorption of HBCD to glass during sample preparation and subsequent adsorption to vials used in the LC-MS analysis. Glass or vial-bound fraction of HBCD probably reduced the fraction of HBCD measured. In the absence of DOC and solids

such as sediments, hydrophobic organic contaminants may adsorb to glass or plastic during sample preparation, leading to reduction in the aqueous fraction of contaminants. The assumption of a complete mass balance without consideration for possible adsorption of hydrophobic contaminants to glass or plastic could lead to a considerable underestimation of the glass or plastic-water distribution of contaminants.

At constant concentration (10 mg/L) of humic acid with increasing concentration of HBCD, both humic acid-bound and freely dissolved fractions of HBCD increased (Figure 4.8). However, the concentration of humic acid-bound HBCD was less than that of freely dissolved HBCD with no significant difference between them ($t = -1.78$, $p = 0.05$). The partition coefficient in Table 4.8 determined from both humic acid-bound and freely dissolved HBCD exhibited the same trend observed in EE2. There was indication of decreasing partition coefficient with increasing HBCD, probably due to limited binding sites in DOC at higher concentrations of HBCD. The sorption isotherm illustrated in Figure 4.9 indicated binding of HBCD to humic acid with a partition coefficient of 14100 L/kg (Log K_{DOC} 4.15) derived from the slope of the regression line.

Table 4.8 Bound and freely dissolved fractions of varying concentrations of HBCD at 10 mg/L humic acid

HA (mg/L)	Nominal HBCD (µg/L)	pH			HBCD before SPE (µg/L)			HBCD bound to HA (µg/L)			HBCD freely dissolved (µg/L)			Total HBCD (µg/L)	Log K_p
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)		
10.0	50.0	6.7	6.4±0.26	4.06	25.30	24.27±1.38	5.69	7.71	8.33±0.96	11.52	35.75	43.33±9.33	21.53	51.66	4.28
		6.3			22.70			9.43			40.50				
		6.2			24.80			7.85			53.75				
	37.5	6.2	6.2±0.00	0.00	9.65	15.22±5.90	38.76	5.23	5.78±0.48	8.30	18.45	25.28±7.94	31.41	31.06	4.36
		6.2			14.60			5.99			23.40				
		6.2			21.40			6.11			34.00				
	25.0	6.5	6.4±0.12	1.88	10.00	9.88±0.20	2.02	3.77	3.85±0.16	4.16	6.63	9.36±2.61	27.88	13.21	4.61
		6.3			9.65			3.74			11.83				
		6.3			9.99			4.03			9.63				
	12.5	6.2	6.2±0.00	0.00	6.22	6.76±0.48	7.10	3.72	3.15±0.50	15.87	8.55	7.78±1.97	25.32	10.93	4.61
		6.2			6.93			2.90			5.55				
		6.2			7.14			2.82			9.25				
	5.0	5.8	6.0±0.15	2.50	2.61	2.84±0.26	9.15	1.59	1.66±0.20	12.05	2.83	3.33±0.52	15.62	4.99	4.70
		6.0			2.79			1.89			3.87				
		6.1			3.13			1.50			3.29				
0.0	50.0	5.8	5.7±0.06	1.05	1.97	0.99±0.85	85.86	3.01	2.00±0.94	47.00	10.78	12.59±1.57	12.47	14.59	na
		5.7			0.46			1.15			13.53				
		5.7			0.53			1.83			13.48				
0.0	5.0	5.9	5.9±0.00	0.00	0.76	0.80±0.12	15.00	0.70	0.54±0.15	27.78	2.77	4.66±1.64	35.19	5.20	na
		5.9			0.70			0.40			5.70				
		5.9			0.94			0.53			5.50				
10.0	0.0	6.3	6.2±0.06	0.97	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na
		6.2			nd			nd			nd				
		6.2			nd			nd			nd				

HBCD, hexabromocyclododecane; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; Log K_p, logarithmic partition coefficient; nd, not detected; na, not applicable.

LOD = 0.28 µg/L; LOQ = 0.83 µg/L

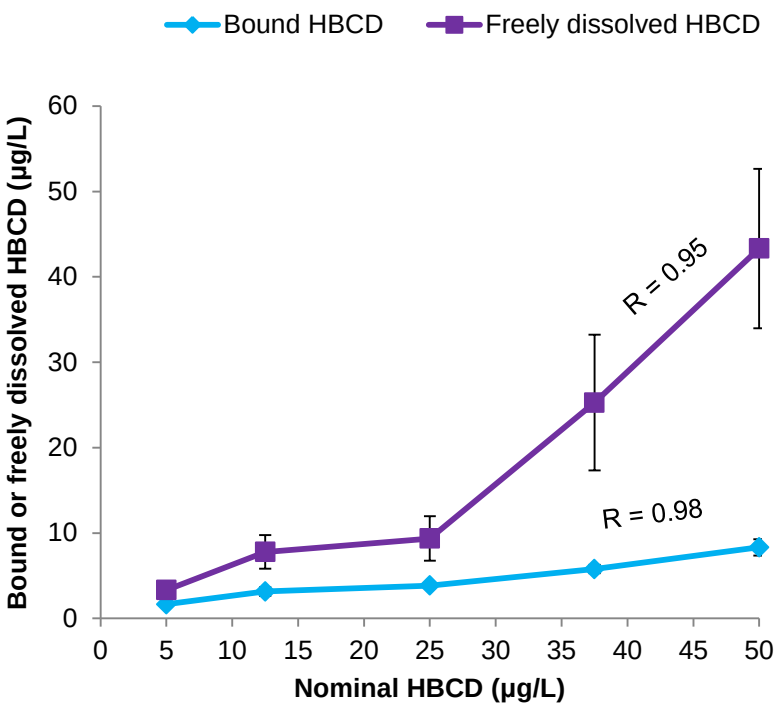


Figure 4.8 The impact of constant concentration (10 mg/L) of humic acid on bound and freely dissolved fractions of increasing concentration of HBCD in deionized water

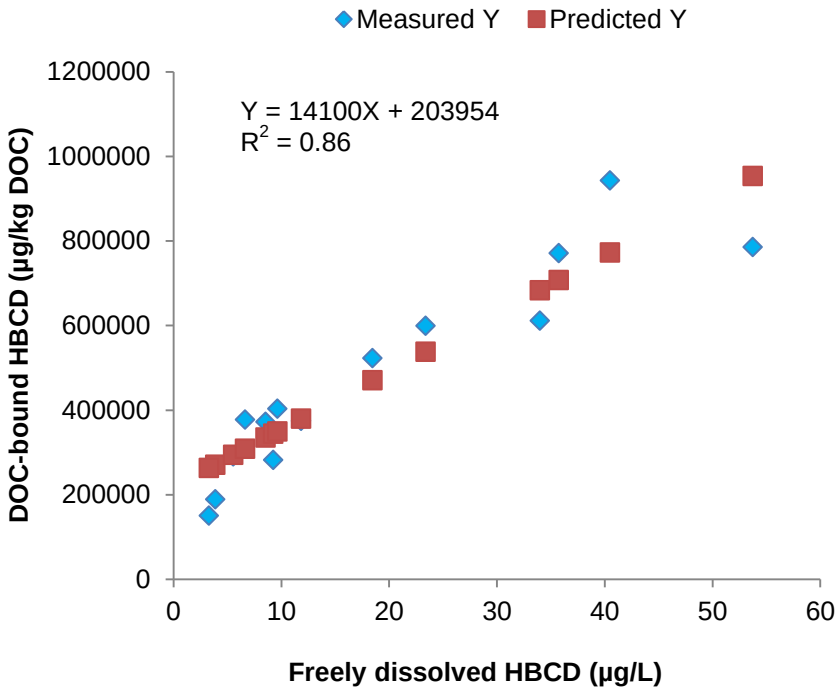


Figure 4.9 Sorption isotherm of increasing concentration of HBCD at 10 mg/L humic acid in deionized water (DOC, dissolved organic carbon; measured Y, measured DOC-bound HBCD; predicted Y, predicted DOC-bound HBCD)

To further evaluate the impact of constant concentration of humic acid on the partition coefficient for HBCD, the concentration of humic acid was increased to 100 mg/L while maintaining the same range of concentrations of HBCD used at 10 mg/L humic acid. Results of the partition coefficient for HBCD based on humic acid-bound and freely dissolved fractions of increasing concentration of HBCD at 100 mg/L humic acid are presented in Table 4.9. The mean pH values of the analysed samples ranged from 5.9-6.9. Prior to SPE, LC-MS analysis showed high mean measurements of ≥92.4% of nominal concentrations (5-50 μg/L) of HBCD. However, in controls of 5 and 50 μg/L HBCD without humic acid, lower measurements of 34.4 and 38.7%, respectively, were observed. HBCD was not detected in the eluate of all controls. This confirmed the assumption that passage of hydrophobic chemicals through SPE cartridge is facilitated by binding to DOC.

The impact of constant concentration (100 mg/L) of humic acid on humic acid-bound and freely dissolved fractions of HBCD at increasing concentration of HBCD is shown in Figure in 4.10. There was significant, positive, linear correlation between increasing concentration of HBCD and humic acid-bound HBCD ($R = 0.99$, $p<0.05$) or freely dissolved HBCD ($R = 0.95$, $p<0.05$) at 100 mg/L humic acid. However, humic acid-bound HBCD was significantly higher in magnitude than freely dissolved HBCD ($t = 2.09$, $p = 0.05$) unlike at 10 mg/L humic acid, implying greater binding of HBCD to DOC at higher concentration of humic acid. In Table 4.9, the partition coefficient estimated from humic acid-bound and freely dissolved fractions of HBCD did not show consistent variation in relation to increasing concentration of nominal HBCD. Figure 4.11 shows the sorption isotherm of increasing concentration of HBCD at 100 mg/L humic acid. There was significant binding of HBCD to DOC at 100 mg/L humic acid ($R^2 = 0.86$, $p<0.05$). A partition coefficient of 25466 L/kg (log K_{DOC} 4.41) for HBCD was derived from the slope of the regression line in Figure 4.11.

Table 4.9 Bound and freely dissolved fractions of varying concentrations of HBCD at 100 mg/L humic acid

HA (mg/L)	Nominal HBCD (µg/L)	pH			HBCD before SPE (µg/L)			HBCD bound to HA (µg/L)			HBCD freely dissolved (µg/L)			Total HBCD (µg/L)	Log K_p
		Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)	Replicates	Mean (+SD)	RSD (%)		
100.0	50.0	6.9	6.9±0.00	0.00	49.90	49.80±0.10	0.20	37.00	36.83±0.29	0.79	13.70	13.13±1.07	8.15	49.96	4.45
		6.9			49.70			36.50			13.80				
		6.9			49.80			37.00			11.90				
	37.5	6.8	6.9±0.06	0.87	37.20	37.23±0.06	0.16	27.70	27.83±0.15	0.54	6.85	7.71±1.57	20.36	35.54	4.56
		6.9			37.20			27.80			6.76				
		6.9			37.30			28.00			9.52				
	25.0	6.9	6.9±0.06	0.87	24.60	24.57±0.06	0.24	19.50	19.53±0.25	1.28	4.98	3.84±1.18	30.73	23.37	4.71
		6.9			24.50			19.30			3.91				
		6.8			24.60			19.80			2.63				
	12.5	6.8	6.9±0.06	0.87	12.20	12.27±0.06	0.49	8.78	8.94±0.16	1.79	2.57	2.41±0.29	12.03	11.35	4.57
		6.9			12.30			8.96			2.08				
		6.9			12.30			9.09			2.58				
	5.0	6.9	6.8±0.06	0.88	4.36	4.62±0.27	5.84	3.55	3.50±0.07	2.00	1.73	2.36±0.90	38.14	5.86	4.17
		6.8			4.60			nd			3.40				
		6.8			4.90			3.45			1.94				
0.0	50.0	6.1	5.9±0.20	3.39	20.10	19.37±1.45	7.49	nd	nd	nd	39.40	39.33±0.60	1.53	39.33	na
		5.9			20.30			nd			38.70				
		5.7			17.70			nd			39.90				
0.0	5.0	6.7	6.2±0.45	7.26	1.39	1.72±0.44	25.58	nd	nd	nd	4.96	4.39±0.91	20.73	4.39	na
		5.8			1.54			nd			4.87				
		6.2			2.22			nd			3.34				
100.0	0.0	6.9	6.8±0.10	1.47	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na
		6.8			nd			nd			nd				
		6.7			nd			nd			nd				

HBCD, hexabromocyclododecane; HA, humic acid; SD, standard deviation; RSD, relative standard deviation; SPE, solid-phase extraction; Log K_p, logarithmic partition coefficient; nd, not detected; na, not applicable.

LOD = 0.20 µg/L; LOQ = 0.60 µg/L.

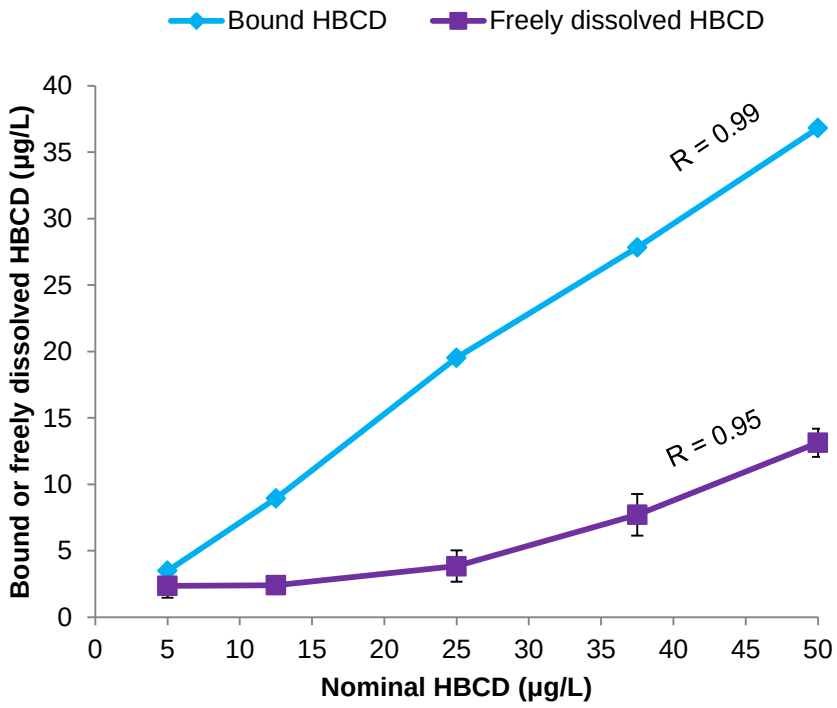


Figure 4.10 The impact of constant concentration (100 mg/L) of humic acid on bound and freely dissolved fractions of varying concentrations of HBCD in deionized water

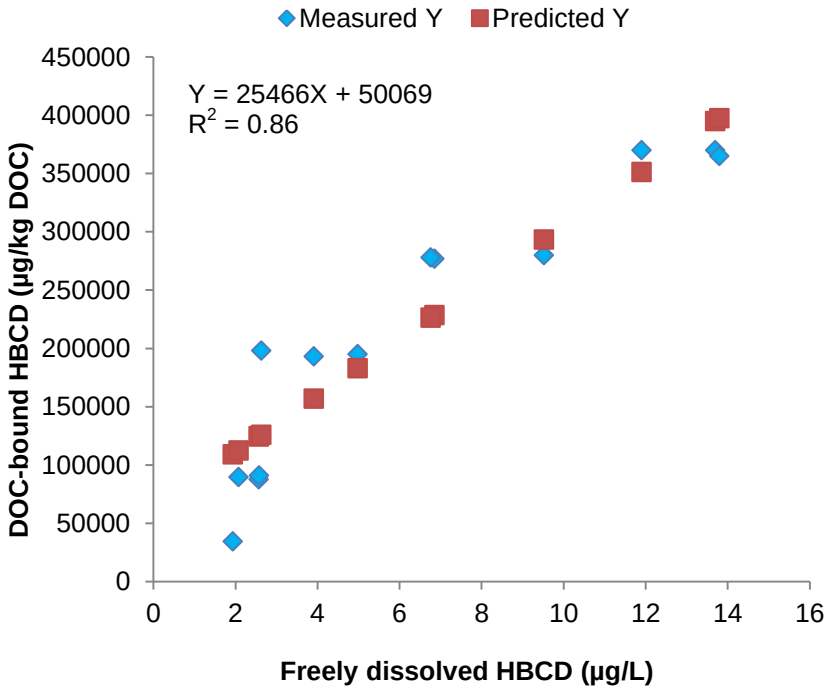


Figure 4.11 Sorption isotherm of increasing concentration of HBCD at 100 mg/L humic acid in deionized water (DOC, dissolved organic carbon; measured Y, measured DOC-bound HBCD; predicted Y, predicted DOC-bound HBCD)

4.3 Biological determination of bioavailability

To extend the chemical assessment of bioavailability, the YES was used as an end point to determine if the presence of humic acid influenced the estrogenic effect of chemicals in the assay. The use of the YES assay in this way, with humic acid being added alongside the chemical of interest was novel, and it required some consideration of how the chemical and humic acid could be added to the yeast, as it was felt important to allow a period of equilibration between chemical and humic acid before the assay took place. In addition, the chemical was added in serial dilution, but a constant concentration of humic acid was required, and there was a need for additional blanks to ensure that humic acid did not affect the assay.

A further challenge was that the assay determines estrogenicity, and one of the compounds so far studied, HBCD, is not known to be estrogenic. Therefore o,p'-DDT was used alongside EE2 as a high log K_{OW} compound rather than HBCD. This substitution was not considered significant in light of the objective of the work, which was to determine if a more hydrophobic compound would bind more strongly to the humic acid. HBCD has a log K_{OW} of 5.62 while that of o,p'-DDT is 6.79.

4.3.1 The use of the YES assay to determine the effect of humic acid on bioavailability of EE2

The YES assay was carried out in 96 well microplates, and the cells which showed positive results (an estrogenic effect) changed colour from yellow to red. Figure 4.12 shows the response of the yeast to EE2 and mixtures of EE2 and humic acid and their controls following 4 days of incubation. There was colour change from yellow to red in wells containing EE2 and EE2 with humic acid at all concentrations tested (1, 10, 50 and 100 mg/L), indicating estrogenicity. In wells containing assay medium only and controls of deionized water or humic acid (1-100 mg/L), there was no change in the original yellow colour, indicating lack of estrogenicity. The response of the yeast to concentrations of EE2 in each experimental unit was reproducible. Greater response was observed in wells with higher concentrations (12.50-0.39 µg/L) than those with lower concentrations (0.200-0.006 µg/L) of EE2.

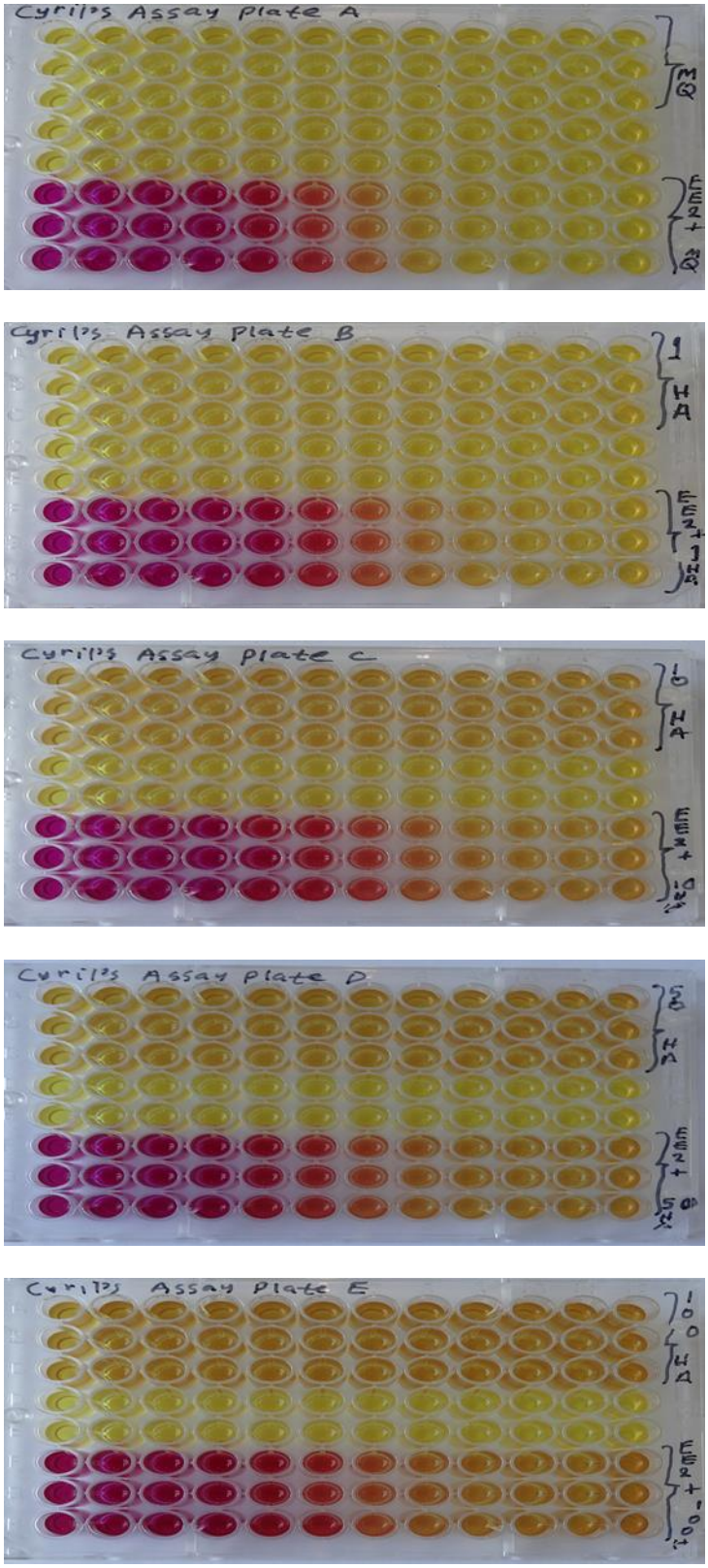


Figure 4.12 Microplates showing response in genetically modified yeast, *Saccharomyces* cerevisiae to exposure to EE2 and mixtures of EE2 and humic acid and their controls (microplates A, B, C, D and E contained 12.5-0.0061 µg/L EE2 each mixed with 0, 1, 10, 50 and 100 mg/L humic acid, respectively, in experimental units in triplicates)

Although the visual condition of the plates was an indication of the outcome of the assay, they were read on a plate reader to record absorbance of light, and then data corrected for blanks to produce dose-response curves. Figure 4.13 (a, b or c) illustrates the measurement of bioavailability of EE2 by yeast response (estrogenicity). Compared to response of the yeast to EE2 alone, the magnitude of estrogenic potency of EE2 at 1, 10, 50 and 100 mg/L humic acid was not significantly different. ANOVA did not indicate significant difference in the estrogenicity at day 2 ($F = 0.14$, $p>0.05$), day 3 ($F = 0.07$, $p>0.05$) and day 4 ($F = 0.10$, $p>0.05$) of incubation. The YES assay, therefore, clearly demonstrated that humic acid had no effect on the bioavailability of the EE2 to the yeast.

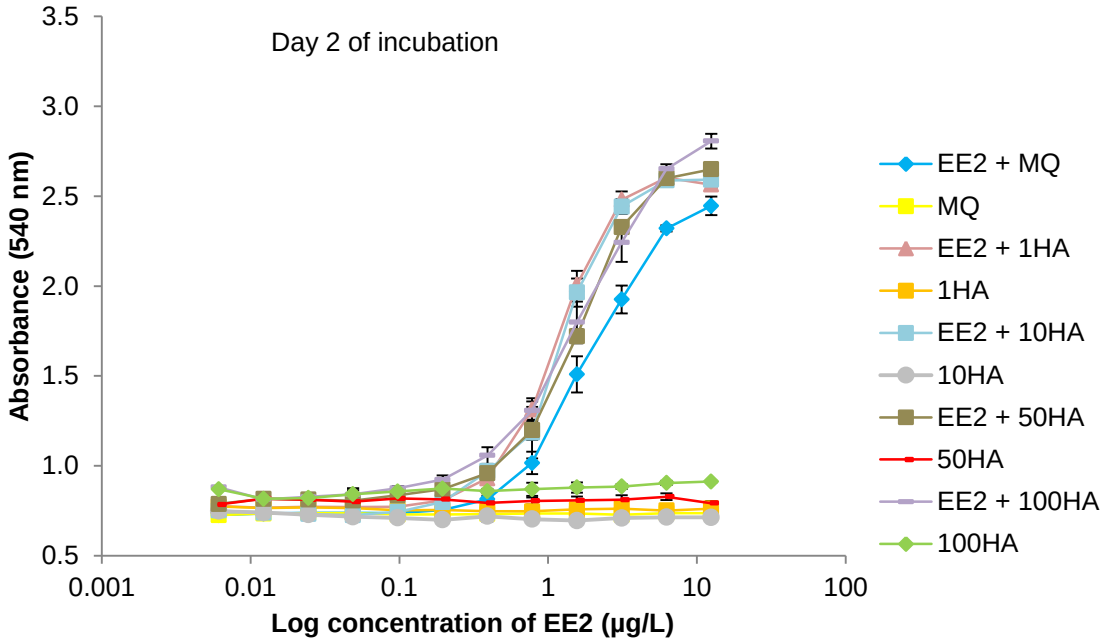


Figure 4.13a Measurement of bioavailability of EE2 in genetically modified yeast, *Saccharomyces cerevisiae*, by estrogenicity on the second day of incubation in a YES assay (EE2, ethinylestradiol; +, with; MQ, deionized water; IHA, 10HA, 50HA and 100HA = 1, 10, 50 and 100 mg/L humic acid, respectively)

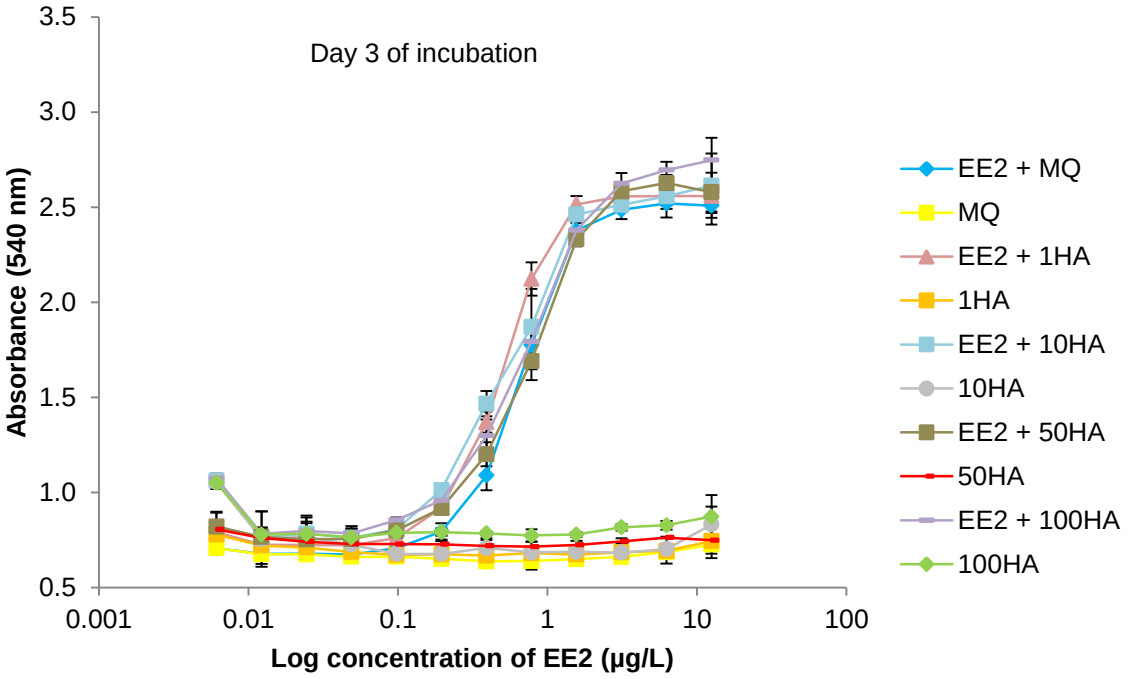


Figure 4.13b Measurement of bioavailability of EE2 in genetically modified yeast, *Saccharomyces cerevisiae*, by estrogenicity on the third day of incubation in a YES assay (EE2, ethinylestradiol; +, with; MQ, deionized water; IHA, 10HA, 50HA and 100HA = 1, 10, 50 and 100 mg/L humic acid, respectively)

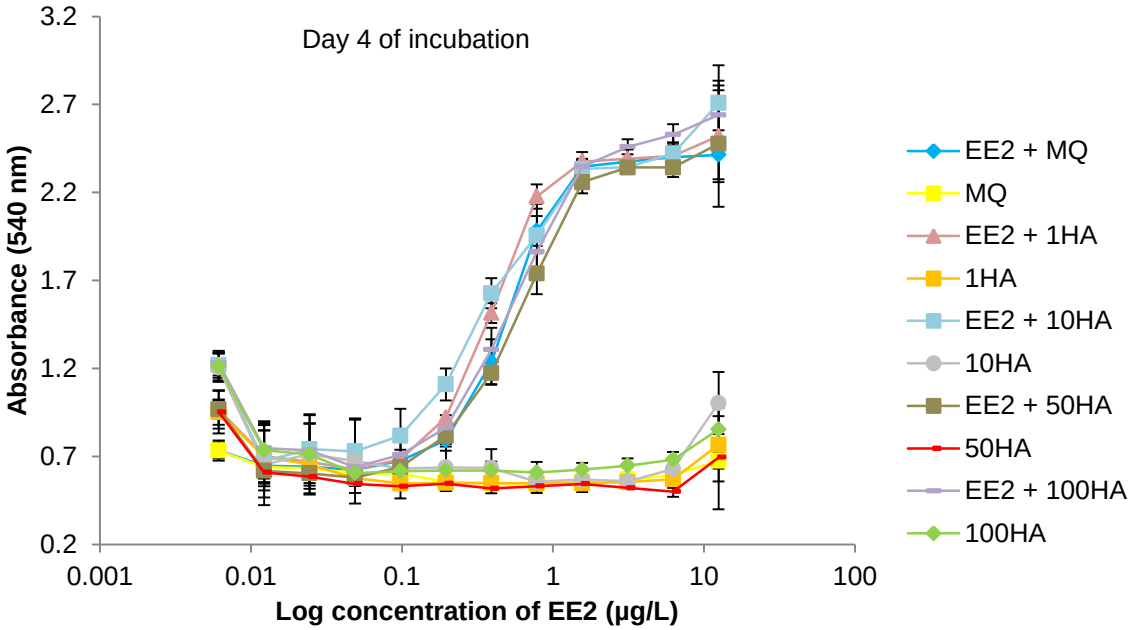


Figure 4.13c Measurement of bioavailability of EE2 in genetically modified yeast, *Saccharomyces cerevisiae*, by estrogenicity on the fourth day of incubation in a YES assay (EE2, ethinylestradiol; +, with; MQ, deionized water; 1HA, 10HA, 50HA and 100HA = 1, 10, 50 and 100 mg/L humic acid, respectively)

4.3.2 The use of the YES assay to determine the effect of humic acid on bioavailability of DDT

The use of the YES with DDT proved to be a significant challenge, as a result of the high hydrophobicity of the compound and the affinity it displayed to bind to the plastic material that the microplates were manufactured from. The standard approach to undertaking the assay is to undertake serial dilutions of the chemicals dissolved in ethanol, allowing the solvent to evaporate from the wells before re-dissolving in water and subsequently adding to the yeast medium. Initial work with DDT indicated that there were problems with positive controls (Figure 4.14).

