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An integrated assessment of estrogenic endocrine disruption in the Irish marine environment, with particular focus on chemical measurements

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Jennifer Ronan

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Supervisors:

Dr. Brendan McHugh

Professor James G. Wilson

Co-supervisor

Dr. Michelle Giltrap

Department of Zoology

School of Natural Sciences

University of Dublin

Trinity College

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work and includes the work of partners in the Seachange project who have been duly cited and acknowledged where relevant within the text of this thesis.

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Summary

Scientific awareness of the presence and effects of endocrine disrupting compounds (EDCs) has increased in recent decades. Many gaps in the knowledge still exist, particularly in relation to concentrations and potential for effects in marine environments. Recent legislation has led to the development of environmental quality standards (EQS) for many EDCs, some of which are extremely low concentrations which are difficult to attain with traditional analytical methods. Challenges also exist in extrapolating the potential for effects in exposed organisms due to concentrations detected in the water phase. This research forms part of a larger collaborative four year project entitled 'Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters' (SeaChange).

Two highly sensitive and selective liquid chromatography tandem mass spectrometry (LC-MS/MS) methods were developed and validated for the detection of steroid estrogens estrone (E1), 17 β -estradiol (E2) and 17 α -ethynyl estradiol (EE2) in seawater and biota and 4-n-nonylphenol (4-NP) and 4-*tert*-octylphenol (4-*t*-OP) in biota. Data from the polar organic chemical integrative sampler (POCIS), the biological effect alkali-labile phosphate (ALP) and estrogen luciferase reporter gene (ER-LUC) assays which were completed externally to this thesis are incorporated to support an assessment of EDC effects.

A study examining the effects of EE2 in *Mytilus* spp. *in vivo* was completed using both a sub-tidal and a pilot scale simulated intertidal regime. A significant increase in ALP response was noted in mussels which underwent EE2 exposure in the simulated intertidal regime ($p < 0.05$). The concentration of EE2 increased from <LOD (limit of detection, 0.11 ng/g wet weight) to 0.77 ± 0.3 ng/g ww in mussel tissue in both exposure regimes after seven days exposure.

Two separate approaches combining chemical and biological effects measurements were used to make an assessment of EDCs and endocrine disruption (ED) in selected study sites. Firstly, a three month field based caging study using transplanted *Mytilus* spp. was conducted. Secondly, a two tiered spatial and temporal study of EDC levels was conducted initially focussing on levels in native shellfish, and secondly, on levels in three species of marine flatfish and in passive samplers.

Results from this multi parameter study indicate that the presence and effects of EDCs at the selected sites are generally low, however combined chemical and biological effects measurements identified Dublin Bay as being at potential risk, thus further monitoring of steroid and EDC levels in water and ED effects in resident species is recommended. The steroids E1 and E2 were detected in Dublin Bay water samples above the LOD (0.07 ng/L). EE2 was not detected at any site (LOD 0.11 ng/L). E1 and E2 were not detected in water from any other site. Selected EDCs were not detected above the LOD in biota (0.4, 0.9, 0.3, 4.0 and 6.6 ng/g wet weight for E1, E2, EE2, NP and OP respectively). POCIS acted as a valuable screening tool with maximum E1 and E2 concentrations of 15.3 and 3.9 ng/device detected in Galway Bay.

The combination of passive sampling, chemical analysis and biological effects measurements provides a detailed in depth assessment of the current status of the studied sites, and has highlighted future monitoring and research needs. Information derived from this integrated approach is of value to researchers and environmental managers worldwide, and highlights strengths and limitations of the approaches used. The research is also valuable in terms of derivation of future “criteria” to support future broader ecosystem based assessments such as under the Marine Strategy Framework Directive.

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Abbreviations

4-NP	4-n-Nonylphenol
4- <i>t</i> -OP	4- <i>tert</i> -octylphenol
AC	Assessment Criteria
ACN	Acetonitrile
ALP	Alkali Labile Phosphate Assay
API	Atmospheric Photo-Ionisation
BAC	Background Assessment Criteria
BC	Background Concentration
BCF	Bioconcentration Factor
BPA	Bisphenol A
BQE	Biological Quality Elements
CEMP	Coordinated Environmental Monitoring Programme
CF	Condition Factor
DI	Deionised
DIT	Dublin Institute of Technology
DO	Dissolved Oxygen
DW	Dry Weight
E1	Estrone
E2	17 β -estradiol
EAC	Environmental Assessment Criteria
ED	Endocrine Disrupting
EDC	Endocrine Disrupting Compound
EE2	17 α -ethynyl estradiol
EEQ	Estradiol Equivalents
EPA	Environmental Protection Agency
ER	Estrogen Receptor
ER-LUC	Estrogen Luciferase Reporter Gene Assay

EQS	Environmental Quality Standard
ESI	Electrospray Ionisation
EU	European Union
GAC	Granular Activated Carbon
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GC-MS/MS	Gas Chromatography Tandem Mass Spectrometry
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
ICES	International Council for the Exploration of the Sea
INT	Intertidal
JAMP	Joint Assessment and Monitoring Guidelines
K _{ow}	Octanol Water Partitioning Coefficient
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LC-MS/TOF	Liquid Chromatography Time of Flight Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAC	Maximum Allowable Concentration
MSFD	Marine Strategy Framework Directive
MI	Marine Institute
MS	Mass Spectrometry
NP	Nonylphenol
OSPAR	Oslo and Paris Commission
OP	Octylphenol
pKa	Acid ionization constant
PM	Particulate Matter
POCIS	Polar Organic Chemical Integrative Sampler

PS	Passive Sampling
QC	Quality Control
QS	Quality Standard
RIA	Radioimmunoassay
RLU	Relative Light Unit
RSD	Relative Standard Deviation
SATL	Shannon Aquatic Toxicity Laboratory
SIRBD	Shannon International River Basin District
SPE	Solid Phase Extraction
SMPD	Semi Permeable Membrane Device
SUB	Sub-tidal
SWD	Shellfish Waters Directive
TCD	Trinity College Dublin
UK	United Kingdom
UPT	Uptake
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
V _n	Vitellin
VTG	Vitellogenin
WFD	Water Framework Directive
WGBEC	ICES Working Group on the Biological Effects of Contaminants
WKIMON	ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their effects in Coastal and Open Sea Areas
WHO	World Health Organisation
WOE	Weight of Evidence
WP	Work Package
WW	Wet weight
WWTP	Wastewater Treatment Plant
WWTPE	Wastewater Treatment Plant Effluent

YES

Yeast Estrogen Screen

Scope of thesis

Contaminants of both natural and anthropogenic origin may enter the marine environment via a number of sources, including municipal and industrial waste water treatment plant effluent (WWTPE), agricultural and terrestrial run off, and accidental release. There is increasing concern as to the fate and effects of some of these compounds, for example hormones, pharmaceuticals, pesticides, herbicides and industrial products. Many of these have been shown to affect reproduction, growth, development, and immune system response in exposed organisms, including humans, and they have thus been termed endocrine disrupting compounds (EDCs). They may be natural or synthetic in origin. The increasing body of scientific data which has demonstrated the negative effects of EDCs in the environment has led to the development of monitoring programmes and legislation which aim to reduce the amount of EDCs present in the environment. The Water Framework Directive (WFD, 2000/60/EC) identifies a list of priority and relevant pollutants. There is a requirement for substantial monitoring of these pollutants in transitional and coastal waters to achieve “good ecological and chemical status” by 2015. A number of the pollutants on this list have endocrine disrupting (ED) properties. Irish coastal and transitional waters have been classified for WFD monitoring purposes with respect to certain priority and relevant pollutants; however they are yet to be monitored for a number of these EDCs.

The isolated generation of chemical data is generally accepted as being insufficient to thoroughly assess pollution impacts within the environment. An integrated approach, using chemical analysis and biological effects monitoring has been recommended within the WFD, the Oslo and Paris Convention (OSPAR), the International Council for the Exploration of the Sea (ICES)/OSPAR Workshop on Integrated Monitoring of Contaminants and their effects in Coastal and Open Sea Areas (WKIMON), and the ICES Working Group on the Biological Effects of Contaminants (WGBEC). Research to date has primarily focused on freshwater environments. Much less is known as to the presence and effects of these compounds in marine environments, thus there is a substantial need for research in this area.

The aim of this thesis is to apply a range of techniques including ‘traditional’ chemical analysis methods, biological effects monitoring and more recently developed passive

sampling technologies to the assessment of EDCs in the marine environment. The techniques have been applied to the analysis of both transplanted and native shellfish, to native fish, water and sediment from a range of both impacted and non-impacted Irish coastal locations, generating valuable baseline data to fill gaps both nationally and internationally.

This research forms part of a larger collaborative four year project entitled '*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*' (SeaChange). Partners in the project include Trinity College Dublin, the Marine Institute, Shannon Aquatic Toxicity Laboratory (SATL), and the Dublin Institute of Technology (DIT). The aim of this thesis within the SeaChange project was selecting target analytes with respect to EDCs, developing suitable methods and applying the techniques to conduct an assessment of EDCs in Irish marine waters, and investigating the effects of these compounds *in vivo*.

Chapter 1 reviews the current knowledge of EDCs with respect to chemical assessment methodologies, biological effects techniques, sampling techniques, legislative status, detection in marine matrices and contaminant induced biological effects. Target analytes with respect to EDC monitoring are selected based on their documented estrogenic effects, estrogenic potency, and relevance within the legislative context of the WFD. The sources, properties and endocrine effects of these compounds are discussed, along with the current knowledge of their presence and effects within the environment and within Irish waters. Monitoring strategies and the value of an integrated approach are discussed. Suitable marine species for biomonitoring are reviewed and the range of available techniques is reported.

The methods selected for use in this research are detailed in **Chapter 2**. Sensitive and selective liquid chromatography tandem mass spectrometry (LC-MS/MS) methods capable of the detection of selected EDCs at environmentally and ecotoxicologically relevant levels which have been developed within this research are described. These methods target specific analytes with potent estrogenic activity. Validation of methodology is discussed including the following parameters: accuracy, precision, linear range, repeatability, reproducibility and calculation of limits of detection/quantification. The methods and analyses completed under subcontract and by partners in the SeaChange project are detailed. These are biological effects techniques measuring overall estrogenic activity and

estrogenic effects utilised in order to account for the presence and effects of unknown estrogenic compounds and the Polar Organic Chemical Integrative Sampler (POCIS) passive sampling device. A combination of these techniques is applied within **Chapters 3 to 5**, to investigate the effects of selected EDCs *in vivo* and to monitor the presence and effects at a number of sites in the Irish marine environment.

Considerable research has been conducted on the effects of EDCs in vertebrate species. Much less is known as to their effects in invertebrates. The invertebrate *Mytilus* spp. is widespread in Europe, and is used in the monitoring programmes of Oslo and Paris Commission (OSPAR) and in the Shellfish Waters Directive 2006/113/EC (SWD) to assess the status and trends of chemical contamination in estuarine and coastal environments. A seven day *in-vivo* study examining the effects of 17 α -ethynyl estradiol (EE2) in *Mytilus* spp. is presented in **Chapter 3**. Chemical analysis of water and tissue was completed to assess uptake and bioconcentration of EE2, while the biological effects alkali labile phosphate (ALP) assay conducted by partners in the SeaChange project was used to monitor the ED effects of EE2. Toxicity and biological effects criteria have been derived using available contaminant effect data, much of which has come from laboratory studies conducted in a ‘fully submerged’ setting. A pilot ‘intertidal’ regime was applied within this study. This study served as a positive control for further investigations into the presence and effects of selected EDCs in the environment conducted in **Chapters 4 and 5**.

A multi-parameter integrated approach was applied to investigate the presence and effects of selected EDCs using caged *Mytilus* spp. at three coastal locations with varying degrees of anthropogenic input (**Chapter 4**). Targeted analysis was completed using LC-MS/MS and passive sampling, while ALP and the estrogen luciferase reported gene assay (ER-LUC) monitored overall estrogenic effects at each site. Test organisms were taken from a well characterised site, in order that biological effects responses observed after exposure could be better interpreted. Variations in abiotic factors which can also influence biomarker response were accounted for. The application of an integrated approach and the value of using caging of transplanted organisms are discussed.

A detailed assessment of the data generated within this research and data generated by other researchers was conducted in **Chapter 5**. Available assessment criteria are discussed and applied where appropriate. Compliance with respect to WFD assessment criteria for 17 β -estradiol in water is discussed. Levels of EDCs in marine matrices and observed

biomarker responses are reviewed. Chemical and biological analyses are integrated for an estimation of the risk status of a number of coastal sites with respect to estrogenic ED.

A discussion of the major findings of this research is presented in **Chapter 6**, along with future recommendations. This thesis has determined the presence of a number of highly potent EDCs in Irish marine waters. It is the first study to utilise a combined chemical and biological effects approach to assess these compounds in the Irish marine environment. Information derived from this integrated approach is of valuable use to researchers worldwide, highlighting strengths and limitations of the approaches used. The combination of passive sampling, chemical analysis and biological effects measurements provides a detailed in depth assessment of the current status of the studied sites, and has highlighted future monitoring and research needs.

In summary this thesis reports the application of an integrated monitoring approach for the assessment of selected EDCs in the marine environment, resulting in the generation of valuable data with respect to selected EDCs and effects in the Irish marine environment and adding to the current understanding of EDCs in marine environments.

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Chapter 1: General Introduction

1.1 General Introduction

Over the last 50 years the scientific community has become increasingly concerned with the fate and effects of chemicals released into the environment. A wide range of contaminants have been shown to affect reproduction, growth, development, and immune system response in exposed organisms, including humans. These processes are controlled by the endocrine system, thus these compounds have been termed endocrine disrupting compounds (EDCs). They may be natural or synthetic in origin. They are released to the aquatic environment via a number of sources, including municipal and industrial waste water treatment plant effluent (WWTPE), agricultural and terrestrial run off, and accidental release.

Many EDCs which affect reproduction, growth and development are estrogenic in nature. These include the natural and synthetic estrogens, which have been shown to be highly potent, and many other anthropogenic compounds, with a wide range of structures and uses. The scientific community has demonstrated the effects of these compounds in laboratory studies and effects in wild populations, such as feminisation of male fish downstream of waste water treatment plants (WWTP) in the UK (Purdom et al. 1994). This information has prompted the generation of EU legislation concerned with the monitoring and reduction of these compounds within the aquatic environment. The WFD identifies a list of priority and relevant pollutants. There is a requirement for substantial monitoring of these pollutants in transitional and coastal waters to achieve “good ecological and chemical status” by 2015. Environmental quality standards (EQS) have been laid down by directive 2008/150/EC in accordance with the provisions and objectives of the WFD.

It is widely accepted that chemical monitoring is no longer sufficient to assess pollution impacts in the environment. Integrating chemical analysis with biological effects monitoring has been recommended within the WFD, the Oslo Paris Commission (OSPAR), the International Council for the Exploration of the Sea (ICES)/OSPAR Workshop on Integrated Monitoring of Contaminants and their effects in Coastal and Open Sea Areas, and WGBEC, the ICES Working Group on the Biological Effects of Contaminants (Davies and Vethaak, 2012).

Research to date has primarily focused on freshwater environments. Much less is known as to the presence and effects of these compounds in marine environments, thus there is a substantial need for research in this area (Atkinson et al. 2003). Many EQS values are very

low concentrations which are difficult to measure with standard techniques, especially in marine environments where dilution is high. Passive sampling techniques are emerging as a promising tool to detect these compounds in the environment.

Considerable research has been conducted on the effects of EDCs in vertebrate species. Much less is known as to their effects in invertebrates. The invertebrate *Mytilus* spp. is widespread in Europe (Gosling, 1992) and is used in the monitoring programmes of OSPAR and in the Shellfish Waters Directive 2006/113/EC (SWD), to assess the status and trends of chemical contamination in estuarine and coastal environments. Further research on the levels and effects of EDCs in invertebrates is essential.

The objective of this chapter is to complete a comprehensive review of the current knowledge with respect to contaminant induced biological effects, current chemical methodologies, their detection in marine matrices, and the current knowledge of the presence of EDCs in marine waters. This will contain an overview of EDCs, their legislative status and environmental effects. The relevance and status of these compounds in an Irish context will also be reviewed.

1.2 The endocrine system

The endocrine system is a complex system which regulates hypothalamic and pituitary function, reproductive function, growth and development, liver function and inter-renal function within an organism. This system allows an organism to react appropriately to numerous external (environmental) and internal (physiological) signals (Ketata et al. 2008). The endocrine system involves several organs and chemical mediators acting in cascades and thus undergoing many regulatory processes. Accordingly, first, second and third order control systems are defined according to the number of endocrine glands and target tissues (Lafont, 2000; Ketata et al. 2008). Hormones or chemical mediators are specific chemicals produced by organs or tissues of the endocrine system that are transported by the blood or other bodily fluids. Specific actions of hormones may include a whole body response, a regulatory action, a morphogenic action or a permissive or complementary action (Tarrant et al. 2005).

1.2.1 Vertebrates

The vertebrate endocrine system has been extensively studied. In vertebrates, estrogens play a fundamental role in both reproduction and somatic cell function, sexual differentiation, the development of secondary sex characteristics, ovulation, the regulation of mating and breeding behaviours, and the regulation of calcium and water homeostasis (Fairbrother, 2000). Many of the currently reported actions of steroid hormones, including estrogens, androgens, and progestogens are mediated via specific receptors that are most often localised in the nucleus of target cells (Langea et al. 2012). The majority of laboratory-based studies on EDCs have focussed on the effects of estrogenic chemicals, because many of the effects seen in vertebrate wildlife are believed to have resulted from a disruption of this axis (Jobling et al. 2004). Mammals express two estrogen receptor (ER) subtypes, ER α and ER β , with ER α appearing to play a more significant role in major reproductive physiological functions in females (Krege et al. 1998). Each ER is encoded by a separate gene with unique transcriptional activities (Cheung et al. 2003), and the proteins differ significantly in their amino acid sequence, size, and ligand-binding characteristics (Pettersson and Gustafsson, 2001). Teleost fish express at least three distinct types of ER (ER α , ER β 1 and ER β 2) (Hawkins et al. 2000). Both ER isoforms are simultaneously expressed in some target tissues but in others differential expression occurs (Kuiper et al. 1997; Hawkins and Thomas, 2004), indicating that ER α and ER β may have distinct roles in the physiological responses to estrogens (Langea et al. 2012).