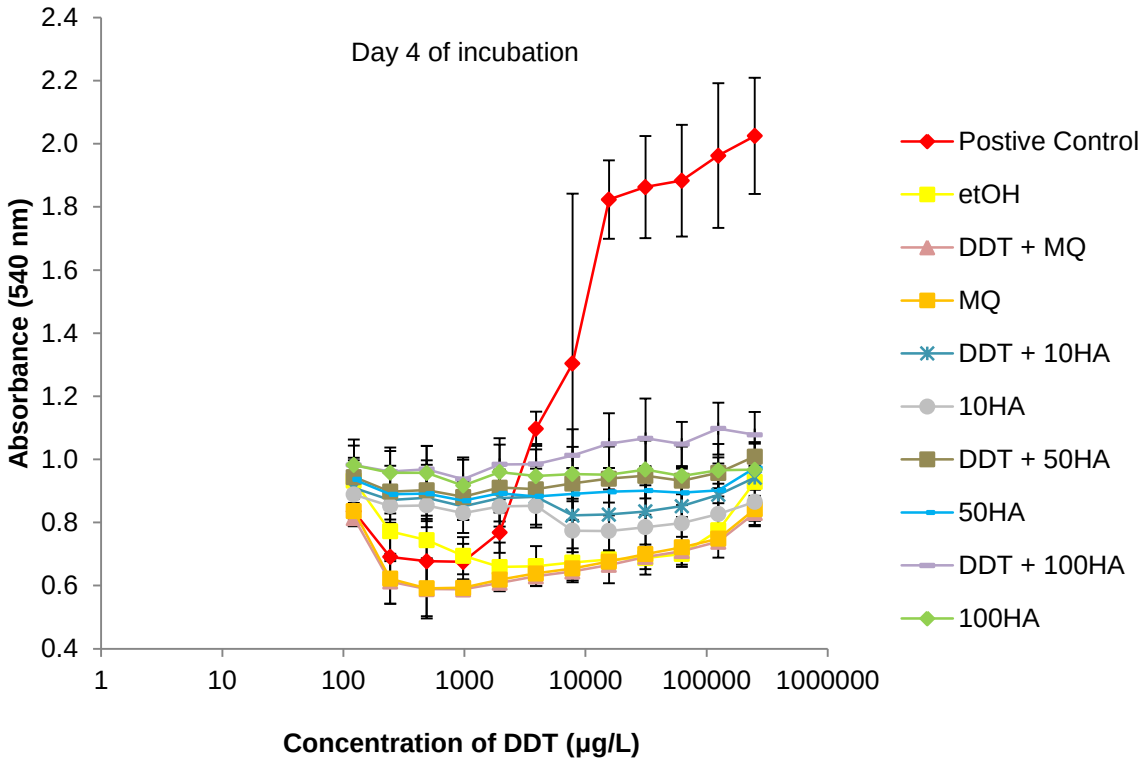


Figure 4.14 The response of yeast to DDT with 10-100 mg/L humic acid in the YES assay, using the approach of evaporating the solvent from the test chemical (YES, yeast estrogen screen; DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; MQ, deionized water; etOH, ethanol; IHA, 10HA and 100HA = 1, 10 and 100 mg/L humic acid, respectively; DDT + MQ, DDT dissolved in deionized water; DDT + 10HA, DDT + 50HA and DDT + 100HA = DDT with 10, 50 and 100 mg/L humic acid, respectively)

Further investigation of this issue, where the approach of allowing the solvent to evaporate and leaving DDT dry in the wells of the plate was compared to addition of DDT in ethanol to water, and direct addition of DDT in ethanol to the yeast medium

were compared (Figure 4.15). It was apparent that the approach of allowing the ethanol to evaporate, and the DDT to dry in the wells, resulted in significant loss of activity. The greatest response was observed when DDT was added directly to the yeast medium. ANOVA indicated significant difference in the estrogenicity of DDT control, dried DDT and DDT dissolved in deionized water ($F = 6.77$, $p<0.05$) (Figure 4.15). However, the experimental design was such that some equilibration of the DDT with humic acid was needed to replicate the EE2 experiments, and allow for comparison of results. It was clear that some response in the assay was obtained by adding the ethanol to water in the wells. This approach was further investigated to determine suitability for the experiment.

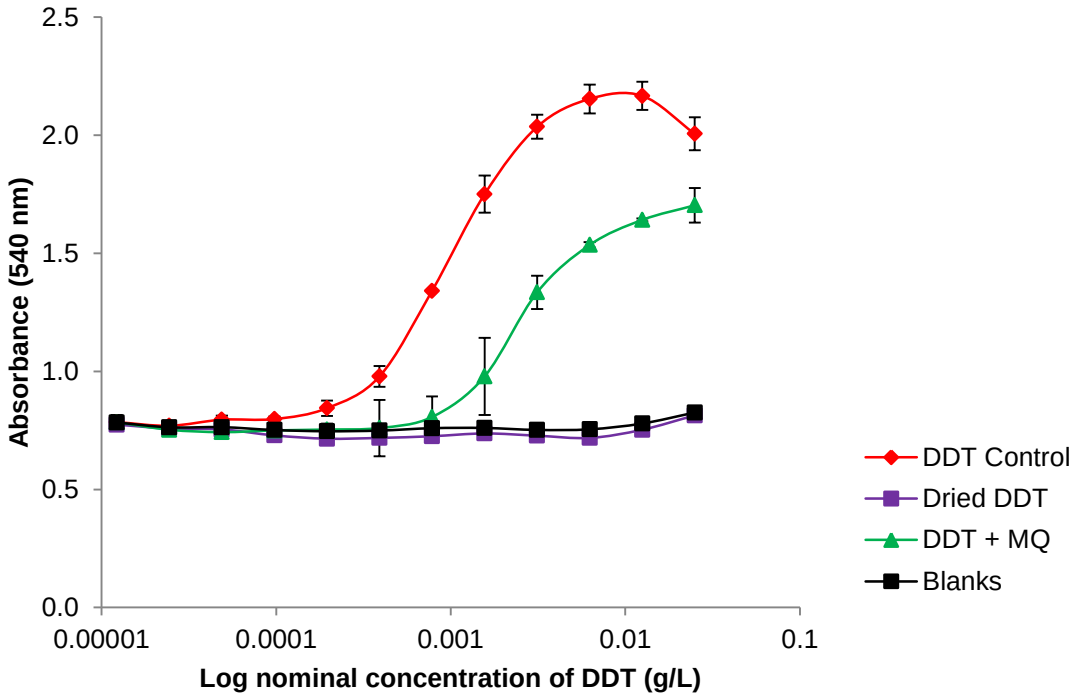


Figure 4.15 Comparison of different approaches for adding DDT to yeast in YES assay (DDT, dichlorodiphenyltrichloroethane; YES, yeast estrogen screen; DDT Control, DDT added directly to the yeast medium; dried DDT, DDT from evaporated ethanol; DDT + MQ, DDT dissolved in deionized water; blanks, ethanol and deionized water)

The hypothesis that the way of mixing the DDT within the wells was having an impact on how much was transferred to the yeast medium was further tested by repeating the above work, but with the addition of a chemical measurement of DDT concentrations transferred from wells where equilibration with the humic acid took place. The results of the assay are shown in Figure 4.16a, where overall a greater

response was observed for the DDT from dried wells and with deionized water than previously.

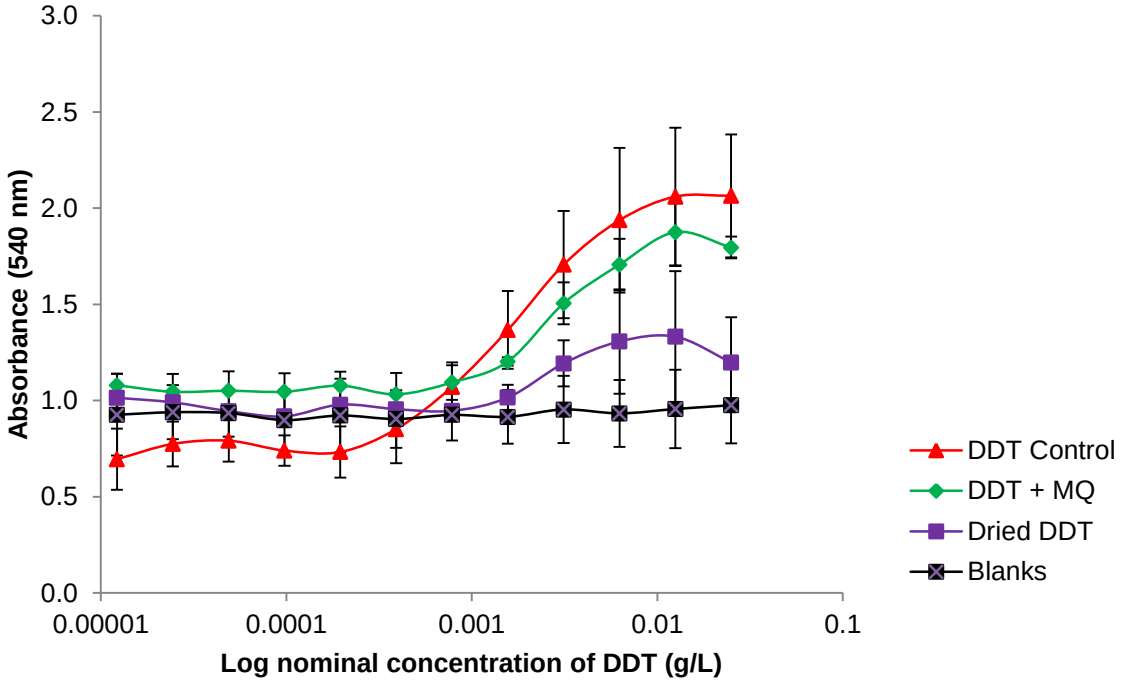


Figure 4.16a Estrogenic response in yeast to DDT exposure in a YES assay using different approaches (DDT, dichlorodiphenyltrichloroethane; YES, yeast estrogen screen; DDT Control, DDT added directly to the yeast medium; dried DDT, DDT from evaporated ethanol; DDT + MQ, DDT dissolved in deionized water; blanks, ethanol and deionized water)

The wells where DDT was allowed to dry (ethanol evaporated) showed less response than where the ethanol was added to deionized water. The deionized water approach was close to the positive control, where DDT was added directly to the yeast culture. However, there was no significant difference in their estrogenicity (ANOVA: $F = 1.11$, $p>0.05$). Figure 4.16a shows nominal concentrations of DDT with the yeast response. The determination of DDT in the equilibration wells by gas chromatography-mass spectrometry (GC-MS) revealed that concentrations of DDT from wells where the ethanol was evaporated were at least an order of magnitude below those where the DDT was added to deionized water, and the small volume of ethanol retained throughout the assay (Figure 4.16b). It was therefore concluded that some modification of the assay was needed to allow equilibration of the DDT with

humic acid, and the step of equilibration was further checked without the drying stage.

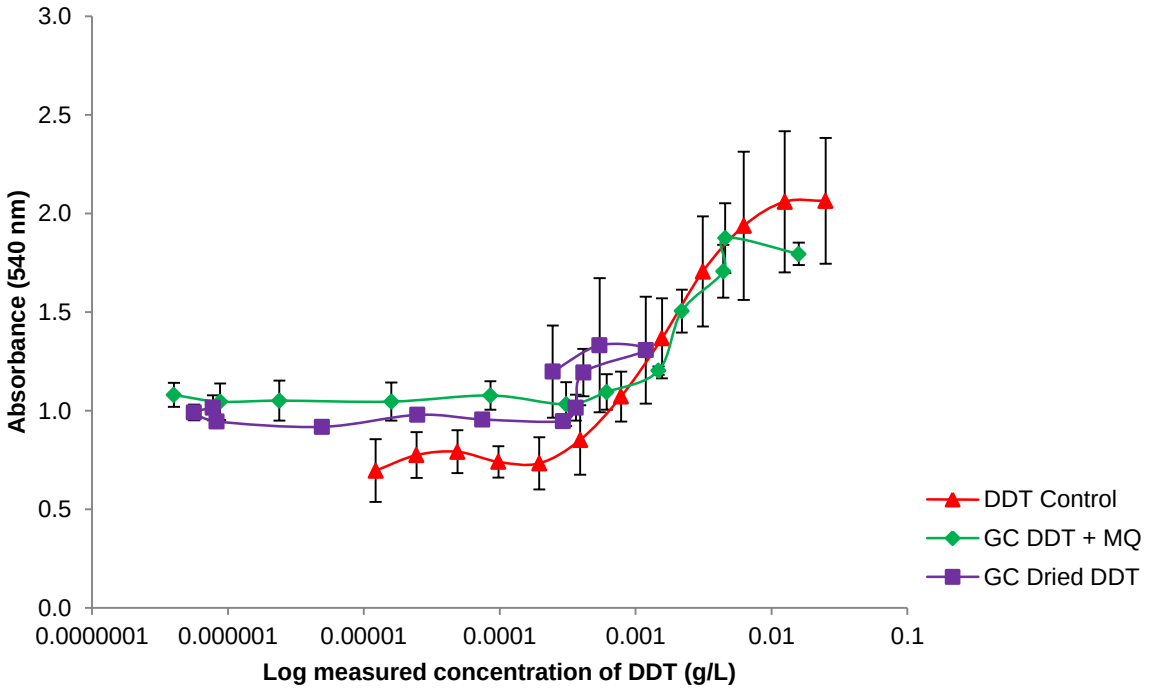


Figure 4.16b Absorbance plotted against GC-MS measured concentrations of DDT in YES assay (GC-MS, gas chromatography-mass spectrometry; DDT, dichlorodiphenyltrichloroethane; DDT Control, DDT added directly to the yeast medium; GC DDT + MQ, GC-MS measured DDT dissolved in deionized water; GC Dried DDT, GC-MS measured DDT from evaporated ethanol)

4.3.2.1 Final method and results for the effect of humic acid on bioavailability of DDT

Before undertaking a study with humic acid, a final check was undertaken to repeat the previous observation that addition of the DDT in ethanol to water in the wells in the plate, rather than drying and re-dissolving, was a valid approach. The evaluation also acted as a check on measured against nominal DDT concentrations. When plotting the dose-response curve for measured and nominal concentrations (Figure 4.17), it was apparent that the measured values corresponded well with the positive control where DDT was added directly to the yeast culture. DDT was not determined from the yeast culture medium, as samples were considered too complex for the

simple liquid / liquid extraction technique used, and were also much lower than in equilibration wells.

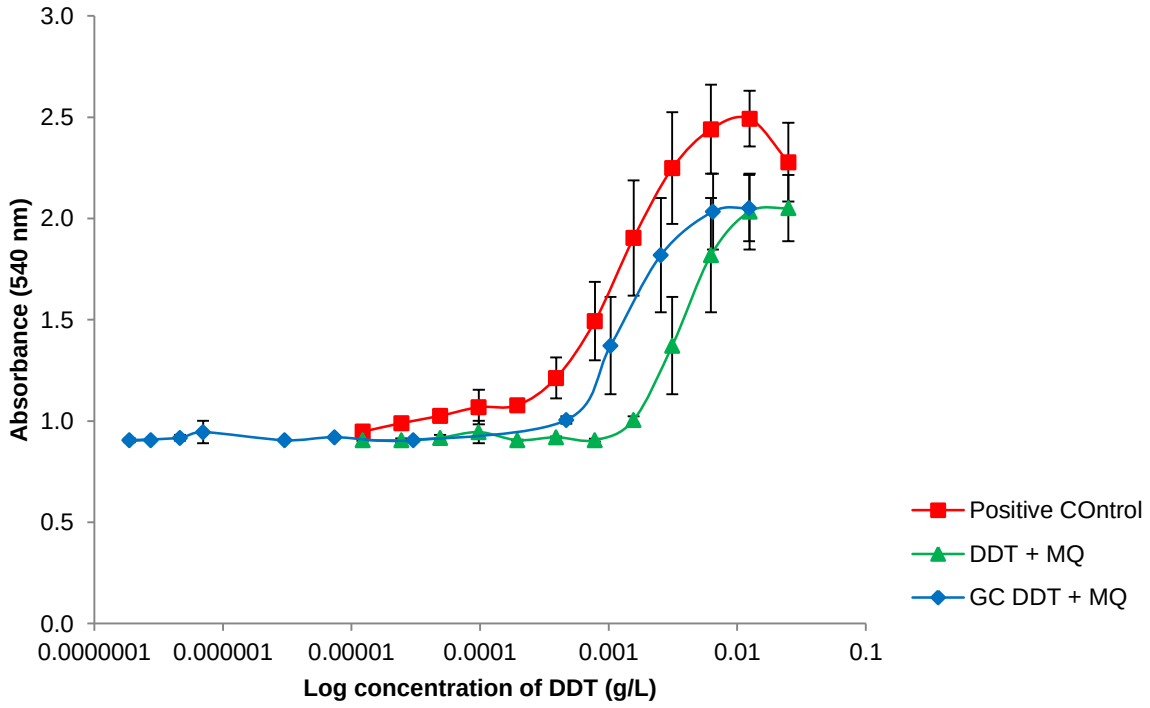


Figure 4.17 The nominal dose-response curves for the positive control and addition of DDT to deionized water with measured (GC-MS) concentrations in YES assay [GC-MS, gas chromatography-mass spectrometry; DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; DDT + MQ, nominal DDT dissolved in deionized water; GC DDT + MQ, GC-MS measured DDT]

Following validation that using measured concentrations of DDT from the equilibration wells gave good matches with positive controls, the effect of humic acid on the availability of DDT to the yeast was investigated. This used three different concentrations of humic acid (1, 10 and 100 g/L) and the DDT was added to these without drying the equilibration plate. Following transfer to the yeast medium, these gave the appropriate concentrations of DDT and humic acid in the exposure wells. The responses obtained, with nominal concentrations of DDT, are shown in Figure 4.18.

All samples with DDT gave a positive response, with no clear difference due to the presence of humic acid (ANOVA: $F = 2.50$, $p = 0.05$), although overall responses were below those of the controls, with curves shifted to the right of the positive control. Blanks with just humic acid as expected, showed no response. In

comparison to the nominal concentrations, when plotting the measured concentrations, the curves moved to the left, more closely matching the positive control (Figure 4.19). There is here some indication that the deionized water and DDT has a curve shifted farthest to the right, which would possibly imply that the presence of humic acid had acted to increase the amount of DDT present, possibly by enhancing its solubility. However, ANOVA did not indicate significant difference in nominal and GC-MS measured concentrations of DDT ($F = 0.83$, $p>0.05$). There is no evidence from the assay that humic acid limits the bioavailability of the DDT.

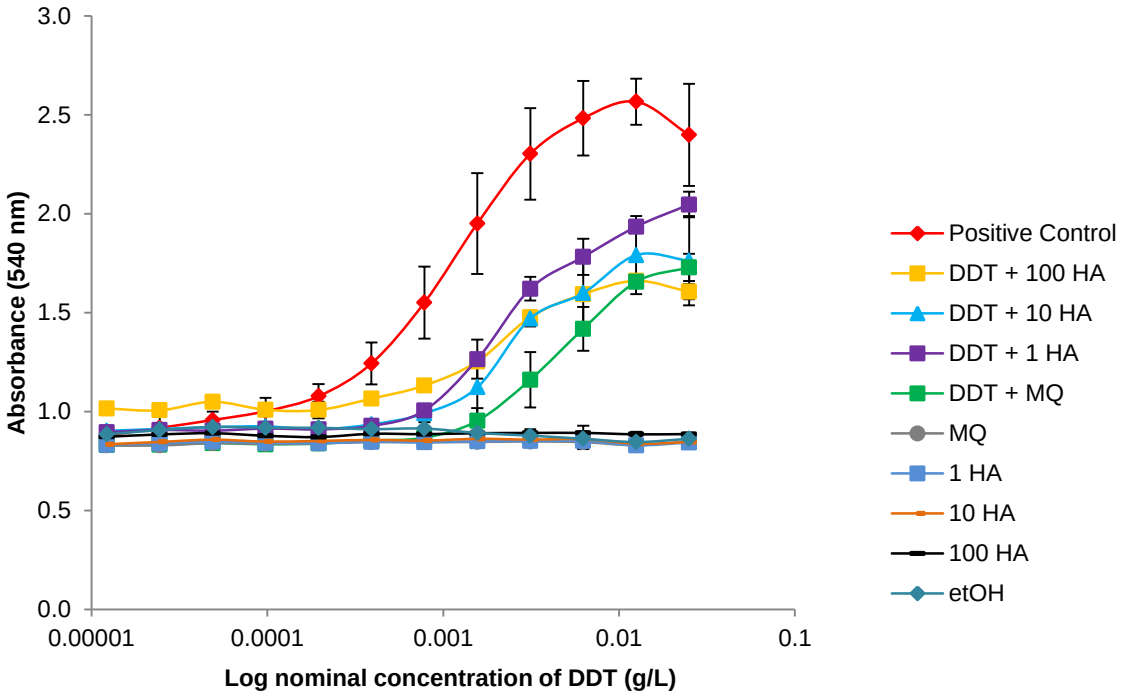


Figure 4.18 The response of the YES to nominal concentrations of DDT with 1-100 mg/L humic acid (YES, yeast estrogen screen; DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; MQ, deionized water; etOH, ethanol; I HA, 10 HA and 100 HA = 1, 10 and 100 mg/L humic acid, respectively; DDT + MQ, DDT dissolved in deionized water; DDT + 1 HA, DDT + 10 HA and DDT + 100 HA = DDT with 1, 10 and 100 mg/L humic acid, respectively)

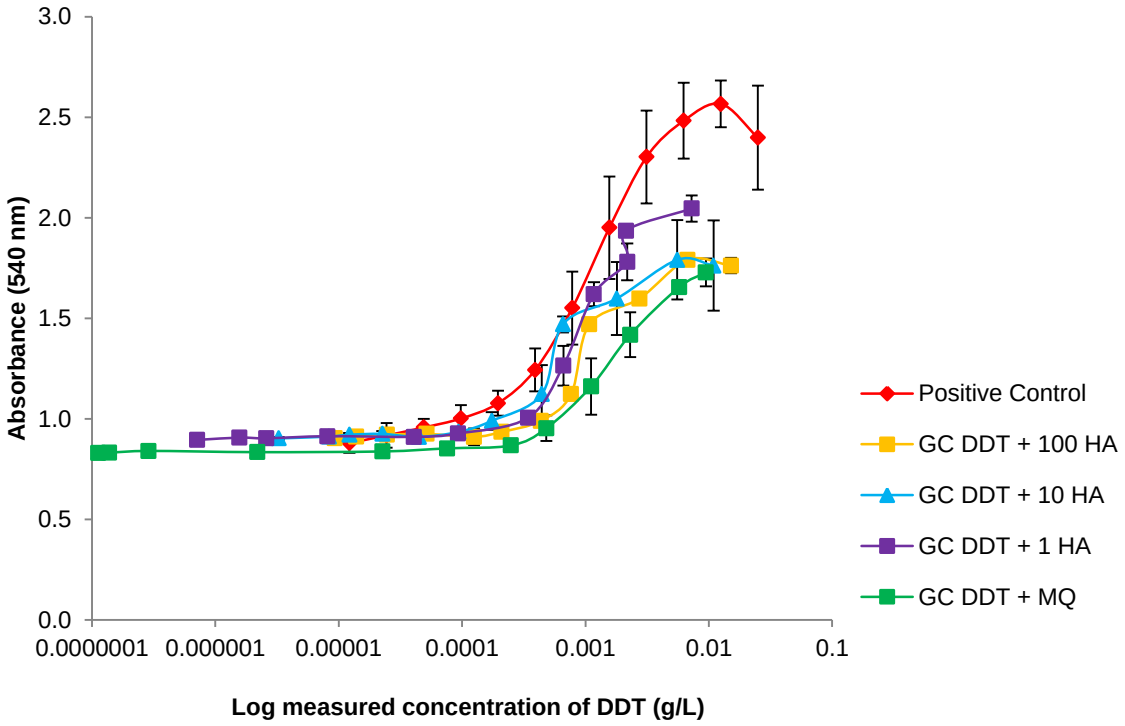


Figure 4.19 The response of the YES to measured concentrations of DDT with 1-100 mg/L humic acid (YES, yeast estrogen screen; DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; GC DDT + MQ, GC-MS measured DDT dissolved in deionized water; GC DDT + 1 HA, GC DDT + 10 HA and GC DDT + 100 HA = GC-MS measured DDT with 1, 10 and 100 mg/L humic acid, respectively)

Chapter Five: Discussion

5.1 Occurrence of EE2, PFOS and HBCD in wastewater effluents

The occurrence of EE2, PFOS and HBCD in WWTP effluents in countries around the world has been reported. A concentration range of 0.4-3.3 ng/L EE2 was reported in the analysis of effluents of four WWTPs in Germany in the study by Hintemann *et al*. (2006). In forty five WWTPs serving both rural and regional municipalities in Victoria, Australia, concentrations of EE2 ranging from not detected (nd) to 0.6 ng/L were measured in the effluents (Allinson *et al.*, 2010). Kanda and Churchley (2008) reported a mean concentration of 0.63 ng/L EE2 in WWTP effluent in Warwickshire, UK. Also, Grover *et al*. (2011) reported concentrations of EE2 in effluents ranging from <0.4-1.7 ng/L in Swindon, UK. In an assessment of steroidal estrogens in effluents of WWTPs in Ontario, Canada, EE2 was detected at concentrations ranging from 0.6-9.8 ng/L (Atkinson *et al.*, 2012). Qiang *et al*. (2013) reported detection of EE2 at concentrations of <2.3-69.1 ng/L in effluents samples collected from 20 rural WWTPs located in Zhejiang Province, China.

In South Sweden, EE2 was not detected in effluent samples analysed at LOQ of 10 ng/L (Zorita *et al.*, 2009). In a comparison of the concentrations of EE2 in WWTP influents (raw or untreated wastewater) and final effluent (treated wastewater) in France, lower concentrations of EE2 were detected in effluent than in the influent. Concentrations of 1.0-3.3 ng/L and <1.0-2.1 ng/L EE2 were measured in the influent and effluent, respectively (Janex-Habibi *et al.*, 2009). Wastewater treatment process may enhance removal or reduction in the concentration of contaminants in final effluent (Bain *et al.*, 2014). Concentrations of EE2 in effluents reported in this study (0.05-0.67 ng/L) fall within the ranges reported in effluents from different WWTPs around the world.

Several studies have indicated the occurrence of PFOS in effluents from WWTPs in different countries of the world. Loganathan *et al*. (2007) reported the occurrence of PFOS in effluents from two WWTPs in Kentucky and Georgia, USA, at concentrations ranging from 1.8-28 ng/L. PFOS was also detected at a range of 106-336 ng/L in the effluents of a municipal WWTP discharging into River Roter Main in Bayreuth, Germany (Becker *et al.*, 2010). In an evaluation of the fate of

PFOS and PFOS-related compounds in WWTPs in Korea, receiving municipal, agricultural and industrial wastewater, Guo *et al*. (2010) observed a tendency to decrease in the concentrations of PFOS after treatment of the wastewater. The authors reported the detection of higher concentrations (nd-68.1 ng/L) and lower concentrations (nd-8.9 ng/L) of PFOS in the influent and effluent, respectively. Effluents from four main municipal WWTPs in Shenyang, China were reported to have concentrations of PFOS in the range of 1.69-3.85 ng/L (Sun *et al.*, 2011). In Greece, concentrations ranging from <0.18-21 ng/L PFOS were determined in the effluents of WWTPs receiving both municipal and industrial wastewater (Arvaniti *et al.*, 2012). PFOS was among many organic contaminants frequently detected in the analysis of effluents from ninety WWTPs across Europe by Loos *et al*. (2013). In their study, the authors reported concentrations of PFOS ranging from <0.5-2101 ng/L with a detection frequency of 93%. Concentrations of PFOS in effluents reported in this study are within the range of concentrations reported in other studies reviewed.

Unlike EE2 and PFOS, there is limited information on the occurrence of HBCD in WWTP effluents. Due to the much more hydrophobic character and high affinity of HBCD for solids, it is more common to detect HBCD in sewage sludge than in effluents. However, in a survey of the distribution and concentrations of HBCD in WWTPs in Japan, concentrations of HBCD measured in the influent and effluent ranged from 16-400 ng/L and 0.39-12 ng/L, respectively. There was removal of more than 90% of HBCD from the wastewater by the WWTPs due to the sorption of HBCD to suspended solids (Ichihara *et al.*, 2014).

It is therefore apparent that EE2, PFOS and HBCD are discharged to the environment. With stringent standards, an assessment of bioavailability and its relevance is important.

5.2 Chemical determination of bioavailability

5.2.1 Determination of adsorption to DOC using SPE

DOC has been shown to be one of the factors affecting bioavailability of contaminants in the aquatic environment (Stanley *et al.*, 2012). In surface waters such as rivers, humic substances cannot be directly measured due to their complex, heterogenous chemical structure. Therefore, TOC is used as a measure of the concentration of humic substances in water (Rodrigues *et al.*, 2009).

The adsorption of organic contaminants to DOC using SPE has been studied. Landrum *et al*. (1984) reported adsorption of the organic contaminants, benzo[a]pyrene, anthracene, biphenyl, p,p'-DDT, hexachlorobiphenyl, tetrachlorobiphenyl and bis(2-ethylhexyl) phthalate to DOC in aqueous solution. In the study in which Sep-Pak C18 cartridges were used for SPE, DOC-bound contaminants passed through the cartridge while unbound or freely dissolved contaminants were retained in the cartridge column. Li and Lee (2001) observed that significant association of hydrophobic polycyclic aromatic hydrocarbons (PAHs) with humic acid in surface water was responsible for the non-retention of humic acid-bound fractions of the compounds in reverse-phase SPE cartridges. In a similar study on the partitioning of PAHs with C18 SPE cartridges, Brown and Peake (2003) reported the adsorption of unbound or freely dissolved PAHs onto the cartridge sorbent and the passing of DOC-bound PAHs through the cartridge. The authors remarked that DOC complexes hydrophobic organic contaminants and allow them to pass through C18 SPE cartridge.

In this study, the range of pH values reported is within the range of pH suitable for the water solubility of humic acid. Humic acid is soluble in water at pH>2 (Shaffer and von Wandruszka, 2015). Though humic acid alone was used, the results are in agreement with the findings in other studies that DOC passes through the cartridge. The insignificant difference in the TOC of the humic acid before and after SPE indicated that the humic acid passed through the cartridge.

5.2.2 Binding to DOC and the partition constants of EE2, PFOS and HBCD

There are limited studies on binding of EE2, PFOS and HBCD to DOC. In this study, all ranges of pH values reported are also within the range of pH at which humic acid is soluble in water. The results indicated that the binding of EE2, PFOS and HBCD was probably dependent on the degree of hydrophobicity and the concentration of humic acid rather than the analytes' range of concentrations tested. DOC-bound EE2 increased with increasing concentration of humic acid, but there were higher fractions of freely dissolved EE2 than DOC-bound EE2 at both constant concentrations (10 and 100 mg/L) of humic acid. This indicated moderate binding of EE2 to DOC and potential bioavailability of EE2, probably due to the moderate hydrophobicity of EE2. The study by Serrano *et al*. (2013) indicated that EE2 has greater affinity for particulate matter than for dissolved and colloidal matter (DCM). The authors reported a Log K_{DCM} of 1.65 for EE2. However, Yamamoto *et al*. (2003) reported K_{DOC} of 4.78 and 4.80 for EE2 with Aldrich humic acid and Suwannee River humic acid, respectively, and observed that hydrogen bonding rather than hydrophobic interaction was the main binding mechanism for EE2 due phenolic group composition and aromaticity of DOM. The authors reported higher correlation of Log K_{DOC} of three steroid estrogens (including EE2) with phenolic functional group factor than hydrophobicity, and concluded that the interaction between phenolic groups of steroid estrogens and DOM contributed considerably to overall binding and K_{DOC}. Neale *et al*. (2008) also reported higher affinity of the steroid estrogen, E2, for DOM containing phenolic functional group than for DOM predominantly composed of carboxylic functional groups. Overall Log K_{DOC} of 2.36 estimated for EE2 in this study is within the range of partition coefficient values reported in other studies.

Results from this study did not indicate significant binding of PFOS to humic acid, probably due to poor hydrophobic interaction between PFOS and humic acid. Therefore, K_{DOC} for PFOS was not determined. However, Xia *et al*. (2015) reported reduction in bioavailability and bioaccumulation of PFOS in *D. magna* by DOM. The authors observed higher partition coefficient of PFOS between albumin (protein) and water (Log K_{ALB} 5.29) than between humic acid and water (Log K_{HA} 4.61), and concluded that in spite of the proteinophilic nature of PFOS, DOM has impact on its

bioavailability in the aquatic environment. In rivers, PFOS may dissolve freely in water, adsorb to sediments or accumulate in biota (Campo *et al.*, 2015).

The impact of hydrophobicity on binding to DOM was clearly seen in the interaction of HBCD with humic acid in this study. Decreasing concentration of freely dissolved HBCD with increased concentration of humic acid indicated binding of HBCD to DOM and potential reduction in the bioavailability of HBCD. It has been observed that organic contaminants with high hydrophobicity have a high tendency to complex with DOM which may influence the bioavailability of the contaminants (Wei-Haas *et al.*, 2014). In literature, measurements of the Log K_{DOC} of HBCD are lacking. To the best of our knowledge, this is the first attempt to measure the parameter for HBCD. Overall Log K_{DOC} of HBCD was higher at higher concentration (100 mg/L) of humic acid than at lower concentration (10 mg/L) of humic acid. The overall Log K_{DOC} values were 4.15 and 4.41 at 10 and 100 mg/L humic acid, respectively. The high K_{DOC} values indicate strong affinity of HBCD for humic acid.

5.3 Biological determination of bioavailability

Several studies have been carried out to assess the estrogenicity of EE2 and other organic contaminants, using YES assays. However, a vast majority of the studies lack consideration of the impact of DOM on bioavailability of the test organic compounds. In the study of estrogenicity of surfactants with YES, weak estrogenicity of the degradation products of alkylphenol polyethoxylates in genetically modified yeast, *Saccharomyces cerevisiae* was reported (Routledge and Sumpter, 1996). The study did not determine the impact of DOM on the test compounds. Similarly, Leusch *et al*. (2010) using YES, measured the estrogenicity of EE2 in river water without determination of the impact of DOM on bioavailabilty of the contaminant. Wang *et al*. (2011a) reported risks of the estrogenic contaminants, estrone (E1), E2, EE2, diethylstibestrol, bisphenol-A, nonylphenol and tert-octyphenol to organisms in Liao River, China, using YES as a biological tool for determination of estrogenicity. The same authors observed significant positive correlation between the contaminants' total estrogenic activity measured by EEQ (estradiol equivalent) and the TOC of sediments, indicating sorption of the contaminants to sediments due to organic

carbon. Apparently, sorption to sediments implied reduction in the freely dissolved fractions of the contaminants.

This study incorporated determination of the impact of humic acid on bioavailability into the YES assay to measure estrogenicity of EE2 and DDT. However, the results did not indicate that hydrophobicity of the test compounds and the presence of humic acid had impact on bioavailability and estrogenicity in the YES assay. The absence of humic acid and increasing concentration of humic acid did not also have any impact on estrogenic response of the yeast to EE2 or DDT. Rather, estrogenicity increased with increasing concentration of EE2 or DDT. However, in another study with YES, binding to humic acid was reported to reduce the estrogenicity and hence bioavailability of 3.5-15 µg/L E2 at 130 mg/L humic acid (Bedard *et al.*, 2014). It has been observed that in YES, binding of organic contaminants to humic acid may be influenced by the intrinsic chemical properties of the contaminant and the condition of the yeast medium (Bedard *et al.*, 2014). In this study, humic acid did not have any effect on the bioavailability of DDT. There was estrogenic response in the yeast to both DDT alone and DDT with humic acid. This indicated that hydrophobicity of DDT did not probably have any effect on its binding to DOC in the yeast medium. In literature, data on the determination of the impact of DOC on the bioavailability of DDT, using YES, are lacking.

Conclusions

The application of the chemical and biological approaches to determine the effect of humic acid on bioavailability indicates that although binding to humic acid occurs, and is related to the hydrophobic nature of the contaminant, there is no overall impact on bioavailability.

1. EE2, PFOS and HBCD were used as model compounds in the study of bioavailability of organic contaminants in rivers. Using chemical analysis, the chemicals were detected in UK wastewater effluents, suggesting that wastewaters are a source of the contaminants in rivers. Concentrations of the

chemicals in the effluents ranged from 0.05-0.67 ng/L EE2, 2-19 ng/L PFOS and 1-33 ng/L HBCD.

2. Both chemical and biological methods were developed and used to determine bioavailability of the test chemicals. The method of Landrum *et al* was found to be appropriate for EE2 and HBCD, but the water solubility of PFOS meant that the method could not be applied to this compound.

3. Using the chemical method, both EE2 and HBCD were observed to bind to DOC. Binding to DOC depended on the concentration of humic acid rather than the concentration of EE2 or HBCD. There was greater binding to DOC at higher concentration (100 mg/L) of humic acid than at lower concentration (10 mg/L) of humic acid. There was no observable binding of PFOS to DOC. Being more hydrophobic, HBCD showed greater binding to DOC than EE2. Greater binding to DOC implied reduction in the freely dissolved or potentially bioavailable fractions of HBCD.

4. The Log K_{DOC} for EE2 was 2.36 at 100 mg/L humic acid. HBCD had Log K_{DOC} values of 4.15 and 4.41 at 10 and 100 mg/L humic acid, respectively. The Log K_{DOC} of EE2 at 10 mg/L humic acid was not computed because there were no DOC-bound fractions of EE2 at this concentration of humic acid. The higher Log K_{DOC} values of HBCD than EE2 indicated higher affinity of HBCD for DOC. Comparison of K_{DOC} of EE2 and HBCD derived from this study with values in literature is difficult due to little or no information on the K_{DOC} of the chemicals in literature. However, it has been observed that DOC occurs naturally in rivers and may limit the freely dissolved or bioavailable fractions of organic contaminants.

5. Application of the YES to determine bioavailability presented significant challenges, particularly when working with the more hydrophobic DDT. The assay required modification to allow for equilibrium between DDT and humic acid to be reached, while at the same time minimising binding of the DDT to the wells of the assay microplates.

6. The YES was used as a biological method to measure the effect of DOC on the bioavailability of EE2 and the more hydrophobic compound, DDT. The YES indicated that the presence of DOC had no effect on bioavailability of the chemicals. There are no previous data on the effect of DOC on the bioavailability of DDT in YES. There is need for further investigation on the impact of DOC on the bioavailability of estrogenic organic contaminants, using YES as a biological tool

Future work

Although this study evaluated bioavailability of organic contaminants in rivers, further work in a number of ways could be carried out to extend it. Future work could include collection and chemical analysis of natural river water samples for measurement of DOC and fractions of organic contaminants bound to DOC or freely dissolved. Data from such work could be compared with laboratory-derived data on bioavailability.

It is thought that organisms in rivers are exposed to mixtures of contaminants and that the fate (including bioavailability and toxicity) of single chemicals may be different from their fate in mixtures. Therefore, there is need for future study on the effects of the interaction of chemicals in a mixture on their bioavailability and toxicity in comparison with single chemicals.

Hydrophobic organic contaminants may exhibit affinity for solids, particularly sediments in rivers, resulting in their distribution between solid and water phases. Therefore, in future work, the impact of sorption of organic contaminants to sediments on bioavailability could be evaluated. Such study could determine the impact of sediments alone and the impact of mixtures of sediments and humic acid on bioavailability.

REFERENCES

Abdallah, M.A., Harrad, S. and Covaci, A. (2008) 'Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, UK: Implications for human exposure', *Environmental Science & Technology,* 42(18), pp. 6855-6861.

ACS (2014) *CAS Registry and CAS Registry Number FAQs. American Chemical Society. Available: http://www.cas.org/content/chemical-substances/faqs (accessed 21 September 2014).*

Ahrens, L. (2011) 'Polyfluoroalkyl compounds in the aquatic environment: A review of their occurrence and fate', *Journal of Environmental Monitoring,* 13(1), pp. 20-31.

Akkanen, J., Slootweg, T., Mäenpää, K., Leppänen, M.T., Agbo, S., Gallampois, C. and Kukkonen, J.V. (2012) 'Bioavailability of organic contaminants in freshwater environments', in *Emerging and Priority Pollutants in Rivers.* Springer, pp. 25-53.

Akkanen, J., Vogt, R.D. and Kukkonen, J.V. (2004) 'Essential characteristics of natural dissolved organic matter affecting the sorption of hydrophobic organic contaminants', *Aquatic Sciences,* 66(2), pp. 171-177.

Alaee, M., Arias, P., Sjödin, A. and Bergman, Å. (2003) 'An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release', *Environment International,* 29(6), pp. 683-689.

Al-Ansari, A.M., Atkinson, S.K., Doyle, J.R., Trudeau, V.L. and Blais, J.M. (2013) 'Dynamics of uptake and elimination of 17α-ethinylestradiol in male goldfish (*Carassius auratus*)', *Aquatic Toxicology,* 132-133, pp. 134-140.

Al-Ansari, A.M., Saleem, A., Kimpe, L.E., Sherry, J.P., McMaster, M.E., Trudeau, V.L. and Blais, J.M. (2010) 'Bioaccumulation of the pharmaceutical 17α-ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River, Canada', *Environmental Pollution,* 158(8), pp. 2566-2571.

Allinson, M., Shiraishi, F., Salzman, S. and Allinson, G. (2010) 'In vitro and immunological assessment of the estrogenic activity and concentrations of 17β-estradiol, estrone, and ethinyl estradiol in treated effluent from 45 wastewater treatment plants in Victoria, Australia', *Archives of Environmental Contamination and Toxicology,* 58(3), pp. 576-586.

Ankley, G.T., Kuehl, D.W., Kahl, M.D., Jensen, K.M., Linnum, A., Leino, R.L. and Villeneuve, D.A. (2005) 'Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (*Pimephales promelas*)', *Environmental Toxicology and Chemistry,* 24(9), pp. 2316-2324.

Aris, A.Z., Shamsuddin, A.S. and Praveena, S.M. (2014) 'Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review', *Environment International,* 69, pp. 104-119.

Arnot, J.A. and Gobas, F.A. (2006) 'A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms', *Environmental Reviews,* 14(4), pp. 257-297.

Arvaniti, O.S., Ventouri, E.I., Stasinakis, A.S. and Thomaidis, N.S. (2012) 'Occurrence of different classes of perfluorinated compounds in Greek wastewater treatment plants and determination of their solid–water distribution coefficients', *Journal of Hazardous Materials,* 239, pp. 24-31.

Atkinson, S.K., Marlatt, V.L., Kimpe, L.E., Lean, D.R., Trudeau, V.L. and Blais, J.M. (2012) 'The occurrence of steroidal estrogens in south-eastern Ontario wastewater treatment plants', *Science of the Total Environment,* 430, pp. 119-125.

Bain, P.A., Williams, M. and Kumar, A. (2014) 'Assessment of multiple hormonal activities in wastewater at different stages of treatment', *Environmental Toxicology and Chemistry,* 33(10), pp. 2297-2307.

Banihashemi, B. and Droste, R.L. (2014) 'Sorption–desorption and biosorption of bisphenol A, triclosan, and 17α-ethinylestradiol to sewage sludge', *Science of the Total Environment,* 487, pp. 813-821.

Beach, S.A., Newsted, J.L., Coady, K. and Giesy, J.P. (2006) 'Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS)', in *Reviews of Environmental Contamination and Toxicology.* Springer, pp. 133-174.

Becker, A.M., Gerstmann, S. and Frank, H. (2008) 'Perfluorooctanoic acid and perfluorooctane sulfonate in the sediment of the Roter Main River, Bayreuth, Germany', *Environmental Pollution,* 156(3), pp. 818-820.

Becker, A.M., Suchan, M., Gerstmann, S. and Frank, H. (2010) 'Perfluorooctanoic acid and perfluorooctane sulfonate released from a waste water treatment plant in Bavaria, Germany', *Environmental Science and Pollution Research,* 17(9), pp. 1502-1507.

Becker, A.M., Gerstmann, S. and Frank, H. (2008) 'Perfluorooctane surfactants in waste waters, the major source of river pollution', *Chemosphere,* 72(1), pp. 115-121.

Bedard, M., Giffear, K.A., Ponton, L., Sienerth, K.D. and Del Gaizo Moore, V. (2014) 'Characterization of binding between 17β-estradiol and estriol with humic acid via NMR and biochemical analysis', *Biophysical Chemistry,* 189, pp. 1-7.

Blewett, T.A., Chow, T.L., Maclatchy, D.L. and Wood, C.M. (2014) 'A species comparison of 17-α-ethynylestradiol uptake and tissue-specific distribution in six

teleost fish', *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* 161(1), pp. 33-40.

Boltes, K., Rosal, R. and García-Calvo, E. (2012) 'Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators', *Chemosphere,* 86(1), pp. 24-29.

Bondarenko, S. and Gan, J. (2009) 'Simultaneous measurement of free and total concentrations of hydrophobic compounds', *Environmental Science & Technology,* 43(10), pp. 3772-3777.

Bossi, R., Strand, J., Sortkjær, O. and Larsen, M.M. (2008) 'Perfluoroalkyl compounds in Danish wastewater treatment plants and aquatic environments', *Environment International,* 34(4), pp. 443-450.

Boudreau, T.M., Sibley, P., Mabury, S., Muir, D. and Solomon, K. (2003) 'Laboratory evaluation of the toxicity of perfluorooctane sulfonate (PFOS) on *Selenastrum capricornutum*, *Chlorella vulgaris*, *Lemna gibba*, *Daphnia magna*, and *Daphnia pulicaria*', *Archives of Environmental Contamination and Toxicology,* 44(3), pp. 0307-0313.

Brooke, D., Footitt, A. and Nwaogu, T. (2004) *Environmental risk evaluation report: Perfluorooctanesulphonate (PFOS). Environment Agency, UK.*

Brown, J.N. and Peake, B.M. (2003) 'Determination of colloidally-associated polycyclic aromatic hydrocarbons (PAHs) in fresh water using C 18 solid phase extraction disks', *Analytica Chimica Acta,* 486(2), pp. 159-169.

Busch, J., Ahrens, L., Sturm, R. and Ebinghaus, R. (2010) 'Polyfluoroalkyl compounds in landfill leachates', *Environmental Pollution,* 158(5), pp. 1467-1471.

Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R. and Sumpter, J.P. (2012) 'Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol', *Environmental Toxicology and Chemistry,* 31(6), pp. 1396-1406.

Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Länge, R., Heijerick, D., Janssen, C., Anderson, P.D. and Sumpter, J.P. (2008) 'Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17α-ethinyl estradiol', *Environmental Science & Technology,* 42(19), pp. 7046-7054.

Calow, P. and Forbes, V.E. (2003) 'Does ecotoxicology inform ecological risk assessment?', *Environmental Science & Technology,* 37(7), pp. 146A-151A.

Campo, J., Pérez, F., Masiá, A., Picó, Y., la Farré, M. and Barceló, D. (2015) 'Perfluoroalkyl substance contamination of the Llobregat River ecosystem (Mediterranean area, NE Spain)', *Science of the Total Environment,* 503, pp. 48-57.

Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S. and Levi, Y. (2004) 'Assessment of river contamination by estrogenic compounds in Paris area (France)', *Science of the Total Environment,* 324(1), pp. 55-66.

Carloni, D. (2009) 'Perfluorooctane sulfonate (PFOS) production and use: Past and current evidence', *Report for United Nations Industrial Development Organization (UNIDO),* .

Chapman, P.M. (2007) 'Determining when contamination is pollution—weight of evidence determinations for sediments and effluents', *Environment International,* 33(4), pp. 492-501.

Chapman, P.M., Fairbrother, A. and Brown, D. (1998) 'A critical evaluation of safety (uncertainty) factors for ecological risk assessment', *Environmental Toxicology and Chemistry,* 17(1), pp. 99-108.

Chari, B.P. and Halden, R.U. (2012) 'Predicting the concentration range of unmonitored chemicals in wastewater-dominated streams and in run-off from biosolids-amended soils', *Science of the Total Environment,* 440(0), pp. 314-320.

Chen, T.C., Chen, T.S., Yeh, K.J., Lin, Y.C., Chao, H.R. and Yeh, Y.L. (2012) 'Sorption of estrogens estrone, 17ß-estradiol, estriol, 17α-ethinylestradiol, and diethylstilbestrol on sediment affected by different origins', *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering,* 47(12), pp. 1768-1775.

Conder, J.M., Hoke, R.A., Wolf, W.d., Russell, M.H. and Buck, R.C. (2008) 'Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds', *Environmental Science & Technology,* 42(4), pp. 995-1003.

Coquery, M., Morin, A., Becue, A. and Lepot, B. (2005) 'Priority substances of the European Water Framework Directive: Analytical challenges in monitoring water quality', *TrAC Trends in Analytical Chemistry,* 24(2), pp. 117-127.

Covaci, A., Gerecke, A.C., Law, R.J., Voorspoels, S., Kohler, M., Heeb, N.V., Leslie, H., Allchin, C.R. and de Boer, J. (2006) 'Hexabromocyclododecanes (HBCDs) in the environment and humans: A review', *Environmental Science & Technology,* 40(12), pp. 3679-3688.

Croue, J. (2004) 'Isolation of humic and non-humic NOM fractions: Structural characterization', *Environmental Monitoring and Assessment,* 92(1-3), pp. 193-207.

Davis, J.W., Gonsior, S., Marty, G. and Ariano, J. (2005) 'The transformation of hexabromocyclododecane in aerobic and anaerobic soils and aquatic sediments', *Water Research,* 39(6), pp. 1075-1084.

Davis, J.W., Gonsior, S.J., Markham, D.A., Friederich, U., Hunziker, R.W. and Ariano, J.M. (2006) 'Biodegradation and product identification of [14C] hexabromocyclododecane in wastewater sludge and freshwater aquatic sediment', *Environmental Science and Technology,* 40(17), pp. 5395-5401.

de Sá Salomão, André Luís, Soroldoni, S., Marques, M., Hogland, W. and Bila, D.M. (2014) 'Effects of single and mixed estrogens on single and combined cultures of *D. subspicatus* and *P. subcapitata*', *Bulletin of Environmental Contamination and Toxicology,* , pp. 1-7.

de Wit, C.A., Herzke, D. and Vorkamp, K. (2010) 'Brominated flame retardants in the Arctic environment—trends and new candidates', *Science of the Total Environment,* 408(15), pp. 2885-2918.

Dean, J. and Scott, W. (2004) 'Recent developments in assessing the bioavailability of persistent organic pollutants in the environment', *TrAC Trends in Analytical Chemistry,* 23(9), pp. 609-618.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W., Lam, P.K., Wu, R.S. and Zhou, B. (2009) 'Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos', *Aquatic Toxicology,* 93(1), pp. 29-36.

Ding, H., Li, Y., Hou, J., Wang, Q. and Wu, Y. (2014) 'Sorption behavior and modeling of endocrine-disrupting chemicals on natural sediments: Role of biofilm covered on surface', *Environmental Science and Pollution Research,* , pp. 1-9.

Doi, H. (2009) 'Spatial patterns of autochthonous and allochthonous resources in aquatic food webs', *Population Ecology,* 51(1), pp. 57-64.

Dreyer, A., Weinberg, I., Temme, C. and Ebinghaus, R. (2009) 'Polyfluorinated compounds in the atmosphere of the Atlantic and Southern Oceans: Evidence for a global distribution', *Environmental Science & Technology,* 43(17), pp. 6507-6514.

Drottar, K. and Krueger, H. (1998) 'Hexabromocyclododecane (HBCD): A flow-through life-cycle toxicity test with the cladoceran (*Daphnia magna*)', *Final report.439A-108, Wildlife International, Ltd, Easton, Maryland, USA,* .

Drottar, K., MacGregor, J. and Krueger, H. (2001) *Hexabromocyclododecane (HBCD): An early life-stage toxicity test with the rainbow trout (Oncorhynchus mykiss).* Final Report, Wildlife International Ltd., Easton, Maryland, USA.

Du, M., Lin, L., Yan, C. and Zhang, X. (2012) 'Diastereoisomer- and enantiomer-specific accumulation, depuration, and bioisomerization of hexabromocyclododecanes in zebrafish (*Danio rerio*)', *Environmental Science & Technology,* 46(20), pp. 11040-11046.

Du, Y., Shi, X., Liu, C., Yu, K. and Zhou, B. (2009) 'Chronic effects of water-borne PFOS exposure on growth, survival and hepatotoxicity in zebrafish: A partial life-cycle test', *Chemosphere,* 74(5), pp. 723-729.

EC (2013) *Proposal for a Council decision on the position to be adopted, on behalf of the European Union, at the Sixth Conference of the Parties to the Stockholm Convention on Persistent Organic Pollutants with regard to the proposal for an amendment of Annexes A and B. 2013/0075 (NLE), Brussels, 12.3.2013. European Commission. Available: http://www.eu-oplysningen.dk/upload/application/pdf/291f306c/20130134.pdf (accessed 23 September 2014).*

EC (2012) *Proposal for a directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. 2011/0429 (COD), Brussels, 31-1-2012. European Commission.*

EC (2008) *Risk assessment: Hexabromocyclododecane (CAS-No.: 25637-99-4; EINECS-No.: 247-148-4). R044_0805_env_hh_final_ECB.doc. Final report, May 2008. European Commission.*

ECHA (2009) *Data on the manufacture, import, export, uses and releases of HBCDD as well as information on potential alternatives to its use. 1 December 2009. European Chemicals Agency.*

ECHA (2008a) *Agreement of the Member State Committee on identification of hexabromocyclododecane (HBCDD) and all major diastereoisomers identified as a substance of very high concern. 8 October 2008. European Chemicals Agency. 4p.*

ECHA (2008b) *Member State Committee support document for identification of hexabromocyclododecane and all major diastereoisomers identified as a substance of very high concern. Adopted on 8 October 2008. European Chemicals Agency. 43p.*

Eljarrat, E., de la Cal, A., Raldua, D., Duran, C. and Barcelo, D. (2004) 'Occurrence and bioavailability of polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from the Cinca River, a tributary of the Ebro River (Spain)', *Environmental Science & Technology,* 38(9), pp. 2603-2608.

Environment Canada (2011) *Screening assessment report on hexabromocyclododecane. Chemical Abstracts Service Registry Number 3194-55-6. November 2011. Environment Canada/Health Canada.*

Ericson, J.F., Laenge, R. and Sullivan, D.E. (2002) 'Comment on “Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance”', *Environmental Science & Technology,* 36(18), pp. 4005-4006.

EU (2013) *Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. OJ L226/1. European Union*.

EU (2010) *Commission Regulation (EU) No 757/2010 of 24 August 2010 amending Regulation (EC) No 850/2004 of the European Parliament and of the Council on persistent organic pollutants as regards Annexes I and III. OJ L223/29. European Union*.

EU (2009) *Commission Regulation (EC) No 552/2009 of 22 June 2009 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annex XVII. OJ L164/7. European Union*.

EU (2006) *Directive 2006/122/EC of the European Parliament and of the Council of 12 December 2006 amending for the 30th time Council Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (perfluorooctane sulfonates). OJ L372/32. European Union*.

Evans, C., Monteith, D. and Cooper, D. (2005) 'Long-term increases in surface water dissolved organic carbon: Observations, possible causes and environmental impacts', *Environmental Pollution,* 137(1), pp. 55-71.

Fan, L., Harris, J.L., Roddick, F.A. and Booker, N.A. (2001) 'Influence of the characteristics of natural organic matter on the fouling of microfiltration membranes', *Water Research,* 35(18), pp. 4455-4463.

Fernández-Sanjuan, M., Meyer, J., Damásio, J., Faria, M., Barata, C. and Lacorte, S. (2010) 'Screening of perfluorinated chemicals (PFCs) in various aquatic organisms', *Analytical and Bioanalytical Chemistry,* 398(3), pp. 1447-1456.

Flores, C., Ventura, F., Martin-Alonso, J. and Caixach, J. (2013) 'Occurrence of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in N.E. Spanish surface waters and their removal in a drinking water treatment plant that combines conventional and advanced treatments in parallel lines', *Science of the Total Environment,* 461–462(0), pp. 618-626.

Fromme, H., Hilger, B., Kopp, E., Miserok, M. and Völkel, W. (2014) 'Polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and “novel” brominated flame retardants in house dust in Germany', *Environment International,* 64, pp. 61-68.

Gallen, C., Baduel, C., Lai, F.Y., Thompson, K., Thompson, J., Warne, M. and Mueller, J.F. (2014) 'Spatio-temporal assessment of perfluorinated compounds in the Brisbane River system, Australia: Impact of a major flood event', *Marine Pollution Bulletin,* .

Giesy, J.P. and Kannan, K. (2002) 'Perfluorochemical surfactants in the environment', *Environmental Science & Technology,* 36(7), pp. 146A-152A.

Graves, W. and Swigert, J. (1997a) *Hexabromocyclododecane (HBCD): A 48-hour flow-through acute toxicity test with the cladoceran (Daphnia magna).* Final Report, 439A-102, Wildlife International Ltd., Easton, Maryland, USA.

Graves, W. and Swigert, J. (1997b) *Hexabromocyclododecane (HBCD): A 96-hour flow-through acute toxicity test with the rainbow trout (Oncorhynchus mykiss).* Final Report, 439A-101, Wildlife International Ltd., Easton, Maryland, USA.

Gros, M., Rodríguez-Mozaz, S. and Barceló, D. (2012) 'Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry', *Journal of Chromatography A,* 1248, pp. 104-121.

Grover, D., Zhou, J., Frickers, P. and Readman, J. (2011) 'Improved removal of estrogenic and pharmaceutical compounds in sewage effluent by full scale granular activated carbon: Impact on receiving river water', *Journal of Hazardous Materials,* 185(2), pp. 1005-1011.

Grund, S., Higley, E., Schönenberger, R., Suter, M.J., Giesy, J.P., Braunbeck, T., Hecker, M. and Hollert, H. (2011) 'The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by in vitro bioassays and chemical analysis', *Environmental Science and Pollution Research,* 18(3), pp. 446-460.

Guerra, P., Cal, A.D.L., Marsh, G., Eljarrat, E. and Barceló, D. (2009) 'Transfer of hexabromocyclododecane from industrial effluents to sediments and biota: Case study in Cinca river (Spain)', *Journal of Hydrology,* 369(3), pp. 360-367.

Guerra, P., Eljarrat, E. and Barceló, D. (2010) 'Simultaneous determination of hexabromocyclododecane, tetrabromobisphenol A, and related compounds in sewage sludge and sediment samples from Ebro River basin (Spain)', *Analytical and Bioanalytical Chemistry,* 397(7), pp. 2817-2824.

Guo, R., Sim, W., Lee, E., Lee, J. and Oh, J. (2010) 'Evaluation of the fate of perfluoroalkyl compounds in wastewater treatment plants', *Water Research,* 44(11), pp. 3476-3486.

Hagenaars, A., Knapen, D., Meyer, I.J., van der Ven, K., Hoff, P. and De Coen, W. (2008) 'Toxicity evaluation of perfluorooctane sulfonate (PFOS) in the liver of common carp (*Cyprinus carpio*)', *Aquatic Toxicology,* 88(3), pp. 155-163.

Hale, R.C., La Guardia, M.J., Harvey, E., Gaylor, M.O. and Mainor, T.M. (2006) 'Brominated flame retardant concentrations and trends in abiotic media', *Chemosphere,* 64(2), pp. 181-186.

Han, S., Zhang, Y., Masunaga, S., Zhou, S. and Naito, W. (2014) 'Relating metal bioavailability to risk assessment for aquatic species: Daliao River watershed, China', *Environmental Pollution,* 189, pp. 215-222.

Han, J. and Fang, Z. (2010) 'Estrogenic effects, reproductive impairment and developmental toxicity in ovoviparous swordtail fish (*Xiphophorus helleri*) exposed to perfluorooctane sulfonate (PFOS)', *Aquatic Toxicology,* 99(2), pp. 281-290.

Hannah, R., D'Aco, V.J., Anderson, P.D., Buzby, M.E., Caldwell, D.J., Cunningham, V.L., Ericson, J.F., Johnson, A.C., Parke, N.J. and Samuelian, J.H. (2009) 'Exposure assessment of 17α-ethinylestradiol in surface waters of the United States and Europe', *Environmental Toxicology and Chemistry,* 28(12), pp. 2725-2732.

Hansen, K., Johnson, H., Eldridge, J., Butenhoff, J. and Dick, L. (2002) 'Quantitative characterization of trace levels of PFOS and PFOA in the Tennessee River', *Environmental Science & Technology,* 36(8), pp. 1681-1685.

Hardy, M.L. (2004) 'A comparison of the fish bioconcentration factors for brominated flame retardants with their nonbrominated analogues', *Environmental Toxicology and Chemistry,* 23(3), pp. 656-661.

Haukås, M., Mariussen, E., Ruus, A. and Tollefsen, K.E. (2009) 'Accumulation and disposition of hexabromocyclododecane (HBCD) in juvenile rainbow trout (*Oncorhynchus mykiss*)', *Aquatic Toxicology,* 95(2), pp. 144-151.

Hayward, S.J., Lei, Y.D. and Wania, F. (2006) 'Comparative evaluation of three high-performance liquid chromatography–based Kow estimation methods for highly hydrophobic organic compounds: Polybrominated diphenyl ethers and hexabromocyclododecane', *Environmental Toxicology and Chemistry,* 25(8), pp. 2018-2027.

He, M., Luo, X., Yu, L., Wu, J., Chen, S. and Mai, B. (2013) 'Diasteroisomer and enantiomer-specific profiles of hexabromocyclododecane and tetrabromobisphenol A in an aquatic environment in a highly industrialized area, South China: Vertical profile, phase partition, and bioaccumulation', *Environmental Pollution,* 179, pp. 105-110.

Heeb, F., Singer, H., Pernet-Coudrier, B., Qi, W., Liu, H., Longrée, P., Müller, B. and Berg, M. (2012) 'Organic micropollutants in rivers downstream of the megacity Beijing: Sources and mass fluxes in a large-scale wastewater irrigation system', *Environmental Science & Technology,* 46(16), pp. 8680-8688.

Heeb, N.V., Schweizer, W.B., Kohler, M. and Gerecke, A.C. (2005) 'Structure elucidation of hexabromocyclododecanes—a class of compounds with a complex stereochemistry', *Chemosphere,* 61(1), pp. 65-73.

Higgins, C.P. and Luthy, R.G. (2006) 'Sorption of perfluorinated surfactants on sediments', *Environmental Science & Technology,* 40(23), pp. 7251-7256.

Hintemann, T., Schneider, C., Schöler, H.F. and Schneider, R.J. (2006) 'Field study using two immunoassays for the determination of estradiol and ethinylestradiol in the aquatic environment', *Water Research,* 40(12), pp. 2287-2294.

Hloušková, V., Lanková, D., Kalachová, K., Hrádková, P., Poustka, J., Hajšlová, J. and Pulkrabová, J. (2014) 'Brominated flame retardants and perfluoroalkyl substances in sediments from the Czech aquatic ecosystem', *Science of the Total Environment,* 470, pp. 407-416.

Hloušková, V., Lanková, D., Kalachová, K., Hrádková, P., Poustka, J., Hajšlová, J. and Pulkrabová, J. (2013) 'Occurrence of brominated flame retardants and perfluoroalkyl substances in fish from the Czech aquatic ecosystem', *Science of the Total Environment,* 461, pp. 88-98.

Hoffmann, F. and Kloas, W. (2012) 'Estrogens can disrupt amphibian mating behavior', *PloS One,* 7(2), pp. e32097.

Holthaus, K.I., Johnson, A.C., Jürgens, M.D., Williams, R.J., Smith, J.J. and Carter, J.E. (2002) 'The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers', *Environmental Toxicology and Chemistry,* 21(12), pp. 2526-2535.

HSDB (2012a) *Ethinylestradiol. CASRN: 57-63-6. Hazardous Substances Data Bank. Database of TOXNET (Toxicology Data Network). US National Library of Medicine. Available:http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~8f6xM5:1 (accessed 17 September 2014).*

HSDB (2012b) *Perfluorooctane sulfonic acid. CASRN:1763-23-1. Hazardous Substances Data Bank. Database of TOXNET (Toxicology Data Network). US National Library of Medicine. Available:http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~t0tPHD:1 (accessed 17 September 2014).*

HSDB (2011) *Hexabromocyclododecane. CASRN:25637-99-4. Hazardous Substances Data Bank. Database of TOXNET (Toxicology Data Network). US National Library of Medicine. Available: http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~jSk7PC:1 (accessed 17 September 2014).*

Hu, J., Liang, Y., Chen, M. and Wang, X. (2009) 'Assessing the toxicity of TBBPA and HBCD by zebrafish embryo toxicity assay and biomarker analysis', *Environmental Toxicology,* 24(4), pp. 334-342.

Hutchinson, T.H. (2002) 'Reproductive and developmental effects of endocrine disrupters in invertebrates: *In vitro* and *in vivo* approaches', *Toxicology Letters,* 131(1), pp. 75-81.

Ichihara, M., Yamamoto, A., Takakura, K., Kakutani, N. and Sudo, M. (2014) 'Distribution and pollutant load of hexabromocyclododecane (HBCD) in sewage treatment plants and water from Japanese Rivers', *Chemosphere,* 110, pp. 78-84.

Ilyas, M., Sudaryanto, A., Setiawan, I.E., Riyadi, A.S., Isobe, T., Takahashi, S. and Tanabe, S. (2011) 'Characterization of polychlorinated biphenyls and brominated flame retardants in sediments from riverine and coastal waters of Surabaya, Indonesia', *Marine Pollution Bulletin,* 62(1), pp. 89-98.

Inoue, Y., Hashizume, N., Yakata, N., Murakami, H., Suzuki, Y., Kikushima, E. and Otsuka, M. (2012) 'Unique physicochemical properties of perfluorinated compounds and their bioconcentration in common carp *Cyprinus carpio* L.', *Archives of Environmental Contamination and Toxicology,* 62(4), pp. 672-680.

Janák, K., Covaci, A., Voorspoels, S. and Becher, G. (2005) 'Hexabromocyclododecane in marine species from the Western Scheldt Estuary: Diastereoisomer-and enantiomer-specific accumulation', *Environmental Science & Technology,* 39(7), pp. 1987-1994.

Janex-Habibi, M., Huyard, A., Esperanza, M. and Bruchet, A. (2009) 'Reduction of endocrine disruptor emissions in the environment: The benefit of wastewater treatment', *Water Research,* 43(6), pp. 1565-1576.

Janoš, P. (2003) 'Separation methods in the chemistry of humic substances', *Journal of Chromatography A,* 983(1), pp. 1-18.