1.2.2 Invertebrates

Knowledge of invertebrate endocrinology has thus far been limited. In the better studied taxa (the crustacean and insecta), it seems that many of the physiological functions that are under hormonal control have no vertebrate comparison (DeFur et al. 1999). In molluscs, the endocrine system and especially the reproductive axis generally includes only neurosecretory cells and other endocrine glands such as gonads (Ketata et al. 2008). The mollusc endocrine system may be considered as the most diverse hormonal system of the invertebrate phyla. It differs among the various classes of molluscs and even among the major group of gastropods, reflecting considerable differences in morphology and life history (Ketata et al. 2008). Evidence for the presence and capabilities of metabolising steroids with various enzymes has been brought for nearly all groups of molluscs (Lafont and Mathieu, 2007). Estrogen receptor orthologs have been found in *Mytilus edulis* (Kishida et al. 2005) and *Crassostrea gigas* (Matsumoto et al. 2007). Vertebrate-like steroids have been identified in mussels (Reis-Henriques et al. 1990), though their exact modes of action, endogenous origin and physiological role remain to be elucidated (Ketata et al. 2008). Invertebrate models for assessing endocrine effects are much needed, both for developing knowledge on potential impacts of EDCs on invertebrate populations, and to determine comparative differences between vertebrate and invertebrate responses to EDCs (Jobling et al. 2004). The effects of EE2 on exposed *Mytilus* spp. is investigated during the course of this research in collaboration with partners in the SeaChange project, thus adding to the current knowledge of the effects of EDCs in invertebrates.

1.3 Endocrine disruption

Endocrine disrupting compounds, which may be natural or synthetic in nature, interfere with the hormonal system of exposed organisms in the following ways: (Sonnenschein and Soto, 1998).

- They may mimic the biological activity of a hormone by binding to a cellular receptor, leading to an unwarranted response by initiating the cell's normal response to the naturally occurring hormone at the wrong time or to an excessive extent (agonistic effect).
- They may bind to the receptor but not activate it. Instead the presence of the chemical on the receptor will prevent binding of the natural hormone (antagonistic effect).
- They may bind to transport proteins in the blood, thus altering the amounts of natural hormones that are present in the circulation.

- They may interfere with the metabolic processes in the body, affecting the synthesis or breakdown rates of the natural hormones.

1.4 Main classes of endocrine disrupting compounds

EDCs have been grouped into five main classes, based on the systems with which they interact (Tarrant et al. 2005):

1. Estrogens and anti-estrogens
2. Androgens and anti-androgens
3. Progestins and anti-progestins
4. Aryl hydrocarbon receptor agonists
5. Thyroid hormone disruptors

They may be natural or synthetic in nature. They do not have structural similarities, so they exert their effects by different mechanisms (Norrgren et al. 1999; Laganà et al. 2004). Discovery of the modes of action of EDCs is made difficult by the fact that a range of factors including exposure duration, level and timing; nutritional status; age and gender of an organism; and target cell or tissue type may result in different effects being produced (Lister and Van Der Kraak, 2001). They reach the environment via a number of sources, including municipal and industrial wastewater effluent, agricultural and terrestrial run off and accidental release (Desbrow et al. 1998; Terzic et al. 2008; Liu et al. 2011). At present, the EDCs receiving most attention are those that, despite their diverse chemical structures, mimic estrogens (Laganà et al. 2004). Most of the research to date has focussed on freshwater environments. Far less is known as to the presence and effects of these compounds in marine and estuarine environments (Atkinson et al. 2003; Noppe et al. 2005), thus there is a substantial need for research in this area. The research conducted within this thesis aims to add to the current understanding of EDCs in marine environments, and generates baseline data on EDCs in Irish coastal and estuarine sites.

1.4.1 Endocrine disrupting compounds relevant to this study

Pesticides, polycyclic aromatic hydrocarbons, certain polychlorinated biphenyls, dioxins, furans, alkylphenols and steroids can be considered EDCs (Depledge and Billingham, 1999; Norrgren et al. 1999). It is not the purpose of this review to detail the effects of all

known EDCs. The following EDCs, which are estrogenic in nature, have been selected as the target compounds in this research, due to their relevance in terms of potency and legislative requirements for monitoring under the WFD and the Marine Strategy Framework Directive 2008/105/EC (MSFD). All compound groups discussed below have been highlighted as ‘target compounds’ by the Irish EPA (Tarrant et al. 2005). Domestic wastewaters are recognized as a main source of contamination for these compounds, and they are likely to be present in Irish wastewaters. Targeted analysis of water from a number of coastal waters will add to the current knowledge of the presence of selected EDCs in the marine environment.

1.4.1.1 Natural and synthetic steroid estrogens

Of the myriad compounds which have been shown to exert estrogenic effects, the natural and synthetic steroid estrogens have the highest potencies (Desbrow et al. 1998; Streck, 2009). Estrogens are a group of steroid hormones defined by their chemical structure and by their effect in the estrous cycle (Gabet et al. 2007). These include estrone (E1) and 17 β estradiol (E2), the naturally occurring estrogens, and 17 α ethynylestradiol (EE2), the synthetic estrogen used in the contraceptive pill (Desbrow et al. 1998; Streck, 2009). They have been shown to exert estrogenic effects in the laboratory (Labadie et al. 2007; Andrew et al. 2008), and in the environment (Solé et al. 2001; Gagné et al. 2004). Between 10 and 100 μ g of E1 and E2 are excreted daily depending on the phase of the cycle, while pregnant women may excrete up to 80 mg of estrogen (mainly as E3) per day (Andreolini et al. 1987; Purdom et al. 1994; Tyler and Routledge, 1998). Modern contraceptive pills typically contain between 20 to 35 μ g of EE2 per pill (Tarrant et al. 2005).

When measured using the *in vitro* yeast estrogen screen (YES), EE2 and E2 are equipotent, although in terms of the *in vivo* vitellogenin response, EE2 is an order of magnitude more potent than E2 (Metcalf et al. 2001; Thorpe et al. 2003). The increased potency of EE2 *in vivo* depends on the 17 α -ethinyl moiety, which increases EE2’s persistence in organisms by reducing its rate of metabolism compared to the endogenous steroids (Routledge et al. 1998).

1.4.1.2 Nonylphenol and octylphenol

Nonylphenol and octylphenol are synthetic estrogenic compounds used in the manufacture of antioxidants, lubricating oil additives, and the production of alkylphenol ethoxylates.

Alkylphenol ethoxylates (APEs) are one of the most widely used classes of non-ionic surfactants. They are used as detergents, emulsifiers, solubilisers, wetting agents and dispersants. They have been used in domestic detergents, cosmetics, pesticide formulations and industrial products. Octylphenol ethoxylates (OPEs) and nonylphenol ethoxylates (NPEs) are two of the most common surfactants in the marketplace (Ying et al. 2002). Nonylphenol polyethoxylates (NPEOs) represent 80-90% of APEO usage with octylphenol polyethoxylates making up the remaining 10-20% (Renner, 1997). Concerns first emerged in 1983 to 1984 when Giger et al. (1984) established that products of NPE degradation were more toxic to aquatic life than their precursors.

Primary degradation of APEs in wastewater treatment plants or in the environment by loss of ethoxy groups generates more persistent shorter-chain APEs and alkylphenols (APs) such as nonylphenol (NP), octylphenol (OP) and AP mono- to triethoxylates (APE1, APE2 and APE3) (Giger et al. 1984). NP has been found to mimic E2 by competing for the binding site of the receptor for the natural estrogen (White et al. 1994; Lee and Lee, 1996). 4-*n*-NP and 4-*t*-OP are reported to be more estrogenic than NPE (White et al. 1994). Log K_{ow} values for NP and OP are 4.48 and 4.12 respectively, they have a low solubility in water and tend to partition favourably to organic matter (Ahel et al. 1993; John et al. 2000). APEs and their metabolites have been shown to degrade much faster in the water column than in sediment (Ying et al. 2002), thus sediments may act as potential sinks for these compounds.

Several systems have been developed to assess the effectiveness of nonylphenol in triggering responses of the endocrine system. These include recombinant yeast assays, human breast cancer cell line (MCF-7) and fish cell hepatocytes. The relative potency of nonylphenol in relation to E2 in such systems presents a high variability (Soares et al. 2008). There is evidence to indicate that the toxicities of nonylphenolic and octylphenolic compounds are equivalent, and based in the current understanding of the toxic mode of action of alkylphenolic compounds, it has been suggested that additive toxicity will result when both nonylphenolic and octylphenolic compounds are present at the same time (Whitehouse, 2002).

In 2003 the European Commission (EC) issued Directive 2003/53/EC which places restrictions on the marketing and use of NP and NPE (EU, 2003). Particular focus is on the cessation or phasing out of discharges, emissions and losses to the environment.

Industrial, agricultural and domestic products, cosmetics and personal care products which contain more than 0.1% by mass of NP and NPE must not be placed on the market. This includes their use as co-formulants in pesticides and biocides. Although restricted for use, NP is still detected in the environment given the high usage of these products. 4-*n*-NP and 4-*t*-OP have been selected as target compounds within this study.

The relative characteristics of the EDCs of concern are previously well documented therefore only summary relevant physicochemical characteristics and associated legislative information are presented in Table 1.1

Table 1.1: Summary of physicochemical properties, relevant legislative criteria and bioconcentration factors for compounds of interest.

	E1	E2	EE2	4-NP	4-<i>t</i>-OP
CAS	53167	50282	57636	104-40-5	140-66-9
Molecular formula	C ₁₈ H ₂₂ O ₂	C ₁₈ H ₂₄ O ₂	C ₁₉ H ₂₄ O ₂	C ₁₅ H ₂₄ O	C ₁₄ H ₂₂ O
pKa	10.4 [~]	10.4 [~]	10.7 [~]	10.7	10.38
Solubility in water mg/L	13 ^{*^}	13 ^{*^}	4.8 ^{*^}	6	4.9
Log K _{ow}	3.01	4.01	3.67	5.5	5.3
AA EQS other surface waters WFD	n/a	0.08 (ng/L)	0.007 (ng/L)	0.3 µg/L	0.012 µg/L
MAC EQS other surface waters WFD	n/a	not derived	not derived	2 µg/L	0.13 µg/L
Relative potency to E2 by YES	0.38 [~]	1 [~]	1.19 [~]	0.00014 ^{**}	0.007 - 0.001 [*]
WFD QS to prevent secondary poisoning of predators	n/a	0.7 ng/g	0.07 ng/g	10 µg/g	10 µg/g
WFD QS human health via consumption of fishery products	n/a	3 ng/g	0.06 ng/g	8.7 µg/g	8.7 µg/g
BCF mussels	not defined	660 ^{*~}	not defined	2000-3000 [^]	not defined
BCF fish	not defined	6.5	610 [^]	1280 ^{^^}	260-1190 ^{^^^}

^{*^} (Ying et al. 2002) note solubility is at 20°C, ^{*} (Routledge and Sumpter, 1997),

^{**} (Routledge and Sumpter, 1996), [^] (Länge R. et al. 2001), ^{^^} (EU, 2005),

^{^^^} (Ferreira-Leach and Hill, 2001)

[~] (Rutishauser et al. 2004), ^{~~} (Ivashechkin et al. 2004), ^{*~} (Peck et al. 2007)

Not defined = during a comprehensive literature search no data could be found.

Not derived = MAC for E2 and EE2 are not given.

1.4.1.3 Degradation in the environment and behaviour in wastewater treatment plants

Estrogens may be excreted and released into the environment as inactive conjugates of glucuronic and sulphuric acids (Baronti et al. 2000). They typically have rapid degradation rates, with reported half lives ranging from hours to days (Williams et al. 2003; Andersen et al. 2004; Tarrant et al. 2005); being degraded into inactive conjugated forms. Secondary treatment processes, such as activated sludge, have been shown to remove up to 90 % of

E1, E2 and EE2 from wastewater during treatment (Janex-Habibi et al. 2009; Gabet-Giraud et al. 2010) while UV sterilization has been demonstrated to remove between 70 and 100 % of E1, E2 and EE2 during treatment (Gabet-Giraud et al. 2010). Deconjugation by bacterial enzymes in WWTPs and in the environment reactivates these conjugates into biologically active parent compounds (Johnson et al. 2000; D'Ascenzo et al. 2003) and they have been detected in surface waters and in wastewater influent and effluent (Fotsis et al. 1980; Fotsis and Adlercreutz, 1987; Jürgens et al. 1999; Baronti et al. 2000; Liu et al. 2009). E1 is often detected in higher concentrations in the environment (Table 1.2) due to the fact that E2 is degraded into E1, and E1 is the most abundant estrogen excreted by menstruating and pregnant women (Beck et al. 2005). Desbrow et al. (1998) found E1 and E2 to be the main contributing compounds to the estrogenicity of WWTP effluent. The synthetic estrogen EE2 is designed to resist degradation in order to be effective as an oral contraceptive with degradation occurring at a lower rate than that of the natural estrogens E1, E2 and estriol (E3) (Young et al. 2004).

Table 1.2: Concentrations of selected EDCs typically detected in the environment.

Matrix	Country	Analytical method	Sample volume (L)	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)	NP (ng/L)	OP (ng/L)	Reference
Coastal surface water	Germany	LC-ESI-MS/MS	49	0.53	nd	17	13.8	0.6	Beck et al. 2005
Coastal surface water	Italy	LC-ESI-MS/MS	1	10	175	34	211	n/a	Pojana et al. 2007
Surface water (estuarine)	UK	GC-MS/MS	2.5	14.3	21.4	1.5	5.8	37.6	Hibberd et al. 2009
Surface water (estuarine)	Netherlands	GC-EI-MS	2	8	nd	nd	n/a	n/a	Noppe et al. 2005
Surface water (estuarine)	Netherlands	GC-EI-MS/MS	2	10	nd	nd	n/a	n/a	Noppe et al. 2007
Surface water (estuarine)	Italy	LC-ESI-MS/MS	0.15-4	1.5	0.11	0.04	n/a	n/a	Baronti et al. 2000
Surface water (freshwater)	Italy	LC-MS/MS	0.25	6	1	nd	11	10	Vigano et al. 2006
Surface water (freshwater)	France	GC-EI-MS	1	30	1.2	nd	n/a	n/a	Labadie and Budzinski 2005
Surface water (freshwater)	Italy	LC-ESI-MS/MS	0.1-1	12	6	1	1466	n/a	Lagana et al. 2004
Surface water (freshwater)	UK	LC-ESI-MS/MS	2.5	10.8	7.6	1.9	n/a	n/a	Grover et al. 2011
Surface water (freshwater)	France	GC-MS	2	0.5-3	0.5-3	0.5-3	n/a	n/a	Mouatassim-Souali et al. 2003
Influent	Italy	LC-ESI-MS/MS	0.1-1	60	31	nd	8768	n/a	Lagana et al. 2004
Influent	France	GC-MS	2	7.2	7.6	2	n/a	n/a	Mouatassim-Souali et al. 2003
Influent	Italy	LC-ESI-MS/MS	0.15-4	132	25	13	n/a	n/a	Baronti et al. 2000
Influent and effluent	UK and Spain	GC-MS	0.1	86.9	93.3	nd	n/a	43.5	Hernando et al. 2004
Influent and effluent	UK and Spain	GC-MS/MS	0.1	94.7	101.3	nd	n/a	47.1	Hernando et al. 2004
Effluent	France	GC-EI-MS	1	71.4	4.4	nd	n/a	n/a	Labadie and Budzinski 2005
Effluent	Italy	LC-ESI-MS/MS	0.1-1	30	8	nd	2235	n/a	Lagana et al. 2004
Effluent	France	GC-MS	2	17.7	18.01	8.7	n/a	n/a	Mouatassim-Souali et al. 2003
Effluent	Italy	LC-ESI-MS/MS	0.15-4	1.7	2.9	1.7	n/a	n/a	Baronti et al. 2000
Effluent	UK	LC-ESI-MS/MS	2.5	10.8	7.6	1.9	n/a	n/a	Grover et al. 2011

APEs are highly cost effective surfactants with exceptional performance and are therefore used widely. Due to their extensive use they reach WWTP in substantial amounts (Soares et al. 2008). The principle pathway for removal of APs from wastewater is by sorption to sludge solids (Scrimshaw and Lester, 2002). APs have been detected in effluent, and surface water samples (Table 1.2). They may still leach into the environment from sludge which has been recycled to land or placed in landfill. The removal of APs from wastewater can be enhanced by adding activated carbon filters, UV treatment and ozonation to existing treatment processes (Johnson and Sumpter, 2001; Jones et al. 2007). Removal of longer chain APEs in WWTP have been reported at between 62 and >99%, however the removal efficiency is much lower for shorter chain APEs with NP accumulated in effluents as a stable degradation product (Yu et al. 2009).

Concentrations of APs reported in seawater, marine sediments and organisms indicate that coastal lagoons, estuaries and bays are more impacted than deep sea areas because of the distance from AP sources, such as WWTP inputs (David et al. 2009). The estrogenic activities in Tokyo Bay sediments collected downstream of a WWTP outlet measured with the yeast estrogen screen (YES) were 0.173 to 84.3 estradiol equivalents (EEQ) in ng/g dry weight (dw) in the sediments. NP was considered to be the most estrogenic compound (Kurihara et al. 2007). Sediments appear to be a sink for APs, and AP concentrations were higher in seawater at sites affected by WW discharge. AP concentrations in marine organisms were higher in benthic and pelagic organisms that live close to the sediment, than fish (David et al. 2009). Analysis in this study therefore focussed on levels of APs in marine biota, particularly shellfish due to their increased risk of contamination as detailed above.

1.5 Legislation and monitoring

The increasing body of scientific data which has demonstrated the negative effects of EDCs has led to the development of monitoring programmes and legislation which aim to reduce their presence in the environment.

1.5.1 The Water Framework Directive

The WFD establishes a framework for the protection of all water bodies, which aims to prevent further degradation of water resources, promotes sustainable water use and ensures

the progressive reduction of pollution of water bodies (Sanchez and Porcher, 2008). The WFD requires all Member States to prevent deterioration of the status of surface water and groundwater and to achieve ‘good status’ by 2015. In addition, pollution by priority substances has to progressively be reduced, emissions of hazardous substances ceased or phased out, and any significant upward trend in pollution in groundwater to be reversed by appropriate means.

Article 8 and Annex V of the WFD specify three main monitoring regimes:

1. Surveillance monitoring, to provide information for the assessment of long-term changes in natural conditions, and changes resulting from widespread anthropogenic activity. The results of this monitoring are used along with impact assessment to determine requirements for future monitoring programs.
2. Operational monitoring, to establish the status of water bodies identified as being at risk of failing to meet their environmental objectives, and to assess any changes in the status of such bodies resulting from the programs of measures.
3. Investigative monitoring, to understand the causes of failure when operational monitoring showed that environmental objectives are not likely to be met, and to allow accidental pollution assessment.

The application of the WFD for the surveillance of chemical contamination of surface waters involves two main objectives:

1. To assess the chemical status of the water bodies, by determining whether contamination levels are compliant with regulatory EQS.
2. To assess the temporal trends in the different environmental compartments of aquatic systems.

A list of priority and relevant pollutants and hazardous substances which have detrimental effects in the environment has been defined within the WFD. There is a requirement for substantial monitoring of these pollutants in transitional and coastal waters to achieve “good ecological and chemical status” by 2015. EQS have been defined for priority substances and certain other pollutants, and priority hazardous substances in water, with some EQS values defined for biota. They are defined as “the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment”. Two forms of EQS have been defined: annual average (AA) EQS and maximum allowable concentrations (MAC) EQS.

Where MAC values have not been defined for a compound, the AA EQS values are considered protective against short-term pollution peaks in continuous discharges since they are significantly lower than the values derived on the basis of acute toxicity. Many priority pollutants have endocrine disrupting (ED) properties. E2 and EE2 have recently been included in the WFD priority pollutant list and provisional annual average (AA) EQS in other surface waters as defined by the WFD have been proposed at 0.08 and 0.007 ng/L respectively. NPs and OPs are included on the WFD priority pollutant list. Their AA EQS values for other surface waters are 0.3 µg/L for NP and 0.01 µg/L for OP. A MAC of 2.0 µg/L has been defined for NP in other surface waters.

1.5.2 The Marine Strategy Framework Directive

The Marine Strategy Framework Directive 2008/105/EC (MSFD) builds on the WFD and lays down a strategy against pollution of water and requires further specific measures for pollution control and EQS. The MSFD aims to achieve ‘good environmental status’ in the European marine environment by 2020, and lays down the EQS in accordance with the provisions and objectives of Directive 2006/60/EC. The objective of this Directive is to establish common quality rules for chemical analysis and monitoring of water, sediment and biota carried out by Member States. The implementation of this Directive is assisted through the WFD Common Implementation Strategy and a series of guidance documents, which include chemical monitoring of surface waters. The MSFD requires an assessment of the current status of the marine environment by 2012, and a detailed description of the targets and indicators which will measure and define ‘good environmental status’ for each Member State’s water bodies. A monitoring programme to achieve good status must be in place by 2014, and a programme of measures for achieving ‘good environmental status’ should be established by 2016. It reinforces aims of the WFD, the progressive reduction of pollution from priority substances, and ceasing or phasing out emissions, discharges and losses of priority hazardous substances.

1.5.3 The Oslo Paris Commission

Ireland is a contracting party to the OSPAR Joint Assessment and Monitoring Programme (JAMP) to evaluate the status and trends of hazardous substances in the marine environment. The OSPAR hazardous substances strategy works to identify substances which are hazardous to the marine environment, to prevent, reduce, and ultimately eliminate pollution with these substances, and to monitor the effectiveness of measures to

achieve this (McGovern et al. 2011). OSPAR ultimately seeks to move towards the cessation of discharges, emissions and losses of hazardous substances by 2020 with the ultimate aim to achieve concentrations of hazardous substances in the marine environment near background values for naturally occurring substances and close to zero for man-made substances (McGovern et al. 2011). Hazardous substances are defined as substances which are persistent, liable to bioaccumulate and toxic (PBT substances), or which give rise to an equivalent level of concern as the PBT substances. The OSPAR co-ordinated monitoring programme (CEMP) monitors the presence of certain priority hazardous substances, with particular focus on coastal areas as they are close to discharge and emission sources. Octylphenol, nonylphenol and nonylphenol ethoxylates have been identified as hazardous substances on the OSPAR list of chemicals for priority action (OSPAR, 2009). 17β estradiol and 17α ethynylestradiol have been included on the OSPAR list of substances of possible concern due to their potential as endocrine disruptors according to the OSPAR Strategy with regard to Hazardous Substances (OSPAR, 2003). There is a lack of data regarding E2, EE2, NP and OP in Irish marine waters, and there is a substantial need for research. Techniques to detect certain hazardous substances and chemicals for priority action in the Irish marine environment and within marine matrices have been developed within this research.

1.6 Integrated monitoring approaches

A number of different techniques exist for the purposes of monitoring the presence and effects of pollutants in the environment. The isolated generation of chemical and biological effects data allows for limited assessments to be made on overall ecosystem health. Analysis using biologically based assays, such as whole organism assays, cellular bioassays and non cellular assays are very useful in determining the qualitative effects aspects of EDCs, providing overall estrogenic effects, and taking into account the synergistic, or anti-estrogenic properties of a sample (Chang et al. 2009). Chemical analysis is generally still required in order to identify the causative chemicals, as a specific response to a single compound may not yet be defined. Integration of chemical and biological effects measurements is seen as the way forward for monitoring of pollution in the marine environment (Law et al. 2010).

The value of an integrated approach has recently been accepted within ICES and OSPAR, who are developing tools for integrated chemical and biological effects monitoring to

provide a more comprehensive assessment of the status of the marine environment. These include WKIMON, the ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their effects in Coastal and Open Sea Areas, and WGBEC, the ICES Working Group on the Biological Effects of Contaminants. Integrated monitoring has also been recommended within the WFD.

An integrated approach has been successfully applied in a number of international studies. The 'Endocrine Disruption in the Marine Environment' (EDMAR) study was the first large-scale interdisciplinary study to investigate the possible effects of EDCs in the UK marine environment, and was conducted between 1991 and 2001 (Matthiessen et al. 2002). The researchers found several androgens and estrogens present in large estuaries, primarily associated with sediment. Only the estrogens appeared to have a major biological significance; with four species of fish experiencing feminisation in industrialised estuaries (Matthiessen et al. 2002). The authors concluded that while some reduction in reproductive success could probably occur, the effects on a population level remained unclear. Estrogenic activity in sediments was measured at 0.5 to 5.5 µg estradiol equivalents/kg, with the active substances remaining largely unidentified (Grover et al. 2011a). The Endocrine Disruption in Catchments (EDCAT) research programme followed, beginning in 2006. It aimed to measure and predict the concentrations of EDCs in 66 rivers, to discover if addition of granular activated carbon (GAC) at WWTP reduced EDC levels, and to investigate if fish from UK Rivers were at risk from sewage-derived EDCs. The authors found significant reductions in the concentrations of estrogenic and pharmaceutical compounds in both water and sediment in a number of UK rivers after the addition of GAC (Grover et al. 2011b). They also found a reduction in estrogenic and anti-androgenic activities of spot water and passive sampler extracts from river water and effluent sites as determined by chemical analysis and *in vitro* bioassay after the addition of GAC (Grover et al. 2011b). Arditoglou and Voutsas (2012) used an integrated approach to determine the presence of phenolic and steroid EDCs in the North Aegean Sea. They found NP and NPEs to be the dominant pollutants, with their presence in sediments presenting a significant risk to biota. NORMAN, the network of reference laboratories and related organisations for monitoring and biomonitoring of emerging environmental pollutants, developed a protocol for the integration of chemical and biological techniques to enable linkage of chemical and biological data (Leonards, 2005-2008). In the United States, the United States Geological Survey (USGS) has conducted a number of studies to investigate the presence and effects of EDCs using integrated chemical and biological measurements predominantly in

freshwater environments. For example, Lozano et al. (2012) found an apparent positive correlation between increased vitellogenin (VTG) levels in male largemouth bass downstream of a WWTP in Chicago, and concentrations of NPEs, with NPE tissue concentrations reported up to 5.42 µg/g. Chemical analysis conducted on WWTP effluent in Colorado using chemical analysis revealed a complex mixture of APs, E2 and EE2, with estrogen equivalence of up to 31 ng/L E2 (Vajda et al. 2008). The authors found a female biased sex ratio in *Catostomus commersoni* in the effluent discharge site, with males comprising 17-22% of the population compared with 36-46% in the upstream site. Reduced gonad size, VTG induction and disrupted ovarian and testicular histopathology were also identified in *C. commersoni* downstream of the WWTP.

1.7 Analytical methods of assessing the presence and effects of EDCs

A number of different techniques have been developed to assess the presence and effects of EDCs in the environment, and are described in detail below.

1.7.1 Sample collection and handling

Solid matrices such as sludge and biota may be freeze-dried before extraction (López de Alda and Barceló, 2001; Dévier et al. 2010). A number of different techniques have been employed for the extraction of solid samples, including ultra-sonication (López de Alda and Barceló, 2001; Vigano et al. 2006), microwave-assisted extraction (Matejíček et al. 2007) and accelerated solvent extraction (Noppe et al. 2007). The sample extraction/preconcentration is the most critical step in the analytical procedure (Streck, 2009). Sample preconcentration is generally completed using solid phase extraction (Gabet et al. 2007), which may be followed by a further sample purification step using gel permeation chromatography (GPC), depending on the matrix (Gabet et al. 2007; Liu et al. 2012).

In order to detect trace levels (ng/L) of EDCs in water samples, concentration of aqueous samples is necessary. Depending on the sensitivity of the technique, large volumes of water may be required. Sample volumes of up to 50 L have been reported in the literature (Beck et al. 2005; Gabet et al. 2007), with most studies sampling between 100 mL and 4 L (Table 1.2). Sample pre-treatment usually involves filtration, followed by a sample

preconcentration step using solid phase extraction with Oasis hydrophilic-lipophilic balanced polymer (HLB) cartridges the most commonly used (Gabet et al. 2007).

Marine environments are dynamic systems, subject to factors such as drought and flooding, and to tidal dilution effects particularly in estuarine areas. Detection of EDCs at biologically and legislatively relevant levels can prove difficult, and requires highly sensitive and specific techniques. Much of the information relating to aqueous concentrations of EDCs reported in the literature has been obtained by the collection of spot water samples, followed by extraction, clean up and analysis in the laboratory using a suitable mass based technique such as liquid or gas chromatography (LC or GC) coupled with mass spectrometry (MS) (Gabet et al. 2007, Streck et al. 2009). The detection of these compounds at and below the existing and proposed EQS values using traditional spot water samples and MS methods has proved challenging due to issues with instrument sensitivities and the volume of water required to detect low ng/L concentrations. Data derived from spot samples may also be unrepresentative as spot samples provide only a snapshot of a single moment in what is generally a dynamic environment. A study by Williams et al. (2003) showed that daily spot samples of waters taken from a river had a wide variance in daily E1 concentrations, ranging from 0.32 to 2.5 ng/L. They may not capture transient pollution events and as such may not be adequate. The cost of such a sampling method precludes the collection and analysis of a sufficient number of samples to mitigate such effects.

Passive sampling (PS) technologies are emerging as sensitive techniques to concentrate trace levels of pollutants in water. They are cost effective, easy to deploy and manage and do not show the same variability as traditional spot sampling. Passive sampling devices have been suggested as complementary methods which can be used in WFD surveillance, operational and investigative monitoring in a 2009 Guidance on Surface Water Chemical Monitoring under the WFD (EC, 2009). They may be used to corroborate or contradict spot sampling data, or as screening tools to identify problem and non problematic areas. Recently the ICES Working Group on Biological Effects of Contaminants (WGBEC 2007) acknowledged the advantages of combining the use of PS and “bioanalyses” as important links between the WFD and the MSFD and recommends their use.

The polar organic chemical integrative sampler (POCIS) is a passive sampler capable of sequestering polar compounds ($\log K_{ow} < 4$) from the water phase (Alvarez et al. 2004). E1,

E2, EE2, NP and OP have been shown to concentrate in POCIS (Alvarez et al. 2007). Their use for WFD purposes requires additional performance criteria such as the calculation of accurate uptake rates in order to calculate time weighted average contaminant concentrations in water, and strict protocols for in situ deployment. Calibration data and sampling rates for POCIS have been reported and reviewed for many EDC groups (Morin et al. 2012), however it has proved extremely difficult to accurately assess sampling rates which consider all the environmental factors in a sampling site, thus POCIS is most commonly accepted for use as a screening device. It has been selected for use within this research and was deployed at a number of test locations.

1.7.2 Detection methods

Mass spectrometry (MS) is a highly specific technique capable of detecting target analytes with high accuracy. Sample molecules are volatilized and ionised using a beam of charged particles or ions. The beam of ions is focused in a high vacuum so that particles of the same mass to charge ratio are separated from other ions present in the sample and are forwarded to a detector (Clayden et al. 2001). In recent years, the use of MS coupled to gas chromatography (GC) or to high-performance liquid chromatography (HPLC) has developed rapidly to provide better systems for identifying compounds in the environment. The types of systems employed differ in their detectors and, for LC-MS and LC-MS/MS, in their ionization techniques (Streck, 2009). They are considered the gold standard methods for sex-steroid identification (Gust et al. 2010) and they are the main methods used for the analysis of EDCs in environmental matrices. The fragmentation pattern generated by tandem mass spectrometry (MS/MS) is a powerful tool for obtaining confidence in compound identification. In addition, the use of MS/MS detection allows analysis without complete chromatographic separation between the analytes, and therefore shortening of the chromatographic run time (Borba da Cunha et al. 2004; Petrovic et al. 2010). The use of GC necessitates a derivatisation step, which is time consuming and labour intensive (Baronti et al. 2000).

LC-MS/MS has emerged as the leading method of analysis for the EDCs due to the high sensitivity and specificity and rapid sample throughput (Croley, 2000) with ionisation in negative electrospray mode (ESI) the most commonly used (Gabet et al. 2007). The presence of endogenous steroids in biota and seasonal fluctuations in the steroid levels present may complicate the interpretation of estrogenic bioassay results. The ability to

accurately identify endogenous and exogenous estrogens is a major advantage of LC-MS/MS. LC-MS/MS was selected as a suitable technique for the detection of selected EDCs in this study. The low limits of detection attainable are particularly valuable for monitoring in the marine environment, where dilution effects may be high, and for detection of EDCs at environmentally and toxicologically relevant levels.

1.7.3 Biological measurements

A number of biological measurement techniques have been developed which examine the effects of contaminants within exposed organisms. They range from cellular to organism and population level effects.

1.7.3.1 Biomarkers

Biomarkers can be defined as measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or magnitude of the host response to such contaminants (Livingstone et al. 2000). Biomarkers may be considered as biomarkers of exposure, which indicate that an organism has been exposed to pollutants, and effect biomarkers, which indicate the magnitude of the organism's response to the pollutant (Ortiz-Zarragoitia and Cajaraville, 2006). Importantly biomarkers can act as short-term indicators of long-term biological effects due to their potential to anticipate changes at higher levels of biological organisation, *i.e.*, populations, communities or ecosystems (Cajaraville et al. 2000). Biomarkers have been developed for both vertebrate and invertebrate species. A number of studies have demonstrated the impact of EDCs on exposed organisms using molecular, chemical, genotoxic, cellular and immunological techniques (Pandian and Sheela, 1995; Allen et al. 1999; Livingstone et al. 2000; Quinn et al. 2004; Peck et al. 2007; Canesi et al. 2008). A number of commonly used *in vitro* bioassays which have been developed for determination of the biological activity of individual compounds, environmental samples or fractions thereof are described below.

1.7.3.2 Ligand binding assays

Ligand binding assays measure the binding of ligands to receptors isolated from animal tissues, human or animal cell lines, or those produced by genetically-modified bacteria (Jacobs et al. 2008). Ligand-binding assays are simple and are applicable to high-throughput screening (Mueller, 2004). With ligand binding assays, the implication is that

binding results in a subsequent effect on biological activity and as such, these assays are attractive as they have the potential for high throughput screening (Matthews and Zacharewski, 2000). Ligand binding assays are limited in that, although a compound may bind to a receptor, agonistic or antagonistic effects cannot be determined (Zacharewski, 1997; Holmes et al. 1998). Furthermore, the binding of compounds to the receptor does not necessarily mean that endocrine disruption takes place *in vivo* or that an organism is affected adversely (Mueller, 2004).