Jeong, G.H., Hwang, N.R., Hwang, E., Lee, B. and Yoon, J. (2014) 'Hexabromocyclododecanes in crucian carp and sediment from the major rivers in Korea', *Science of the Total Environment,* 470, pp. 1471-1478.

Ji, K., Kim, Y., Oh, S., Ahn, B., Jo, H. and Choi, K. (2008) 'Toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid on freshwater macroinvertebrates (*Daphnia magna* and *Moina macrocopa*) and fish (*Oryzias latipes*)', *Environmental Toxicology and Chemistry,* 27(10), pp. 2159-2168.

Jobling, S. and Owen, R. (2013) 'Ethinyl oestradiol in the aquatic environment', *Late Lessons from Early Warnings: Science, Precaution, Innovation. Eds. European Environment Agency, EEA Report No.1/2013, EEA, Copenhagen, Denmark,* .

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G. and Sumpter, J.P. (1998) 'Widespread sexual disruption in wild fish', *Environmental Science & Technology,* 32(17), pp. 2498-2506.

Jobling, S. and Owen, R.J. (eds.) (2010) *Ethinyl oestradiol: Bitter pill for the precautionary principle.*European Environment Agency.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., Aerle, R., Santos, E. and Brighty, G. (2006) 'Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations', *Environmental Health Perspectives,* 114, pp. 1.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R. and Sumpter, J.P. (2002) 'Wild intersex roach (*Rutilus rutilus*) have reduced fertility', *Biology of Reproduction,* 67(2), pp. 515-524.

Johnson, A.C., Dumont, E., Williams, R.J., Oldenkamp, R., Cisowska, I. and Sumpter, J.P. (2013) 'Do concentrations of ethinylestradiol, estradiol, and

diclofenac in European rivers exceed proposed EU environmental quality standards?', *Environmental Science & Technology,* 47(21), pp. 12297-12304.

Jones, P.D., Hu, W., De Coen, W., Newsted, J.L. and Giesy, J.P. (2003) 'Binding of perfluorinated fatty acids to serum proteins', *Environmental Toxicology and Chemistry,* 22(11), pp. 2639-2649.

Jürgens, M.D., Holthaus, K.I.E., Johnson, A.C., Smith, J.J.L., Hetheridge, M. and Williams, R.J. (2002) 'The potential for estradiol and ethinylestradiol degradation in English rivers', *Environmental Toxicology and Chemistry,* 21(3), pp. 480-488.

Kanda, R. and Churchley, J. (2008) 'Removal of endocrine disrupting compounds during conventional wastewater treatment', *Environmental Technology,* 29(3), pp. 315-323.

Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S. and Van Leeuwen, J. (2006) 'Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems', *Environmental Science & Technology,* 40(21), pp. 6537-6546.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J. and Aït-Aïssa, S. (2010) 'Bioanalytical characterisation of multiple endocrine-and dioxin-like activities in sediments from reference and impacted small rivers', *Environmental Pollution,* 158(1), pp. 74-83.

Koh, Y.K., Chiu, T.Y., Boobis, A.R., Scrimshaw, M.D., Bagnall, J.P., Soares, A., Pollard, S., Cartmell, E. and Lester, J.N. (2009) 'Influence of operating parameters on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes', *Environmental Science & Technology,* 43(17), pp. 6646-6654.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and Buxton, H.T. (2002) 'Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance', *Environmental Science & Technology,* 36(6), pp. 1202-1211.

Konwick, B.J., Tomy, G.T., Ismail, N., Peterson, J.T., Fauver, R.J., Higginbotham, D. and Fisk, A.T. (2008) 'Concentrations and patterns of perfluoroalkyl acids in Georgia, USA surface waters near and distant to a major use source', *Environmental Toxicology and Chemistry,* 27(10), pp. 2011-2018.

Kresinová, Z., Moeder, M., Ezechiáš, M., Svobodová, K. and Cajthaml, T. (2012) 'Mechanistic study of 17α-ethinylestradiol biodegradation by pleurotus ostreatus: Tracking of extracelullar and intracelullar degradation mechanisms', *Environmental Science and Technology,* 46(24), pp. 13377-13385.

Kuch, H.M. and Ballschmiter, K. (2001) 'Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range', *Environmental Science & Technology,* 35(15), pp. 3201-3206.

Kuiper, R.V., Cantón, R.F., Leonards, P.E.G., Jenssen, B.M., Dubbeldam, M., Wester, P.W., van den Berg, M., Vos, J.G. and Vethaak, A.D. (2007) 'Long-term exposure of European flounder (*Platichthys flesus*) to the flame-retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD)', *Ecotoxicology and Environmental Safety,* 67(3), pp. 349-360.

Labadie, P. and Chevreuil, M. (2011) 'Partitioning behaviour of perfluorinated alkyl contaminants between water, sediment and fish in the Orge River (nearby Paris, France)', *Environmental Pollution,* 159(2), pp. 391-397.

Labadie, P. and Hill, E.M. (2007) 'Analysis of estrogens in river sediments by liquid chromatography–electrospray ionisation mass spectrometry: Comparison of tandem mass spectrometry and time-of-flight mass spectrometry', *Journal of Chromatography A,* 1141(2), pp. 174-181.

Lai, K., Johnson, K., Scrimshaw, M. and Lester, J. (2000) 'Binding of waterborne steroid estrogens to solid phases in river and estuarine systems', *Environmental Science & Technology,* 34(18), pp. 3890-3894.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. (2002) 'Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems', *Science of the Total Environment,* 289(1-3), pp. 159-168.

Landrum, P.F., Nihart, S.R., Eadie, B.J. and Gardner, W.S. (1984) 'Reverse-phase separation method for determining pollutant binding to Aldrich humic acid and dissolved organic carbon of natural waters', *Environmental Science & Technology,* 18(3), pp. 187-192.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H. and Sumpter, J.P. (2001) 'Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*)', *Environmental Toxicology and Chemistry,* 20(6), pp. 1216-1227.

Langston, W., Burt, G., Chesman, B. and Vane, C. (2005) 'Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment', *Journal of the Marine Biological Association of the United Kingdom,* 85(01), pp. 1-31.

Larcher, S. and Yargeau, V. (2013) 'Biodegradation of 17α-ethinylestradiol by heterotrophic bacteria', *Environmental Pollution,* 173, pp. 17-22.

Law, K., Palace, V.P., Halldorson, T., Danell, R., Wautier, K., Evans, B., Alaee, M., Marvin, C. and Tomy, G.T. (2006) 'Dietary accumulation of hexabromocyclododecane diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) I: Bioaccumulation parameters and evidence of bioisomerization', *Environmental Toxicology and Chemistry,* 25(7), pp. 1757-1761.

Law, R.J., Kohler, M., Heeb, N.V., Gerecke, A.C., Schmid, P., Voorspoels, S., Covaci, A., Becher, G., JanAk, K. and Thomsen, C. (2005) 'Hexabromocyclododecane challenges scientists and regulators', *Environmental Science & Technology,* 39(13), pp. 281A-287A.

Legler, J. (2008) 'New insights into the endocrine disrupting effects of brominated flame retardants', *Chemosphere,* 73(2), pp. 216-222.

Lei, B., Huang, S., Zhou, Y., Wang, D. and Wang, Z. (2009) 'Levels of six estrogens in water and sediment from three rivers in Tianjin area, China', *Chemosphere,* 76(1), pp. 36-42.

Lein, N.P.H., Fujii, S., Tanaka, S., Nozoe, M. and Tanaka, H. (2008) 'Contamination of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in surface water of the Yodo River basin (Japan)', *Desalination,* 226(1), pp. 338-347.

Leusch, F.D., De Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S. and Chapman, H.F. (2010) 'Comparison of five *in vitro* bioassays to measure estrogenic activity in environmental waters', *Environmental Science & Technology,* 44(10), pp. 3853-3860.

Li, F., Zhang, C., Qu, Y., Chen, J., Chen, L., Liu, Y. and Zhou, Q. (2010) 'Quantitative characterization of short-and long-chain perfluorinated acids in solid matrices in Shanghai, China', *Science of the Total Environment,* 408(3), pp. 617-623.

Li, H., Shang, H., Wang, P., Wang, Y., Zhang, H., Zhang, Q. and Jiang, G. (2013) 'Occurrence and distribution of hexabromocyclododecane in sediments from seven major river drainage basins in China', *Journal of Environmental Sciences,* 25(1), pp. 69-76.

Li, M. (2010) 'Chronic effects of perfluorooctane sulfonate and ammonium perfluorooctanoate on biochemical parameters, survival and reproduction of *Daphnia magna*', *Journal of Health Science,* 56(1), pp. 104-111.

Li, M. (2009) 'Toxicity of perfluorooctane sulfonate and perfluorooctanoic acid to plants and aquatic invertebrates', *Environmental Toxicology,* 24(1), pp. 95-101.

Li, N. and Lee, H.K. (2001) 'Solid-phase extraction of polycyclic aromatic hydrocarbons in surface water: Negative effect of humic acid', *Journal of Chromatography A,* 921(2), pp. 255-263.

Lima, D.L., Schneider, R.J. and Esteves, V.I. (2012) 'Sorption behavior of EE2 on soils subjected to different long-term organic amendments', *Science of the Total Environment,* 423, pp. 120-124.

Lin, A.Y.-. and Reinhard, M. (2005) 'Photodegradation of common environmental pharmaceuticals and estrogens in river water', *Environmental Toxicology and Chemistry,* 24(6), pp. 1303-1309.

Lindstrom, A.B., Strynar, M.J., Delinsky, A.D., Nakayama, S.F., McMillan, L., Libelo, E.L., Neill, M. and Thomas, L. (2011a) 'Application of WWTP biosolids and resulting perfluorinated compound contamination of surface and well water in Decatur, Alabama, USA', *Environmental Science & Technology,* 45(19), pp. 8015-8021.

Lindstrom, A.B., Strynar, M.J. and Libelo, E.L. (2011b) 'Polyfluorinated compounds: Past, present, and future', *Environmental Science & Technology,* 45(19), pp. 7954-7961.

Linnik, P., Ivanechko, Y.S., Linnik, R. and Zhezherya, V. (2013) 'Humic substances in surface waters of the Ukraine', *Russian Journal of General Chemistry,* 83(13), pp. 2715-2730.

Liu, R., Zhou, J. and Wilding, A. (2004) 'Microwave-assisted extraction followed by gas chromatography–mass spectrometry for the determination of endocrine disrupting chemicals in river sediments', *Journal of Chromatography A,* 1038(1), pp. 19-26.

Liu, W., Chen, S., Quan, X. and Jin, Y. (2008) 'Toxic effect of serial perfluorosulfonic and perfluorocarboxylic acids on the membrane system of a freshwater alga measured by flow cytometry', *Environmental Toxicology and Chemistry,* 27(7), pp. 1597-1604.

Liu, Y., Sun, W. and Ni, J. (2011) 'Biodegradation of bisphenol A, 17ß-estradiol, and 17α-ethynylestradiol in river water', *International Journal of Environment and Pollution,* 45(1-3), pp. 225-236.

Loganathan, B.G., Sajwan, K.S., Sinclair, E., Kumar, K.S. and Kannan, K. (2007) 'Perfluoroalkyl sulfonates and perfluorocarboxylates in two wastewater treatment facilities in Kentucky and Georgia', *Water Research,* 41(20), pp. 4611-4620.

Loos, R., Carvalho, R., Antonio, D.C., Comero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T. and Blaha, L. (2013) 'EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents', *Water Research,* 47(17), pp. 6475-6487.

Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S. and Bidoglio, G. (2009) 'EU-wide survey of polar organic persistent pollutants in European river waters', *Environmental Pollution,* 157(2), pp. 561-568.

Mackay, D. and Fraser, A. (2000) 'Bioaccumulation of persistent organic chemicals: Mechanisms and models', *Environmental Pollution,* 110(3), pp. 375-391.

Magnér, J.A., Alsberg, T.E. and Broman, D. (2009) 'The ability of a novel sorptive polymer to determine the freely dissolved fraction of polar organic compounds in the presence of fulvic acid or sediment', *Analytical and Bioanalytical Chemistry,* 395(5), pp. 1525-1532.

Martin, J.W., Mabury, S.A., Solomon, K.R. and Muir, D.C. (2003) 'Bioconcentration and tissue distribution of perfluorinated acids in rainbow trout (*Oncorhynchus mykiss*)', *Environmental Toxicology and Chemistry,* 22(1), pp. 196-204.

Marvin, C.H., Tomy, G.T., Alaee, M. and MacInnis, G. (2006) 'Distribution of hexabromocyclododecane in Detroit River suspended sediments', *Chemosphere,* 64(2), pp. 268-275.

Marvin, C.H., Tomy, G.T., Armitage, J.M., Arnot, J.A., McCarty, L., Covaci, A. and Palace, V. (2011) 'Hexabromocyclododecane: Current understanding of chemistry, environmental fate and toxicology and implications for global management', *Environmental Science & Technology,* 45(20), pp. 8613-8623.

McDonald, S., Bishop, A.G., Prenzler, P.D. and Robards, K. (2004) 'Analytical chemistry of freshwater humic substances', *Analytica Chimica Acta,* 527(2), pp. 105-124.

Meng, X., Duan, Y., Yang, C., Pan, Z., Wen, Z. and Chen, L. (2011) 'Occurrence, sources, and inventory of hexabromocyclododecanes (HBCDs) in soils from Chongming Island, the Yangtze River Delta (YRD)', *Chemosphere,* 82(5), pp. 725-731.

Mézin, L.C. and Hale, R.C. (2004) 'Combined effects of humic acids and salinity on solid-phase microextraction of DDT and chlorpyrifos, an estimator of their bioavailability', *Environmental Toxicology and Chemistry,* 23(3), pp. 576-582.

Moermond, C., Verbruggen, E. and Smit, C. (2010) *Environmental risk limits for PFOS: A proposal for water quality standards in accordance with the Water Framework Directive. RIVM Report 601714013/2010. National Institute for Public Health and the Environment (RIVM), The Netherlands.* Rijksinstituut voor Volksgezondheid en Milieu RIVM.

Moreira, M., Aquino, S., Coutrim, M., Silva, J. and Afonso, R. (2011) 'Determination of endocrine-disrupting compounds in waters from Rio das Velhas, Brazil, by liquid chromatography/high resolution mass spectrometry (ESI-LC-IT-TOF/MS)', *Environmental Technology,* 32(12), pp. 1409-1417.

Morose, G. (2006) 'An overview of alternatives to tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD)', *Lowell Center for Sustainable Production, University of Massachusetts, Lowell* *http://sustainableproduction.org/downloads/AternativestoTBBPAandHBCD.pdf*, .

Morris, S., Allchin, C.R., Zegers, B.N., Haftka, J.J., Boon, J.P., Belpaire, C., Leonards, P.E., Van Leeuwen, S.P. and de Boer, J. (2004) 'Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs', *Environmental science & technology,* 38(21), pp. 5497-5504.

Motwani, S.K., Chopra, S., Ahmad, F.J. and Khar, R.K. (2007) 'Validated spectrophotometric methods for the estimation of moxifloxacin in bulk and

pharmaceutical formulations', *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 68(2), pp. 250-256.

Mudumbi, J., Ntwampe, S., Muganza, F. and Okonkwo, J. (2014) 'Perfluorooctanoate and perfluorooctane sulfonate in South African river water', *Water Science & Technology,* 69(1), pp. 185-194.

Murakami, M., Imamura, E., Shinohara, H., Kiri, K., Muramatsu, Y., Harada, A. and Takada, H. (2008) 'Occurrence and sources of perfluorinated surfactants in rivers in Japan', *Environmental Science & Technology,* 42(17), pp. 6566-6572.

Nagpal, N.K. and Meays, C.L. (2009) *Water quality guidelines for pharmaceutically-active-compounds (PhACs):17α-ethinylestradiol (EE2). September 2009. Ministry of Environment Province of British Columbia.*

Neale, P.A., Escher, B.I. and Schäfer, A.I. (2008) 'Quantification of solute–solute interactions using negligible-depletion solid-phase microextraction: Measuring the affinity of estradiol to bulk organic matter', *Environmental Science & Technology,* 42(8), pp. 2886-2892.

Ng, C.A. and Hungerbühler, K. (2013) 'Bioconcentration of perfluorinated alkyl acids: How important is specific binding?', *Environmental Science & Technology,* 47(13), pp. 7214-7223.

NICNAS (2012) *Hexabromocyclododecane. Priority Existing Chemical Assessment Report No.34. June 2012. National Industrial Chemicals Notification and Assessment Scheme. Austrialian Government Department of Health and Ageing. Available:http://www.nicnas.gov.au/chemical-information/pec-assessments (accessed 15 September 2014).*

Nicolas, J. (1999) 'Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants', *Aquatic Toxicology,* 45(2), pp. 77-90.

Nyholm, J.R., Norman, A., Norrgren, L., Haglund, P. and Andersson, P.L. (2008) 'Maternal transfer of brominated flame retardants in zebrafish (*Danio rerio*)', *Chemosphere,* 73(2), pp. 203-208.

Oakes, K.D., Sibley, P.K., Martin, J.W., Maclean, D.D., Solomon, K.R., Mabury, S.A. and Van Der Kraak, Glen J (2005) 'Short-term exposures of fish to perfluorooctane sulfonate: Acute effects on fatty acyl-CoA oxidase activity, oxidative stress, and circulating sex steroids', *Environmental Toxicology and Chemistry,* 24(5), pp. 1172-1181.

OECD (2002) *Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts. ENV/JM/RD(2002)17/FINAL. 21 November 2002. Organisation for Economic Cooperation and Development.*

Oh, J.K., Kotani, K., Managaki, S. and Masunaga, S. (2014) 'Levels and distribution of hexabromocyclododecane and its lower brominated derivative in Japanese riverine environment', *Chemosphere,* 109, pp. 157-163.

Onogbosele, C.O. and Scrimshaw, M.D. (2014) 'Hexabromocyclododecane and hexachlorocyclohexane: How lessons learnt have led to improved regulation', *Critical Reviews in Environmental Science and Technology,* 44(13), pp. 1423-1442.

Palace, V.P., Pleskach, K., Halldorson, T., Danell, R., Wautier, K., Evans, B., Alaee, M., Marvin, C. and Tomy, G.T. (2008) 'Biotransformation enzymes and thyroid axis disruption in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane diastereoisomers', *Environmental Science & Technology,* 42(6), pp. 1967-1972.

Palace, V., Park, B., Pleskach, K., Gemmill, B. and Tomy, G. (2010) 'Altered thyroxine metabolism in rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane (HBCD)', *Chemosphere,* 80(2), pp. 165-169.

Paul, A.G., Jones, K.C. and Sweetman, A.J. (2008) 'A first global production, emission, and environmental inventory for perfluorooctane sulfonate', *Environmental Science & Technology,* 43(2), pp. 386-392.

Pawlowski, S., Van Aerle, R., Tyler, C. and Braunbeck, T. (2004) 'Effects of 17α-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay', *Ecotoxicology and Environmental Safety,* 57(3), pp. 330-345.

Pérez, M.R., Fernandino, J.I., Carriquiriborde, P. and Somoza, G.M. (2012) 'Feminization and altered gonadal gene expression profile by ethinylestradiol exposure to pejerrey, *Odontesthes bonariensis*, a South American teleost fish', *Environmental Toxicology and Chemistry,* 31(5), pp. 941-946.

Petersen, E.J., Huang, Q. and Weber, W.J. (2010) 'Relevance of octanol–water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes', *Environmental Toxicology and Chemistry,* 29(5), pp. 1106-1112.

Petrović, M., Škrbić, B., Živančev, J., Ferrando-Climent, L. and Barcelo, D. (2014) 'Determination of 81 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap in different types of water in Serbia', *Science of the Total Environment,* 468, pp. 415-428.

Pistocchi, A. and Loos, R. (2009) 'A map of European emissions and concentrations of PFOS and PFOA', *Environmental Science & Technology,* 43(24), pp. 9237-9244.

Qiang, Z., Dong, H., Zhu, B., Qu, J. and Nie, Y. (2013) 'A comparison of various rural wastewater treatment processes for the removal of endocrine-disrupting chemicals (EDCs)', *Chemosphere,* 92(8), pp. 986-992.

Quinn, C.L., Van Der Heijden, S.A., Wania, F. and Jonker, M.T.O. (2014) 'Partitioning of polychlorinated biphenyls into human cells and adipose tissues:

Evaluation of octanol, triolein, and liposomes as surrogates', *Environmental Science and Technology,* 48(10), pp. 5920-5928.

Rani, M., Shim, W.J., Han, G.M., Jang, M., Song, Y.K. and Hong, S.H. (2014) 'Hexabromocyclododecane in polystyrene based consumer products: An evidence of unregulated use', *Chemosphere,* 110, pp. 111-119.

Reiley, M.C. (2007) 'Science, policy, and trends of metals risk assessment at EPA: How understanding metals bioavailability has changed metals risk assessment at US EPA', *Aquatic Toxicology,* 84(2), pp. 292-298.

Remberger, M., Sternbeck, J., Palm, A., Kaj, L., Strömberg, K. and Brorström-Lundén, E. (2004) 'The environmental occurrence of hexabromocyclododecane in Sweden', *Chemosphere,* 54(1), pp. 9-21.

Renner, R. (2006) 'The long and the short of perfluorinated replacements', *Environmental Science & Technology,* 40(1), pp. 12-13.

Renner, R. (2000) 'Scotchgard ban highlights unknowns', *Environmental Science & Technology,* 34(17), pp. 371A-373A.

Ritson, J., Graham, N., Templeton, M., Clark, J., Gough, R. and Freeman, C. (2014) 'The impact of climate change on the treatability of dissolved organic matter (DOM) in upland water supplies: A UK perspective', *Science of the Total Environment,* 473, pp. 714-730.

Roberts, C. and Swigert, J. (1997) 'Hexabromocyclododecane (HBCD): A 96-hour toxicity test with the freshwater alga (*Selenastrum capricornutum*)', *Final Report. 439A-103, Wildlife International Ltd., Easton, Maryland, USA.,* .

Rodrigues, A., Brito, A., Janknecht, P., Proença, M.F. and Nogueira, R. (2009) 'Quantification of humic acids in surface water: Effects of divalent cations, pH, and filtration', *Journal of Environmental Monitoring,* 11(2), pp. 377-382.

Ronisz, D., Finne, E.F., Karlsson, H. and Förlin, L. (2004) 'Effects of the brominated flame retardants hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA), on hepatic enzymes and other biomarkers in juvenile rainbow trout and feral eelpout', *Aquatic Toxicology,* 69(3), pp. 229-245.

Rose, J., Holbech, H., Lindholst, C., Nørum, U., Povlsen, A., Korsgaard, B. and Bjerregaard, P. (2002) 'Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (*Danio rerio*)', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 131(4), pp. 531-539.

Routledge, E.J. and Sumpter, J.P. (1996) 'Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen', *Environmental Toxicology and Chemistry,* 15(3), pp. 241-248.

Rüdel, H., Müller, J., Quack, M. and Klein, R. (2012) 'Monitoring of hexabromocyclododecane diastereomers in fish from European freshwaters and estuaries', *Environmental Science and Pollution Research,* 19(3), pp. 772-783.

Ruiz, S.H., Wickramasekara, S., Abrell, L., Gao, X., Chefetz, B. and Chorover, J. (2013) 'Complexation of trace organic contaminants with fractionated dissolved organic matter: Implications for mass spectrometric quantification', *Chemosphere,* 91(3), pp. 344-350.

Sanderson, H., Boudreau, T.M., Mabury, S.A. and Solomon, K.R. (2004) 'Effects of perfluorooctane sulfonate and perfluorooctanoic acid on the zooplanktonic community', *Ecotoxicology and Environmental Safety,* 58(1), pp. 68-76.

Schmitt, C., Balaam, J., Leonards, P., Brix, R., Streck, G., Tuikka, A., Bervoets, L., Brack, W., van Hattum, B. and Meire, P. (2010) 'Characterizing field sediments from three European river basins with special emphasis on endocrine effects – A recommendation for *Potamopyrgus antipodarum* as test organism', *Chemosphere,* 80(1), pp. 13-19.

Schuetze, A., Heberer, T., Effkemann, S. and Juergensen, S. (2010) 'Occurrence and assessment of perfluorinated chemicals in wild fish from Northern Germany', *Chemosphere,* 78(6), pp. 647-652.

Scott, B.F., Spencer, C., Lopez, E. and Muir, D.C. (2009) 'Perfluorinated alkyl acid concentrations in Canadian rivers and creeks', *Water Quality Research Journal of Canada,* 44(3), pp. 263.

Segner, H., Caroll, K., Fenske, M., Janssen, C., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G., Watts, M. and Wenzel, A. (2003) 'Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: Report from the European IDEA project', *Ecotoxicology and Environmental Safety,* 54(3), pp. 302-314.

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A. and Harms, H. (2004) 'Peer reviewed: Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated', *Environmental Science & Technology,* 38(12), pp. 228A-231A.

Senthil Kumar, K., Zushi, Y., Masunaga, S., Gilligan, M., Pride, C. and Sajwan, K.S. (2009) 'Perfluorinated organic contaminants in sediment and aquatic wildlife, including sharks, from Georgia, USA', *Marine Pollution Bulletin,* 58(4), pp. 621-629.

Senthilkumar, K., Ohi, E., Sajwan, K., Takasuga, T. and Kannan, K. (2007) 'Perfluorinated compounds in river water, river sediment, market fish, and wildlife samples from Japan', *Bulletin of Environmental Contamination and Toxicology,* 79(4), pp. 427-431.

Serrano, D., Omil, F., Lema, J. and Patureau, D. (2013) 'Application of a three-compartment model as a tool to understand the partition of 17α-ethinylestradiol

in mixed liquor systems', *Environmental Progress & Sustainable Energy,* 32(2), pp. 257-262.

Shaffer, L. and von Wandruszka, R. (2015) 'Temperature induced aggregation and clouding in humic acid solutions', *Advances in Environmental Chemistry,* 2015.

Sharpe, R.L., Benskin, J.P., Laarman, A.H., MacLeod, S.L., Martin, J.W., Wong, C.S. and Goss, G.G. (2010) 'Perfluorooctane sulfonate toxicity, isomer-specific accumulation, and maternal transfer in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*)', *Environmental Toxicology and Chemistry,* 29(9), pp. 1957-1966.

Shi, X., Du, Y., Lam, P.K.S., Wu, R.S.S. and Zhou, B. (2008) 'Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS', *Toxicology and Applied Pharmacology,* 230(1), pp. 23-32.

Shi, X., Liu, C., Wu, G. and Zhou, B. (2009) 'Waterborne exposure to PFOS causes disruption of the hypothalamus-pituitary-thyroid axis in zebrafish larvae', *Chemosphere,* 77(7), pp. 1010-1018.

Shrivastava, A. and Gupta, V.B. (2011) 'Methods for the determination of limit of detection and limit of quantitation of the analytical methods', *Chronicles of Young Scientists,* 2(1), pp. 21.

Silva, C.P., Otero, M. and Esteves, V. (2012) 'Processes for the elimination of estrogenic steroid hormones from water: A review', *Environmental Pollution,* 165, pp. 38-58.

So, M., Miyake, Y., Yeung, W., Ho, Y., Taniyasu, S., Rostkowski, P., Yamashita, N., Zhou, B., Shi, X. and Wang, J. (2007) 'Perfluorinated compounds in the Pearl River and Yangtze River of China', *Chemosphere,* 68(11), pp. 2085-2095.

Souza, M.S., Hallgren, P., Balseiro, E. and Hansson, L. (2013) 'Low concentrations, potential ecological consequences: Synthetic estrogens alter life-history and demographic structures of aquatic invertebrates', *Environmental Pollution,* 178, pp. 237-243.

Stanley, E.H., Powers, S.M., Lottig, N.R., Buffam, I. and Crawford, J.T. (2012) 'Contemporary changes in dissolved organic carbon (DOC) in human-dominated rivers: Is there a role for DOC management?', *Freshwater Biology,* 57(s1), pp. 26-42.

Stubbings, W.A. and Harrad, S. (2014) 'Extent and mechanisms of brominated flame retardant emissions from waste soft furnishings and fabrics: A critical review', *Environment International,* 71, pp. 164-175.

Sun, H., Li, F., Zhang, T., Zhang, X., He, N., Song, Q., Zhao, L., Sun, L. and Sun, T. (2011) 'Perfluorinated compounds in surface waters and WWTPs in Shenyang, China: Mass flows and source analysis', *Water Research,* 45(15), pp. 4483-4490.

Takazawa, Y., Nishino, T., Sasaki, Y., Yamashita, H., Suzuki, N., Tanabe, K. and Shibata, Y. (2009) 'Occurrence and distribution of perfluorooctane sulfonate and perfluorooctanoic acid in the rivers of Tokyo', *Water, Air, and Soil Pollution,* 202(1-4), pp. 57-67.

Tomy, G.T., Halldorson, T., Danell, R., Law, K., Arsenault, G., Alaee, M., MacInnis, G. and Marvin, C.H. (2005) 'Refinements to the diastereoisomer-specific method for the analysis of hexabromocyclododecane', *Rapid Communications in Mass Spectrometry,* 19(19), pp. 2819-2826.

Tomy, G.T., Palace, V., Marvin, C., Stapleton, H.M., Covaci, A. and Harrad, S. (2011) 'Biotransformation of HBCD in biological systems can confound temporal-trend studies', *Environmental Science and Technology,* 45(2), pp. 364-365.

UNEP (2013) *Report of the Conference of the Parties to the Stockholm Convention on Persistent Organic Pollutants on the work of its sixth meeting. UNEP/POPS/COP.6/33. Geneva, 28 April - 10 May 2013. United Nations Environment Programme.*

UNEP (2012) *Report of the Persistent Organic Pollutants Review Committee on the work of its eighth meeting: Addendum to the risk management evaluation on hexabromocyclododecane. UNEP/POPS/POPRC.8/16/Add.3. Geneva, 15-19 October 2012. United Nations Environment Programme.*

UNEP (2010) *Draft risk profile: Hexabromocyclododecane. UNEP/POPS/POPRC.6/10. Geneva, 11-15 October 2010. United Nations Environment Programme.*

UNEP (2009) *Report of the Conference of the Parties of the Stockholm Convention on Persistent Organic Pollutants on the work of its fourth meeting. UNEP/POPS/COP.4/38. Geneva, 4-8 May 2009. United Nations Environment Programme.*

UNEP (2006) *Draft risk profile: Perfluorooctane sulfonate (PFOS). UNEP/POPS/POPRC.2/11. Geneva, 6-10 November 2006. United Nations Environment Programme.*

UNEP (2001) *Final act of the Conference of Plenipotentiaries on the Stockholm Convention on Persistent Organic Pollutants. UNEP/POPS/CONF/4. Stockholm, 22-23 May 2001. United Nations Environment Programme.*

USEPA (2014a) *Emerging contaminants - Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). March 2014. United States Environmental Protection Agency. Available: http://www2.epa.gov/sites/production/files/2014-04/documents/factsheet_contaminant_pfos_pfoa_march2014.pdf (accessed 23 September 2014).*

USEPA (2014b) *Flame retardants alternatives for hexabromocyclododecane (HBCD). Chapter 4: Hazard evaluation of HBCD and alternatives. Final report, June 2014. United States Environment Protection Agency. Available:*

http://www.epa.gov/dfe/pubs/projects/hbcd/hbcd-full-report-508.pdf (accessed 23 September 2014)
.

USEPA (2008) *Supporting documents for initial risk-based prioritization of high production volume chemicals. 3/18/2008. United States Environmental Protection Agency. Available: http://www.epa.gov/hpvis/rbp/HBCD.3194556.Web.SupportDocs.31408.pdf (accessed 23 September 2014).*

Veith, G.D., DeFoe, D.L. and Bergstedt, B.V. (1979) 'Measuring and estimating the bioconcentration factor of chemicals in fish', *Journal of the Fisheries Board of Canada,* 36(9), pp. 1040-1048.

Viganò, L., Benfenati, E., Cauwenberge, A.v., Eidem, J.K., Erratico, C., Goksøyr, A., Kloas, W., Maggioni, S., Mandich, A. and Urbatzka, R. (2008) 'Estrogenicity profile and estrogenic compounds determined in river sediments by chemical analysis, ELISA and yeast assays', *Chemosphere,* 73(7), pp. 1078-1089.

Vignati, D.A., Ferrari, B.J. and Dominik, J. (2007) 'Laboratory-to-field extrapolation in aquatic sciences', *Environmental Science & Technology,* 41(4), pp. 1067-1073.

Völker, J., Richter, S., Borchardt, D. and Mohaupt, V. (2013) 'Risk and monitoring based indicators of receiving water status: Alternative or complementary elements in IWRM?', *Integrated Water Resources Management in a Changing World: Lessons Learnt and Innovative Perspectives,* , pp. 133.

Vorkamp, K., Bossi, R., Bester, K., Bollmann, U.E. and Boutrup, S. (2014) 'New priority substances of the European Water Framework Directive: Biocides, pesticides and brominated flame retardants in the aquatic environment of Denmark', *Science of the Total Environment,* 470, pp. 459-468.