1.7.3.3 Recombinant receptor-reporter assays

Recombinant receptor-reporter assays are more complex than binding assays as they give more information on the effects of binding to the receptor (Scrimshaw and Lester, 2004), thus they can measure the potency of a substance or sample. The most widely used recombinant receptor reporter assays are the yeast screen and luciferase reporter assays. The yeast estrogen screen (YES) developed by Routledge and Sumpter (1996) provides an inexpensive means of assessing estrogenicity. The human estrogen receptor (hER) is integrated into the yeast genome along with an expression plasmid which also contains estrogen responsive sequences (ERE) controlling the expression of the reporter gene *lac-Z*. *Lac-Z* encodes the enzyme β -galactosidase and in the presence of estrogens, β -galactosidase is synthesised and secreted into the growth medium, resulting in a colour change which can be measured by absorbance at 540 nm. It has been validated for the detection of a wide range of estrogen receptor agonists including E1, E2, EE2 as well as NP and OP (Routledge and Sumpter, 1996; Routledge and Sumpter, 1997).

Stably transfected luciferase reporter cell lines which are used to indicate binding to the estrogen receptor (ER) are widely used in reporter gene assays, the most common of which are the estrogen receptor (ER)-mediated chemical activated luciferase gene expression (ER-CALUX) assay (Legler et al. 1999; Legler et al. 2002) which uses human breast adenocarcinoma cell lines expressing endogenous ER α and β , the MELN cell line containing human breast cancer (MCF-7) cells (Michallet-Ferrier et al. 2004), and the estrogen luciferase reporter gene assay (ER-LUC) which uses a recombinant human ovarian carcinoma (BG1) cell line (Rogers and Denison, 2000). In each of these assays, the cells have been stably transfected with an estrogen responsive luciferase reporter plasmid. Luciferase induction measures the amount of luciferase induced from estrogen receptors and response elements with luminescence after cell lysing and the addition of luciferin

(Rogers and Denison, 2000; Legler et al. 2002; Michallet-Ferrier et al. 2004), and indicates the presence of estrogenicity in a sample. These reporter gene assays have been used in a large number of studies to investigate the estrogenic activity of marine and freshwater environmental samples, and to assess the estrogenicity (expressed as estradiol equivalents or EEQ) of potential EDCs (Murk et al. 2002; Michallet-Ferrier et al. 2004; Soto et al. 2006; van der Burg et al. 2010; Schmitt et al. 2011). ER-LUC has been selected for use in this research to investigate the estrogenicity of samples collected at a number of marine sites.

1.7.3.4 Cell proliferation assays

Cell proliferation assays are based on the measurement of cell proliferation induced by an EDC. They are predominantly based on human derived cell lines, with the estrogen responsive MCF-7 or T47-D human breast cancer cells being the most commonly used (Zacharewski, 1997). The E-SCREEN assay was developed by Soto et al. (1992). It compares cell yields in both positive and negative controls with those from samples exposed to test compounds. The presence of active chemicals is sensed by an increase of cell proliferation in comparison to the positive control. Cell proliferation tests therefore determine the action of an EDC by means of a physiological endpoint relative to a control (Streck, 2009). A range of cell lines for MCF-7 exist and these display different responses leading to possible problems with reproducibility (Scrimshaw and Lester, 2004).

1.7.3.5 Immunoassay techniques

Two of the most common immunoassay techniques, which are based on the use of specific antibodies, include radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). While generally rapid and inexpensive, immunoassays are less specific than mass spectrometry methods (Gust et al. 2010). Radioimmunoassays have been validated for different fish species and are accepted as sensitive, quantitative and specific. Competitive or non competitive ELISAs using either monoclonal or polyclonal antibodies are widely used, and allow quantitation once in the right dilution range (Solé et al. 2001). Their specificity when applied to molluscs may be limited by cross-reactivity or subjected to interferences from co-extracted matrix components (Zhu et al. 2003; Gust et al. 2010).

1.7.3.6 Vitellogenin induction and the alkali labile phosphate assay

Vitellogenin (VTG) is an egg yolk precursor protein produced by females during gametogenesis. Estrogenic compounds are the only known stimulants of VTG (Kime, 1999). Although VTG is understood to have a specific function in female fish, it is well known that male fish have the physiological potential to produce as much VTG as females, provided they receive estrogenic stimulation (Carlson et al. 2000). The production of VTG by male or immature fish provides a marker for detecting the presence of environmental pollutants with estrogenic activity (Tyler et al. 1996). VTG has successfully been used to assess ED in a large number of marine and estuarine fish species including dab, *Limanda limanda* (Scott et al. 2007), flounder, *Platichthys flesus* (Allen et al. 1999; Matthiessen et al. 2002; Vethaak et al. 2005), plaice, *Pleuronectes platessa* (Scott et al. 2000), viviparous blenny, *Zoarces viviparous*, and sand gobies, *Pomatoschistus minutus* and *P. lozanoi* (Matthiessen et al. 2002) to name but a few.

Recent work has identified the presence of vitellin-like protein (Vn), an egg yolk precursor protein produced in female molluscs during oogenesis. It may become elevated in females and produced by males upon exposure to endogenous estrogenic compounds (Gagné and Blaise, 2000). The alkali-labile phosphate assay (ALP) is widely used to indirectly determine Vn-like protein (Blaise et al. 1999; Gagné and Blaise, 2000; Marin and Matozzo, 2004). The ALP assay is considered simple, cost-efficient and is not species specific (Porte et al. 2006). The Bradford assay (Bradford, 1976) is a method for the determination of microgram quantities of protein. It is used in conjunction with ALP, to determine the total gonadal protein concentration, in order to normalise ALP data to the total protein content of the visceral mass. The ALP assay has been significantly correlated with other VTG assays (Gagné and Blaise, 1998). VTG as a measure of ED exposure in molluscs has been tested in different laboratory studies, but the results are often contradictory (Matozzo et al. 2008), thus standardised testing protocols are required in order to allow comparability between studies.

1.7.4 Limitations of biological effects techniques

While the biological effects measurements detailed above provide valuable information regarding the effects of EDCs and mechanisms of action and are gaining international acceptability, there are a number of limitations to their use, predominantly related to the lack of definitive assessment criteria, which would allow for more direct comparison of

results. The lack of quality assurance programmes, proficiency testing schemes and resultant lack of directly comparable data diminishes the value of measurements and makes it difficult to standardise testing. Cross-reactivity and interferences are also an issue. In terms of bioassays in mussels, the results of in vitro assays are difficult to interpret as endogenous ligands potentially implicated in mussel reproduction could induce false positives responses in cell bioassays (David et al. 2008). More data is required in order to improve data confidence and generate valuable baseline data. The strengths and weaknesses of a number of these techniques are summarised in Table 1.3. This project seeks to add to the current understanding of selected biological effects techniques, which are described in more detail in Chapter 2.

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Table 1.3: Strengths and weaknesses of biological assays: reproduced and adapted from Tarrant et al. (2005)

Assay type	Examples	Sensitivity	Advantages	Limitations
Ligand binding assays	Generally use ER isolate from human, animal or genetically modified bacterial cell lines,	Variable	Direct assessment of chemical binding to the estrogen receptor.	Does not measure ER activation; Cannot distinguish between agonists and antagonists; Inability to account for metabolism; Unsuitability to automation; Non-competitive displacement at high concentrations. No standardised assay conditions.
Recombinant reporter assays (human and vertebrate cell lines)	ER-LUC*, ER-CALUX	6×10^{-11} M of estradiol.	Can detect agonists and antagonists; Easy to perform. Can screen compounds over a wide concentration range.	Non-estrogenic mitogen interference. Assay duration. Presence of endogenous estrogens. MCF-7 cell metabolic capacity, Reproducibility: a range of cell lines exist and these display different responses leading to possible problems with reproducibility
Recombinant reporter assays (yeast cells)	YES	1×10^{-11} M of estradiol.	Can detect agonists and antagonists; Partial metabolic competence; Reliable, robust, cost-effective. Generally rapid	Toxicity of certain chemicals. Limited ability of some chemicals to cross the yeast cell wall. Chemical sorption to the plastic plates; Creeping of certain chemicals.
Immunoassay techniques	RIA ELISA	1×10^{-9} M of estradiol.	Generally rapid. Cost effective.	Specificity may be limited by cross-reactivity or subjected to interferences from co-extracted matrix component. No standardised assay conditions.
Cell proliferation assays	E-SCREEN	1×10^{-3} M of estradiol.	Can determine the action of an EDC by means of a physiological endpoint relative to a control	Reproducibility: a range of cell lines for MCF-7 exist and these display different responses leading to possible problems with reproducibility. No standardised assay conditions.
Vitellogenin assays	ALP VTG	1×10^{-9} M of estradiol.	Specific response to estrogenic compounds	Limited detection of vitellogenin protein, similarities in responsiveness, issues with endogenous VTG and Vn in females.

1.8 Bioindicators

A number of vertebrate and invertebrate species have been used as bioindicators to assess environmental pollution impacts.

1.8.1 Invertebrate molluscs

The invertebrate species most commonly used in environmental monitoring programmes are *Mytilus* spp., sessile filter feeding bivalves which are widely distributed in temperate regions of the world (Gosling, 1992). They are abundant, have a primary benthic life stage, filter large amounts of water, bioaccumulate many compounds and have a relatively stable population which enables repeated sampling. They are easily transplanted and maintained, making them suitable for use in monitoring studies when indigenous populations may not be present, or for investigations of point sources of contaminants. Their ubiquity makes them a suitable indicator organism for use in environmental monitoring programs to assess the status and trends of chemical contamination in estuarine and coastal environments. They were introduced as biomonitoring organisms in the mid-1970s with the establishment of the Mussel Watch monitoring program to assess spatial and temporal trends in chemical contamination in estuarine and coastal environments (Goldberg, 1975). Since then mussels have been widely used to assess the biological effects of pollution. They are used in the OSPAR CEMP and in monitoring of the quality of shellfish growing waters via the Shellfish Waters Directive 2006/113/EC (SWD).

Mussels are considered resilient animals, but little is known about the acute and chronic biological effects of EDC exposure. Endogenous hormones may complicate the interpretation of bioassays which measure estrogenic responses in *Mytilus* spp. Bayen et al. (2004) found difficulties in interpreting the results of *Perna viridis* extracts screened for androgenic and estrogenic activities using ER- α and ER- β , and suggested that naturally occurring estrogens could induce false positive responses. A report by Porte et al. (2006) indicates that selected molluscan taxa are able to synthesise sex steroids, including 17 β -estradiol, via catalysis by cytochrome P450s and steroid dehydrogenases (Porte et al. 2006). Seasonal E2 levels, measured using an enzyme linked immunosorbent assay have been reported in *Mytilus galloprovincialis*, ranging from 1.71 to 5.42 ng/g dry dw (David et al. 2008).

Egg-yolk protein (vitellin) accumulates in molluscan oocytes during gonadal maturation, synthesised from its precursor vitellogenin (Andrew et al. 2008). Xenobiotics which mimic the structure of estrogens (and exert effects through direct receptor mediated genomic action) or contaminants that alter sex steroid function by interacting with steroidogenesis, metabolism and/or non-genomic action of endogenous sex steroids are likely to affect processes possibly linked to endocrine action in molluscan models such as oysters and mussels (Porte et al. 2006; Andrew et al. 2008). The relative Vn content in female *Crassostrea gigas* as measured with ELISA showed an apparent increase as the oocyte developed (Li et al. 1998). A similar trend was observed in ovarian tissues cultured in the presence of E2. The authors concluded that E2 is one of the major factors which control vitellogenesis in the oyster, and that the ovary is the site of synthesis of vitellin in female oysters. Canesi et al. (2004) investigated the possible effects and the mechanisms of action of the natural estrogen E2 in the hemocytes of *Mytilus galloprovincialis*. Their results showed that nanomolar concentrations of E2 rapidly induced cell shape changes, lysosomal destabilization and hydrolytic enzyme release, and stimulation of bactericidal activity (Canesi et al. 2004). Exposure to E2 *in vivo* caused significant increases in oocyte diameter and vitellin content in *C. gigas* (Li et al. 1998). In a study of the effects of NP and EE2 on the Sydney rock oyster *Saccostrea glomerata* it was found that male individuals in 1 µg/L and 100 µg/L NP exposures and 5 ng/L EE2 were at earlier stages of spermatogenic development than corresponding controls (Andrew et al. 2008). The authors also found that their results suggest NP may be accumulated in tissue and is partly resistant to biotransformation; maintaining its potential for chronic estrogenic action, while EE2, although exhibiting greater estrogenic potency on biological endpoints possibly exerts its estrogenic action before being rapidly metabolised and/or excreted. Another limitation to using *Mytilus* spp. as a monitoring tool is their marked seasonality (Gabbot and Peek, 1991), thus natural seasonal patterns in biomarker response should be understood in order to interpret responses observed in impacted environments (Blaise et al. 2002; Petrovic et al. 2004).

1.8.2 Vertebrates-Fish

A number of international studies have shown that disruption of the endocrine system of some wild fish populations is occurring (Solé et al. 2001; Matthiessen and Law, 2002; Kidd et al. 2007). Purdom et al. (1994) published one of the first studies on endocrine disruption in fish exposed to WWTPE, after the presence of hermaphroditic fish in lagoons

receiving WWTP was noted. They exposed caged male trout to WWTP and noted the induction of VTG after exposure. Many species of fish have been used to assess the effects of EDCs.

The marine flatfish *Limanda limanda* (dab), *Pleuronectes platessa* (plaice) and *Platichthys flesus* (flounder) are suitable species for monitoring ED effects in marine systems. They are among the species of fish which are used as biomonitoring species within WGBEC, ICES, OSPAR and WKIMON. They are widely distributed in the North Eastern Atlantic (Scott et al. 2007). Dab are an offshore sentinel species of the Clean Seas Environment Monitoring Programme (CSEMP) in the UK. Flounder are the most ideal species for monitoring ED effects near point source discharges as they spend more time in estuaries, and are thus exposed to higher levels of anthropogenic input. Dab and plaice are generally offshore species, thus the presence of EDCs in these species may indicate a higher contaminant burden and would be of greater concern. These flatfish species were chosen as core species within this research to add to the growing dataset of EDC levels in marine species.

1.9 Caging studies

Typically, in OSPAR and the SWD a sample of indigenous shellfish is collected and analysed. Caging shellfish in areas under investigation is an emerging technique. A review of the literature relating to biomonitoring studies by Besse et al. (2012) found that biomonitoring for WFD purposes using transplanted organisms was more suitable than passive approaches (using indigenous species), as they implement reproducible strategies, control biotic confounding factors and provide robust, comparable results. In areas without an indigenous shellfish population, caging may be used as a tool to assess the effects of pollution providing a time-integrated means of assessment. If the test organisms are taken from a well characterised unpolluted site, they can be employed in spatial surveys, enabling a more direct comparison of different test sites. Caging as a technique is relatively simple and inexpensive to set up and deploy. Cages can be deployed for long periods of time and this technique has been used successfully in a number of international studies from assessing responses to pollution (Martel et al. 2003; Tsangaris et al. 2010) and the endocrine response of freshwater bivalves placed downstream of wastewater effluent using biomarkers (Gagné et al. 2004), to using chemical techniques to examine contaminant uptake and bioavailability (Gagnon et al. 2006) and trends in coastal water quality (Hunt

and Slone, 2010). Research has primarily focussed on freshwater environments, with little data existing on integrated studies in marine environments.

1.10 *In vivo* studies

In vivo tests are much more reliable in assessing the true effects of EDCs because it is possible to consider many variables such as survival, growth, secondary sexual development, organ weight, plasma vitellogenin, sexual steroids in the plasma, fecundity, gamete viability and histology (Soares et al. 2008). Elevated vitellogenin levels have been reported in both male and female Pacific Rock oysters (*Saccostrea glomerata*) when exposed to 50 ng/L EE2 for 8 weeks with a number of individuals showing the presence of intersex (ovotestis) when examined using histology, highlighting the value of vitellogenin induction as a marker of biological effects. Organisms may be more susceptible to the effects of EE2 depending on the stage of gametogenesis. Ciocan et al. (2010) found a significant increase in VTG and ER2 mRNA expression in *Mytilus edulis* exposed to E2 and EE2 at the early stage of gametogenesis, whereas mature mussels displayed no statistically significant changes.

Most of the information on biological effects and mechanisms of action of EDCs on marine organisms come from studies on vertebrates (Porte et al. 2006), and they are too numerous to detail in this review. Hormonal induction of sex reversal in fish using steroids has been used within the aquaculture industry and is possible in at least 47 species (Pandian and Sheela, 1995). The most striking example of the effects of EDCs in fish was the collapse of a fathead minnow (*Pimephales promelas*) population following seven years exposure to low level concentrations of EE2 in the Experimental Lakes Area in Ontario Canada (Kidd et al. 2007). The authors assessed the sub-cellular to population level effects of EE2 and observed elevated VTG levels in both males and females, the presence of intersex and decreased gonadosomatic indices within the first year of exposure, culminating in the collapse of the population after seven years. Significantly elevated VTG levels were detected in flounder exposed in three separate experiments to 333 ng/L E2, 20 ng/L EE2 and 100 µg/L NP respectively for ten days (Kirby et al. 2007). Significant VTG mRNA induction was recorded in roach, stickleback, rainbow trout, medaka, zebrafish and fathead minnow exposed to between 2 and 10 ng/L EE2. A study by Yokota et al. (2001) on the effects of nonylphenol on two generations of the fish *Oryzias latipes* suggested that the toxic effects of nonylphenol can be enhanced by continuous exposure. The numerous

studies which demonstrate the ED effect of steroids and APs at environmentally relevant concentrations *in vivo* highlights the need for detailed monitoring of the levels and effects of these compounds in marine environments.