Wang, L., Ying, G., Zhao, J., Liu, S., Yang, B., Zhou, L., Tao, R. and Su, H. (2011a) 'Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools', *Environmental Pollution,* 159(1), pp. 148-156.

Wang, M., Chen, J., Lin, K., Chen, Y., Hu, W., Tanguay, R.L., Huang, C. and Dong, Q. (2011b) 'Chronic zebrafish PFOS exposure alters sex ratio and maternal related effects in F1 offspring', *Environmental Toxicology and Chemistry,* 30(9), pp. 2073-2080.

Wang, P., Wong, M. and Tam, N. (2013) 'Antioxidant responses of two microalgae, *Selenastrum capricornutum* and *Chlorella* sp., to estradiol and ethinylestradiol', *Journal of Applied Phycology,* 25(3), pp. 891-903.

Wang, W., Delgado-Moreno, L., Ye, Q. and Gan, J. (2011) 'Improved measurements of partition coefficients for polybrominated diphenyl ethers', *Environmental Science & Technology,* 45(4), pp. 1521-1527.

Wang, Y., Arsenault, G., Riddell, N., McCrindle, R., McAlees, A. and Martin, J.W. (2009) 'Perfluorooctane sulfonate (PFOS) precursors can be metabolized enantioselectively: Principle for a new PFOS source tracking tool', *Environmental Science & Technology,* 43(21), pp. 8283-8289.

Wei-Haas, M.L., Hageman, K.J. and Chin, Y. (2014) 'Partitioning of polybrominated diphenyl ethers to dissolved organic matter Isolated from Arctic surface waters', *Environmental Science & Technology,* 48(9), pp. 4852-4859.

Whidbey, C.M., Daumit, K.E., Nguyen, T.-., Ashworth, D.D., Davis, J.C.C. and Latch, D.E. (2012) 'Photochemical induced changes of *in vitro* estrogenic activity of steroid hormones', *Water Research,* 46(16), pp. 5287-5296.

Williams, R.J., Johnson, A.C., Smith, J.J. and Kanda, R. (2003) 'Steroid estrogens profiles along river stretches arising from sewage treatment works discharges', *Environmental Science & Technology,* 37(9), pp. 1744-1750.

Wise, A., O'Brien, K. and Woodruff, T. (2010) 'Are oral contraceptives a significant contributor to the estrogenicity of drinking water?', *Environmental Science & Technology,* 45(1), pp. 51-60.

Wu, J., Guan, Y., Zhang, Y., Luo, X., Zhi, H., Chen, S. and Mai, B. (2011) 'Several current-use, non-PBDE brominated flame retardants are highly bioaccumulative: Evidence from field determined bioaccumulation factors', *Environment International,* 37(1), pp. 210-215.

Wu, J., Guan, Y., Zhang, Y., Luo, X., Zhi, H., Chen, S. and Mai, B. (2010) 'Trophodynamics of hexabromocyclododecanes and several other non-PBDE brominated flame retardants in a freshwater food web', *Environmental Science & Technology,* 44(14), pp. 5490-5495.

Xia, X., Dai, Z., Rabearisoa, A.H., Zhao, P. and Jiang, X. (2015) 'Comparing humic substance and protein compound effects on the bioaccumulation of perfluoroalkyl substances by *Daphnia magna* in water', *Chemosphere,* 119, pp. 978-986.

Xian, Q., Ramu, K., Isobe, T., Sudaryanto, A., Liu, X., Gao, Z., Takahashi, S., Yu, H. and Tanabe, S. (2008) 'Levels and body distribution of polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs) in freshwater fishes from the Yangtze River, China', *Chemosphere,* 71(2), pp. 268-276.

Xu, H., Yang, J., Wang, Y., Jiang, Q., Chen, H. and Song, H. (2008) 'Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*)', *Aquatic Toxicology,* 88(1), pp. 1-8.

Yamamoto, H., Liljestrand, H.M., Shimizu, Y. and Morita, M. (2003) 'Effects of physical-chemical characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates', *Environmental Science & Technology,* 37(12), pp. 2646-2657.

Yang, J., Li, H., Ran, Y. and Chan, K. (2014) 'Distribution and bioconcentration of endocrine disrupting chemicals in surface water and fish bile of the Pearl River Delta, South China', *Chemosphere,* 107, pp. 439-446.

Ye, X., Strynar, M.J., Nakayama, S.F., Varns, J., Helfant, L., Lazorchak, J. and Lindstrom, A.B. (2008) 'Perfluorinated compounds in whole fish homogenates from the Ohio, Missouri, and Upper Mississippi Rivers, USA', *Environmental Pollution,* 156(3), pp. 1227-1232.

Ying, G., Kookana, R.S. and Ru, Y. (2002) 'Occurrence and fate of hormone steroids in the environment', *Environment International,* 28(6), pp. 545-551.

Ying, G., Kookana, R.S., Kumar, A. and Mortimer, M. (2009) 'Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland', *Science of the Total Environment,* 407(18), pp. 5147-5155.

Yu, Z., Chen, L., Mai, B., Wu, M., Sheng, G., Fu, J. and Peng, P. (2008) 'Diastereoisomer-and enantiomer-specific profiles of hexabromocyclododecane in the atmosphere of an urban city in South China', *Environmental Science & Technology,* 42(11), pp. 3996-4001.

Zhang, X., Yang, F., Zhang, X., Xu, Y., Liao, T., Song, S. and Wang, J. (2008) 'Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD)', *Aquatic Toxicology,* 86(1), pp. 4-11.

Zhang, Y., Ruan, Y., Sun, H., Zhao, L. and Gan, Z. (2013) 'Hexabromocyclododecanes in surface sediments and a sediment core from rivers and harbor in the northern Chinese city of Tianjin', *Chemosphere,* 90(5), pp. 1610-1616.

Zhang, Z., Ren, N., Kannan, K., Nan, J., Liu, L., Ma, W., Qi, H. and Li, Y. (2014a) 'Occurrence of endocrine-disrupting phenols and estrogens in water and sediment of the Songhua River, Northeastern China', *Archives of Environmental Contamination and Toxicology,* 66(3), pp. 361-369.

Zhang, Y., Sun, H. and Ruan, Y. (2014b) 'Enantiomer-specific accumulation, depuration, metabolization and isomerization of hexabromocyclododecane (HBCD) diastereomers in mirror carp from water', *Journal of Hazardous Materials,* 264, pp. 8-15.

Zhao, Z., Xie, Z., Möller, A., Sturm, R., Tang, J., Zhang, G. and Ebinghaus, R. (2012) 'Distribution and long-range transport of polyfluoroalkyl substances in the Arctic, Atlantic Ocean and Antarctic coast', *Environmental Pollution,* 170, pp. 71-77.

Zorita, S., Mårtensson, L. and Mathiasson, L. (2009) 'Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden', *Science of the Total Environment,* 407(8), pp. 2760-2770.

Zushi, Y., Hogarh, J.N. and Masunaga, S. (2012) 'Progress and perspective of perfluorinated compound risk assessment and management in various countries and institutes', *Clean Technologies and Environmental Policy,* 14(1), pp. 9-20.

APPENDICES

Appendix 1 Experimental design for determination of bound and freely dissolved fractions of constant concentration of EE2 at varying concentrations of humic acid

EE2 (mg/L)	HA (mg/L)	Replicates		
		A	B	C
5	100	1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water
	10	0.1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water
	1	0.01 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 500 mg/L EE2 in 100 ml deionized water
	0	1 ml 500 mg/L EE2 in 100 ml deionized water	1 ml 500 mg/L EE2 in 100 ml deionized water	1 ml 500 mg/L EE2 in 100 ml deionized water

EE2, ethinylestradiol; HA, humic acid.
Concentrations of LC-MS calibration: 0.5, 1.0, 2.5, 5.0 and 10.0 mg/L EE2.

Appendix 2 Experimental design for determination of bound and freely dissolved fractions of varying concentrations of EE2 at 10 mg/L humic acid

HA (mg/L)	EE2 (mg/L)	Replicates		
		A	B	C
10	0.5	0.1 ml 10 g/L HA and 5 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 5 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 5 ml 10 mg/L EE2 in 100 ml deionized water
	0.4	0.1 ml 10 g/L HA and 4 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 4 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 4 ml 10 mg/L EE2 in 100 ml deionized water
	0.3	0.1 ml 10 g/L HA and 3 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 3 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 3 ml 10 mg/L EE2 in 100 ml deionized water
	0.2	0.1 ml 10 g/L HA and 2 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 2 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 2 ml 10 mg/L EE2 in 100 ml deionized water
	0.1	0.1 ml 10 g/L HA and 1 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 10 mg/L EE2 in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 10 mg/L EE2 in 100 ml deionized water
Controls				
0.5 mg/L EE2		5 ml 10 mg/L EE2 in 100 ml deionized water	5 ml 10 mg/L EE2 in 100 ml deionized water	5 ml 10 mg/L EE2 in 100 ml deionized water
0.1 mg/L EE2		1 ml 10 mg/L EE2 in 100 ml deionized water	1 ml 10 mg/L EE2 in 100 ml deionized water	1 ml 10 mg/L EE2 in 100 ml deionized water
10 mg/L HA		0.1 ml 10 g/L HA in 100 ml deionized water	0.1 ml 10 g/L HA in 100 ml deionized water	0.1 ml 10 g/L HA in 100 ml deionized water

HA, humic acid; EE2, ethinylestradiol.
Concentrations of LC-MS calibration: 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/L EE2.

Appendix 3 Experimental design for determination of bound and freely dissolved fractions of varying concentrations of EE2 at 100 mg/L humic acid

HA (mg/L)	EE2 (mg/L)	Replicates		
		A	B	C
100	5	1 ml 10 g/L HA and 0.5 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.5 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.5 ml 1000 mg/L EE2 in 100 ml deionized water
	4	1 ml 10 g/L HA and 0.4 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.4 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.4 ml 1000 mg/L EE2 in 100 ml deionized water
	3	1 ml 10 g/L HA and 0.3 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.3 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.3 ml 1000 mg/L EE2 in 100 ml deionized water
	2	1 ml 10 g/L HA and 0.2 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.2 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.2 ml 1000 mg/L EE2 in 100 ml deionized water
	1	1 ml 10 g/L HA and 0.1 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.1 ml 1000 mg/L EE2 in 100 ml deionized water	1 ml 10 g/L HA and 0.1 ml 1000 mg/L EE2 in 100 ml deionized water
Controls				
5 mg/L EE2		0.5 ml 1000 mg/L EE2 in 100 ml deionized water	0.5 ml 1000 mg/L EE2 in 100 ml deionized water	0.5 ml 1000 mg/L EE2 in 100 ml deionized water
1 mg/L EE2		0.1 ml 1000 mg/L EE2 in 100 ml deionized water	0.1 ml 1000 mg/L EE2 in 100 ml deionized water	0.1 ml 1000 mg/L EE2 in 100 ml deionized water
100 mg/L HA		1 ml 10 g/L HA in 100 ml deionized water	1 ml 10 g/L HA in 100 ml deionized water	1 ml 10 g/L HA in 100 ml deionized water

HA, humic acid; EE2, ethinylestradiol.
Concentrations of LC-MS calibration: 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 5.5 mg/L EE2.

Appendix 4 Experimental design for determination of bound and freely dissolved fractions of constant concentration of PFOS at varying concentrations of humic acid

PFOS (mg/L)	HA (mg/L)	Replicates		
		A	B	C
5	100	1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water
	10	0.1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water
	1	0.01 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 500 mg/L PFOS in 100 ml deionized water
	0	1 ml 500 mg/L PFOS in 100 ml deionized water	1 ml 500 mg/L PFOS in 100 ml deionized water	1 ml 500 mg/L PFOS in 100 ml deionized water

PFOS, perfluorooctane sulfonate; HA, humic acid.
Concentrations of LC-MS calibration: 0.5, 1.0, 2.5, 5.0 and 10.0 mg/L PFOS.

Figure 5 Experimental design for determination of bound and freely dissolved fractions of constant concentration of HBCD at varying concentrations of humic acid

HBCD (μg/L)	HA (mg/L)	Replicates		
		A	B	C
50	100	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water
	10	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water
	1	0.01 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.01 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water
	0	1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water

HBCD, hexabromocyclododecane; HA, humic acid.
Concentrations of LC-MS calibration: 5, 10, 25, 50 and 100 μg/L HBCD.

Appendix 6 Experimental design for determination of bound and freely dissolved fractions of varying concentrations of HBCD at 10 mg/L humic acid

HA (mg/L)	HBCD (μg/L)	Replicates		
		A	B	C
10.0	50.0	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water
	37.5	0.1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water
	25.0	0.1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water
	12.5	0.1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water
	5.0	0.1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water
Controls				
50.0 μg/L HBCD		1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water
5.0 μg/L HBCD		0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 5000 μg/L HBCD in 100 ml deionized water
10.0 mg/L HA		0.1 ml 10 g/L HA in 100 ml deionized water	0.1 ml 10 g/L HA in 100 ml deionized water	0.1 ml 10 g/L HA in 100 ml deionized water

HA, humic acid; HBCD, hexabromocyclododecane.
Concentrations of LC-MS calibration: 1, 2, 5, 10, 25, 50 and 100 μg/L HBCD.

Appendix 7 Experimental design for determination of bound and freely dissolved fractions of varying concentrations of HBCD at 100 mg/L humic acid

HA (mg/L)	HBCD (μg/L)	Replicates		
		A	B	C
100.0	50.0	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 1 ml 5000 μg/L HBCD in 100 ml deionized water
	37.5	1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.75 ml 5000 μg/L HBCD in 100 ml deionized water
	25.0	1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.5 ml 5000 μg/L HBCD in 100 ml deionized water
	12.5	1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.25 ml 5000 μg/L HBCD in 100 ml deionized water
	5.0	1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 10 g/L HA and 0.1 ml 5000 μg/L HBCD in 100 ml deionized water
Controls				
50.0 μg/L HBCD		1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water	1 ml 5000 μg/L HBCD in 100 ml deionized water
5.0 μg/L HBCD		0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 5000 μg/L HBCD in 100 ml deionized water	0.1 ml 5000 μg/L HBCD in 100 ml deionized water
100.0 mg/L HA		1 ml 10 g/L HA in 100 ml deionized water	1 ml 10 g/L HA in 100 ml deionized water	1 ml 10 g/L HA in 100 ml deionized water

HA, humic acid; HBCD, hexabromocyclododecane.
Concentrations of LC-MS calibration: 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/L HBCD.

Appendix 8 Absorbance readings in the YES assay used to determine the effect of humic acid on bioavailability of EE2

Nominal EE2 (µg/L)	12.5	6.25	3.125	1.5625	0.7813	0.3906	0.1953	0.0977	0.0488	0.0244	0.0122	0.0061
			Raw absorbance (540 nm) at 22.7-23.2 °C on day 2 of incubation									
Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.7281	0.7291	0.7165	0.7222	0.7222	0.7185	0.7247	0.7195	0.7131	0.7315	0.7267	0.7296
	0.7460	0.7462	0.7255	0.7352	0.7286	0.7382	0.7256	0.7245	0.7386	0.7417	0.7368	0.7216
	0.7343	0.7375	0.7396	0.7460	0.7528	0.7393	0.7366	0.7451	0.7573	0.7397	0.7400	0.7271
EE2 + MQ	2.7089	2.4992	1.9822	1.4841	0.9956	0.8107	0.7693	0.7262	0.7228	0.7246	0.7228	0.7249
	2.5905	2.4729	1.9893	1.5368	0.9966	0.7952	0.7417	0.7116	0.7294	0.7199	0.7183	0.7278
	2.6192	2.4661	2.1398	1.6907	1.0809	0.8068	0.7411	0.7054	0.7119	0.7249	0.7067	0.7245
1HA	0.7429	0.7375	0.7312	0.7436	0.7385	0.7472	0.7368	0.7420	0.7527	0.7548	0.7645	0.7794
	0.7733	0.7570	0.7720	0.7562	0.7405	0.7407	0.7648	0.7359	0.7796	0.7791	0.7799	0.7780
	0.7703	0.7588	0.7848	0.7739	0.7634	0.7571	0.7610	0.7795	0.7631	0.7741	0.7538	0.7635
EE2 + 1HA	2.7416	2.7399	2.6060	2.0726	1.3522	0.9602	0.8047	0.7766	0.7608	0.7648	0.7610	0.7739
	2.7695	2.7399	2.6248	2.1568	1.4103	0.9591	0.8094	0.7774	0.7413	0.7582	0.7680	0.7747
	2.7314	2.6823	2.5083	1.9417	1.2473	0.8775	0.7599	0.7335	0.7498	0.7450	0.7550	0.8037
10HA	0.7326	0.7244	0.7030	0.6844	0.7046	0.7038	0.7013	0.6934	0.7319	0.7227	0.7401	0.7370
	0.6987	0.7016	0.7009	0.7027	0.7022	0.7025	0.7080	0.7283	0.7222	0.7115	0.7361	0.7501
	0.7089	0.7154	0.7248	0.6980	0.7038	0.7532	0.6899	0.7094	0.6965	0.7517	0.7445	0.7573
EE2 + 10HA	2.8113	2.7740	2.5740	2.0570	1.0575	0.9596	0.7972	0.7509	0.6853	0.7042	0.6974	0.7267
	2.7682	2.7336	2.5227	2.1194	1.3773	0.9900	0.8014	0.7399	0.7192	0.7092	0.7037	0.7443
	2.8241	2.6878	2.5913	1.9602	1.1569	0.9433	0.7749	0.7280	0.6968	0.6587	0.6819	0.8042
50HA	0.7790	0.8189	0.7876	0.8034	0.7961	0.7779	0.8216	0.8155	0.7828	0.8023	0.8207	0.7918
	0.7957	0.8492	0.8377	0.8303	0.8267	0.8255	0.8059	0.8376	0.8018	0.8375	0.8214	0.8013
	0.8035	0.8173	0.8072	0.7886	0.7910	0.7776	0.8132	0.8006	0.8171	0.7970	0.8097	0.7598
EE2 + 50HA	2.8988	2.7392	2.4713	1.7913	1.2637	1.0092	0.8566	0.7808	0.8102	0.8154	0.7987	0.8140
	2.8695	2.6914	2.3664	1.7865	1.2226	0.9585	0.8375	0.7997	0.7820	0.7935	0.7584	0.7902
	2.8360	2.6441	2.3839	1.7425	1.1783	0.9248	0.8369	0.7771	0.7666	0.7880	0.7803	1.0983
100HA	0.9172	0.8948	0.8878	0.8927	0.8483	0.8452	0.8827	0.8497	0.8570	0.8162	0.8209	0.8713
	0.9082	0.9020	0.8713	0.8459	0.8492	0.8622	0.8610	0.8379	0.8155	0.7976	0.7927	0.8650
	0.9134	0.9183	0.8952	0.8995	0.9118	0.8709	0.8768	0.8856	0.8543	0.8499	0.8356	0.8746
EE2 + 100HA	3.0452	2.8260	2.3239	2.0014	1.4016	1.1207	0.9196	0.9032	0.8244	0.8202	0.8071	0.8622
	2.9702	2.6939	2.1803	1.7651	1.2731	0.9819	0.8727	0.8759	0.7790	0.8442	0.8590	0.8948
	2.9662	2.7582	2.4341	1.8560	1.2983	1.1086	0.8558	0.8464	0.8182	0.8543	0.8356	0.9487

Appendix 8 (continued)

Raw absorbance (620 nm) at 22.7-23.2 °C on day 2 of incubation

Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.6586	0.6564	0.6443	0.6488	0.6498	0.6450	0.6527	0.6471	0.6403	0.6585	0.6546	0.6576
	0.6736	0.6725	0.6513	0.6610	0.6546	0.6651	0.6515	0.6509	0.6632	0.6666	0.6620	0.6503
	0.6575	0.6609	0.6642	0.6699	0.6761	0.6625	0.6603	0.6690	0.6811	0.6630	0.6641	0.6528
EE2 + MQ	0.8613	0.8206	0.7496	0.7101	0.6721	0.6630	0.6672	0.6437	0.6456	0.6487	0.6469	0.6527
	0.8464	0.8217	0.7565	0.7134	0.6638	0.6419	0.6453	0.6313	0.6537	0.6459	0.6442	0.6558
	0.8623	0.8248	0.7884	0.7413	0.6699	0.6382	0.6388	0.6239	0.6353	0.6489	0.6328	0.6518
1HA	0.6710	0.6568	0.6528	0.6627	0.6581	0.6678	0.6582	0.6631	0.6715	0.6741	0.6838	0.7039
	0.6965	0.6719	0.6892	0.6728	0.6565	0.6570	0.6800	0.6524	0.6944	0.6949	0.6961	0.6976
	0.6932	0.6759	0.6987	0.6876	0.6784	0.6723	0.6758	0.6942	0.6779	0.6886	0.6695	0.6814
EE2 + 1HA	0.8448	0.7897	0.7738	0.7406	0.7011	0.6926	0.6702	0.6731	0.6698	0.6770	0.6757	0.6924
	0.8846	0.7839	0.7906	0.7393	0.6898	0.6676	0.6668	0.6698	0.6498	0.6696	0.6818	0.6922
	0.8822	0.7832	0.7739	0.6948	0.6744	0.6418	0.6290	0.6369	0.6615	0.6592	0.6722	0.7224
10HA	0.6333	0.6228	0.6023	0.5826	0.6051	0.6023	0.6012	0.5928	0.6294	0.6200	0.6357	0.6372
	0.5988	0.5977	0.5950	0.5962	0.5961	0.5967	0.6010	0.6190	0.6135	0.6035	0.6270	0.6459
	0.6055	0.6081	0.6185	0.5932	0.5985	0.6440	0.5820	0.6013	0.5903	0.6426	0.6357	0.6506
EE2 + 10HA	0.8093	0.7823	0.7264	0.6832	0.6082	0.6084	0.5985	0.6124	0.5815	0.5947	0.5909	0.6226
	0.7902	0.7460	0.7200	0.6693	0.6350	0.6309	0.5919	0.5984	0.5987	0.5976	0.5965	0.6368
	0.8674	0.7344	0.7296	0.6632	0.5942	0.5837	0.5714	0.5888	0.5783	0.5510	0.5754	0.6960
50HA	0.6603	0.6946	0.6655	0.6793	0.6752	0.6553	0.6981	0.6904	0.6642	0.6782	0.6950	0.6696
	0.6755	0.7196	0.7047	0.6979	0.6950	0.6942	0.6755	0.7074	0.6724	0.7043	0.6895	0.6739
	0.6807	0.6907	0.6778	0.6653	0.6714	0.6601	0.6853	0.6744	0.6893	0.6744	0.6866	0.6406
EE2 + 50HA	0.8785	0.8090	0.7776	0.7288	0.6931	0.7056	0.6662	0.6266	0.6757	0.6833	0.6691	0.6856
	0.8846	0.7899	0.7567	0.7381	0.7065	0.6662	0.6537	0.6564	0.6505	0.6683	0.6329	0.6661
	0.9100	0.7800	0.7492	0.7318	0.7110	0.6529	0.6605	0.6390	0.6388	0.6657	0.6561	0.9704
100HA	0.7703	0.7418	0.7350	0.7438	0.6992	0.6992	0.7327	0.7006	0.7083	0.6702	0.6735	0.7239
	0.7583	0.7480	0.7173	0.6990	0.7015	0.7130	0.7079	0.6865	0.6646	0.6552	0.6410	0.7171
	0.7605	0.7592	0.7371	0.7384	0.7512	0.7100	0.7182	0.7239	0.6942	0.6889	0.6739	0.7161
EE2 + 100HA	0.9629	0.8953	0.8125	0.8302	0.7666	0.7541	0.7025	0.7278	0.6609	0.6674	0.6582	0.6992
	0.9360	0.8201	0.7654	0.7814	0.7250	0.6868	0.6724	0.6973	0.6373	0.6881	0.7048	0.7359
	0.9522	0.8586	0.8220	0.7966	0.7106	0.7186	0.6620	0.6822	0.6650	0.6946	0.6851	0.7819

Appendix 8 (continued)

Raw absorbance (540 nm) at 23.5-23.8 °C on day 3 of incubation

Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.7423	0.6977	0.6615	0.6432	0.6407	0.6444	0.6505	0.6676	0.6777	0.6926	0.6900	0.7226
	0.7172	0.6771	0.6547	0.6454	0.6306	0.6330	0.6325	0.6582	0.6504	0.6683	0.6774	0.6895
	0.7170	0.6821	0.6664	0.6588	0.6452	0.6350	0.6692	0.6602	0.6571	0.6607	0.6589	0.7089
EE2 + MQ	2.7472	2.7340	2.6849	2.5132	1.7653	1.0604	0.7787	0.6779	0.6576	0.6506	0.6749	0.7067
	2.6536	2.6386	2.5903	2.4734	1.8141	1.0971	0.7911	0.7211	0.6428	0.6369	0.6784	0.7197
	2.6175	2.6531	2.6350	2.5760	2.0517	1.2298	0.8406	0.7335	0.6627	0.6693	0.7108	0.7216
1HA	0.7727	0.7413	0.6953	0.6955	0.7359	0.7011	0.6781	0.6761	0.6868	0.6977	0.7090	0.7429
	0.7516	0.6748	0.6992	0.6645	0.6555	0.6447	0.6772	0.6592	0.6956	0.7321	0.7405	0.7858
	0.7068	0.6618	0.6610	0.6613	0.6526	0.6567	0.6687	0.6805	0.6787	0.7020	0.7123	0.8070
EE2 + 1HA	2.7708	2.6820	2.6666	2.6409	2.1655	1.3622	0.8791	0.7326	0.6852	0.6713	0.6991	0.8538
	2.7786	2.7002	2.6826	2.6312	2.2753	1.4767	0.9233	0.7673	0.6991	0.6962	0.6902	0.8605
	2.7376	2.6796	2.6571	2.5540	2.0879	1.3466	0.8853	0.7240	0.6965	0.6965	0.7033	0.9891
10HA	1.0093	0.7250	0.6995	0.6915	0.6984	0.7420	0.6923	0.7106	0.8203	0.8720	0.9234	1.0903
	0.7407	0.6755	0.6492	0.6949	0.6643	0.6483	0.6510	0.6492	0.6855	0.6701	0.6704	1.0173
	0.7459	0.7047	0.7014	0.6772	0.6915	0.7328	0.6871	0.6700	0.6713	0.7576	0.6720	1.0546
EE2 + 10HA	2.8073	2.8248	2.6579	2.5817	1.6514	1.4322	1.0179	0.8177	0.6400	0.6976	0.6653	0.9130
	2.7977	2.7053	2.6460	2.5742	2.1491	1.5530	1.0288	0.8191	0.7297	0.7484	0.7679	0.8236
	2.7914	2.6814	2.6144	2.5303	1.9651	1.4959	1.0421	0.8177	0.7843	0.7024	0.6802	1.1430
50HA	0.7752	0.7610	0.7231	0.7213	0.7260	0.7385	0.7457	0.7471	0.7573	0.7600	0.7766	0.9032
	0.7500	0.7597	0.7537	0.7471	0.7335	0.7347	0.7192	0.7231	0.7200	0.7528	0.7414	0.7823
	0.7189	0.7681	0.7494	0.7024	0.6856	0.6836	0.7182	0.7140	0.7088	0.7100	0.7636	0.7321
EE2 + 50HA	2.9223	2.8212	2.7736	2.4916	1.8274	1.2774	0.9116	0.7949	0.7542	0.7376	0.7009	0.8624
	2.8984	2.8244	2.7628	2.4538	1.7496	1.2215	0.9396	0.7551	0.7220	0.7464	0.7298	0.8998
	2.8028	2.7397	2.6490	2.3974	1.6877	1.2036	0.9147	0.7735	0.7789	0.8260	0.8446	1.2968
100HA	0.9270	0.8409	0.8320	0.7797	0.7547	0.7761	0.7973	0.7888	0.7851	0.8179	0.8156	1.0775
	0.8714	0.8010	0.7982	0.7655	0.7554	0.7966	0.7697	0.7633	0.7339	0.7183	0.7468	1.0498
	0.8211	0.8393	0.8191	0.7899	0.8103	0.7797	0.8073	0.8105	0.7710	0.8095	0.7731	1.0237
EE2 + 100HA	3.0270	2.8790	2.7757	2.6338	1.9720	1.3399	0.9399	0.8412	0.8532	0.8190	0.7818	1.0620
	2.9277	2.7950	2.7102	2.4597	1.8127	1.2150	0.8916	0.8421	0.7320	0.8735	0.7877	1.1281
	2.8924	2.8268	2.7674	2.4806	1.8928	1.4034	0.9010	0.7762	0.7746	0.8262	0.8888	1.2053

Appendix 8 (continued)

Raw absorbance (620 nm) at 23.5-23.8 °C on day 3 of incubation

Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.6258	0.5754	0.5392	0.5203	0.5179	0.5175	0.5287	0.5440	0.5542	0.5712	0.5701	0.6068
	0.6011	0.5509	0.5296	0.5216	0.5084	0.5095	0.5085	0.5327	0.5270	0.5444	0.5544	0.5718
	0.5923	0.5556	0.5400	0.5344	0.5192	0.5093	0.5416	0.5365	0.5343	0.5365	0.5350	0.5865
EE2 + MQ	0.7545	0.7046	0.6824	0.6528	0.5965	0.5448	0.5197	0.5088	0.5221	0.5220	0.5503	0.5856
	0.7715	0.7112	0.6755	0.6581	0.6062	0.5490	0.5440	0.5569	0.5106	0.5131	0.5561	0.6017
	0.7860	0.7322	0.7013	0.6953	0.6512	0.5617	0.5415	0.5600	0.5254	0.5414	0.5883	0.6064
1HA	0.6369	0.5969	0.5537	0.5519	0.5914	0.5604	0.5388	0.5360	0.5469	0.5572	0.5712	0.6191
	0.6099	0.5266	0.5478	0.5179	0.5061	0.4979	0.5288	0.5107	0.5444	0.5825	0.5918	0.6417
	0.5674	0.5144	0.5123	0.5120	0.5053	0.5094	0.5214	0.5317	0.5300	0.5513	0.5634	0.6569
EE2 + 1HA	0.7792	0.6519	0.6290	0.6427	0.5904	0.5527	0.5112	0.5064	0.5096	0.5135	0.5475	0.7041
	0.7996	0.6712	0.6561	0.6205	0.6017	0.5499	0.5143	0.5176	0.5132	0.5341	0.5398	0.7084
	0.8471	0.7011	0.6655	0.6008	0.5719	0.5414	0.4955	0.4954	0.5159	0.5367	0.5542	0.8358
10HA	0.7688	0.5351	0.5095	0.4957	0.5080	0.5516	0.5040	0.5208	0.6216	0.6710	0.7237	0.8900
	0.5449	0.4843	0.4527	0.4945	0.4701	0.4551	0.4570	0.4545	0.4857	0.4753	0.4729	0.8130
	0.5419	0.5136	0.5084	0.4875	0.4978	0.5345	0.4919	0.4774	0.4761	0.5573	0.4741	0.8421
EE2 + 10HA	0.7717	0.7472	0.6254	0.6107	0.5068	0.5118	0.5251	0.5044	0.4422	0.4884	0.4670	0.7072
	0.7886	0.6590	0.6210	0.5868	0.5735	0.6096	0.4929	0.4988	0.4847	0.5331	0.5616	0.6137
	0.8522	0.6671	0.6042	0.5833	0.5496	0.4993	0.4847	0.4860	0.5282	0.4796	0.4696	0.9172
50HA	0.5911	0.5734	0.5343	0.5339	0.5373	0.5464	0.5528	0.5507	0.5573	0.5613	0.5744	0.6934
	0.5676	0.5664	0.5554	0.5473	0.5360	0.5345	0.5175	0.5248	0.5112	0.5433	0.5351	0.5772
	0.5339	0.5735	0.5526	0.5139	0.4968	0.4911	0.5219	0.5173	0.5151	0.5103	0.5628	0.5339
EE2 + 50HA	0.8428	0.7288	0.7089	0.6661	0.5841	0.5751	0.5125	0.5052	0.5241	0.5188	0.4945	0.6469
	0.8673	0.7569	0.7243	0.6527	0.5794	0.5319	0.5495	0.4931	0.4968	0.5342	0.5234	0.6834
	0.8681	0.7316	0.6450	0.6235	0.6017	0.5661	0.5425	0.5140	0.5521	0.6116	0.6327	1.0702
100HA	0.7248	0.6233	0.6156	0.5675	0.5434	0.5634	0.5833	0.5748	0.5670	0.6000	0.5997	0.8550
	0.6606	0.5818	0.5740	0.5484	0.5378	0.5753	0.5516	0.5436	0.5170	0.5078	0.5305	0.8287
	0.6112	0.6222	0.5984	0.5736	0.5935	0.5635	0.5903	0.5888	0.5502	0.5889	0.5552	0.7977
EE2 + 100HA	0.8863	0.7631	0.7148	0.7414	0.6866	0.5979	0.5410	0.5537	0.5987	0.5909	0.5634	0.8186
	0.8420	0.7228	0.7190	0.7061	0.6525	0.5818	0.5106	0.5447	0.5040	0.6392	0.5678	0.8837
	0.8734	0.7558	0.7354	0.6790	0.6322	0.5869	0.5297	0.5023	0.5383	0.5913	0.6651	0.9517