The biological effects techniques described above are advantageous in that:

1. They provide a measure of the total estrogenicity at a site, and account for many compounds which may possibly be present. This is especially valuable as targeting all known estrogenic compounds by chemical analysis would be prohibitively expensive, would involve the collection of large sample volumes, and may be extremely time consuming during sample preparation and analysis.
2. While chemical analysis may only detect low concentrations of estrogenic compounds, the biological effects techniques described above can account for additive or synergistic effects, taking account of the presence of mixtures of compounds.

The ER-LUC was selected as a suitable indicator of estrogenicity for use within this research project, and the ALP assay was selected by partners in the SeaChange project as a marker of estrogenic endocrine disruption *in vivo*. These assays are described in more detail in Chapter 2. Data on the presence and effects of EDCs in Irish marine waters is currently lacking. The resulting data derived from these assays is integrated with chemical analysis data generated within this research. A comprehensive assessment of the risks posed to the Irish marine environment due to the presence and effects of selected EDCs is conducted in Chapter 5 and contributes to the current understanding of levels and effects within marine systems.

1.11 Irish context

Irish research on EDCs has thus far been limited and has concentrated primarily on freshwater environments, with very little information on estuarine and marine habitats available. The Irish Environmental Protection Agency (EPA) has published two main reports, in 1998 and 2005. The first, by Dempsey and Costello (1998), is a review of estrogen mimicking chemicals in relation to water quality in Ireland in the freshwater environment only. The potential endocrine disrupting effects of natural and synthetic hormones, phytoestrogens, organochlorine pesticides, non- organochlorine pesticides,

polychlorinated biphenyls (PCBs), dioxins and furans, alkylphenol ethoxylates (APEs), phthalates, butylated hydroxyanisole (BHA) and bisphenol A (BPA) and mixtures of estrogen mimicking chemicals were reviewed, using data from international studies, particularly biological assessments. The report recommended that biological assessment be carried out in Irish waters; on a variety of wastewater treatment works discharges and receiving waters, containing both industrial and municipal effluent. The report also advised that technical experience in the study of endocrine disrupting chemicals should be developed nationally, perhaps in collaboration with overseas experts. The application of these recommendations in Irish waters has thus far been limited. Due to higher dilution in marine waters, the concentrations of EDCs are expected to be low and thus direct effects of estrogenic substances have been assumed to be of minor or negligible relevance. As such, aquatic ecosystems have received only little attention in recent years and thus there is a lack of knowledge concerning the occurrence and fate of these chemicals in marine environments (Atkinson et al. 2003). Insufficiently sensitive detection capabilities of analytical procedures for the determination of estrogenic substances in marine waters are another reason why only few data concerning contamination levels in marine environments are available (Beck et al. 2005).

Recent advances in MS techniques have improved the sensitivity of analysis. Aquatic ecosystems near coasts, estuaries, gulfs or relatively closed seas are highly influenced by the inland waters flowing into them via rivers or via direct wastewater effluent releases. Coastal areas are often dilution zones of freshwater associated with anthropogenic contaminant inputs resulting from domestic, industrial and agricultural activities.

The 2005 EPA report, by Tarrant et al. (2005) addressed some of the suggestions made in the 1998 report. The presence of, and risks posed by EDCs in Irish rivers and the related aquatic ecosystems were investigated. The following compounds were designated as ‘target compounds’ for monitoring in the Irish context, having being selected using the following criteria; they are shown to be highly estrogenic by a recombinant yeast assay (YES) or they are weakly estrogenic but are likely to be present at relatively high concentrations in Irish wastewaters, leachates and surface waters:

Group 1: Alkylphenol ethoxylates and their neutral metabolites

Group 2: Steroid sex hormones and related synthetic compounds

Group 3: Bisphenol A

Group 4: Phthalates

Group 5: Pesticides and Herbicides

This study by Tarrant et al. (2005) used biological effects techniques to assess estrogenic ED in the Irish (predominantly) freshwater environment in the following case studies:

1. The measurement of VTG levels, using RIA, in caged brown trout, which were placed in the River Lee upstream and downstream of Ballincollig WWTP (15,000 PE). The authors found no evidence of raised VTG in caged trout and concluded that estrogenic compounds emitted from Ballincollig WWTP are not emitted in sufficient quantities to be a threat at this location.
2. The measurement of VTG levels in wild brown trout from 11 freshwater locations. Elevated VTG levels were determined in wild brown trout sampled in the River Liffey, downstream of Osberstown WWTP (68,000 PE).
3. A detailed investigation into the estrogenicity of effluents from ten WWTPs and receiving waters using YES. The effluents from Osberstown and Ringsend WWTPs were the highest measured in the study, at 17.2 and 16.0 ng estradiol equivalents (EEQ) per litre. Estrogenicity was not determined (not detected) in all receiving waters studied.

1.12 Ireland and the Water Framework Directive

The EPA, Marine Institute and Inland Fisheries Ireland have classified 121 Irish coastal and transitional waters for WFD purposes between 2007 and 2009. Of these, 55 were classed as either high (16%) or good (30%) status, with the remainder classified as moderate or worse. In terms of surface area, just over 64% of the total monitored area was found to be at high or good ecological status (EPA, 2010). The EPA also assessed 101 Irish transitional and coastal waters for WFD purposes, using biological quality elements (BQEs) (Lucey, 2009). Approximately 60% were classified as having high (29.7%) or good (29.7%) status, with the remainder (40.6%) classified as moderate or worse. BQEs used in this case for WFD assessment were phytoplankton (biomass and bloom frequency), aquatic flora (rocky shore seaweed biodiversity and opportunistic macroalgal abundance) and fish (structure of the fish community). The upper Liffey in Dublin and Lough Mahon and the Lee in Cork received a poor status for fish. Kinvara Bay lagoons were classified as bad based on the fish community. An initial assessment of dangerous substances in WFD coastal and transitional waters was conducted in Ireland between 2007 and 2009. It found

most waters to have ‘good status’ (McGovern et al. 2011). The steroid estrogens and APs were not included in this assessment.

1.13 Ireland and the Shellfish Waters Directive

The value of the Irish molluscan shellfish industry, which includes mussels, oysters, clams and scallops, among other molluscan species, was estimated at €47 million in 2007 (Lucey, 2009). Contamination of these areas with treated and untreated wastewater could have serious implications for consumers. A shellfish sanitation monitoring programme has been in place since 1985, classifying shellfish growing waters based on a number of microbiological criteria and levels of *Escherichia coli*. The SWD also requires substantial monitoring of shellfish production areas. In 2008, 102 production areas were classed as either Class A, (26%, can be sold for direct human consumption), Seasonal A (12%), or Class B (62%, can be sold for human consumption following purification or cooking by an approved method) (Gilmartin and Silke, 2009). Shellfish from the 63 designated shellfish waters for SWD purposes have also been analysed for trace metals and organohalogens in accordance with the SWD (codified version Directive 79/923/EEC). Monitoring was completed in accordance with OSPAR monitoring guidelines, with mussels and oysters the preferred species. Irish shellfish growing waters were generally found to be of high quality for the substances monitored (Lucey, 2009). No monitoring has been conducted with respect to levels of the steroid estrogens and APs. If these compounds are present, they may potentially impact this valuable industry, by affecting growth, development and reproduction of exposed shellfish.

1.14 Project description

Research on the presence and effects of EDCs in Irish waters has predominantly focussed on freshwater environments (Tarrant et al. 2005; Reid et al. 2006, 2007), with the exception of a measurement of the estrogenicity of receiving waters in the Liffey estuary using a biological effects technique (Tarrant et al. 2005). No other information as to the presence and effects of EDCs in Irish marine waters was found during the course of this literature review.

Much of the treated and untreated effluent produced is discharged into estuarine and coastal waters yet few studies to determine the presence of specific estrogenic EDCs or the

potential ED effects resulting from these discharges have been reported. A number of the largest WWTP in Ireland, including Ringsend WWTP, Dublin, Cork City WWTP and Galway City WWTP discharge into marine environments. There is a strong potential for estrogenic effects particularly in the Liffey estuary and Dublin Bay due to the high anthropogenic inputs at this site. A large proportion of untreated waste also enters Irish waters. Sources include direct discharge, release from domestic sources (septic tanks), and agricultural run off. In addition to direct discharge of effluents and untreated waste to coastal and estuarine environments, these sites are the final discharge point for effluents discharged upstream in river systems. The potential for ED effects within Irish marine locations is currently unknown, thus a detailed investigation is required. In order address this, an applied integrated approach has been used within the course of this research, generating data to fill gaps both nationally and internationally.

There is a requirement for substantial monitoring of a number of EDCs under the WFD and MSFD, and they have been highlighted within OSPAR, WGBEC and WKIMON. Techniques developed in the course of this research focus on WFD priority pollutants, and the data generated adds to the current dataset on levels of EDCs within marine environments, and assesses compliance of the Irish waters studied with WFD EQS and available assessment criteria where appropriate.

This research forms part of a larger collaborative four year project entitled '*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*'. Partners in the project include Trinity College Dublin, the Marine Institute, Shannon Aquatic Toxicity Laboratory (SATL), and the Dublin Institute of Technology (DIT). This project was divided into five main work packages (WP). WP1 involved the selection and development of biomarkers in marine biota, and sediment bioassays. WP2 was a two tiered analysis of a number of study sites using a range of chemical and biological techniques. WP3 involved an investigation into the biological effects of endocrine disruption. WP4 was the development of chemical analysis techniques, and selection and use of suitable passive sampling technologies. WP5 related to the integration of data derived from the project. The research presented in this thesis forms part of WPs 2-5, selecting target analytes with respect to EDCs, developing suitable methods, and applying the techniques to conduct an assessment of EDCs in Irish marine waters, and investigating the effects of these compounds *in vivo*.

1.15 Research questions

The following research questions will be addressed within this thesis:

Question 1: What are the presence and effects of selected endocrine disrupting compounds in the Irish Marine Environment?

Hypothesis: Endocrine disrupting compounds of both natural and synthetic origin may be present in the Irish marine environment. Deleterious effects on resident organisms and populations may result.

Question 2: What is a suitable means of detecting these compounds and assessing their impacts?

Hypothesis: Multi-parameter integrated analyses can provide a clearer insight into the presence and effects of these compounds within marine environments. Chemical analysis methods, passive sampling technology and assessment of compound uptake in caged indicator species will be utilised to conduct research presented in this PhD. Biological effects measurements data obtained by partners in '*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*' project will be integrated with chemical data obtained in this PhD. This should give a clearer indication of ecosystem health, which will be beneficial in environmental monitoring programmes, for example, in the potential for effects monitoring of wastewater treatment plant effluent.

Question 3: What are the cause and effect relationships of these compounds?

Hypothesis: Using *in vivo* exposure studies, the cause and effects of selected EDCs on selected indicator organisms will be elucidated. Caging studies and monitoring techniques such as passive sampling can be utilised to estimate exposure levels and effects of EDCs detected in the marine environment. Chemical assessment methods developed in this PhD will be used to assess the presence and concentrations of selected compounds. This data will be integrated with results from biological effects measurements results obtained by partners in the '*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*' project to derive cause and effect relationships.

Data generated within this research provides the first comprehensive integrated assessment of estrogenic endocrine disrupting compounds, and endocrine disruption in Irish marine

waters. A range of techniques including ‘traditional’ chemical analysis methods, and more recently developed passive sampling technologies are applied. Specific analytes with potent estrogenic activity have been selected for targeted chemical analysis, while biomarker techniques measuring overall estrogenic activity and estrogenic effects are utilised in order to account for the presence and effects of unknown estrogenic compounds. The techniques were applied to the analysis of both transplanted and native shellfish, to native fish, water and sediment from a range of both impacted and non-impacted Irish coastal locations, generating valuable data to fill gaps both nationally and internationally.

Chapter 2: Methods

2.1 Introduction

The integration of chemical analysis data with biological effects monitoring is becoming increasingly relevant for the assessment of pollution in the environment, and has been recommended within the WFD, OSPAR, WKIMON and WGBEC. Following a detailed literature review (Chapter 1), liquid chromatography tandem mass spectrometry (LC-MS/MS) was chosen as the primary chemical assessment technique, to be conducted on both spot water and biota samples. The polar organic chemical integrative sampler (POCIS) was selected as an alternative method to spot water sample collection. The estrogen luciferase reporter gene assay (ER-LUC) was selected as a complementary biological effects method, used along with the alkali labile phosphate assay (ALP). The combination of techniques was deemed suitable for an integrated assessment of the presence and effects of EDCs as presented in Chapters 3 to 5 of this thesis.

This chapter firstly reports selection of EDCs of interest followed by “generic” sample collection and preparation information which is common regardless of the analytical technique employed. Details of the development and validation of two ultra-trace LC-MS/MS methods for the detection of selected EDCs in marine matrices are then discussed and finally other supporting methodologies which were completed external to this thesis are reported.

2.1.1 *Selection of compounds of interest*

In recent decades, scientific and legislative communities have become increasingly aware of the presence and effects of EDCs in the environment (Botham et al. 2003). Compounds which mimic estrogens are currently receiving the most attention, given their documented biological effects (Laganà et al. 2004). The steroid estrogens and the alkylphenols were selected as target compounds of interest in an Irish context and are thus studied within this thesis. The use, legislation and effects of these compounds are discussed in detail in Chapter 1.

2.1.2 *Selection and preparation of marine biota (shellfish and fish)*

Mytilus spp. were selected as target organisms due to their use within the monitoring programmes of OSPAR, the WFD and their continuing use in support of MSFD objectives. *Crassostrea gigas* (oysters) are among a number of shellfish species commercially produced in Irish waters and were selected as a target species for monitoring of EDC levels within designated SWD production waters.

Mussels and oysters from Redbank hatchery, New Quay, Co. Clare (N 53°09'27.28; W 09°04'03.84, Figure 2.1) were used for method development and validation purposes. New Quay is situated on the west coast of Ireland and has been defined as 'Class A' for the purposes of EC Regulation 854/2004. This means there is no indication of faecal contamination and wastewater, a major source of EDCs, in the area (DEHLG, 2007). Mussels measuring 4-6 cm and commercially sized oysters were collected and dissected, the whole soft tissue weight recorded and shell length measured to the nearest ± 0.1 mm using a callipers. Tissue was homogenised using a Waring blender (Waring Commercial, New Hartford, CT, USA), freeze dried and frozen at -30 °C prior to analysis. It should be noted that the Irish coastal zone contains a mixture of pure, hybrid and introgressed mussels (Coghlan and Gosling, 2007), and since there is no single morphological characteristic that can be reliably used to separate this mixed population, classification of the exact *Mytilus* species used was not possible.

Limanda limanda (dab), *Pleuronectes platessa* (plaice) and *Platichthys flesus* (flounder) were selected as target organisms as they are among the species of fish which are used as biomonitoring species within WGBEC, ICES, OSPAR and WKIMON. Samples of dab flounder and plaice were collected at four locations (Figure 2.1). Fish were sacrificed, the weight and length measured. Muscle and liver samples were collected and pooled from between 20-50 individuals. All biota were collected where present in accordance with OSPAR CEMP sampling protocols (OSPAR, 1999). Tissue was homogenised, freeze dried and frozen at -30 °C prior to analysis.

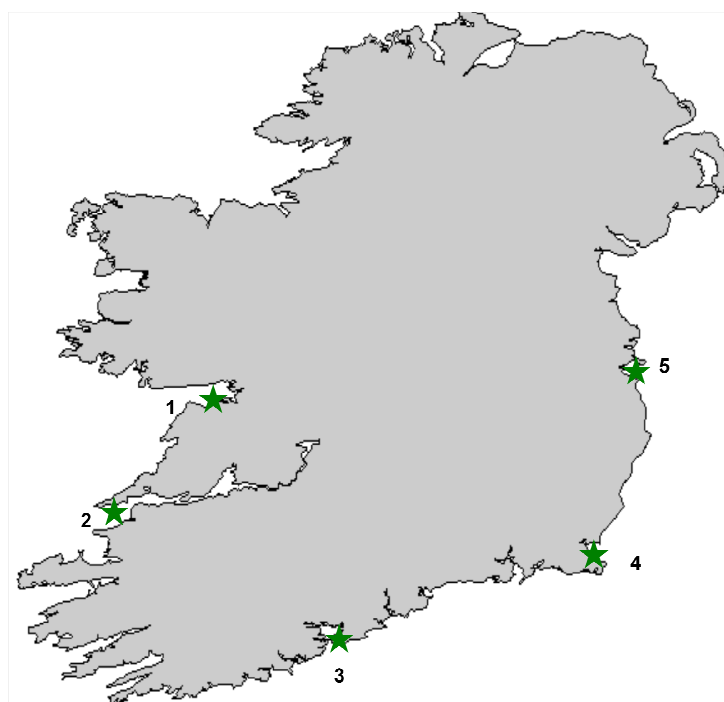


Figure 2.1: Sampling locations

1. Mussels, New Quay. 2-5 Fish, 2. Shannon, 3. Cork, 4. Wexford, 5. Dublin.

Samples collected as per the above protocols were then analysed utilising either of the two LC-MS/MS methods further described and validated below.

2.2 Development and validation of two sensitive LC-MS/MS methods for the determination of selected EDCs in environmental samples.

This section details the development and validation of two quality assured sensitive LC-MS/MS methods, the first for the detection of steroid estrogens at sub ng/L concentrations in seawater and at low ng/g concentrations in marine biota and the second, for the detection of 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) at ng/g concentrations in biota. As a result of their higher log K_{ow} values relative to the estrogens and their higher bioaccumulation factors in organisms (see Table 1.1) biota was selected as the target matrix for NP and OP analysis. Initially development of a multi-residue method for the steroid estrogens, NP and OP was attempted; however differences in optimal analysis conditions necessitated the development of two methods. The first method developed was for the steroid estrogens in water and biota, with the method for NP and OP in biota building upon this method, with some modifications discussed in detail below. The potential influence of marine focused co-factors on analytical results and application for the purposes of WFD screening/surveillance monitoring of E2 in coastal and transitional waters are evaluated. A schematic of the method is presented in Figure 2.2.

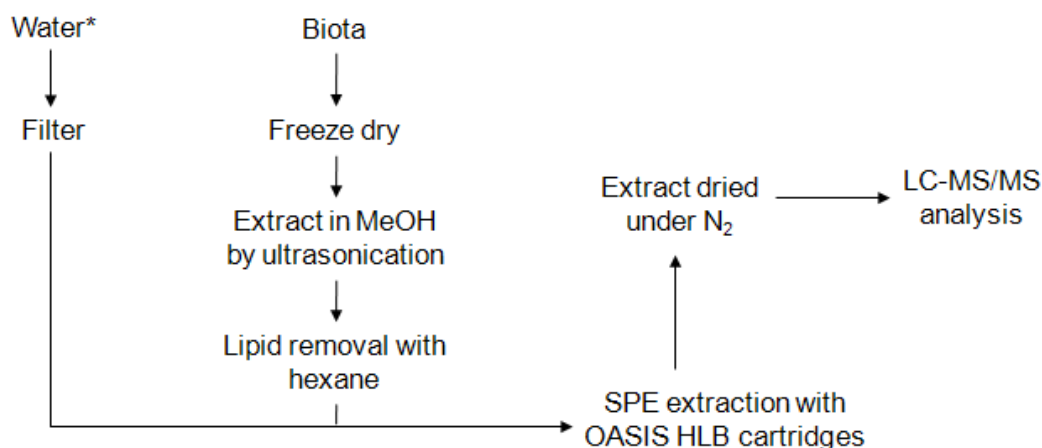


Figure 2.2: Schematic of extraction procedure.

* Water analysis was completed for E1, E2 and EE2 only. Biota analysis was completed for E1, E2, EE2, 4-NP and 4-t-OP

2.2.1 Chemicals

Pestiscan grade solvents acetonitrile, methanol, methyl tert butyl ether, ethyl acetate, and n-hexane (99% purity) were supplied by Fisher Scientific (Dublin, Ireland). Triethylamine, sodium chloride, sodium hydrogen carbonate, formic and acetic acids, formalin, E1, E2, 17 β -estradiol d₂ (E2-d₂), EE2 and warfarin (WF) standards were obtained from Sigma Aldrich (Dublin, Ireland). NP, OP and 4-NP-d₈ (NP-d₈) standards were obtained from Analytical Standards, (Augsburg, Germany). Purified deionised (DI) water was provided using a Barnstead water purification system (Thermo Scientific, Leicestershire, UK).

2.2.2 Standard preparation

Stock standard solutions of E1, E2, EE2, NP, OP and NP-d₈ were prepared in MeOH at 1 μ g per μ L, with the exception of E2-d₂ which was prepared at 200 ng per μ L. Working solutions of each analyte, mixtures and spiking solutions were prepared at different concentrations by appropriate dilution of the stock solution in MeOH. Final calibration standards for E1, E2 and EE2 in water analysis were prepared in 20:80 MeOH: H₂O while those for tissue were prepared in MeOH. Final calibration standards of NP and OP for tissue were prepared in MeOH. All solutions were stored at <4°C in the dark and used within one month.

2.2.3 Preparation of water samples for estrogen analysis

Deionised water was used for the development of the method for analysis of water samples. Artificial seawater of varying salinities were prepared using dilutions of a stock solution containing 175 g sodium chloride, 2.5 g sodium hydrogen carbonate and 5 L deionised water. Salinity was measured using a Sondes multi-parameter probe (YSI Inc. Ohio, USA). Water samples (five L) were filtered with 0.45 μ m glass fibre filters (Whatman GF-F, General Electric Company, New Jersey, US) prior to solid phase extraction (SPE). To each sample 50 ng/L internal standard E2-d₂ was added. As UV light has been demonstrated to degrade the steroid estrogens (Gabet-Giraud et al. 2010), the potential effects of UV degradation were minimised during development and sample analysis by ensuring all samples and extraction steps were protected from light. Samples were stored in the dark prior to analysis. Amber glassware was used in sample collection and storage, and during extraction and analysis. Aluminium foil was used to cover the glass lid of the Turbovap during sample concentration.

A summary of recoveries related to each development step is presented in Table 2.1, with detailed information on each step presented below.

Table 2.1 summary of method development stages-analyte recoveries (%) and RSDs (%)

	E1 recovery	RSD	E2 recovery	RSD	EE2 recovery	RSD	n=	NP recovery	RSD	OP recovery	RSD	n=
Water 100 mL (spiked at 5 ng/L)	65.4	27.1	72.2	31.4	69.4	27.4	5	-	-	-	-	-
Water 250 mL (spiked at 5 ng/L)	127.3	16.4	132.1	15.9	129.3	33.8	5	-	-	-	-	-
Water 500 mL (spiked at 5 ng/L)	117.2	18.8	114.7	13.2	109.4	19.4	5	-	-	-	-	-
Water 1000 mL (spiked at 5 ng/L)	102.5	7.7	93.6	9.4	107.7	13.1	5	-	-	-	-	-
Water 5000 mL (spiked at 5 ng/L)	97.3	6.7	95.4	8.7	102.2	9.2	5	-	-	-	-	-
SPE method 1	193.0	79.3	197.9	77.7	207.0	78.1	3	-	-	-	-	-
SPE method 2	145.7	20.9	146.0	23.2	152.8	13.8	3	-	-	-	-	-
SPE method 3	101.0	13.7	101.8	12.9	113.2	10.6	3	-	-	-	-	-
SPE method 4	136.9	13.3	145.2	18.1	153.9	9.0	3	-	-	-	-	-
SPE method 5	100.8	9.6	100.7	7.4	96.8	6.2	3	98.8	6.1	103.2	7.7	5
Analyte spiked on wet tissue	40.4	16.3	43.1	16.2	47.4	20.4	4	-	-	-	-	-
Analyte spiked on dry tissue	57.8	9.7	63.5	12.6	60.1	8.3	4	-	-	-	-	-
Extracted by ultra-sonic bath 1.A	44.7	6.8	48.2	9.9	50.3	8.1	4	100.8	5.3	105.4	8.3	4
Extracted by ultra-sonic bath 1.B	38.2	8.1	47.3	10.8	41.1	13.8	4	-	-	-	-	-
Extracted by ultra-turrax mixing	19.7	6.3	22.4	8.9	16.6	6.6	4	-	-	-	-	-
Extracted by ultra-sonic probe	50.8	3.9	56.4	8.2	59.5	6.2	4	102.7	2.1	103.4	5	4
Instrument repeatability	96.6	2.4	95.3	5.2	100.4	6.1	20	97.4	1.5	96.2	4	20
Between batch repeatability	98.0	7.3	101.4	7.5	99.7	6.2	25	99.3	4.9	99.8	6.2	10

Development work in grey was conducted using aqueous standards for quantitation

Development work in white was conducted using matrix-matched standards for quantitation

n= number of replicates for each treatment. - = not done.

2.2.4 Development of solid phase extraction (SPE) method

The selection of an appropriate solid sorbent to the target analyte as well as the use of solvents for washing and elution is fundamental to EDC analysis (Chang et al. 2009). Oasis HLB cartridges were selected for use in these methods as they are the most commonly used cartridges for the extraction of estrogens from environmental samples with a number of elution methods, and reported recoveries generally ranging from 75-100% (Gabet et al. 2007), and they are also widely used for preconcentration of APs (Ferreira-Leach and Hill, 2001; Vigano et al. 2006). Development of the SPE method was first conducted for the steroid estrogens in water samples. Five different elution methods were trialled for the SPE step with deionised water spiked with 60 ± 1 ng/L of E1, E2 and EE2. Three were existing methods (Waters Corporation, 2003; Viganò et al. 2006; Miège et al. 2009), with a fourth and fifth adapted from these methods. Based on the bed capacity of the SPE cartridges 2 mL of the MeOH elution solvent was selected, which reduced drying and sample processing time (method 5). Table 2.2 shows that the elution method as developed in this method showed the most reproducible and accurate results of the methods investigated (method 5).

Table 2.2: SPE validation parameters (recovery (%) and RSD (%)) in water

Method	E1 recovery	RSD	E2 recovery	RSD	EE2 recovery	RSD	Reference
1	193.0	79.3	197.9	77.7	207.0	78.1	Waters Corporation, 2003
2	145.7	20.9	146.0	23.2	152.8	13.8	Miège et al. 2009
3	101.0	13.7	101.8	12.9	113.2	10.6	Viganò et al. 2006
4	136.9	13.3	145.2	18.1	153.9	9.0	Waters Corporation, 2003; Viganò et al. 2006
5	100.8	9.6	100.7	7.4	96.8	6.2	Current method modified from above

The final SPE method used Oasis HLB 3 mL, 60 mg SPE cartridges (Waters, Milford, USA). Cartridges were rinsed and conditioned with 3 mL pestiscan grade methanol and deionised water, prior to addition of the sample extract. Following this, cartridges were

rinsed with 3 mL 5% MeOH in deionised water and eluted in 2 mL methanol under a gentle vacuum. This was reduced to dryness under nitrogen at 35°C and resuspended in 130 µL of 1:4 methanol: water by vortex mixing for 30 seconds. 10 ng of external standard Warfarin (WF) was added. 20 µL of this solution was injected into the HPLC system.

2.2.5 Influence of sample volume on method performance

The impact of sample volume was examined by extraction of spiked water volumes ranging from 100 mL to five L. Recoveries at each volume (100, 250, 500, 1000 and 5000 mL) were between 65-127% for E1, 72-132% for E2, and 69-129% for EE2 (see Table 2.1). At low concentrations and low sample volumes (e.g. 0.5 ng in 100 mL, equivalent to 5 ng/L) recoveries were highly variable. Increasing the sample volume to five L improved the reproducibility of results and enhanced detection capacity. Although analyte recoveries and RSDs at 1 L were also satisfactory, a five L sample size was selected as the optimal volume for analysis purposes in order to enhance the detection capacity of the method for marine samples, where the concentrations of selected EDCs are expected to be low.

2.2.6 Development of tissue extraction method

The tissue extraction method was modified from a number of existing extraction methods for water, sediment and biota (Labadie and Budzinski, 2005; Viganò et al. 2006; Urbatzka et al. 2007; Baugros et al. 2008; Viganò et al. 2008; Zafra-Gómez et al. 2008), and further developed and validated to be specific to the extraction of complex shellfish tissues as detailed below.

For steroid estrogens, three extraction methods for biological tissues were trialled, with E1, E2 and EE2 spiked at 20 ng/g dry weight (dw) as follows:

1. Ultrasonication using a sonic bath

MeOH was added to a 50 mL glass centrifuge tube containing 0.25 g of freeze dried tissue and extracted by ultrasonication in a sonic bath as follows:

- A. Ten mL MeOH for 15 minutes, samples centrifuged at 3000 rpm for 5 minutes and supernatant collected. A further 10 mL MeOH was added to the pellet and re-extracted for 15 min, and centrifuged as above. The supernatants were pooled.
- B. 20 mL MeOH was added and the pellet sonicated for 30 minutes.

Recoveries using method 1.A were 45, 48 and 50% with RSDs of 7, 10 and 8% for E1, E2 and EE2, respectively. Recoveries using method 1.B were 38, 47 and 41% with RSDs of 8, 11 and 14% for E1, E2 and EE2, respectively.

2. Mixing by ultra turrax

Ten mL MeOH was added to a 50 mL glass centrifuge tube containing 0.25 g of freeze dried tissue and extracted by mixing with an ultra turrax for one minute. The probe was rinsed with 10 mL MeOH for 30 seconds. The samples were centrifuged at 3000 rpm for 5 minutes. The supernatant was collected. 10 mL MeOH was added to the pellet and re-extracted for 1 min using the ultra turrax, with centrifugation as above. The supernatants were pooled. Recoveries for all analytes using this method were 19.7, 22.4 and 16.6% with RSDs of 6.3, 8.9 and 6.6% for E1, E2 and EE2, respectively.

3. Ultrasonication by ultrasonic probe

Ten mL MeOH was added to a 50 mL glass centrifuge tube containing 0.25 g of freeze dried tissue and extracted by mixing with an ultrasonic probe for one minute. The probe was rinsed with 10 mL MeOH for 30 seconds. The samples were centrifuged at 3000 rpm for 5 minutes. The supernatant was collected. 10 mL MeOH was added to the pellet and re-extracted for 1 min using the ultrasonic probe, with centrifugation as above. The supernatants were pooled. Recoveries using this method were 51, 56 and 59% with RSDs of 4, 8 and 6% for E1, E2 and EE2, respectively.

The ultrasonic probe was selected for use in the method as it gave the most reproducible results and highest analyte recoveries.

Two of the above extraction methods were trialled for 4-NP and 4-*t*-OP:

1. Sonication in an ultrasonic bath conducted as per the method 1.A for estrogens.
2. Sonication by ultrasonic probe conducted as per the method 3 for estrogens.

Recoveries for NP and OP using the ultrasonic probe were 102.7 and 103.4% with RSDs of 2.1 and 5%, respectively. Recoveries for NP and OP using the sonic bath were 100.8 and 105.4% with RSDs of 5.3 and 8.3%, respectively. While RSDs were slightly higher using extraction in the sonic bath, they were still low and were deemed acceptable. There was no statistically significant difference in recoveries ($p > 0.5$), thus sonication in an

ultrasonic bath (2) was selected for NP and OP, as this step reduced the overall extraction time improving the efficiency in terms of sample throughput.

For each treatment, lipid removal was conducted after extraction by centrifugation of the extract at 3000 rpm for 5 minutes with 20 mL of n-hexane (99%). The methanol fraction was reduced to incipient dryness under nitrogen in a Turbopap at 35°C and resuspended with 9 mL deionised water prior to SPE.

2.2.7 Clean up by solid phase extraction-tissue

The SPE method developed and selected in section 2.2.4 was applied to the analysis of estrogens in biota. The following modifications were applied for NP and OP: After the sample eluted in 2 mL methanol, the extract was reduced to approximately 40 µL under nitrogen at 35°C. The weight of the final extract was recorded. 20 µL of this solution was injected into the HPLC system. 20 µL was retained for repeat analysis and stored in the dark at < 4°C. The solvent transfer to 20:80 MeOH:H₂O was not necessary for NP and OP, as their ionisation was most efficient in MeOH.

2.2.8 Wet versus dry tissue

Fortified tissue was prepared in two ways: A: by adding spiking solutions gravimetrically to dry homogenised tissue or B: by adding spiking solutions gravimetrically to wet homogenised tissue, and allowing to sit for 10 minutes prior to extraction. Fortified tissue was taken through the extraction procedure. The optimal recoveries were obtained using freeze dried tissue ($57.8 \pm 9.7\%$, $63.5 \pm 12.6\%$ and $60.1 \pm 8.3\%$ for E1, E2 and EE2 in dry tissue vs. $40.4 \pm 16.3\%$, $43.1 \pm 16.2\%$ and $47.4 \pm 20.4\%$ for E1, E2 and EE2 in wet tissue). All tissues were therefore freeze-dried before extraction and analysis.

2.2.9 Calibration: matrix effects and external standardisation.

Ion suppression, and less frequently, enhancement, due to matrix interference from co-extracted matrix components is a common problem in ESI LC-MS/MS analysis when analysing biological matrices (Gomez et al. 2006). As no certified reference materials were available, relative retention times were attained by assessing recovery of additions of known amounts of the target analytes to spiked blank tissue/water. Aqueous standards were used during development of the method for the detection of E1, E2 and EE2 in water. Calibration standards in 20:80 MeOH: H₂O were injected prior to samples and the

calibration curve generated from these standards was used to quantify analytes present in the samples. E2-d₂ was added to each sample prior to extraction and was used to quantify losses during the extraction. WF was used as an external standard. This method of calibration was found to be satisfactory, with recoveries of target analytes in fortified artificial seawater found to be in the range of 80-110%. Initial method development for these compounds in biota also used aqueous standards (sections 2.2.6 to 2.2.8), but recoveries using this means of quantitation were poor (19-64%).

Two different methods of standardization were trialled for steroid estrogens in biota. The first used E2-d₂ as internal standard and the second used WF as external standard. Six blank mussel tissues were spiked with 25 ng/g E1, E2, EE2 and E2-d₂ and taken through the extraction procedure as documented in section 2.2.6 to 2.2.9. WF was used as external standard added to each vial added directly before LC-MS/MS analysis at an injected concentration of 10 ng/mL. Using WF to quantify sample concentrations produced more reproducible results than those calculated using E2-d₂ (88 to 102%, RSD 3.2 to 9% using WF compared with 59 to 70% RSD of 35 to 42% using E2-d₂). WF was then selected as external standard for quantification, with E2-d₂ used to confirm retention times and to quantify any losses throughout the extraction and clean-up. In each batch, blank tissue was fortified gravimetrically with target analytes over a concentration range of 1.5 to 500 ng/g ww and taken through the extraction procedure as detailed above. A standard curve was generated from the spikes and was used to quantify analytes present in the samples/spiked in-house material. Recoveries of target analytes in a further 20 spikes using this method were $99.1 \pm 9.2\%$, $101.8 \pm 9.5\%$, and $99.6 \pm 7.9\%$ for E1, E2, and EE2, respectively.

Quantitation of NP and OP was performed using 4-Nonylphenol-d₈ (ring D4- ethylD4) as internal standard. Blank tissue was fortified gravimetrically with NP and OP over a concentration range of 16 to 500 ng/g ww. 25 ng/g dw NP-d₈ was added, and the spikes taken through the extraction procedure as detailed above. A standard curve was generated from the spikes and was used to quantify analytes present in the samples/spiked in-house material. Recoveries of target analytes using this method were $100.4\% \pm 5.7\%$ and $99.2 \pm 7.3\%$ for NP and OP, respectively.