Appendix 8 (continued)

Raw absorbance (540 nm) at 23.0-23.4 °C on day 4 of incubation

Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.7242	0.6264	0.5873	0.5552	0.5668	0.5388	0.5877	0.6267	0.6370	0.7011	0.6779	0.7108
	0.6649	0.5815	0.5494	0.5566	0.5426	0.5392	0.5276	0.5734	0.5927	0.6060	0.6251	0.6911
	0.6374	0.5814	0.5619	0.5592	0.5314	0.5613	0.5532	0.6052	0.5889	0.5942	0.6197	0.7983
EE2 + MQ	2.6617	2.5834	2.5497	2.5027	1.9968	1.1905	0.7786	0.6205	0.5492	0.5671	0.6156	0.8160
	2.6197	2.4803	2.4704	2.4384	2.0289	1.2341	0.7542	0.6764	0.5853	0.5736	0.7551	0.9129
	2.5563	2.5427	2.5113	2.5049	2.2300	1.4340	0.8626	0.6629	0.5924	0.6907	0.8848	0.7260
1HA	1.0093	0.6261	0.5843	0.5676	0.5935	0.5841	0.5661	0.5643	0.5948	0.7218	0.8734	0.8720
	0.6582	0.5419	0.5444	0.5457	0.5193	0.5195	0.5414	0.5279	0.5762	0.6323	0.6136	0.9771
	0.6348	0.5443	0.5339	0.5267	0.5294	0.5457	0.5411	0.5489	0.5604	0.6083	0.6068	1.0089
EE2 + 1HA	2.6827	2.5348	2.4976	2.4962	2.2353	1.5041	0.8969	0.6443	0.5640	0.5514	0.5752	1.0064
	2.7614	2.5256	2.4961	2.4618	2.3147	1.6894	0.9599	0.7048	0.6030	0.5754	0.5745	1.0135
	2.6909	2.5044	2.4906	2.3957	2.1364	1.4954	0.8913	0.6329	0.5444	0.5654	0.7729	1.1831
10HA	1.1937	0.7413	0.5282	0.5109	0.5543	0.7584	0.7865	0.8278	0.9528	0.9675	0.9217	1.2911
	0.9716	0.5679	0.5733	0.6046	0.5468	0.5639	0.5256	0.5202	0.5552	0.5284	0.5182	1.1902
	0.8457	0.5765	0.5755	0.5908	0.5713	0.5803	0.6008	0.5476	0.5170	0.6408	0.5251	1.1316
EE2 + 10HA	2.6993	2.5060	2.4919	2.4371	1.8153	1.5790	1.0353	0.8020	0.5687	0.5302	0.5568	1.0280
	2.6971	2.4882	2.4533	2.4690	2.2100	1.7109	1.1079	0.7562	0.6988	0.8184	0.6874	1.0923
	2.7300	2.4582	2.3811	2.3533	2.0242	1.6595	1.0958	0.8070	0.7731	0.8240	0.8687	1.3332
50HA	1.0349	0.5326	0.5149	0.5852	0.5676	0.5486	0.5962	0.5516	0.6037	0.6935	0.7266	1.0904
	0.5509	0.4728	0.5290	0.5507	0.5379	0.4968	0.5159	0.5250	0.5209	0.5357	0.5539	0.9000
	0.4995	0.4976	0.5204	0.4961	0.4895	0.5082	0.5277	0.5162	0.5097	0.5267	0.5486	0.8665
EE2 + 50HA	2.9202	2.5654	2.5117	2.3688	1.8730	1.2281	0.8019	0.6395	0.5247	0.5260	0.4983	0.9663
	2.8823	2.5327	2.4957	2.3831	1.8057	1.2208	0.7789	0.6052	0.5266	0.5308	0.4832	1.0327
	2.7587	2.7137	2.4652	2.3510	1.7226	1.1399	0.7567	0.6018	0.6058	0.8512	0.9200	1.4261
100HA	0.9218	0.6984	0.6243	0.5862	0.5499	0.5628	0.5833	0.5804	0.5498	0.9111	0.9135	1.3001
	0.8681	0.6344	0.6246	0.6278	0.6066	0.6448	0.6194	0.6163	0.5841	0.5684	0.6002	1.1809
	0.7743	0.7148	0.6960	0.6626	0.6703	0.6544	0.6598	0.6557	0.6853	0.6567	0.6950	1.1536
EE2 + 100HA	2.9232	2.7253	2.6327	2.6056	2.0550	1.4712	0.8787	0.6529	0.8886	0.6523	0.8580	1.1345
	2.8229	2.5762	2.5495	2.3868	1.9999	1.2386	0.8121	0.6995	0.6942	0.8891	0.7169	1.1341
	2.8463	2.7181	2.5315	2.5216	2.0017	1.4682	0.8803	0.6384	0.5209	0.9303	1.0014	1.3561

Appendix 8 (continued)

Raw absorbance (620 nm) at 23.0-23.4 °C on day 4 of incubation

Well number	1	2	3	4	5	6	7	8	9	10	11	12
MQ	0.5814	0.4782	0.4402	0.4083	0.4205	0.3888	0.4399	0.4758	0.4869	0.5539	0.5335	0.5709
	0.5208	0.4357	0.4040	0.4105	0.3987	0.3957	0.3839	0.4246	0.4458	0.4589	0.4787	0.5476
	0.4920	0.4338	0.4155	0.4135	0.3858	0.4128	0.4068	0.4585	0.4441	0.4477	0.4720	0.6455
EE2 + MQ	0.6808	0.5647	0.5350	0.5227	0.4834	0.4403	0.4159	0.3977	0.3864	0.4133	0.4677	0.6644
	0.7480	0.5607	0.5494	0.5374	0.4975	0.4409	0.4088	0.4607	0.4232	0.4266	0.6020	0.7645
	0.7613	0.6286	0.5786	0.5800	0.5373	0.4635	0.4231	0.4299	0.4203	0.5283	0.7276	0.5856
1HA	0.8201	0.4538	0.4161	0.3989	0.4249	0.4208	0.4020	0.3994	0.4298	0.5491	0.6951	0.7144
	0.4851	0.3696	0.3687	0.3740	0.3454	0.3488	0.3698	0.3552	0.3992	0.4579	0.4420	0.7910
	0.4312	0.3727	0.3609	0.3546	0.3581	0.3746	0.3714	0.3769	0.3879	0.4316	0.4339	0.8134
EE2 + 1HA	0.6940	0.4997	0.4618	0.4734	0.4317	0.4231	0.3884	0.3517	0.3487	0.3636	0.3996	0.8135
	0.7931	0.5031	0.4848	0.4473	0.4550	0.4613	0.3929	0.3782	0.3726	0.3835	0.4014	0.8180
	0.8226	0.5345	0.5102	0.4334	0.3953	0.4023	0.3437	0.3301	0.3231	0.3753	0.5880	0.9875
10HA	0.9060	0.5075	0.3093	0.2835	0.3305	0.5239	0.5448	0.5829	0.6969	0.7123	0.6790	1.0366
	0.7131	0.3414	0.3376	0.3661	0.3189	0.3321	0.2979	0.2912	0.3204	0.3013	0.2895	0.9297
	0.5883	0.3542	0.3536	0.3667	0.3473	0.3540	0.3716	0.3246	0.2987	0.4101	0.2983	0.8660
EE2 + 10HA	0.6804	0.4622	0.4624	0.4320	0.3889	0.3883	0.3839	0.3931	0.3286	0.2909	0.3263	0.7680
	0.7067	0.4615	0.4409	0.4570	0.4269	0.5001	0.3919	0.3437	0.3898	0.5477	0.4440	0.8163
	0.8203	0.4685	0.3924	0.3887	0.3610	0.3884	0.3482	0.3727	0.4481	0.5317	0.5908	1.0437
50HA	0.7902	0.3319	0.3101	0.3767	0.3591	0.3390	0.3841	0.3392	0.3803	0.4669	0.4943	0.8271
	0.3619	0.2747	0.3172	0.3345	0.3244	0.2850	0.2986	0.3103	0.2917	0.3095	0.3280	0.6488
	0.2836	0.2945	0.3124	0.2945	0.2836	0.2948	0.3150	0.3036	0.3014	0.3062	0.3318	0.6171
EE2 + 50HA	0.8381	0.4946	0.4566	0.4281	0.3797	0.3306	0.2965	0.2910	0.2690	0.2874	0.2757	0.6999
	0.8561	0.4855	0.4602	0.4536	0.3862	0.3346	0.2922	0.2944	0.2716	0.2978	0.2641	0.7630
	0.8714	0.7065	0.4682	0.4522	0.3861	0.3226	0.2925	0.2953	0.3476	0.5915	0.6597	1.1501
100HA	0.6884	0.4581	0.3934	0.3573	0.3250	0.3348	0.3566	0.3523	0.3217	0.6528	0.6567	1.0349
	0.5674	0.3982	0.3816	0.3886	0.3691	0.4017	0.3816	0.3745	0.3469	0.3375	0.3671	0.9184
	0.4820	0.4718	0.4595	0.4311	0.4388	0.4199	0.4266	0.4169	0.4430	0.4241	0.4592	0.8914
EE2 + 100HA	0.7985	0.6031	0.5457	0.5827	0.5219	0.5303	0.3836	0.3263	0.5907	0.4062	0.6036	0.8580
	0.7588	0.5122	0.5213	0.4837	0.5896	0.4387	0.3442	0.3551	0.4317	0.6216	0.4753	0.8624
	0.8506	0.6466	0.5020	0.5885	0.4869	0.4436	0.4170	0.3231	0.2701	0.6518	0.7368	1.0580

EE2, ethinylestradiol; MQ, deionized water; 1HA, 10HA, 50HA and 100HA = 1, 10, 50 and 100 mg/L humic acid, respectively.

Apendix 9 Absorbance readings in the determination of the response of yeast to DDT with 10-100 mg/L humic acid in the YES assay using the approach of evaporating the solvent from the test chemical

Nominal DDT (µg/L)	250000	125000	62500	31250	15625	7812.5	3906.3	1953.1	976.56	488.28	244.14	122.07
			Raw absorbance (540 nm) at 23.0-23.4 °C on day 4 of incubation									
Well number	1	2	3	4	5	6	7	8	9	10	11	12
Positive control	2.3000	2.3027	2.2635	2.2266	2.1922	0.8868	1.1958	0.8437	0.7195	0.7298	0.8157	0.8200
	1.9637	2.0191	2.0183	2.0231	2.0001	1.8626	1.3114	0.9656	0.8172	0.7667	0.7600	0.7551
	1.9020	2.0696	2.0547	2.0593	2.0403	1.9238	1.3789	0.9876	0.8503	0.7942	0.7836	0.8200
etOH	0.9209	0.8746	0.7393	0.7231	0.7102	0.7243	0.7351	0.7479	0.7530	0.8192	0.8807	0.9595
	0.9373	0.7286	0.6815	0.6526	0.6525	0.6551	0.6273	0.6093	0.6949	0.6911	0.7336	0.9377
	0.9270	0.7226	0.6798	0.6868	0.6855	0.6404	0.6226	0.6207	0.6355	0.7227	0.7026	0.8994
DDT + MQ	0.8031	0.7237	0.7107	0.6893	0.6653	0.6484	0.6055	0.5849	0.5716	0.5891	0.5875	0.7915
	0.6574	0.6676	0.6669	0.6626	0.6590	0.6644	0.6533	0.6575	0.6609	0.6451	0.6630	0.6564
	0.7614	0.7741	0.7661	0.7621	0.7485	0.7531	0.7430	0.7522	0.7492	0.7305	0.7560	0.7681
MQ	0.8733	0.7609	0.7236	0.7052	0.6894	0.6561	0.6384	0.6472	0.6218	0.6996	0.7137	0.8553
	0.8507	0.7424	0.7238	0.7059	0.6744	0.6394	0.6200	0.6070	0.5886	0.5565	0.5757	0.8466
	0.8018	0.7427	0.7164	0.6890	0.6647	0.6685	0.6582	0.6035	0.5693	0.5181	0.5762	0.8077
DDT + 10HA	1.0011	0.9444	0.9379	0.9321	0.9310	0.9237	0.9042	0.9233	0.8922	0.8999	0.8806	0.9456
	1.0063	0.9450	0.9412	0.9332	0.9319	0.9390	0.9330	0.9218	0.8914	0.9245	0.9099	0.9529
	1.0104	0.9478	0.9154	0.9101	0.9049	0.9049	0.9032	0.9043	0.8861	0.9128	0.9056	0.9205
10HA	0.8632	0.8286	0.8126	0.8155	0.8105	0.8138	0.8160	0.8227	0.7885	0.8260	0.8336	0.8595
	0.7938	0.7297	0.6531	0.6222	0.5921	0.5941	0.8213	0.8064	0.7985	0.8249	0.8216	0.8507
	0.9399	0.9221	0.9289	0.9179	0.9164	0.9141	0.9212	0.9236	0.9030	0.9115	0.9016	0.9557
DDT + 50HA	1.0538	1.0223	1.0044	1.0227	1.0171	1.0113	0.9769	0.9967	0.9620	0.9797	0.9699	0.9615
	1.0209	0.9620	0.9510	0.9806	0.9717	0.9487	0.9224	0.9143	0.8739	0.8945	0.8976	0.9989
	0.9185	0.9693	0.9244	0.8969	0.8970	0.9043	0.9022	0.9037	0.9061	0.9345	0.9481	0.9195
50HA	0.9790	0.8862	0.8936	0.8784	0.8788	0.8750	0.8833	0.8752	0.8881	0.9099	0.9107	1.0051
	0.9691	0.8261	0.8184	0.8385	0.8332	0.8177	0.7840	0.8136	0.8017	0.7773	0.7910	0.8893
	0.9700	0.9939	0.9712	0.9863	0.9799	0.9788	0.9801	0.9882	0.9174	0.9863	0.9669	0.9143
DDT + 100HA	1.1393	1.1697	1.1246	1.2030	1.1599	1.1065	1.0486	1.0712	1.0127	1.0354	1.0234	1.0218
	1.1453	1.1440	1.0818	1.0177	1.0826	0.9916	1.0152	0.9802	0.9542	0.9876	0.9412	1.0046
	1.0048	1.0522	1.0699	1.0539	1.0339	1.0492	1.0066	0.9948	0.9456	0.9816	0.9748	0.9241
100HA	1.0624	1.0593	1.0538	1.0741	1.0541	1.0531	1.0447	1.0591	1.0118	1.0556	1.0496	1.0751
	0.9259	0.8991	0.9071	0.9133	0.9019	0.9110	0.9000	0.8996	0.8693	0.9155	0.9093	0.9260
	0.9137	0.9384	0.8820	0.9169	0.8995	0.8972	0.8960	0.9209	0.8708	0.9009	0.9137	0.9458

Appendix 9 (continued)

	Raw absorbance (620 nm) at 23.0-23.4 °C on day 4 of incubation											
Well number	1	2	3	4	5	6	7	8	9	10	11	12
Positive control	0.7198	0.6589	0.6150	0.5800	0.6148	0.5848	0.4499	0.4239	0.4192	0.4830	0.5877	0.6142
	0.6535	0.6303	0.6257	0.6203	0.6142	0.6063	0.5807	0.5491	0.5408	0.5504	0.5682	0.5873
	0.6693	0.6751	0.6423	0.6311	0.6321	0.6252	0.5964	0.5750	0.5769	0.5749	0.5923	0.6739
etOH	0.6522	0.5831	0.4378	0.3973	0.3801	0.3815	0.3956	0.4202	0.4394	0.5086	0.5770	0.6789
	0.6488	0.4322	0.3676	0.3298	0.3269	0.3288	0.3060	0.3005	0.3778	0.3982	0.4425	0.6588
	0.6504	0.4452	0.3907	0.3834	0.3916	0.3437	0.3307	0.3341	0.3588	0.4435	0.4424	0.6475
DDT + MQ	0.6630	0.5986	0.5888	0.5668	0.5476	0.5317	0.4914	0.4736	0.4621	0.4844	0.4797	0.6698
	0.5556	0.5594	0.5625	0.5575	0.5548	0.5610	0.5513	0.5559	0.5574	0.5498	0.5627	0.5623
	0.6432	0.6503	0.6454	0.6402	0.6288	0.6311	0.6228	0.6327	0.6313	0.6180	0.6414	0.6626
MQ	0.7349	0.6277	0.5899	0.5742	0.5591	0.5280	0.5134	0.5149	0.5008	0.5834	0.5849	0.7212
	0.7192	0.6113	0.5935	0.5778	0.5467	0.5130	0.4992	0.4873	0.4689	0.4481	0.4595	0.7154
	0.6755	0.6214	0.5971	0.5723	0.5495	0.5517	0.5407	0.4918	0.4631	0.4226	0.4715	0.6855
DDT + 10HA	0.7685	0.7245	0.7183	0.7179	0.7170	0.7141	0.7005	0.7201	0.6941	0.7001	0.6896	0.7466
	0.7664	0.7183	0.7253	0.7165	0.7139	0.7218	0.7186	0.7094	0.6983	0.7194	0.7100	0.7481
	0.7657	0.7171	0.7011	0.6994	0.6965	0.6986	0.6934	0.7063	0.6953	0.7172	0.7167	0.7308
10HA	0.7045	0.6636	0.6450	0.6444	0.6417	0.6429	0.6471	0.6562	0.6249	0.6585	0.6667	0.6921
	0.6661	0.6038	0.5332	0.5043	0.4785	0.4771	0.6535	0.6401	0.6371	0.6617	0.6607	0.6866
	0.7383	0.7181	0.7269	0.7146	0.7155	0.7146	0.7159	0.7211	0.7080	0.7144	0.7067	0.7572
DDT + 50HA	0.7718	0.7442	0.7470	0.7477	0.7515	0.7477	0.7326	0.7470	0.7264	0.7434	0.7407	0.7334
	0.7501	0.6926	0.6942	0.7050	0.7035	0.7015	0.6847	0.6896	0.6631	0.6817	0.6901	0.7875
	0.7029	0.7272	0.6967	0.6683	0.6684	0.6773	0.6808	0.6883	0.7037	0.7213	0.7425	0.7168
50HA	0.7592	0.6736	0.6815	0.6630	0.6666	0.6613	0.6691	0.6635	0.6780	0.6943	0.6969	0.7852
	0.7583	0.6422	0.6299	0.6483	0.6406	0.6277	0.5995	0.6277	0.6156	0.5983	0.6120	0.7096
	0.7407	0.7664	0.7444	0.7534	0.7469	0.7449	0.7450	0.7508	0.7016	0.7518	0.7426	0.6957
DDT + 100HA	0.8003	0.7819	0.7899	0.7985	0.7960	0.7849	0.7741	0.7880	0.7616	0.7752	0.7779	0.7837
	0.7790	0.7646	0.7493	0.7296	0.7538	0.7321	0.7538	0.7367	0.7229	0.7491	0.7231	0.7756
	0.7222	0.7527	0.7592	0.7526	0.7512	0.7597	0.7463	0.7508	0.7249	0.7552	0.7489	0.7168
100HA	0.8051	0.8004	0.7912	0.8073	0.7933	0.7859	0.7846	0.7967	0.7652	0.7923	0.7903	0.8155
	0.7226	0.6963	0.6969	0.6993	0.6936	0.6964	0.6891	0.6859	0.6714	0.7006	0.7007	0.7205
	0.7186	0.7291	0.6795	0.6992	0.6856	0.6840	0.6853	0.6990	0.6734	0.6885	0.7036	0.7362

DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; etOH, ethanol; MQ, deionized water; 10HA, 50HA and 100HA = 10, 50 and 100 mg/L humic acid, respectively.

Appendix 10 Absorbance readings in the comparison of different approaches for adding DDT to yeast in YES assay

Nominal DDT (g/L)	0.025	0.0125	0.00625	0.003125	0.001563	0.000781	0.000391	0.000195	0.0000977	0.0000488	0.0000244	1.2E-05
			Raw absorbance (540 nm) at 21.5-21.6 ^{o}C									
Well number	1	2	3	4	5	6	7	8	9	10	11	12
DDT control	1.7166	1.6711	1.5368	1.3215	0.9365	0.7919	0.7528	0.7491	0.8059	0.7798	0.7439	0.7689
	1.7984	1.7297	1.6206	1.3846	1.0555	0.7947	0.7843	0.7825	0.7733	0.7796	0.7443	0.7531
Dried DDT	0.8203	0.7590	0.7220	0.7446	0.7292	0.7352	0.7484	0.7757	0.7802	0.7497	0.7985	0.7869
	0.8038	0.7419	0.7275	0.7355	0.7099	0.7175	0.7183	0.7317	0.7367	0.7675	0.7628	0.7874
DDT + MQ	2.2288	2.2893	2.2704	2.0903	1.7049	1.2729	0.9140	0.8270	0.8121	0.8051	0.7985	0.8059
	2.0590	2.3097	2.2689	2.2071	1.9413	1.4409	1.0624	0.8780	0.8299	0.8222	0.8103	0.7977
Blank for DDT control	0.8246	0.7840	0.7685	0.7570	0.7697	0.7666	0.7868	0.7317	0.7433	0.7568	0.7578	0.8070
	0.8394	0.7899	0.7513	0.7615	0.7608	0.7544	0.7243	0.7780	0.7554	0.7313	0.7478	0.7650
Blank for dried DDT	0.8120	0.7644	0.7171	0.7268	0.7547	0.7188	0.7255	0.7136	0.7265	0.7672	0.7567	0.7693
	0.8235	0.7434	0.7257	0.7325	0.7258	0.7347	0.7110	0.7134	0.7342	0.7472	0.7662	0.7841
Blank for DDT + MQ	0.8085	0.7993	0.7802	0.7710	0.7743	0.8101	0.7536	0.7777	0.7666	0.7934	0.7812	0.8006
	0.8457	0.7934	0.7844	0.7627	0.7761	0.7715	0.7935	0.7637	0.7832	0.7861	0.7687	0.7750
			Raw absorbance (620 nm) at 21.5-21.6 ^{o}C									
DDT control	0.7829	0.7490	0.7058	0.6739	0.6783	0.6466	0.6448	0.6466	0.7009	0.6764	0.6430	0.6685
	0.7818	0.7298	0.6864	0.6684	0.6752	0.6370	0.6729	0.6750	0.6670	0.6737	0.6386	0.6497
Dried DDT	0.7207	0.6530	0.6209	0.6409	0.6254	0.6316	0.6433	0.6713	0.6720	0.6475	0.6946	0.6825
	0.7005	0.6394	0.6244	0.6330	0.6080	0.6137	0.6137	0.6234	0.6338	0.6634	0.6591	0.6837
DDT + MQ	0.8558	0.7878	0.7701	0.7427	0.7131	0.6706	0.6376	0.6450	0.6641	0.6675	0.6746	0.6885
	0.8337	0.8066	0.7530	0.7458	0.7146	0.6733	0.6533	0.6436	0.6602	0.6764	0.6802	0.6808
Blank for DDT control	0.7200	0.6772	0.6612	0.6509	0.6649	0.6624	0.6827	0.6280	0.6376	0.6515	0.6555	0.7049
	0.7361	0.6834	0.6445	0.6547	0.6538	0.6484	0.6178	0.6691	0.6509	0.6245	0.6434	0.6602
Blank for dried DDT	0.7073	0.6612	0.6115	0.6208	0.6477	0.6147	0.6211	0.6084	0.6211	0.6618	0.6495	0.6631
	0.7170	0.6360	0.6205	0.6282	0.6207	0.6284	0.6055	0.6086	0.6252	0.6403	0.6583	0.6777
Blank for DDT + MQ	0.6841	0.6693	0.6455	0.6380	0.6431	0.6774	0.6178	0.6441	0.6329	0.6570	0.6492	0.6776
	0.7290	0.6592	0.6458	0.6245	0.6391	0.6351	0.6537	0.6274	0.6453	0.6502	0.6360	0.6583

DDT, dichlorodiphenyltrichloroethane, MQ, deionized water; DDT control, DDT added directly to the yeast medium; dried DDT, DDT from evaporated ethanol; DDT + MQ, DDT dissolved in deionized water.

Appendix 11 Absorbance readings in estrogenic response in yeast to DDT exposure in a YES assay using different approaches

Nominal DDT (g/L)	0.025	0.0125	0.00625	0.003125	0.001563	0.000781	0.000391	0.000195	9.77E-05	4.88E-05	2.44E-05	1.22E-05
				Raw absorbance (540 nm) at 22.6 °C								
Well number	1	2	3	4	5	6	7	8	9	10	11	12
DDT control	2.4552	2.4982	2.3269	1.9112	1.4762	0.8812	0.8139	0.6407	0.7570	0.6138	0.6327	0.8247
	1.8274	1.9720	1.8281	1.6723	1.1897	1.0138	0.7825	0.6905	0.7767	0.6939	0.7256	0.6562
DDT + MQ	1.5764	1.6357	1.5113	1.3297	1.2043	1.0251	1.0076	1.0403	0.9843	1.0345	1.0075	1.0668
	1.6906	1.8054	1.6364	1.4316	1.1059	0.9363	0.9287	0.9502	0.8783	0.9082	1.0247	0.9148
Dried DDT	0.9572	1.0401	1.0441	0.8965	0.7973	0.7891	0.8875	0.8152	0.8208	0.8097	0.9495	1.0213
	1.3608	1.5261	1.4743	1.2229	1.0333	0.8895	0.8674	0.8870	0.8779	0.8513	0.9323	1.0506
Blank for DDT control	0.8468	0.8316	0.8472	0.8135	0.8368	0.8678	0.8725	0.8184	0.8009	0.9045	0.8805	0.8067
	0.6745	0.6496	0.6566	0.7170	0.6924	0.7089	0.6000	0.5981	0.6602	0.7065	0.6798	0.5650
Blank for DDT + MQ	1.1317	1.1277	1.1214	1.1247	1.0970	1.1414	1.1082	1.1326	1.1183	1.1242	1.1130	1.1237
	1.1681	1.1606	1.0735	1.0985	0.9814	0.9636	0.9394	1.0233	0.9765	0.9782	0.9794	1.0401
Blank for dried DDT	1.0342	0.9856	0.9368	0.9982	0.9343	0.9255	0.9563	1.0023	0.9163	0.9310	0.9622	1.0577
	1.0002	0.9827	0.9664	0.9715	0.9519	0.9474	0.9490	0.9608	0.9191	0.9674	1.0216	0.9660
				Raw absorbance (620 nm) at 22.6 °C								
DDT control	0.9120	0.8895	0.8303	0.6846	0.6638	0.4473	0.5682	0.4934	0.6315	0.5074	0.5333	0.7209
	0.5923	0.7215	0.7221	0.7745	0.5573	0.6418	0.5646	0.5598	0.6595	0.5923	0.6252	0.5695
DDT + MQ	0.8583	0.9123	0.9219	0.9245	0.9816	0.9051	0.9040	0.9403	0.8856	0.9339	0.9079	0.9686
	0.9247	0.8595	0.8043	0.8421	0.7955	0.7666	0.8119	0.8423	0.7735	0.8027	0.9204	0.8173
Dried DDT	0.8586	0.8302	0.7602	0.6800	0.6587	0.6820	0.7809	0.7121	0.7171	0.7088	0.8441	0.9186
	0.8797	0.8287	0.8351	0.8123	0.8170	0.7676	0.7575	0.7806	0.7715	0.7479	0.8287	0.9447
Blank for DDT control	0.7467	0.7048	0.7064	0.6769	0.6977	0.7273	0.7308	0.6797	0.6707	0.7621	0.7574	0.7044
	0.6034	0.5564	0.5656	0.6114	0.5915	0.6100	0.5090	0.5084	0.5672	0.6129	0.5922	0.4964
Blank for DDT + MQ	1.0377	1.0271	1.0225	1.0234	0.9954	1.0380	1.0076	1.0288	1.0153	1.0218	1.0117	1.0244
	1.0696	1.0546	0.9697	0.9926	0.8774	0.8610	0.8371	0.9178	0.8730	0.8741	0.8758	0.9390
Blank for dried DDT	0.9341	0.8818	0.8317	0.8920	0.8304	0.8240	0.8525	0.8942	0.8115	0.8265	0.8573	0.9562
	0.8829	0.8751	0.8597	0.8677	0.8460	0.8404	0.8420	0.8540	0.8141	0.8600	0.9151	0.8644

DDT, dichlorodiphenyltrichloroethane, MQ, deionized water; DDT control, DDT added directly to the yeast medium; dried DDT, DDT from evaporated ethanol; DDT + MQ, DDT dissolved in deionized water.

Appendix 12 GC-MS data on absorbance plotted against GC-MS measured concentrations of DDT in YES assay

Well number	1	2	3	4	5	6	7	8	9	10	11	12
Nominal DDT (g/L)	0.025	0.0125	0.00625	0.003125	0.001563	0.000781	0.000391	0.000195	0.0000977	0.0000488	0.0000244	0.0000122
				GC-MS measured concentrations of DDT (g/L)								
GC DDT + MQ	0.015858	0.004557	0.004417	0.002186	0.001474	0.000612	0.000308	0.0000853	0.0000159	0.00000239	0.000000875	0.0000004
GC Dried DDT	0.000245	0.000544	0.001188	0.000413	0.000363	0.000291	7.46E-05	0.0000248	4.93E-06	8.25E-07	0.000000563	0.000000775

DDT, dichlorodiphenyltrichloroethane; MQ, deionized water; GC-MS, gas chromatography-mass spectrometry; GC DDT + MQ, GC-MS measured DDT dissolved in deionized water; GC Dried DDT, GC-MS measured DDT from evaporated ethanol.