As a quality control measure in every batch completed, blank tissue was spiked and taken through the extraction procedure, with recoveries of these spikes used to assess the batch

performance. Tissue concentrations were reported on a wet weight basis, with conversion factors calculated based on the tissue wet weight divided by dry weight for each sample.

2.2.10 Liquid chromatography

The HPLC system consisted of an Agilent 1200 Series Game Pad, Agilent 1200 Binary Pumps, an Agilent 1200 High Performance Autosampler and an Agilent 1200 thermostatted column compartment (Agilent Technologies Deutschland GmbH, Böblingen, Germany). For LC separation a Kinetex 4.6 x 50 mm, 2.1 mm id C18 2.5 µm particle size column and a Luna 5 µm C8 100 Å 50 x 2.0 mm column were tested. The sharpest peaks and best separation were achieved with the Kinetex C18 along with a Krudcatcher® guard column. The mobile phases selected were deionised water (A) and 5:95 water: acetonitrile (B). Triethylamine (TEA) was selected as the mobile phase buffer. Percentages ranging from 0.001 to 0.1% TEA were trialled, with 0.025% TEA in each mobile phase producing the most intense signal responses for the steroids. Formic acid and ammonium formate were also tested as buffers, but failed to produce sufficiently intense signals. MeOH was tested in place of ACN in mobile phase B, but resulted in poor separation of target analytes. Full separation is not necessary for the selective MS/MS detection (Gomez et al. 2006), but was achieved for most of the compounds analysed in this method (see Figure 2.3). The final mobile phases used for E1, E2 and EE2 were 0.025% TEA in deionised water (A) and 0.025% TEA in 5:95 water: acetonitrile (B) flowing at 300 µL per minute with a gradient as reported in Table 2.3. The column oven temperature was 30°C. For NP and OP the mobile phases were 0.025% TEA and 0.2% ACN in deionised water (A) and 0.025% TEA in acetonitrile (B) flowing at 300 µL per minute with a gradient as per Table 2.4. The column oven temperature was 40°C.

Table 2.3: LC gradient for the measurement of steroid estrogens in water and marine biota.

Time (min)	% mobile phase A	% mobile phase B
0.00	95	5
2.00	68.5	31.5
7.00	66	34
7.01	0	100
9.00	0	100
9.01	95	5
12.00	95	5

Table 2.4: LC gradient for the measurement of NP and OP in marine biota.

Time (min)	% mobile phase A	% mobile phase B
0.00	60	40
1.50	55	45
2.00	55	45
4.00	45	55
5.00	45	55
5.50	40	60
6.50	40	60
7.00	30	70
9.50	20	80
10.00	60	40
14.00	60	40

2.2.11 Electrospray tandem mass spectrometry

For tandem mass spectrometry (MS/MS) experiments, an Applied Biosystems 3200 Q-TRAP was used (Applied Biosystems, Foster City, CA, USA). Due to the complexity of the biological matrix tandem MS was selected. Interpreting the relative retention time and the precursor and product ions in the mass spectrum provided the specificity of this method for detecting the analytes studied. Optimisation of the ion source and MS/MS settings was performed by the automatic optimisation function of the MS software (Analyst 1.4, Applied Biosystems, Foster City, CA, USA), and fine tuned manually using infusion with a syringe pump and flow injection analysis of standard solutions. Optimisation was conducted with solutions of target analytes in 20:80 MeOH: H₂O, 50:50 MeOH: H₂O and 100% MeOH. For quantification the multiple reaction monitoring mode (MRM) was selected. The relevant instrument settings for each precursor-product ion transition and method performance parameters are detailed in Table 2.5.

Table 2.5: Summary LC-MS/MS instrument and method performance parameters in water and biota

	E1	E2	EE2	4-NP	4-t-OP
Precursor ion (m/z)	269	271	295	219	205
Product ions (m/z)	145, 142	145, 183	145, 199	106, 119	133,92.9
Water parameters (ng/L)					
LOD	0.07	0.07	0.11	n/a	n/a
LOQ	0.12	0.12	0.15	n/a	n/a
Linear range	0.07-60	0.07-60	0.11-60	n/a	n/a
R ² Standard curve water	0.999	0.999	0.999	n/a	n/a
Recovery (%) at LOQ	97.2	97	102	n/a	n/a
Precision (RSD %) at LOQ	9	3.1	4.3	n/a	n/a
Tissue parameters (ng/g ww) shellfish					
LOD	0.4	0.9	0.3	4.00	6.6
LOQ	0.7	1.7	0.6	6.80	11.6
Linear range	0.4 - 200	0.9 - 201	0.3 - 202	1.4-406	3.6-538
R ² Standard curve	0.999	0.996	0.999	0.997	0.999
Recovery (%) at LOQ	88	100	93.2	102.4	96.1
Precision (RSD %) at LOQ	8.7	8.2	3.2	5.3	4.1
Tissue parameters (ng/g ww) fish muscle					
LOD	0.4	0.9	0.3	34	34
LOQ	0.7	1.7	0.6	68	68
Linear range	0.4 - 200	0.9 - 201	0.3 - 202	34-700	34-700
R ² Standard curve	0.999	0.996	0.999	0.999	0.993
Recovery (%) at LOQ	88	100	93.2	99.6	99.7
Precision (RSD %) at LOQ	8.7	8.2	3.2	4.37	5.47
Tissue parameters (µg/g ww) fish liver					
LOD	0.05	0.09	0.2	0.18	0.18
LOQ	0.1	0.2	0.4	0.31	0.31
Linear range	0.05-0.9	0.09-0.9	0.2-0.9	0.18-0.85	0.18-0.85
R ² Standard curve	0.997	0.991	0.999	0.997	0.999
Recovery (%) at LOQ	104.9	101	100.3	102.9	100.5
Precision (RSD %) at LOQ	6.8	1.4	3.4	11.1	3.13

The electrospray ion source (Turbo-Ionspray, Applied Biosystems) was operated in negative mode at 550 °C. Nitrogen was used as nebulizer, drying, curtain and collision gas. Gas one (GS1-nebuliser gas) was adjusted to 35 psi. Gas two (GS2-drying gas) was adjusted to 50 psi. Curtain gas pressure was optimised to 44 psi. The dwell time for each parent-product ion was 75 ms. For NP and OP ionisation was most intense in MeOH. The electrospray ion source was operated at 575 °C. GS1 was adjusted to 65 psi. GS2 was adjusted to 25 psi. Curtain gas pressure was optimised to 44 psi. The dwell time for each parent-product ion was 100 ms. A LC-MS/MS trace of NP and OP matrix matched standard is presented in Figure 2.4. Optimal signal intensities for the APs were found for the compounds in MeOH, while for the estrogens, optimal intensities were observed in 20:80 MeOH: H₂O.

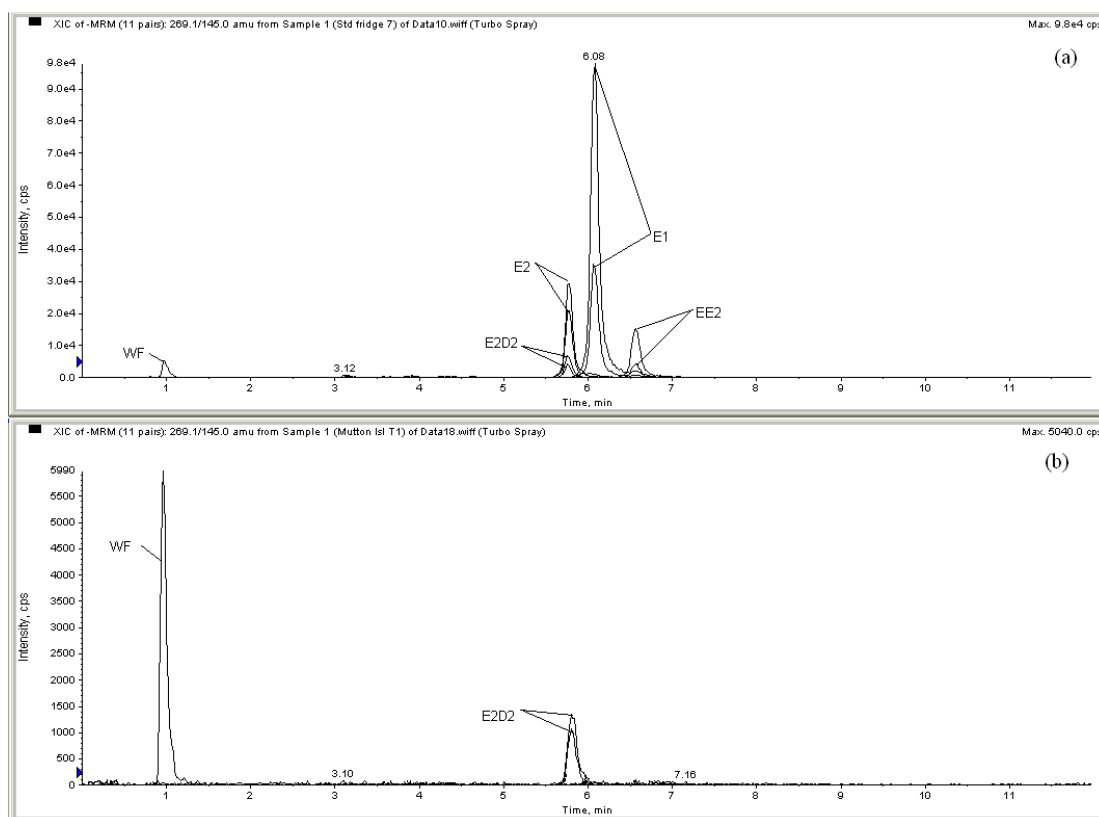


Figure 2.3: (a) Separation of external standard Warfarin (WF), followed by E2, E2d₂, E1 and EE2 by LC-MS/MS, (b) chromatogram of a formalin treated water sample.

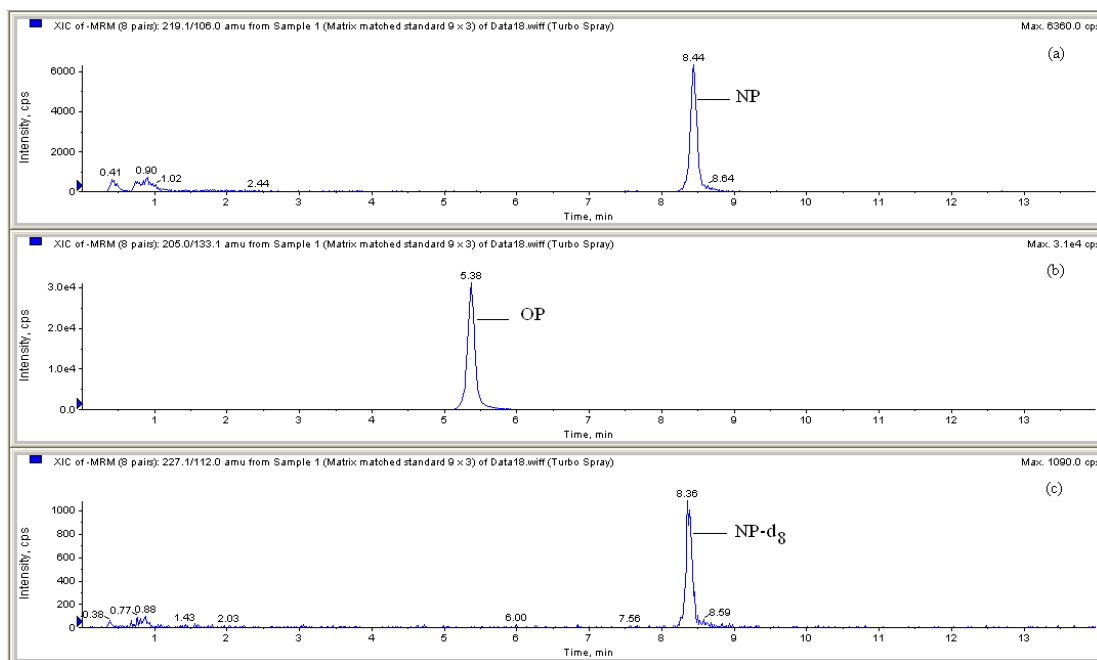


Figure 2.4: Trace of (a) NP, (b) OP and (c) NP-d₈ in fortified mussel tissue.

2.2.12 Quality control of analysis.

For each analysis of both water and biota a solvent blank, procedural blank and fortified quality control (QC) samples along with repeat injections of a working standard were analysed as quality controls. Recovery of additions of known amounts of the target analytes to QC samples (artificial seawater-estrogens and fortified blank tissue-estrogens and APs) were used to assess batch performance as no appropriate certified reference materials were available. Method performance based on the recoveries of fortified water and mussel tissue are presented in Table 2.5.

2.2.13 Method performance characteristics

A variety of performance parameters were evaluated in order to quantify the fitness for purpose of the analytical methods. Details are presented below and summarised in Table 2.5.

2.2.13.1 Limits of detection and quantitation (LOD and LOQ)

Method detection limits were determined using the response factors of each analyte at a range of concentrations, plotted against the analyte concentration (see Figure 2.5 for an example of a response factor plot used). Ten analyses were completed at each concentration, with the standard deviation of the mean response factor at each concentration plotted against the analyte concentration and calculated at a 99% confidence

interval. The lowest concentration within the 99% confidence interval was selected as the limit of detection. The second lowest concentration was accepted as the LOQ. Although peaks were detected at lower concentrations, they failed to fit within the 99 % confidence interval and had a greater variability in response. Response factors were calculated as follows:

$$RF = \text{analyte peak area} / \text{external standard peak area}$$

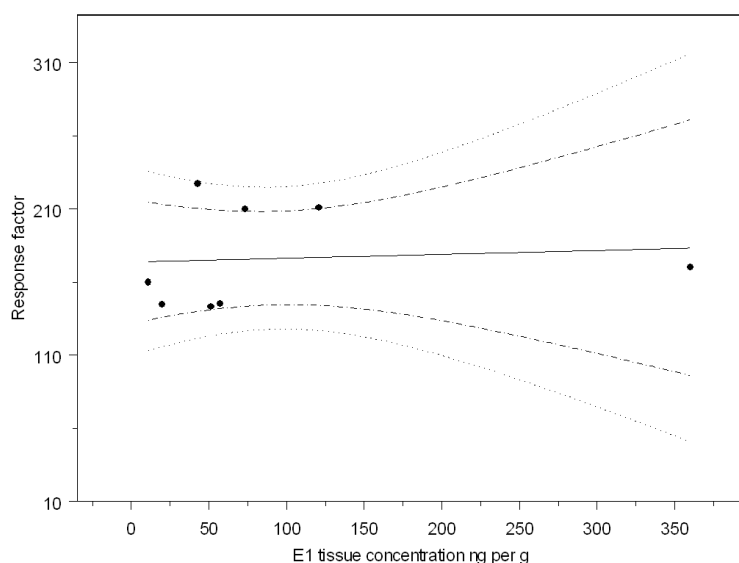


Figure 2.5: Response factor (RF) plot for E1 in tissue with concentration in ng/g ww.

Each point represents the mean RF at each standard concentration. Depicted are 95% confidence interval (inner dashed line) and 99% confidence interval (outer dotted line) for each of the parameters in both water and biota.

2.2.13.2 Linearity

Linearity was assessed for E1, E2 and EE2 over a concentration range of 0.2 to 500 ng/g ww for biota, and 0.2 to 60 ng/L for water samples using fortified blank samples. Linearity was assessed for NP and OP over a concentration range of 1 to 850 ng/g ww for biota using fortified blank samples. Data for the linear range of each analyte is presented in Table 2.5. Acceptable regression for the standard curve was set at > 0.99. The standard curve for E1 in water is shown in Figure 2.6.

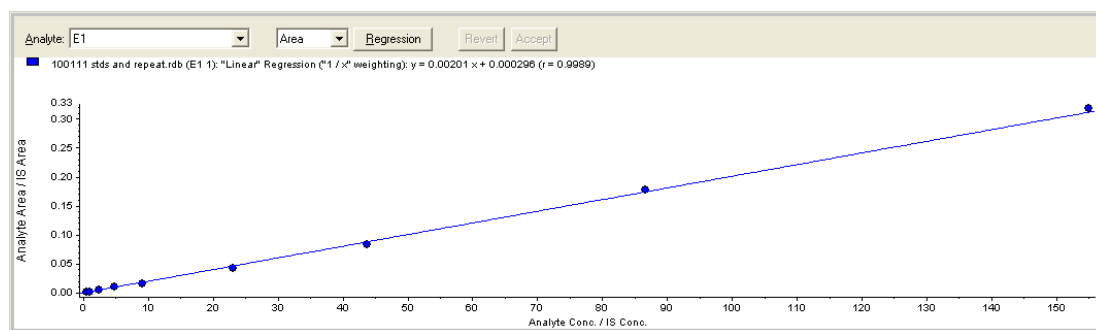


Figure 2.6: Linearity of E1 in water. Regression ($r > 0.99$).

2.2.13.3 Repeatability and precision.

Instrument repeatability was assessed using repeat injections of standard solutions ($n=20$). RSD for E1, E2 and EE2 standards in 20:80 MeOH: H₂O were 2.4, 5.2 and 6.1%, respectively. RSDs for NP and OP in MeOH were 1.45 and 3.99%, respectively. In the absence of a certified reference material, between-batch repeatability of the full analytical methods was assessed using injections of fortified blank material ($n=25$ for E1, E2, EE2, $n=10$ 4-NP and 4-*t*-OP). Recoveries were $98.6 \pm 7.3\%$, $101.4 \pm 7.5\%$ and $99.7 \pm 6.2\%$ and for E1, E2 and EE2 respectively. Recoveries were $99.3 \pm 4.9\%$ and $99.8 \pm 6.2\%$ in shellfish, $100 \pm 3.6\%$ and $96.4 \pm 9.4\%$ in fish muscle and $100 \pm 9\%$ and 100 ± 2.1 in liver for 4-NP and 4-*t*-OP respectively. Precision or accuracy was completed for each compound using determinations of fortified blank material. Precision (expressed as relative standard deviation) is presented in Table 2.5.