Appendix 13 Absorbance readings in the response of the YES to nominal concentrations of DDT with 1-100 mg/L humic acid

Nominal DDT (g/L)	0.025	0.0125	0.00625	0.00313	0.001563	0.000781	0.0003906	0.000195	9.77E-05	4.88E-05	2.44E-05	1.22E-05
			Raw absorbance (540 nm) at 24.5-24.9 ^{o}C									
Well number	1	2	3	4	5	6	7	8	9	10	11	12
Positive control	2.3867	2.6639	2.4971	2.2572	1.8486	1.4665	1.1817	1.0302	0.9541	0.9478	0.8752	0.8378
	2.7835	2.8280	2.7587	2.5661	2.2171	1.7064	1.3381	1.1199	1.0316	0.9595	0.9323	0.8940
DDT + 100HA	1.6495	1.6414	1.6022	1.4714	1.2122	1.0989	0.9855	0.9633	0.9405	0.9445	0.9197	0.8946
	1.5568	1.6151	1.5847	1.4572	1.2029	1.0858	0.9996	0.9634	0.9549	0.9692	0.9647	0.9230
DDT + 10HA	1.6154	1.6729	1.4996	1.4224	0.9789	0.9002	0.8524	0.8227	0.8258	0.8173	0.8220	0.7944
	1.9838	1.9744	1.7398	1.4693	1.1676	0.9376	0.8799	0.8408	0.8228	0.8237	0.8324	0.7844
DDT + 1HA	2.0455	1.9348	1.7375	1.5801	1.1957	0.9617	0.9005	0.8874	0.8765	0.8721	0.8705	0.8705
	2.1208	1.0792	1.8589	1.6813	1.3287	1.0048	0.9120	0.8974	0.8941	0.8924	0.8831	0.8837
DDT + MQ	1.7203	1.7151	1.3687	1.0679	0.8926	0.8530	0.8495	0.8395	0.8416	0.8280	0.8255	0.8507
	1.8186	1.6974	1.5045	1.2560	0.9848	0.8717	0.8518	0.8411	0.8411	0.8372	0.8410	0.8296
MQ	0.8357	0.8360	0.8206	0.8310	0.8376	0.8387	0.8423	0.8350	0.8368	0.8304	0.8186	0.8131
	0.8609	0.8237	0.8701	0.8660	0.8548	0.8515	0.8486	0.8434	0.8367	0.8491	0.8388	0.8428
1HA	0.8809	0.9150	0.9051	0.9145	0.9034	0.9173	0.9056	0.8953	0.9072	0.8885	0.8864	0.8802
	0.9146	0.9220	0.9158	0.9111	0.9140	0.9089	0.9188	0.9124	0.9184	0.9118	0.9170	0.9014
10HA	0.8617	0.8529	0.8502	0.8834	0.8972	0.9068	0.9025	0.9047	0.9111	0.9058	0.8947	0.8913
	0.8509	0.8575	0.8679	0.8973	0.9105	0.9226	0.9136	0.9084	0.9220	0.9216	0.9049	0.9048
100HA	0.9885	1.0200	0.9477	0.9806	1.0278	0.9868	1.0051	0.9437	0.9909	1.0410	0.9964	0.9941
	1.0007	1.0164	1.0038	1.0076	1.0023	1.0122	1.0314	1.0155	1.0038	1.0366	1.0018	1.0132
etOH	0.8647	0.8481	0.8616	0.8786	0.9006	0.9065	0.9118	0.9288	0.9235	0.9243	0.9018	0.8829
	0.8593	0.8434	0.8631	0.8773	0.8829	0.9211	0.9098	0.9063	0.9126	0.9188	0.9105	0.8865

Appendix 13 (continued)

	Raw absorbance (620 nm) at 24.5-24.9 °C											
Well number	1	2	3	4	5	6	7	8	9	10	11	12
Positive control	0.8601	0.8530	0.8174	0.7980	0.7643	0.7336	0.7107	0.6979	0.6998	0.7274	0.6958	0.7080
	0.9174	0.8582	0.8391	0.8062	0.7872	0.7489	0.7330	0.7100	0.7022	0.6974	0.7041	0.7261
DDT + 100HA	0.8738	0.8544	0.8334	0.8401	0.8255	0.8196	0.7961	0.8011	0.7820	0.7860	0.7640	0.7384
	0.8632	0.8580	0.8628	0.8521	0.8373	0.8268	0.8152	0.8038	0.8089	0.8132	0.8075	0.7670
DDT + 10HA	0.7877	0.7907	0.7833	0.7621	0.7412	0.7409	0.7240	0.7082	0.7069	0.6963	0.7006	0.6835
	0.8336	0.8205	0.7843	0.7659	0.7477	0.7365	0.7363	0.7205	0.7020	0.7020	0.7066	0.6728
DDT + 1HA	0.8470	0.8433	0.8370	0.8192	0.7999	0.7878	0.7845	0.7790	0.7683	0.7655	0.7616	0.7595
	0.8608	0.8120	0.8435	0.8376	0.8168	0.8025	0.7984	0.7887	0.7896	0.7841	0.7746	0.7773
DDT + MQ	0.7915	0.7973	0.7713	0.7458	0.7313	0.7375	0.7490	0.7402	0.7457	0.7354	0.7320	0.7473
	0.8153	0.8142	0.7942	0.7683	0.7433	0.7484	0.7403	0.7406	0.7415	0.7362	0.7401	0.7276
MQ	0.7508	0.7606	0.7416	0.7396	0.7467	0.7452	0.7440	0.7308	0.7319	0.7334	0.7240	0.7067
	0.7743	0.7497	0.7864	0.7723	0.7567	0.7513	0.7504	0.7450	0.7412	0.7538	0.7451	0.7485
1HA	0.8019	0.8430	0.8163	0.8173	0.7987	0.8171	0.8039	0.7920	0.7970	0.7818	0.7822	0.7790
	0.8320	0.8460	0.8306	0.8190	0.8222	0.8173	0.8202	0.8115	0.8175	0.8099	0.8136	0.7938
10HA	0.7755	0.7691	0.7535	0.7816	0.7826	0.7998	0.7919	0.7861	0.7961	0.7936	0.7830	0.7869
	0.7715	0.7766	0.7716	0.7949	0.8050	0.8199	0.8059	0.8003	0.8141	0.8057	0.7931	0.7960
100HA	0.8574	0.8871	0.8232	0.8417	0.8795	0.8488	0.8633	0.8161	0.8423	0.8985	0.8427	0.8487
	0.8847	0.8901	0.8727	0.8753	0.8735	0.8757	0.8934	0.8773	0.8707	0.8857	0.8564	0.8689
etOH	0.6891	0.6732	0.6696	0.6797	0.6861	0.6895	0.6970	0.7012	0.6997	0.7066	0.6945	0.7151
	0.7149	0.6789	0.6964	0.7063	0.7008	0.7217	0.7135	0.7111	0.7196	0.7247	0.7327	0.7457

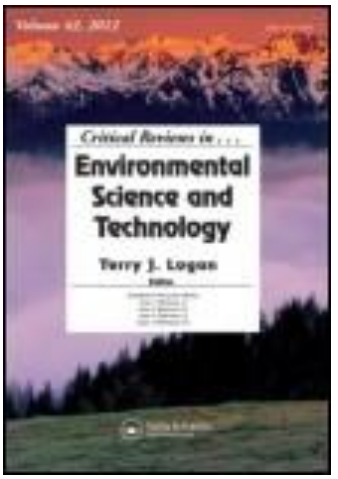
DDT, dichlorodiphenyltrichloroethane; positive control, DDT added directly to the yeast medium; MQ, deionized water; etOH, ethanol; 1HA, 10HA and 100HA = 1, 10 and 100 mg/L humic acid.

Appendix 14 GC-MS data on the response of the YES to measured concentrations of DDT with 1-100 mg/L humic acid

Well number	1	2	3	4	5	6	7	8	9	10	11	12
Nominal DDT (g/L)	0.025	0.0125	0.00625	0.003125	0.001563	0.000781	0.0003906	0.0001953	9.77E-05	4.88E-05	2.44E-05	1.22E-05
				GC-MS measured concentrations of DDT (g/L)								
GC DDT + 100HA	0.015121	0.00671	0.002741	0.001074	0.000765	0.000435	0.0002087	0.0001251	0.0000516	0.00002458	0.00001386	0.000009288
GC DDT + 10HA	0.010929	0.005542	0.001796	0.000657	0.000443	0.000174	0.0000937	0.0000446	0.0000226	0.0000122	0.00000836	0.00000326
GC DDT + 1HA	0.007257	0.002128	0.002182	0.001169	0.000662	0.000343	9.279E-05	0.0000407	8.088E-06	0.000002588	0.000001563	7.125E-07
GC DDT + MQ	0.009471	0.005761	0.002304	0.001111	0.000482	0.000249	7.549E-05	2.266E-05	2.188E-06	2.875E-07	1.375E-07	1.125E-07

DDT, dichlorodiphenyltrichloroethane; GC-MS, gas chromatography-mass spectrometry; GC DDT + MQ, GC-MS measured DDT dissolved in deionized water; GC DDT + 1HA, GC DDT + 10HA and GC DDT + 100HA = GC-MS measured DDT with 1, 10 and 100 mg/L humic acid, respectively.

Appendix 15 Publication



Critical Reviews in Environmental Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/best20

Hexabromocyclododecane and Hexachlorocyclohexane: How Lessons Learnt Have Led to Improved Regulation

Cyril O. Onogbosele[a] & Mark D. Scrimshaw[a]

[a] Institute for the Environment, Brunel University, Uxbridge, Middlesex, UK

Accepted author version posted online: 06 Sep 2013.Published online: 27 May 2014.

To cite this article: Cyril O. Onogbosele & Mark D. Scrimshaw (2014) Hexabromocyclododecane and Hexachlorocyclohexane: How Lessons Learnt Have Led to Improved Regulation, Critical Reviews in Environmental Science and Technology, 44:13, 1423-1442, DOI: 10.1080/10643389.2013.782172

To link to this article: http://dx.doi.org/10.1080/10643389.2013.782172

Critical Reviews in Environmental Science and Technology, 44:1423–1442, 2014

ISSN: 1064-3389 print / 1547-6537 online DOI: 10.1080/10643389.2013.782172

Hexabromocyclododecane and Hexachlorocyclohexane: How Lessons Learnt Have Led to Improved Regulation

CYRIL O. ONOGBOSELE and MARK D. SCRIMSHAW
Institute for the Environment, Brunel University, Uxbridge, Middlesex, UK

The use of chemicals by society has many benefits but contamination of the environment is an unintended consequence. One example is the organochlorine compound hexachlorocyclohexane (HCH). During the 1980s, when HCH was banned in many countries, the brominated flame retardant, hexabromocyclododecane (HBCD), found increasing use. The persistent, bioaccumulative, and toxic characteristics of HBCD are, 30 years later, likely to warrant global action on production and use under the Stockholm Convention on persistent organic pollutants. Historical lessons have taught us that we need to control the use of chemicals and programs are in place worldwide in an attempt to do so.

KEY WORDS: bioaccumulation, chemicals, environment, organochlorine, organobromine, persistence, toxicity

1. INTRODUCTION

Over the years, the growth of the chemical industry and the manufacture and use of a number of chemical substances have resulted in global contamination of the environment with some chemical substances. In particular, those classified as persistent organic pollutants (POPs) have attracted attention due to a growing body of scientific evidence of their PBT properties and the potential for long-range environmental transport (UNEP, 2009). Among POPs are the synthetic organohalogens, hexachlorocyclohexane (HCH), and

Address correspondence to Mark D. Scrimshaw, Institute for the Environment, Brunel University, Uxbridge, Middlesex UB8 3PH, UK. E-mail: mark.scrimshaw@brunel.ac.uk

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/best.

142
3

hexabromocyclododecane (HBCD). The manufacture and use of HCH began much earlier than that of HBCD (Breivik et al., 1999; Alaee et al., 2003). For several years, the environmental fate and toxicological effects of HCH were extensively studied and known before the manufacture and use of HBCD (ATSDR, 2005; EC, 2008).

With the molecular formula $C_6H_6Cl_6$, HCH is an organochlorine first synthesized in 1825 by photochlorination of benzene, and was then known as benzene hexachloride (BHC) (CEC, 2006). Technical HCH is a mix- ture of five isomers: α (alpha)-HCH (55–80%), β (beta)-HCH (5–14%), γ (gamma)-HCH (8–15%), δ (delta)-HCH (2–16%), and ε (epsilon)-HCH (3–5%) (Vijgen et al., 2011). The proportion of the different isomers in technical products varied due to differences in production processes. The most envi- ronmentally significant isomers are the α, β, and γ isomers. The insecticidal property of HCH virtually exhibited by the γ isomer was discovered in 1942. The γ-HCH was then named lindane after Van Linden, the discoverer of α and γ isomers (CEC, 2006). With the exception of γ-HCH, the other isomers of HCH became residues of the production process. Technical HCH was used in the control of insect pests until the late 1970s when it was replaced by lindane ($\geq$99% γ-HCH) (Breivik et al., 1999). The production of 1 tonne of lindane generated approximately 6–10 tonnes of α- and β-HCH and as a result of the waste isomers generated, the production and regulation of lindane was a global problem for many years (IHPA, 2006).

Lindane and technical HCH have been used in the treatment of fruits, food crops, ornamental plants, seeds, forestry products, soil, livestock, and pets to eradicate pests such as insects, ticks, and mites (Li, 1999). The insecticide has also been used as a pharmaceutical formulation in shampoo, lotions, or creams for treatment of head lice and scabies (mite infection) in humans (WHO, 1991). It is estimated that from 1950 to 2000, about 600,000 tonnes of lindane was used globally; on an annual basis, this was about 12,000 tonnes per annum over a period of 50 years. The estimated use in agriculture in Europe, Asia, Africa, and Oceania were 287,160, 73,200, 63,570, 28,540, and 1,030 tonnes, respectively (IHPA, 2006). Breivik et al. (1999) reported that 382,000 tonnes of technical HCH and 81,000 tonnes of lindane were used in Europe from 1970 to 1996. In addition, they observed an estimated cumulative usage of 259,000 tonnes of α-HCH, 135,000 tonnes of γ-HCH and 20,000 tonnes of β-HCH.

Release of HCH to the environment involves several pathways: emissions from manufacturing sites, volatilization to the atmosphere during application in agriculture, atmospheric deposition, leaching in soil and re-

lease from stockpiles of disposed residual HCH isomers (UNEP, 2006). Exposure of biota (including humans) to HCH is mainly through intake of contaminated food and water. In addition, human exposure to lindane may be by direct contact during its application for pharmaceutical and agricultural purposes (CEC, 2006; UNEP, 2006). Because of the adverse effects of

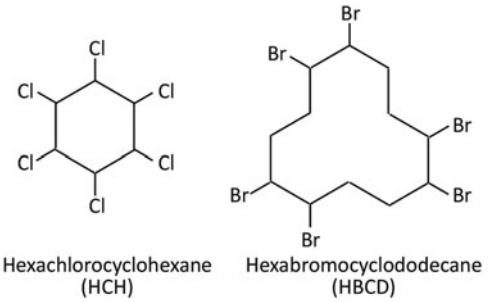


FIGURE 1. The structures of the two halogenated cyclic alkanes, HCH and HBCD.

lindane on the environment and human health, by 2006, the use of lindane had been banned in 52 countries, and restricted in 33 countries (CEC, 2006). The proposal to list lindane and α- and β-HCH on Annex A (elimination) of Stockholm Convention on POPs was made by Mexico in 2005 and 2006, respectively (Vijgen et al., 2011). In 2009, they were finally listed on Annex A of Stockholm Convention on POPs. This implied a global ban on the production and use of lindane, and α- and β-HCH. However, a specific exemption (5 years limit effective from 2009) allows the use of lindane as a human health pharmaceutical for the control of head lice and scabies as second-line treatment (UNEP, 2009).

A halogenated cyclic alkane, similar in structure to HCH (Figure 1), HBCD has a molecular formula of $C_{12}H_{18}Br_6$, and is an additive brominated flame retardant (BFR) produced by bromination of 1,5,9-cyclododecatriene (Heeb et al., 2005). As a flame retardant, it is incorporated into a wide range of consumer products to resist ignition of combustion and prevent or reduce flammability, particularly in materials that are susceptible to combus- tion (BSEF, 2009). Law et al. (2005) described 16 possible stereoisomers of HBCD comprising six pairs of enantiomers and four mesoforms. However, technical HBCD is a mixture of three diastereomers: α-HBCD (10–13%), β - HBCD (1–12%), and γ -HBCD (75–89%) (Covaci et al., 2006). Like HCH, the complex stereochemistry of HBCD and the differential environmental be- havior and fate of its isomers have made chemical analysis and regulation of HBCD difficult (Janak et al., 2005; Law et al., 2005). The production of HBCD for use as a BFR in polystyrene materials commenced in the 1980s, though the chemical had been available on the market since the 1960s (EC, 2008). HCH had been in use for at least, two decades before the global intro- duction of HBCD. HBCD is mainly used in expanded polystyrene, extruded polystyrene and backcoating of textiles for upholstered furniture, upholstery seating in

transportation vehicles, draperies, wall coverings, mattress ticking, and interior textiles such as car cushions and roller blinds (Swedish Chemi- cals Agency, 2008). Polystyrenes are principally used for thermal insulation boards in construction and building industries (Darnerud, 2003). In Europe, in particular, HBCD is also used in high-impact polystyrene for electrical and electronic equipment such as audiovisual equipment cabinets, wire and cable distribution boxes, and refrigerator lining (ECHA, 2009). Deng et al. (2009) observed that the estimated total market demand for HBCD in 2001 globally was over 16,700 tonnes, with 2,800 tonnes from USA, 9,500 tonnes from Europe, 3,900 tonnes from Asia, and 500 tonnes from the rest of the world. In 2002 and 2003, the global demands were 21,447 and 21,951 tonnes, respectively (UNEP, 2010b). The increasing global demand for HBCD has resulted in an annual production of almost twice that historically reached for HCH.

Release of HBCD to the environment may arise from emissions and discharge of HBCD from manufacturing sites (Covaci et al., 2006), and the use and disposal of its products (Wu et al., 2011). HBCD is an additive flame retardant; it is not chemically bound to the material it protects unlike reactive flame retardants. Therefore, it is predisposed to high leaching and release to the environment from its products in use or after disposal (USEPA, 2010). Evidence of the distribution of HBCD in environmental media such as air, soil, sediments, surface water and sewage sludge, and biota (including humans) have been reported (ECHA, 2008; Environment Canada, 2011). Because of its volatility, atmospheric transport is also an important pathway for transport of HBCD within the environment (de Wit et al., 2010). In the European Union, due to the PBT properties of HBCD, HBCD has been identified as a substance of very high concern within the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) framework (ECHA, 2008). In the USA, HBCD is also considered to be of high concern based on its PBT properties, high toxicity to aquatic organisms, and occurrence in remote regions of the world (UNEP, 2010a).

HBCD is among new POPs being considered for global elimination. It was nominated by Norway in 2008 for listing in the annexes of Stockholm Convention on POPs. It has met criteria for inclusion in Annex D of the Convention based on the screening criteria of PBT properties and the potential for long-range environmental transport, and completed the Annex E assessment (UNEP, 2010a). The Persistent Organic Pollutants Review Committee (POPRC), a subsidiary body of Stockholm Convention mandated to assess a given proposal by a Party for listing of a chemical as a POP in Annex(es) A, B (restriction), and/or C (unintentional production), at its sixth meeting held from 11–15 October 2010 in Geneva, Switzerland, considered and adopted the risk profile of HBCD. It was concluded that HBCD should proceed to Annex F (management evaluation). At its seventh meeting held from 10–14 October 2011 in Geneva, the Committee considered a draft risk management plan for possible control measures and socioeconomic considerations and recommended that HBCD should be listed in Annex A as a control measure. However, the recommendation is yet to be adopted by the Convention (UNEP, 2011).

This paper will comparatively review the PBT properties and the potential for long-range environmental transport of HCH and HBCD, and evaluate where the consequences of using HBCD could have been foreseen as a result of the early warnings from HCH.

2. PERSISTENCE

Characteristically, HCH and HBCD are persistent and resistant to degradation. Though degradation by microorganisms may result in the slow removal of HCH from water, photolysis and hydrolysis are not considered to be significant pathways for degradation of HCH isomers (CEC, 2006; Addison et al., 2009; Hu et al., 2010). Once released to the environment, HCH partitions into the air, water, sediments and soil, and accumulates in biota. Technical HCH is no longer used as an insecticide in most parts of the world, but its isomers are still reported to occur in surface waters, sediments, soil and biota in countries where it has long been banned because of its persistence in the environment (Zhao et al., 2009; Hu et al., 2010; Vijgen et al., 2011). Among banned organochlorines, Brun et al. (2008) reported α- and γ -HCH among the most frequently detected chemical substances in wet-precipitation across Atlantic Canada.

Chen et al. (1984) reported half-lives of 91 hours (3.79 days), 152 hours (6.33 days), and 104 hours (4.33 days) for α-HCH, β-HCH, and γ -HCH, respectively, in the air. Hydrolytic half-lives of 0.8 years (292 days) (pH 8.0, 20° C) and 26 years (pH 7.8, 5° C) were estimated for α-HCH by Ngabe et al. (1993). In addition, Harner et al. (1999) estimated a half-life of 63 years for α-HCH in the Arctic Ocean. In natural freshwaters such as rivers and lakes, the estimated half-lives for γ -HCH/lindane range from 3 to 300 days (Mackay et al., 1997). In seawater (pH 8.0, 20° C), a half-life of 1.1 years is estimated while 110 years is estimated in the Arctic Ocean (pH 8.0, 0° C) for lindane (UNEP, 2006). In soils, half-lives of 55 days (Singh et al., 1991) and 161 days (Doelman et al., 1990) for α-HCH, 100 and 184 days for β-HCH (Singh et al., 1991), and 88 to 1,146 days (aerobic conditions) and 12 to 174 days (anaer- obic conditions) for γ -HCH (Slooff and Matthijsen, 1988; IPCS, 1991) have been reported. Information on degradation half-lives of HCH in sediments is limited. However, in aquatic sediments, half-lives of 90 days (WWFC, 1999), and 0.9, 12.6, and 1.26 years for α-, β -, and γ -HCH, respectively, in the Arctic (Helm et al., 2002) have been estimated. In environmental media, β -HCH does not undergo degradation easily. Compared to other HCH isomers, it is detected most commonly in environmental media due to its lower water solubility (higher K_{ow}) and greater chemical stability (Bhatt et al., 2009). HCH persists in biota. Data on the occurrence of HCH in biota are usually in the form of concentrations rather than biological half-lives, although in humans, an estimated half-life of 7 to 10 years for β-HCH, which is the

TABLE 1. A comparison of the persistence of HCH and HBCD in environmental media

Criterion	Regulatory threshold (UNEP, 2001)	HCH	HBCD
Half-life in air	>2 days	3.7 to 6.33 (Chen et al., 1984)	0.4 to 5.2 (Marvin et al., 2011)
Half-life in water	>60 days	3 to 300 days (Mackay et al., 1997)	60 to 130 days (Marvin et al., 2011)
Half-life in aquatic sediments	>180 days	0.9 to 12.6 years (Helm et al., 2002)	125–191 days (EC, 2008)
Half-life in soil	>180 days	<180–1,146 (IPCS, 1991)	6.9 to 63 days (Davis et al., 2005) ≥182 days (Environment Canada, 2011)
Half-life in biota (days/years)	none	7 to 10 years (humans) (Zou and Matsumura, 2003)	23 to 219 days (humans) (Schecter et al., 2012) 1 to 17 days (mice) (Schecter et al., 2012) 53 to 136 days (fish) (Janak et al., 2005)

predominant isomer in mammals, has been reported (Zou and Matsumura, 2003).

HBCD also has the propensity for persistence. Like HCH, half-lives in air and water greater than the regulatory thresholds of >2 and >60 days (UNEP, 2001), respectively, have been reported (Table 1). However, there appears to be a lack of experimental data on the degradation half-life of HBCD in both freshwater and marine water. The range of values (60–130 days) stated in Table 1 for HBCD are rather estimates derived from models. In studies on the biodegradation of HBCD in aquatic sediments, half-lives of 210, 130, and 190 days (aerobic) and 210, 80, and 125 days (anaerobic) for α-, β-, and γ-HBCD, respectively, have been reported. However, using temperature of 12°C as benchmark, the half-life of HBCD in sediments is estimated to be 125–191 days (EC, 2008). Compared to β- and γ-HBCD, α-HBCD is resistant to reductive dehalogenation under anaerobic condition in sediments (EC, 2008). Data on degradation half-lives of HBCD in soils are limited. Davis et al. (2005) determined half-lives of 63 and 6.9 days in aerobic and anaerobic soils, respectively, for HBCD. However, in the study, degradation products were not reported, and only the fate of γ-HBCD was determined. On the basis of empirical data primarily, the half-life of HBCD in soil is $\geq$182 days (Environment Canada, 2011).

When released to the environment, HBCD isomers will adsorb onto solid particles of sediments and soil (Janak et al., 2005). Though there is a predominance (>90%) of γ-HCBD in the environment compared to α- and β-HBCD, α-HBCD often has the highest prevalence in biota, followed by β-HBCD (Birnbaum and Staskal, 2004). This has been attributed to bioisomerization of the diastereomers and differences in the metabolizing capacity of organisms, particularly fish (Law et al., 2004; Janak et al., 2005). Half-lives of 136 and 53 days for α- and β-HBCD, respectively, in *Oncorhynchus mykiss* (Rainbow trout) have been reported (Janak et al., 2005).

The abundance of HBCD in environmental media in remote locations such as the Arctic without demonstrable existing sources of exposure, and its trophic transfer in food webs provide evidence of persistence of HBCD. Concentrations of HBCD measured in dated sediment core samples indicate widespread occurrence and also provide evidence of the persistence of HBCD in the environment (UNEP, 2007a). Generally, HCH is more persistent in environmental media than HBCD (Table 1), however isomers of both HCH and, HBCD exhibit differences in their persistence in environmental media.

3. BIOACCUMULATION

Octanol-water partition coefficient (K_{OW}) and bioconcentration factor (BCF) are used to assess the potential for a chemical to bioaccumulate. Log K_{ow} values of 3.8, 3.78, and 3.72 for α-, β- and γ-HCH, respectively (ATSDR, 2005), indicate a potential for bioaccumulation. A wide range of BCFs for HCH have been reported in several studies. Oliver and Niimi (1985) reported BCF of 1,100–2,800 in fish. In invertebrates, BCFs ranging from 60–2,750 have been estimated (UNEP, 2007b). Due to its lipophilicity, HCH accumulates in

food chains. It has been reported to accumulate rapidly in invertebrates, fish, birds, and mammals (CEC, 2006). In biota, particularly mammals, the variations observed in the isomeric composition of HCH may be due to differences in sources and time of exposure, isomeric uptake, metabolism, and adiposity of species (Willett et al., 1998). Generally, β-HCH being the most persistent and bioaccumulative isomer, may exhibit highest prevalence among HCH isomers detected in mammalian tissues (Solomon and Weiss, 2002; Li and Macdonald, 2005). This is attributable to the greater resistance to metabolism and the much longer half-life of β-HCH than other HCH isomers in adipose tissues of mammals (Li and Macdonald, 2005). Zou and Matsumura (2003) reported the accumulation of β-HCH in the adipose and breast tissues of humans.

HBCD also has the potential for bioaccumulation like HCH. However, the log K_{OW} values of HBCD are higher than those of HCH. For technical HBCD, α-HBCD, β-HBCD, and γ-HBCD, the estimated log K_{ow} values are 5.62, 5.07, 5.12, and 5.47, respectively (ECHA, 2008). HBCD has low water solubility of 66 μg/l (Swedish Chemicals Agency, 2008). Because of its hydrophobicity and lipophilicity, it exhibits partitioning into adipose tissues in biota, followed by accumulation, characteristic of many POPs (de Wit, 2002; Law et al., 2003). The accumulation of HBCD in different organisms such as invertebrates, fish, birds, and mammals (including humans), and its biomagnification in food chains have been reported (Tomy et al., 2004; Law et al., 2006; Covaci et al., 2006). BCFs of 18,100 in *Pimephales promelas* (fathead minnows) (Veith et al., 1979) and 19,200 in *O. mykiss* (Drottar et al., 2001) have been measured. Stereoisomer-specific bioaccumulation has been observed in HBCD. Like HCH, HBCD seems to undergo stereoselective processes such as biotransformation and bioisomerization in the environment, resulting in relative enrichment of different stereoisomers (Janak et al., 2005; Law et al., 2005; Heeb et al., 2008). This has been observed in the preferential accumulation of α-HBCD in relation to the much dominant γ-HBCD in the technical HBCD mixture (Janak et al., 2005). Differences in the water solubility of HBCD stereoisomers (48.8, 14.7, and 2.1 μg/l for α-, β-, and γ-HBCD, respectively) may also be responsible for differences in the metabolism and bioaccumulation of the stereoisomers (Hunziker et al., 2004). The regulatory criteria for bioaccumulation assessment based on K_{ow} and BCF include United Nations Environment Programme (UNEP) (Stockholm Convention on POPs), log $K_{ow} \geq 5$ and BCF $\geq$ 5,000; European Union (REACH), BCFs $\geq$ 2,000 (bioaccumulative), and $\geq$5,000 (very bioaccumulative); United States (Toxic Substances Control Act), BCFs of 1,000–5,000 (bioaccumulative) and $\geq$5,000 (very bioaccumulative), and Environment Canada (Canadian Environment Protection Act), log $K_{ow} \geq 5$ and BCF $\geq$ 5,000 (Arnot and Gobas, 2006). On the basis of these criteria, HBCD is much more bioaccumulative than HCH.

4. TOXICITY

Reported adverse effects of HCH (Table 2) in laboratory animals and humans include carcinogenicity, genotoxicity, neurotoxicity, developmental toxicity,

endocrine disruption, reproductive disorders, hematological alterations, and immunosuppression (ATSDR, 2005; UNEP, 2006). Mathur et al. (2002) reported β-HCH levels to be significantly higher in breast cancer patients, 31–50 years of age in relation to noncancer patients. β-HCH is a risk factor for the progression of breast cancer cells to advanced state of malignancy (Zou and Matsumura, 2003). Studies by Khan et al. (2010) indicated a positive significant association between sperm count and the level of α- and β-HCH in infertile human males as a result of Y chromosome microdeletions by the HCH isomers. HCH is mutagenic, and can cause spermatogenic failure in humans. Neurological effects such as seizures, convulsion and coma in humans, and immunosuppression and suppressed antibody responses in laboratory animals arising from exposure to lindane have been observed (WHO/Europe, 2003). Prenatal exposure to β-HCH has been associated with alteration in thyroid hormone levels and possible adverse brain development in humans (Alvarez-Pedrerol et al., 2008). Studies on rats and rabbits have indicated reproductive disorders such as reduced ovulation, reduction in the number of testicular spermatids and epididymal sperms, degeneration of

TABLE 2. Comparative toxicity of HCH and HBCD. A comparison of values for ecotoxicity is shown in Figure 2

Toxicity	HCH	HBCD
Mammalian toxicity		
Carcinogenicity	β-HCH 29 μg/l *in vitro* (Zou and Matsumura, 2003)	No data
Genotoxicity	α-HCH 130 μg/l; β HCH 300 μg/l *in vitro* (Khan et al., 2010)	No data
Neurotoxicity		
Reproductive toxicity	α-HCH at 23.4 mg/kg/day in rats (WHO Europe, 2003)	13.5 mg/kg/day in mice (Eriksson et al., 2006)
Developmental toxicity	γ-HCH 6 mg/kg/day in male rats (ATSDR, 2005)	2,500 mg/kg/day in rats (Darnerud, 2003)
Immunotoxicity	γ-HCH 13.1 mg/kg/day in rats (ATSDR, 2005)	Has the potential (UNEP, 2010b)
	γ-HCH 6- 25 mg/kg/day in rats (UNEP, 2006)	No data
Endocrine disruption	β- and γ-HCH (UNEP, 2006; Alvarez-Pedrerol et al., 2008)	Has the potential (UNEP, 2010b)
	Technical HCH 3 to 11 mg/l *in vitro* (mammalian cells) (Tiemann, 2008)	α-HBCD 0.064 μg/l *in vitro* (mammalian cells) (Ibhazehiebo et al., 2011)
Ecotoxicity		
Acute toxicity	Highly toxic to freshwater fish (UNEP, 2006)	Toxic to freshwater fish embryos (Deng et al., 2009)
	Highly toxic to aquatic invertebrates (UNEP, 2006)	Toxic to aquatic invertebrates (ECHA, 2008)
	Moderately toxic to birds and mammals (CEC, 2006)	No data on acute toxicity to birds and mammals
	Highly toxic to algae (IPCS, 1992; Schafer et al.,1994)	Highly toxic to algae (Desjardins et al., 2005)
Chronic toxicity	Aquatic biota (UNEP, 2006)	Aquatic biota (EC, 2008)
Inhibition of growth and survival	In daphnids and fish (Gorge and Nagel, 1990; Ferrando et al., 1995)	In daphnids and fish (Drottar and Kruegar, 1998; Drottar et al., 2001)
Inhibition of reproduction	In aquatic invertebrates, birds and mammals (UNEP, 2006)	In daphnids, fish, birds, mammals, and earthworm (UNEP, 2010b)
Terrestrial phytotoxicity	Technical HCH 1,250 mg/kg in soil (Pereira et al., 2010)	No (UNEP, 2010b)
Endocrine disruption	Technical HCH 1 to 10 mg/l in fish (Singh and Canario, 2004)	In fish exposed to 5 μg/l (Palace et al., 2010)

seminiferous tubules and disruption of spermatogenesis as a result of exposure to lindane. Hematological changes such as leukocytosis, granulocytosis, eosinophilia, thrombocytopenia, and leukopenia have also been observed in humans following chronic exposure to lindane (UNEP, 2006). Acute exposure to lindane in humans may cause adverse effects ranging from skin irritation to dizziness, diarrhea, vomiting, headache nausea convulsion, and death (CEC, 2006).

The ecotoxicity of HCH has been extensively studied. Lindane is toxic to aquatic organisms. Schafer et al. (1994) reported lindane's inhibition of growth in the freshwater algae, *Chlamydomonas reinhardi* and *Scenedesmus subspicatus* at 72h EC_{50} of 4.0 mg/l and 72h EC_{50} of 3.2 mg/l, respectively. The LC_{50} (median lethal concentration) for aquatic invertebrates and fish ranges from 10–520 μg/l to 1.7–131 μg/l, respectively (UNEP, 2006). Studies on the chronic toxicity of lindane showed reduction in the growth of freshwater fish larvae at a NOAEC (no observed adverse effect concentration) of 2.9 μg/l, and decline in reproduction in aquatic invertebrates at NOAEC of 54 μg/l (UNEP, 2006). In aquatic birds and mammals generally, chronic exposure to lindane has resulted in reduced rate of growth and survival, decrease in body weight and egg production and endocrine disruption as important endpoints (CEC, 2006). In the terrestrial environment, Pereira et al. (2010) reported on the phytotoxicity of HCH in relation to the germination and growth responses of different plant species.

Unlike HCH, information on the relative toxicity of the different isomers of HBCD in humans and wildlife is virtually lacking. However, extrapolations of toxicological tests on technical HBCD mixture in mammals strongly indicate that HBCD has the potential to cause adverse effects in humans (Table 2). These include endocrine disruption, particularly of the thyroid-hormone system (Ibhazehiebo et al., 2011); neurotoxicity (learning and memory defects) (Eriksson et al., 2006; Reistad et al., 2006); reproductive disorders such as inhibition of oogenesis (Darnerud, 2003), and adverse effect on liver weight and activity (Germer et al., 2006). The possible role of HBCD in carcinogenicity is not known. The limited data indicate that with the exception of endocrine disruption in mammalian cell cultures, where effects occurred at concentrations of mg/l rather than μg/l, risks posed by HBCD to mammals are not greater than those of HCH.

Ecotoxicity studies (Table 2 and Figure 2) have shown that HBCD, like HCH, can potentially produce adverse effects in aquatic organisms, particularly algae, invertebrates, fish, birds and mammals, and terrestrial organisms at environmentally relevant concentrations (Darnerud, 2003; Birnbaum and Staskal, 2004). Generally, laboratory studies on the toxicity of HBCD to aquatic organisms indicate endpoints such as inhibition of survival, growth, development and reproduction, endocrine disruption, histopathological changes, oxidative stress and apoptosis and mortality (Legler, 2008; Deng et al., 2009; UNEP, 2010b; Environment Canada, 2011). HBCD is highly toxic to algae. 72h EC_{50} (effective concentration in 50%) values based on decrease in population density in marine algae range from 9.3–12 μg/l in *Skeletonema costatum*, and 50–370 μg/l in *Thalassiosira pseudonana* (Walsh et al., 1987). In studies by Roberts and Swigert (1997), 72h EC_{50} >2.5 μg/l was observed in the freshwater alga, *Pseudokirchneriella subcapitata* (=*Selenastrum capricornutum*). In the cladoceran crustacean, *Daphnia magna* (water flea), a 21-day chronic exposure to HBCD indicated a NOEC

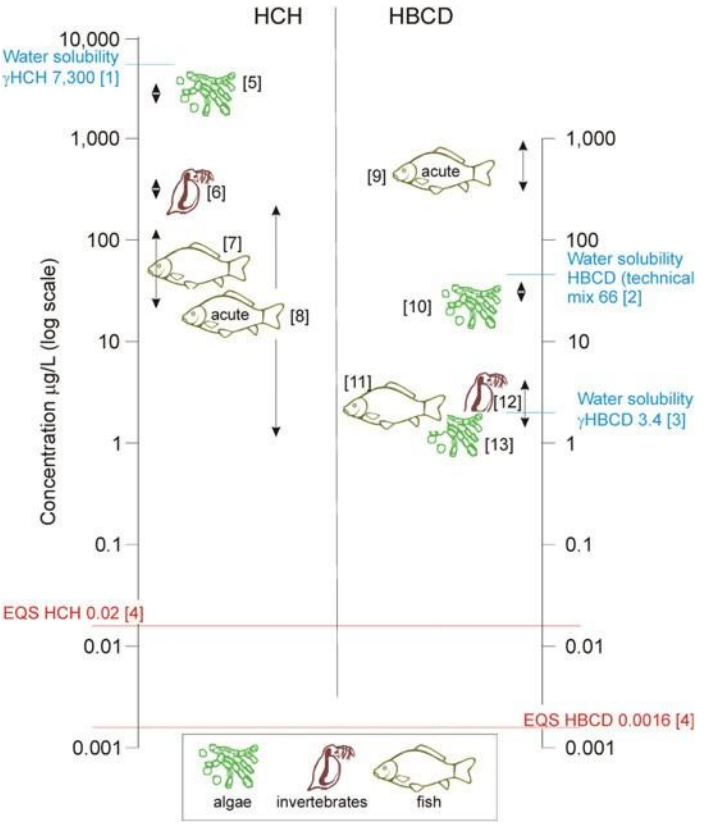


FIGURE 2. Graphical representation of the toxicity of HCH and HBCD in relation to their reported solubility and proposed (annual average) EQS. [1] Stenzel and Markley (1997); [2] HSDB (2009); [3] UNEP (2010b); [4] EC (2012); [5] Schafer et al. (1994); [6] Ferrando et al. (1995); [7] Gorge and Nagel (1990); [8] UNEP (2006); [9] Deng et al. (2009); [10] Desjardins et al. (2005); [11] Drottar et al. (2001); [12] Drottar and Krueger (1998); [13] Roberts and Swigert (1997).

(no observed effect concentration) of 3.1 μg/l and a LOEC (lowest observed effect concentration) of 5.6 μg/l based on significant reduction in growth (Drottar and Krueger, 1998). Thyroid hormone-dependent development effects in tadpoles of *Xenopus laevis* (Schriks et al., 2006) and significant adverse changes in the levels and patterns of circulating thyroid hormones in *Salmo salar* (Atlantic salmon) (Lower and Moore, 2007) and *O. mykiss* (Palace et al., 2010) exposed to HBCD have been observed. HBCD has also been reported to cause malformation and reduction of the survival of embryos of zebrafish, *Danio rerio* at 96 hr exposure to concentrations of 0.5 and 1.0 mg/l (Deng et al., 2009). In the earthworm, *Eisenia fetida*, NOEC for survival and reproduction estimated as 4,190 and 128 mg HBCD/kg dry soil, respectively, have been observed following 56 days exposure (UNEP, 2010b). HBCD has also been evaluated for phytotoxicity in the terrestrial

ecosystem. At NOEC >5,000 mg HBCD/kg dry soil, there was no adverse effect on seedling emergence in *Zea mays* (corn), *Cucumber sativa* (cucumber), *Lycopersicon esculentum* (tomato), and *Glycine max* (soybean) (UNEP, 2010b). Overall, the data on ecotoxicity for HBCD indicate a risk to the environment at lower concentrations (10 to 100 times less) than posed by HCH, which is reflected in the proposed environmental quality standards (EQS) for these compounds (Figure 2).

5. LONG-RANGE ENVIRONMENTAL TRANSPORT

There is evidence of long-range environmental transport of HCH dating several decades. Several studies have reported the transport of HCH over long distances in the environment by air and ocean currents (Li and Macdonald, 2005; Shen et al., 2005; Brun et al., 2008). It is estimated that 12–30% of lindane used in agriculture volatilizes and becomes airborne for long-range transport (USEPA, 2006). In the atmosphere, HCH condenses and deposits on oceans and freshwaters, and tends to accumulate in colder climates, particularly the Arctic where it is trapped by low evaporation rates (CEC, 2006). Far from important pollution sources, the Arctic is a recipient of HCH emitted from other parts of the world. In the Arctic, HCH has been detected in environmental media such as air (Li and Bidleman, 2003) and water (Li and Macdonald, 2005), and biota (Willett et al., 1998; Hoekstra et al., 2002).

HBCD has the potential for long-range environmental transport and trans-boundary threat like HCH. Arnot et al. (2009) observed that HBCD portioning behavior in the atmosphere is such that at higher temperatures (15–35°C), there is gaseous deposition while at lower temperatures (−35–5°C), its association with particles will enhance the rate of dry deposition. Studies have indicated the occurrence of HBCD in water and sediments and biota such as fish, birds, and mammals in remote regions of the world (for example, the Arctic) considered to be far from point sources of emission as a result of atmospheric deposition (Law et al., 2006; de Wit et al., 2010; Letcher et al., 2010). Pollution of the Arctic with POPs such as HCH and HBCD is of great concern because people living in the Arctic are at high health risks due to their consumption of wildlife such as fish, birds, and mammals with considerable quantities of these chemicals (CEC, 2006; UNEP, 2010b). It is concerns about the impacts of chemicals that has led to action by regulatory bodies worldwide.

6. LESSONS LEARNT: REGULATION AND CONTROL

It is apparent that the use and subsequent release of these two chemicals to the environment has resulted in widespread contamination and significant concerns about the consequences of exposure of wildlife and humans. Although they are different chemicals, the two halogenated chemicals which have been discussed in this work are examples of compounds which, because of their toxicity and similar physicochemical characteristics. Experience of chemicals in the environment has led to an approach to prioritize

them based on such characteristics, and for regulators to focus on their PBT properties and the amount of chemicals that are in use, because impact is related to the concentration of a chemical. Perhaps the real lesson that society has learnt from the experience of using these, and similar chemicals, is that their release to the environment was in retrospect unwanted and unwise, and that tighter controls are required to prevent this occurring in future.

Regulators are now using such properties and usage patterns to prioritize chemicals for which control measures on use, or approval for use, are based. In the United Sates, the USEPA HPV Challenge Programme (USEPA, 2007) aims to make available health and environmental effects data for "chemicals produced or imported in the United States in quantities of 1 million pounds or more per year". Within Europe, the REACH system (EC, 2006) came into force in 2007 and those who manufacture or import chemicals are obliged to register information about them in a central database. The REACH regulations also allow for identification of the most hazardous chemicals and for their substitution with alternatives. From a worldwide perspective, it is also important that countries showing strong economic growth are also involved in controlling chemicals. As well as being a signatory to the Stockholm Convention, China has newly enacted regulation, described as "China REACH" (Lau et al., 2012), which is aimed at ensuring the relevant authorities are notified about new chemical substances so that risks they pose can be effectively managed. There is, therefore, evidence that regulatory bodies worldwide are taking action to manage the use of chemicals, and the benefits of sound chemical management are of international concern (UNEP, 2012).

7. CONCLUSION

It is important for society to take stock of, and learn from past experiences in order to better protect the environment and prevent or reduce adverse consequences. The PBT properties and the long-range environmental transport exhibited by both HCH and HBCD have been affirmed by international treaties, including the Stockholm Convention on POPs. It is apparent that our understanding of the fate and behavior of chemicals has led to a number of frameworks where information can be utilized in future to minimize the risks that using chemicals can pose. There are increasing regulatory controls at both national and regional levels and that highlighting the benefits of managing chemicals is being undertaken at an international level.

FUNDING

Cyril Onogbosele is grateful to the Tertiary Education Trust Fund (TET Fund), Abuja, Nigeria.

REFERENCES

Addison, R.F., Muir, D.C.G., Ikonomou, M.G., Harword, L., and Smith, T.G. (2009). Hexachlorocyclohexanes (HCH) in ringed seal (*Phoca hispida*) from Ulukhaktok (Holman), NT: Trends from 1978 to 2006. *Sci. Total Environ.* 407, 5139–5146.

Alaee, M., Arias, P., Sjodin, A., and Bergman, A. (2003). An overview of commercially used brominated flame retardants, their applications, their patterns in different countries/regions and possible modes of release. *Environ. Int.* 29, 683–689.

Alvarez-Pedrerol, M., Ribas-Fito, N., Torrent, M., Carrizo, D., Garcia-Esteban, R., Grimalt, J.O., and Sunyer, J. (2008). Thyroid disruption at birth due to prenatal exposure to β-hexachlorocyclohexane. *Environ. Int.* 34(6), 737–740.

Arnot, J., McCarty, L., Armitage, J., Toose-Reid, L., Wania, F., and Cousin, I. (2009). An evaluation of hexabromocyclododecane (HBCD) for persistent organic pollutant (POP) properties and the potential for adverse effects in the environment. Submitted to European Brominated Flame Retardant Industry Panel, May 26, 2009.

Arnot, J.A. and Gobas, F.A.P.C. (2006). A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ. Rev.* 14, 257–297.

ATSDR. (2005). Toxicological profile for alpha-, beta-, gamma-, delta-hexachlorocyclohexane. U.S. Department of Health and Human Services, Public Health Service. Agency for Toxic Substances and Disease Registry, August 2005.

Bhatt, P., Kumar, M.S., and Chakrabarti, T. (2009). Fate and degradation of POP-hexachlorocyclohexane. *Crit. Rev. Environ. Sci. Technol.* 39, 655–695.

Birnbaum, L.S. and Staskal, D.F. (2004). Brominated flame retardants: Cause for concern? *Environ. Health Perspect.* 112(1), 9–17.

Breivik, K., Pacyna, J.M., and Munch, J. (1999). Use of α-, β- and γ-hexachlorocyclohexane in Europe, 1970–1996. *Sci. Total Environ.* 239, 151–163.

Brun, G.L., MacDonald, R.M., Verge, J., and Aube, J. (2008). Long-term atmospheric deposition of current-use and banned pesticides in Atlantic Canada: 1980–2000. *Chemosphere* 71, 314–327.

BSEF. (2009). *HBCD (hexabromocyclododecane)*. Fact Sheet, June 2009 edition. Bromine Science and Environmental Forum.

CEC. (2006). The North American Regional Action Plan (NARAP) on lindane and other hexachlorocyclohexane (HCH) isomers. Commission for Environmental Cooperation, 30 November 2006.

Chen, Z.M., Zabik, M.J., and Leavitt, R.A. (1984). Comparative study of thin film photodegradative rates for 36 pesticide. *Ind. Eng. Chem. Prod. Res. Dev.* 23, 5–11.

Covaci, A., Gerecke, A.C., law, R.J., Voorspoels, S., Kohler, M., Heeb, N.V., Leslie, H., Allchin, C.R., and De Boer, J. (2006). Hexabromocyclododecanes (HBCDs) in the environment and humans: A review. *Environ. Sci. Technol.* 40, 3679–3688.

Darnerud, P.O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environ. Int.* 29, 841–853.

Davis, J.W., Gonsior, S., Marty, G., and Ariano, J. (2005). The transformation of hexabromocyclododecane in aerobic and anaerobic soils and aquatic sediments. *Water Res.* 39, 1075–1084.

de Wit, C.A. (2002). An overview of brominated flame retardants in the environment.

Chemosphere 46, 583–624.

de Wit, C.A., Herzke, D., and Vorkamp, K. (2010). Brominated flame retardants in the Arctic environment – Trends and new candidates. *Sci. Total Environ.* 408(15), 2885–2918.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W.Y., Lam, P.K.S., Wu, R.S.S., and Zhou, B. (2009). Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. *Aquat. Toxicol.* 93, 29–36.

Desjardins, D., MacGregor, J.A., and Krueger, H.O. (2005). Hexabromocyclododecane (HBCD): A 72-hour toxicity test with the marine diatom (*Skeletonema costatum*) using generator column saturated media. *Chapter 2, Final report*. Wildlife International Ltd., Easton, Maryland, USA. 19pp.

Doelman, P., Haanstra, L., Loonen, H., and Vos, A. (1990). Decomposition of alpha- and beta-hexachlorocyclohexane in soil under field conditions in a temperate climate. *Soil Biol. Biochem.* 22(5), 629–634.

Drottar, K.R., and Krueger, H.O. (1998). Hexabromocyclododecane (HBCD): A flow-through life-cycle toxicity test with the cladoceran (*Daphnia magna*). Final report. *439a-108*. Wildlife International Ltd., Easton, Maryland, USA. p78.

Drottar, K.R., and MacGregor, J.A., and Krueger, H.O. (2001). Hexabromocyclododecane (HBCD): An early life-stage toxicity test with rainbow trout (*Oncorhynchus mykiss*). Final Report. Wildlife International Ltd., Easton, Maryland, USA. pp. 1–102.

EC. (2006). "Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation. European Commission, *Off. J. Eur. Commun.* L395/17 (2006).

EC. (2008). Risk assessment: Hexabromocyclododecane, CAS-NO: 25637-99-4, EINECS NO: 247-148-4, final report. European Commission, May 2008. 492pp.

EC. (2012). Proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy Brussels, 31.1.2012 COM(2011) 876 final 2011/0429 (COD).

ECHA. (2008). Member State Committee support document for identification of hexabromocyclododecane and all major diastereoisomers identified as a substance of very high concern. European Chemicals Agency, 8 October 2008.

ECHA. (2009). Data on the manufacture, import, export, uses and releases of HBCDD as well as information on potential alternatives to its use. European Chemicals Agency, 1 December 2009.

Environment Canada. (2011). Screening assessment report on hexabromocyclododecane. Chemical Abstracts Service Registry Number 3194-55-6. Environment Canada, Health Canada, November 2011.

Eriksson, P., Fischer, C., Wallin, M., Jakobsson, E., and Frederisson, A. (2006). Impaired behaviour, learning and memory in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). *Environ. Toxicol. Pharmacol.* 21, 317–322.

Ferrando, M.D., Sancho, E., and Andreumoliner, E. (1995). Effects of lindane on *Daphnia magna* during chronic exposure. *J. Environ. Sci. Health Part B* 30(6), 815–825.

Germer, S., Pierma, A.H., van der Ven, L., Kamyschnikow, A., Fery, Y., Schmitz, H., and Schrenk, D. (2006). Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. *Toxicology* 218, 229–236.

Gorge, G., and Nagel, R. (1990). Toxicity of lindane, atrazine and deltamethrin to early life stages of zebrafish (*Brachydanio rerio*). *Ecotoxicol. Environ. Saf.* 20,

246–255.

Harner, T., Kylin, H., Bidleman, T.F., and Strachan, W.M.J. (1999). Removal of alpha- and gamma-hexachlorocyclohexane and enantiomers of alpha-hexachlorocyclohexane in the Eastern Arctic Ocean. *Environ. Sci. Technol.* 33(88), 1157–1164.

Heeb, N.V., Schweizer, W.B., Kohler, M., and Gerecke, A.C. (2005). Structure elucidation of hexabromocyclododecanes – A class of compounds with a complex stereochemistry. *Chemosphere* 61, 65–73

Heeb, N.V., Schweizer, W.B., Mattrel, P., Haag, R., Gerecke, A.C., Schmid, P., Zennegg, M., and Vonmont, H. (2008). Regio- and stereoselective isomerisation of hexabromocyclododecanes (HBCDs): Kinetics and mechanism of γ - to α-HBCD isomerisation. *Chemosphere*, 73, 1201–1210

Helm, P.A., Diamond, M.L., Semkin, R., Strachan, W.M.J., Teixeira, C., and Gregor, D. (2002). A mass balance model describing multiyear fate of organochlorine compounds in a high Arctic lake. *Environ. Sci. Technol.* 36(5), 996–1003.

Hoekstra, P.F., O'Hara, T.M., Pallant, S.J., Solomon, K.R., and Muir, D.C.G. (2002). Bioaccumulation of organochlorine contaminants in bowhead whales (*Balaena mysticetus)* from Barrow Alaska. *Arch. Environ. Contam. Toxicol.* 42, 497–507.

HSDB. (2009). Hazardous Substances Data Bank. National Library of Medicine. Available: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB and search on "Lindane". Last accessed on: 14/02/13.

Hu, W., Wang, T., Khim, J.S., Luo, W., Jiao, W., Lu, Y., Naile, J.E., Chen, C., Zhang, X., and Giesy, J.P. (2010). HCH and DDT in sediments from marine and adjacent riverine areas of North Bohai Sea, China. *Arch. Environ. Contam. Toxicol.* 59, 71–79.

Hunziker, R.W., Gonsior, S., MacGregor, J.A., Desjardins, D., Ariano, J., and Friederich, U. (2004). Fate and effect of hexabromocyclododecane in the environment. *Organohalogen Compd.* 66, 2300–2305.

Ibhazehiebo, K., Iwasaki, T., Shimokawa, N., and Koibuchi, N. (2011). 1,2,5,6,9,10-α hexabromocyclododecane (HBCD) impairs thyroid hormone-induced dendrite arborization of Purkinje cells and suppresses thyroid hormone receptor-mediated transcription. *Cerebellum*, 10, 22–31.

IHPA. (2006). The legacy of lindane HCH isomer production: A global overview of residue management, formulation and disposal. International HCH and Pesticides Association. Available: www.ihpa.info (Accessed April 29, 2014).

IPCS. (1991). *Lindane*. Environmental Health Criteria 124. World Health Organization, Geneva. International Programme on Chemical Safety.

IPCS. (1992). *Alpha- and beta-hexachlorocyclohexanes*. Environmental Health Criteria 123. World Health Organization, Geneva. International Programme on Chemical Safety. Available: http://www.pic.int/Portals/5/AIII-Info/HCH/EHC%

20123%20-%20alpha%20and%20beta%20hexachlorocyclohexanes.rtf (Accessed April 29, 2014).

Janak, K., Covaci, A., Voorspoels, S., and Becher, G. (2005). Hexabromocyclododecane in marine species from the Western Scheldt Estuary: Diastereoisomer- and enantiomer-specific accumulation. *Environ. Sci. Technol.* 39, 1987–1994.

Khan, F.H., Ganesan, P., and Kumar, S. (2010). Y chromosome microdeletion and altered sperm quality in human males with high concentration of seminal hexachlorocyclohexane (HCH). *Chemosphere*, 80, 972–977.

Lau, M.H.Y., Leung, K.M.Y., YuWong, S.W., Wang, H., and Yan, Z-G. (2012). Environmental policy, legislation and management of persistent organic pollutants (POPs) in China. *Environ. Pollut.* 165, 182–192.

Law, K., Palace, V.P., Halldorson, T., Danell, R., Wautier, K., Evans, B., Brinkworth, L., Alaee, M., and Tomy, G.T. (2004). Dietary accumulation of hexabromocyclododecane isomers in juvenile rainbow trout (*Oncorhynchus mykiss*). *The Third International Workshop on Brominated Flame Retardants*, Toronto, 2004. Book of Abstracts, 2004, pp. 433–436.

Law, R.J., Alaee, M., Allchin, C.R., Boon, J.P., Lebeuf, M., Lepom, P., and Stern, G.A. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environ. Int.* 29, 757–770.

Law, R.J., Allchin, C.R., de Boer, J., Covaci, A., Herzke, D., Lepom, P., Morris, S., Tronczynsk, J., and de Wit, C.A. (2006). Levels and trends of brominated flame retardants in the European environment. *Chemosphere*, 64, 187–208.

Law, R.J., Kohler, M., Heeb, N.V., Gerecke, A.C., Schmid, P., Voorspoels, S., Covaci, A., Becher, G., Janak, K., and Thomson, C. (2005). Hexabromocyclododecane challenges scientists and regulators. *Environ. Sci. Technol.* 39, 281A–287A.

Legler, J. (2008). New insights into the endocrine disrupting effects of brominated flame retardants. *Chemosphere*, 73, 216–222.

Letcher, R.J., Bustines, J.O., Dietz, R., Jenssen, B.M., Jorgensen, E.H., Sonne, C., Verreault, J., Vijayanm, M., and Gabrielsen, G.W. (2010). Exposure and effects assessment of persistent organohalogen contaminants in Arctic wildlife and fish. *Sci. Total Environ.* 408(15), 2995–3045.

Li, Y.F. (1999). Global technical hexachlorocyclohexane usage and its contamination consequences in the environment: From 1948–1997. *Sci. Total Environ.* 232, 121–128.

Li, Y.F., and Bidleman, T.F. (2003). Correlation between global emissions of α-hexachlorocyclohexane and its concentration in the Arctic air. *J. Environ. Inform.* 1, 52–57.

Li, Y.F., and Macdonald, R.W. (2005). Sources and pathways of selected organochlorine pesticides to the Arctic and the effect of pathway divergence on HCH trends in biota: A review. *Sci. Total Environ.* 342, 87–106.

Lower, N. and Moore, A. (2007). The impact of a brominated flame retardant on smoltification and olfactory function in Atlantic salmon (*Salmo salar* L.) smolts. *Mar. Freshw. Behav. Phy.* 40(4), 267–284.

Mackay, D., Shiu, W.Y., and Ma, K.C. (1997). *Illustrated Handbook of Physical-chemical Properties and Environmental Fate for Organic Chemicals.* CRC Press: Boca Raton, FL

Marvin, C.H., Tomy, G.T., Armitage, J.M., Arnot, J.A., McCarty, L., Covaci, A., and Palace, V. (2011). Hexabromocyclododecane: Current understanding of chemistry, environmental fate and toxicology and implications for global management. *Environ. Sci. Technol.* 45, 8613–8623.

Mathur, V., Bhatnagar, P., Sharma, R.G., Acharya, V., and Sexana, R. (2002). Breast cancer incidence and exposure to pesticides among women originating from Jaipur. *Environ. Int.* 28(5), 331–336.

Ngabe, B., Bidleman, T.F., and Falconer, R.L. (1993). Base hydrolysis of alpha- and gamma-hexachlorocyclohexanes. *Environ. Sci. Technol.* 27, 1930–1933.

Oliver, B.G., and Niimi, A.J. (1985). Bioconcentration factors for some halogenated organics for rainbow trout: Limitations in their use for prediction of environmental residues. *Environ. Sci. Technol.* 19(9), 842–849.

Palace, V., Park, B., Pleskach, K., Gemmill, B., and Tomy, G. (2010). Altered thyroxine metabolism in rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane (HBCD). *Chemosphere*, 80, 165–169.

Pereira, R.C., Monterroso, C., and Macias, F. (2010). Phytotoxicity of hexachlorocyclohexane: Effect on germination and early growth in different plant species. *Chemosphere*, 79, 326–333.

Reistad, T., Fonnum, F., and Mariussen, E. (2006). Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells in vitro. *Arch. Toxicol.* 80, 785–796.

Roberts, C.A. and Swigert, J.P. (1997). Hexabromocyclododecane (HBCD): A 96-hour toxicity test with the freshwater alga (*Selenastrum capricornutum*). Final report, 439A-103. Wildlife International Ltd., Easton, Maryland, USA.

Schafer, H., Hettler, H., Fritsche, G., Pitzen, G., Roderer, G., and Wenzel, A. (1994). Biotests using unicellular algae and ciliates for predicting long-term effects of toxicants. *Ecotoxicol. Environ. Saf.* 27, 64–81.

Schecter, A., Szabo, D.T., Miller, J., Gent, T.L., Malik-Bass, N., Petersen, M., Paepke, O., Colacino, J.A., Hynan, L.S., Harris, T.R., Malla, S., and Birnbaum, L.S. (2012). Hexabromocyclododecane (HBCD) stereoisomers in U.S. Food from Dallas, Texas. *Environ. Health Perspect.* 120(9), 1260–1264.

Schriks, M., Zninavashe, E., Furlow, J.D., and Mark, A.J. (2006). Disruption of thyroid hormone-mediated *Xenopus laevis* tadpole tail tip regression by hexabromocyclododecane (HBCD) and 2,2 ,3,3 ,4,4 ,5,5 ,6-nonabrominated diphenyl ether (BDE206). *Chemosphere*, 65, 1904–1908.

Shen, L., Wania, F., Lei, Y.D., Teixeira, C., Muir, D.C.G., and Bidleman, T.F. (2005). Atmospheric distribution and long-range transport behaviour of organochlorine pesticides in North America. *Environ. Sci. Technol.* 39(2), 409–420.

Singh, G., Kathpal, T.S., Spencer, W.F., and Dhankar, J.S. (1991). Dissipation of some organochlorine insecticides in cropped and uncropped soil. *Environ. Pollut.* 70(3), 1219–1239.

Singh, P.B. and Canario, A.V.M. (2004). Reproductive endocrine disruption in the freshwater catfish, *Heteropneustes fossilis*, in response to the pesticide γ-hexachlorocyclohexane. *Ecotoxicol. Environ. Saf.* 58, 77–83.

Slooff, W., and Mathijsen, A.C.J.M. eds. (1988). Integrated criteria documents: Hexachlorocyclohexanes. Bilthoven National Institute of Public Health and Environmental Protection (RIVM Report No. 758473011).

Solomon, G.M., and Weiss, P.M. (2002). Chemical contaminants in breast milk: Time trends and regional variability. *Environ. Health Perspect.* 110, A339–A347.

Stenzel, J., and Markley, B. (1997). Hexabromocyclododecane (HBCD): Determination of the water solubility. Project Number: 439C-105. Wildlife International Ltd., Easton, MD.

Swedish Chemicals Agency. (2008). Hexabromocyclododecane CAS-NO: 25637-99-45 EINECS-NO: 247-148-4. *Summary risk assessment report. Final report*. 2008, Sweden.

Tiemann, U. (2008). *In vivo* and *in vitro* effects of the organochlorine pesticide DDT, TCPM, methoxychlor and lindane on the female reproductive tract of mammals: A review. *Reprod. Toxicol.* 25, 316–326.

Tomy, G.T., Budakowski, W., Halldorson, T., Whittle, D.M., Keir M.J., Marvin, C., Macinnis, G., and Alaee, M. (2004). Biomagnification of α- and γ-hexabromocyclododecane isomers in Lake Ontario food web. *Environ. Sci. Technol.* 38, 2298–2303.

UNEP. (2001). Final act of the Conference of Plenipotentiaries on the Stockholm Convention on persistent organic pollutants. UNEP/POPS/CONF/4. Stockholm, 22–23 May 2001. United Nations Environment Programme.

UNEP. (2006). Report of the Persistent Organic Pollutants Review Committee on the work of its second meeting. Addendum. Risk profile on lindane. UNEP/POPS/POPRC.2/17/Add.4. 21 November 2006. United Nations Environment Programme.

UNEP. (2007a). Persistent Organic Pollutants Review Committee third meeting. Item 7 of the provisional agenda: Presentation on environmental transport and modelling. The OECD screening tool for overall persistence and long-range transport potential. UNEP/POPS/POPRC.3/INF/7. Geneva, 19–23 November 2007. United Nations Environment Programme.

UNEP. (2007b). Stockholm Convention on Persistent Organic Pollutants: Persistent Organic Pollutants Review Committee third meeting. Additional information related to assessment of bioaccumulation data under Annex D of the Convention. UNEP/POPS/POPRC.3/INF/8. Geneva, 19–23 November 2007. United Nations Environment Programme.

UNEP. (2009). Report of the Conference of the Parties of the Stockholm Convention on Persistent Organic Pollutants on the work of its fourth meeting. UNEP/POPS/COP.4/38. Geneva, 4–8 May 2009. United Nations Environment Programme.

UNEP. (2010a). Report of the Persistent Organic Pollutants Review Committee on the work of its sixth meeting. UNEP/POPS/POPRC.6/13. Geneva, 11–15 October 2010. United Nations Environment Programme.

UNEP. (2010b). Persistent Organic Pollutants Review Committee sixth meeting. Draft risk profile: Hexabromocyclododecane. UNEP/POPS/POPRC.6/10. Geneva, 11–15 October 2010. United Nations Environment Programme.

UNEP. (2011). Persistent Organic Pollutants Review Committee seventh meeting. Draft risk management evaluation on hexabromocyclododecane. UNEP/POPS/POPRC.7/5. Geneva, 10–14 October 2011. United Nations Environment Programme.

UNEP. (2012). Global Chemicals Outlook.
USEPA. (2006). Addendum to the 2002 Lindane Reregistration Eligibility Decision (RED). United States Environmental Protection Agency, July 2006. Available: http://www.epa.gov/oppsrrd1/REDs/lindane_red_addendum. pdf (Accessed April 29, 2014).
USEPA. (2010). Hexabromocyclododecane (HBCD) Action Plan. United States Envi- ronmental Protection Agency, August 2010.
USEPA. (2007). High Production Volume (HPV) Challenge Program. Available: http://www.epa.gov/hpv/index.htm
Veith, G.D., DeFoe, D.L., and Bergstedt, B.V. (1979). Measuring and estimating the bioconcentration factors of chemicals in fish. *J. Fish Res. Board Can.* 36, 1040–1048.
Vijgen, J., Abhilash, P.C., Li, Y.F., Lal, R., Forter, M., Torres, J., Singh, N., Yunus, M., Tian, C., Schaffer, A., and Weber, R. (2011). Hexachlorocylohexane as new Stockholm Convention POPs – A global perspective on the management of lindane and its waste isomers. *Environ. Sci. Pollut. Res.* 18, 152–162.
Walsh, G.E., Yoder, M.J., McLaughlin, L.L., and Lores, E.M. (1987). Responses of marine unicellular algae to brominated organic compounds in six growth media. *Ecotoxicol. Environ. Saf.* 14, 215–222.
WHO. (1991). International Programme on Chemical Safety. Health and Safety Guide No.54. Lindane (gamma-HCH) health and safety guide. *United Nations Environ- ment Programme.* International Labour Organization. World Health Organiza- tion. Geneva, 1991.
WHO Europe. (2003). Health risks of persistent organic pollutants from long- range transboundary air pollution. Joint WHO/Convention Task Force on the Health Aspects of Air Pollution. Chapter 3. Hexachlorocyclohexanes. Avail- able: http://www.unece.org/fileadmin/DAM/env/documents/2002/eb/wg1/eb. air.wg.1.2002.14.e.pdf (Accessed April 29, 2014).
Willett, K.L., Ulrich, E.M., and Hites, R.A. (1998). Differential toxicity and envi- ronmental fates of hexachlorocyclohexane isomers. *Environ. Sci. Technol.* 32, 2197–2207.
Wu, J., Guan, Y., Zhang, Y., Luo, X., Zhi, H., Chen, S., and Mai, B. (2011). Several current-use, non-PBDE brominated flame retardants are highly bioaccumula- tive: Evidence from field determined bioaccumulation factors. *Environ. Int.* 37, 210–215.
WWFC. (1999). Lindane: A review of toxicity and environmental fate. World Wildlife Fund Canada, November 1999.
Zhao, Z., Zhang, L., Wu, J., and Fan, C. (2009). Distribution and bioaccumulation of organochlorine pesticides in surface sediments and benthic organisms from Taihu Lake, China. *Chemosphere*, 77, 1191–1198.
Zou, E., and Matsumura, F. (2003). Long-term exposures to β-hexachlorocyclo- hexane (β-HCH) promotes transformation and invasiveness of MCF-7 human breast cancer cells. *Biochem. Pharmacol.* 66, 831–840.