2.2.14 Proficiency testing schemes

To further support method validation, samples from the independent proficiency testing (PT) scheme managed by Aquacheck LGC Proficiency Standards testing in the UK. Trial Rounds (AS2921, round 137) were completed in the course of this study. This PT scheme was only completed for the steroid estrogens. Although the PT scheme was for the steroid estrogens in effluent samples, and the method developed as part of this research is for water samples, it was the only external independent means of assessing the performance of the method. 400 mL blind spiked artificial effluent samples were analysed for E1, E2 and EE2. Satisfactory results were obtained for E1 samples only, Overestimations of concentrations of 60 and 87% were reported for E2 and EE2 at average concentrations of 8 and 3 ng/L assigned for each respectively.

There are a number of potential reasons for the unsatisfactory E2 and EE2 results. The current method has been validated for water samples with varying salinities, while the proficiency testing material provided was artificial effluent. The method was also further optimised for five L water samples. During validation reproducibility and recoveries were noted to become more variable at low concentrations in small sample volumes with over-recoveries recorded at lower sample volumes (see Table 2.1). Stock solutions provided by Aquacheck were in acetone, whereas all development for this method has been conducted using spiking solutions in MeOH. These factors may have impacted on the performance of the method, thus the proficiency testing results are taken as being indicative of method performance only. During the course of method development, the Aquacheck PT scheme served as the most independent method of assessing the method performance. Participation in future PT schemes specifically for steroids in water samples will further test the suitability of this method. Repeatability and recoveries of the target analytes from spiked in house quality controls analysed in the same batch as the proficiency testing materials were 98.6, 101.4 and 99.7 ± 7.3 , 7.5 and 6.2% for E1, E2 and EE2 respectively.

No proficiency testing schemes or certified reference materials (CRMs) are currently available for the steroid estrogens or APs in mussel or fish tissue, so method performance was assessed based on the recoveries of fortified mussel and fish tissue.

2.2.15 Investigation of potential impacts of physicochemical cofactors on EDC analysis

Due to the fact that seawater has a number of physicochemical differences to that of other riverine and/or ground waters, the potential for analytical interference in respect of particulate matter content and salinity were evaluated under controlled laboratory conditions as detailed below.

2.2.15.1 Evaluation of the effect of particulate matter on EDC concentrations in marine water.

Suspended particulate matter in seawater is typically <15 mg/L (Anderson and Meyer, 1986; Dobrynin et al. 2010), with increases up to 70 mg/L occurring in certain areas, such as river plumes (Ferraria et al. 2003; Dobrynin et al. 2010). EQS values are set for total water, which includes the particulate phase. Water samples in this study were filtered (<0.45 µm) prior to analysis to remove larger debris and some particulate matter which may block the SPE cartridges. To assess the potential impact of filtration/removal of particulate matter loadings on analyte recovery, particulate matter (sediment particle size <63 µm) was added at 10 mg/L and 100 mg/L to both spiked and blank jars containing

deionised water in triplicate. Jars containing both spiked (at 50 ng/L) and unspiked water without PM addition were also analysed. Jars were shaken overnight on an orbital shaker (Stuart Ltd), filtered, extracted and analysed by LC-MS/MS. PM retained on each filter paper was also extracted and analysed. Analysis of the extracted filter papers were <LOD (range 0.4 to 1.7 ng/g dw) for all compounds. Recoveries of dissolved E1, E2 and EE2 in the filtered extracts were 51, 84 and 51% respectively. The fact that levels were not detected in the particulate phase but with reduced recovery in the water phase suggests that all three compounds primarily remained in the water phase, but may be subject to differing degrees of degradation during the overnight shaking process at room temperature. Similar reductions in EE2 concentrations have been noted by Ciocan et al. (2010) and in results presented in Chapter 3 in spiked seawater after 24 hours. Low levels as reported on particulate matter are supported by Lopez de Alda and Barceló (2001) who demonstrated that filtration did not lead to significant losses of estrogens and progestogens from water and sediment.

2.2.15.2 Evaluation of the effect of salinity on recovery of target analytes

As estuarine and marine waters will differ in their salinity profile the potential for saline induced effect on analysis was examined. Saline water was prepared at a range of salinities (12, 24 and 35‰) using deionised water, sodium chloride and sodium hydrogen carbonate. This water was spiked with E1, E2 and EE2 at 80 ± 2 ng/L. Recoveries ranged from 91 to 108% with RSDs of between 3 and 11%. Recoveries were compared using a one way analysis of variance (ANOVA). Assumption tests of normality and equal variance were performed prior to ANOVA. There were no statistically significant differences ($p = 0.14$, 0.77 and 0.13 for E1, E2 and EE2, respectively) between salinities thus salinity alone was deemed not to influence dissolved concentrations of EDCs.

2.2.16 Summary of validated methods

The validated methods were performed as follows:

For steroid estrogens in water, five L water samples were filtered with $0.45 \mu\text{m}$ glass fibre filters (Whatman GF-F, General Electric company, US) prior to SPE. To each sample, 50 ng/L E2-d₂ was added. Bakerbond SPE™ 75 ml reservoirs (JT Baker, Avantor Performance Materials, Centre Valley, PA, USA) were attached to the SPE cartridges to enable application of the sample. Sample clean-up was conducted using Oasis HLB 3 mL, 60 mg SPE cartridges (Waters, Milford, MA, USA). Cartridges were rinsed and

conditioned with 3 mL pestiscan grade methanol and deionised water, prior to addition of the sample extract. Cartridges were rinsed with 3 mL 5% MeOH in deionised H₂O, and samples eluted in 2 mL methanol under a gentle vacuum. This was reduced to dryness under N at 35°C and resuspended in 130 µL of 1:4 methanol: water. Ten ng of external standard Warfarin (Sigma Aldrich, St. Louis, MO 63103, USA) was added. 20 µL of this solution was injected into the HPLC system and quantified as detailed above.

Tissue samples for steroid estrogens were extracted as follows: Ten mL MeOH was added to a 50 mL centrifuge tube containing 0.5 g of freeze dried tissue which had been spiked with 25 ng/g E2-d₂, and extracted by ultrasonication for one min. Samples were centrifuged at 2800 g for five min and the supernatant collected. The probe was rinsed with 10 mL MeOH for 30 seconds. Ten mL MeOH was added to the pellet and re-extracted for one min by ultrasonication, and centrifuged as above. Lipid removal was conducted by centrifugation of the extract at 2800 g for five minutes with 20 mL of n-hexane (99% purity). The MeOH fraction was reduced to incipient dryness under nitrogen at 35°C and resuspended with nine mL deionised water. The extract was then applied to SPE cartridges, with SPE conducted as per water samples. Recoveries of target analytes using this method were 99.1 ± 9.2%, 101.8 ± 9.5% and 99.6 ± 7.9% for E1, E2 and EE2 respectively.

Tissue samples for NP and OP analysis were extracted as follows: Ten mL MeOH was added to a 50 mL centrifuge tube containing 0.5 g of freeze dried tissue which had been spiked with 25 ng/g NP-d₈, and extracted in an ultrasonic bath for 15 minutes. Samples were centrifuged at 2800 g for 5 minutes and the supernatant collected. 10 mL MeOH was added to the pellet and re-extracted for 15 min by ultrasonication in an ultrasonic bath, and centrifuged as above. Lipid removal was conducted by centrifugation of the extract at 2800 g for 5 minutes with 20 mL of n-hexane (99%). The MeOH fraction was reduced to incipient dryness under nitrogen at 35°C and resuspended with 9 mL deionised water. Sample clean-up was conducted using Oasis HLB 3 mL, 60 mg SPE cartridges. Cartridges were rinsed and conditioned with 3 mL pestiscan grade methanol and deionised water, prior to addition of the sample extract. Cartridges were rinsed with 3 mL 5% MeOH in deionised H₂O, and samples eluted in 2 mL methanol under a gentle vacuum. This was reduced to near dryness (approximately 100 µL) under N at 35°C and weighed. 20 µL of this solution was injected into the HPLC system and quantified as detailed above. Recoveries of target analytes using this method were 100.4% ± 5.7% and 99.2 ± 7.3% for NP and OP, respectively.

2.3 External analysis: passive sampling and biological effects techniques

The following methods were used within this research project but were not developed and validated as part of this thesis. The analysis of samples using these methods was performed by a number of researchers, who are cited accordingly. Results derived from these methods form part of the data set analysed within this thesis, and were selected due to the additional information they provide with respect to the presence and effects of EDCs in the marine environment. It should be noted that derivation of the integrated sampling program, sampling design, collection and preparation in addition to final data assessment were all completed as part of this thesis.

2.3.1 Passive sampling

Concentrations of these EDCs reported in the literature have mostly been obtained using the collection of spot water, sediment or biota samples, followed by extraction, clean up and analysis in the laboratory using a suitable mass based technique such as liquid or gas chromatography (LC or GC) coupled with mass spectrometry (MS) (Gabet et al. 2007; Streck, 2009). The analytical difficulties associated with the determination of low estrogen concentrations have proved challenging (Baronti et al. 2000) and the detection of these compounds at and below the existing and proposed EQS values using traditional spot water samples and MS methods has proved difficult.

Passive sampling (PS) technologies are emerging as sensitive techniques to detect trace levels of pollutants in water. They are cost effective, easy to deploy and manage and do not show the same variability as traditional spot sampling. In 2009, the EC published a guidance document for surface water chemical monitoring under the WFD (EC, 2009). Within this document, passive sampling devices are suggested as complementary methods which can be used in surveillance, operational and investigative monitoring. They may be used to corroborate or contradict spot sampling data, as they are less affected by short term fluctuation in concentrations, or as screening tools to identify problem and non problematic areas. Recently the ICES Working Group on Biological Effects of Contaminants (WGBEC 2007) acknowledged the advantages of combining the use of PS and “bioanalyses” as important links between the WFD and MSFD and recommends their use.

The polar organic chemical integrative sampler (POCIS) is a passive sampler capable of sequestering polar compounds ($\log K_{ow} < 4$) from the water phase (Alvarez et al. 2004). E1, E2, EE2, NP and OP have been shown to concentrate in POCIS (Alvarez et al. 2007;

Morin et al. 2012). Based on results to date, POCIS remains in the integrative phase of sampling during exposure periods of at least 30 days (Alvarez et al. 2007). There have been numerous studies on the derivation of time weighted average (TWA) contaminant concentrations in POCIS, with a view to using the device in a qualitative manner. A range of calibration data and sampling rates determined in laboratory studies for POCIS have been reviewed and reported at between 0.02 and 0.85 litres per day for E1, E2 and EE2 (Morin et al. 2012). The average estimated uptake rate from these values is 0.42 L/day. It could be estimated that the approximately 12.6 L of water will pass through a sampler during deployment (based on a 30 day deployment) using 0.42 L/day, but this value could be as low as 0.6 L or as high as 25.5 L sampled in 30 days if using the lowest and highest sampling rates reported, respectively. The average value (0.42 L/day) will be applied to samplers deployed during the course of this research to calculate estimated water concentrations. It is important to note however that it has proven extremely difficult to accurately assess sampling rates which consider all the environmental factors in a sampling site, thus POCIS is most commonly accepted for use as a screening device. Any water concentrations derived using this value (0.42 L/day) and reported in this thesis are extremely tentative and are included to provide indications of levels of compounds which may be present only. Further confirmatory analysis using spot water samples is essential and is recommended where compounds are detected using POCIS.

All POCIS devices were supplied by the National Laboratory Service, Environment Agency in the United Kingdom. Samplers were extracted and analysed by the EA lab as described in section 2.3.1.1 to 2.3.1.3 below. POCIS devices used in this study consisted of a layer of Waters Oasis HLB sorbent (230 mg approx) sandwiched between two membrane layers of polyethersulphone polymer, fixed in place by two circular steel rings secured with stainless steel nuts and bolts. The POCIS were mounted on holders (3 per sampler) and placed inside a protective perforated stainless steel housing (Figure 2.7).

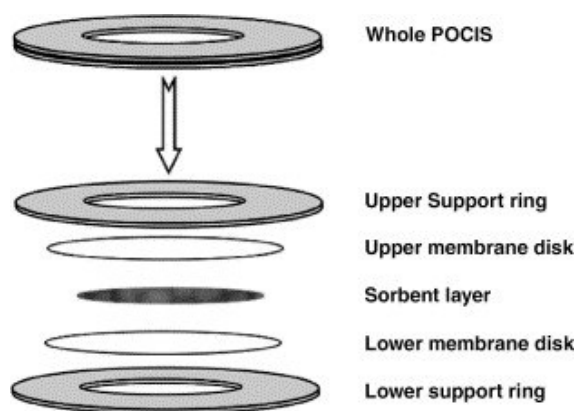


Figure 2.7: Exploded view of a POCIS showing the sorbent layer contained between two membrane disks sandwiched between two support rings.

Figure and caption from Alvarez, et al. (2005).

A POCIS device containing three POCIS disks and one single POCIS disk for use as a field blank were packed in an airtight metal canister and stored at 2-8°C prior to delivery to the Marine Institute. Samplers were stored at -30°C and transported to the site in cold conditions. At each deployment, the canister was opened, and a field blank POCIS disk and POCIS sampler were exposed to the environment in the same manner. The POCIS were attached to a suitable structure, depending on the study involved, and deployed 1m below the surface at each site, for a period of one month. The samplers and field blank were placed back in an airtight metal container at the time of removal. The time period the field blank was exposed to the air exactly matched the time taken for deployment and retrieval of the sampler. Storage and transportation of the devices was conducted in a manner that would not expose them to contamination. Samplers and field blanks were stored at -30°C prior to transport (in cold conditions) to the EA lab for extraction and analysis.

2.3.1.1 Extraction and analysis

Passive sampler extracts were analysed as per the Environment Agency (UK) Blue Book 220, method B (EA, 2008) as follows: On arrival to the laboratory the POCIS disks were cleaned to remove any debris and/or deposits. Any excess water was removed where necessary and the discs allowed to air-dry overnight. The loaded POCIS was carefully dismantled and the sorbent transferred with MeOH into a glass chromatography column fitted with a glass wool plug and a stopcock. This MeOH was collected and pooled with

the extraction solvent. The sample was eluted with 40 mL MeOH (adjusted to near drop-wise flow). The collected eluate and rinse were evaporated by rotary evaporation to approximately 1 mL.

2.3.1.2 Solid phase extraction

The sampler extract in MeOH obtained in section 2.3.1.1 was passed through a styrene divinyl benzene polymer SPE cartridge. The cartridge was washed, dried and the steroids desorbed with dichloromethane. This extract was evaporated to incipient dryness and dissolved in a 90:10 v/v% iso-hexane: propan-2-ol mixture and cleaned-up using normal phase chromatography with an Amino LC column fractionation. The resulting extract was again evaporated to incipient dryness before being dissolved in 50% aqueous methanol. This extract was then fractionated using reverse phase chromatography with a standard C18 phase column. The resulting extract was evaporated to incipient dryness and dissolved in 10% aqueous methanol. 100 µL of this was analysed using high performance liquid chromatography with negative ion atmospheric photo-ionisation interface (API) and time of flight mass spectrometric detection (MS/TOF).

2.2.1.3 Liquid chromatography-time of flight mass spectrometry

The HPLC system consisted of an Agilent 1100 Series Game Pad, Agilent 1100 Binary Pumps, an Agilent 1100 High Performance Autosampler and an Agilent 1100 thermostatted column compartment (Agilent Technologies Deutschland GmbH, Böblingen, Germany). A Luna phenyl hexyl 2 x 150 mm, 3 µm particle size column (Phenomenex, Macclesfield, Cheshire, UK) fitted with a Luna phenyl propyl 4 x 2 mm guard column thermostatted at 60°C was used for chromatographic separation. For liquid chromatography time of flight mass spectrometry (LC-MS/TOF) experiments, an Applied Biosystems 3200 Q-TOF was used (Applied Biosystems, Foster City, CA, USA). The mobile phases were deionised water (A) and 0.025% TEA in 95:5 MeOH: acetone (B) flowing at 300 µL per minute with a gradient as per Table 2.6. LC-MS/TOF analysis was performed as per the Environment Agency Blue Book 220 method B (EA, 2008). Results are expressed as ng per device.

Table 2.6: LC gradient for the analysis of POCIS extracts by LC-MS/TOF

Time (min)	% mobile phase A	% mobile phase B
0.00	95	5
0.50	80	20
1.00	60	40
12.00	20	80
14.50	95	5

2.3.2 Estrogen luciferase reporter gene assay

The estrogen luciferase reporter gene assay (ER-LUC) was developed by Rogers and Denison (2000). It consists of a recombinant human ovarian carcinoma (BG1) cell line, which has been stably transfected with an estrogen responsive luciferase reporter plasmid (Rogers and Denison, 2000). Stably transfected luciferase reporter cell lines which are used to indicate binding to the estrogen receptor (ER) are widely used in reporter gene assays, the most common of which are the estrogen receptor (ER)-mediated chemical activated luciferase gene expression (ER-CALUX) assay (Legler et al. 1999; Legler et al. 2002) which uses human breast adenocarcinoma cell lines expressing endogenous ER α and β , and the MELN cell line containing human breast cancer (MCF-7) cells (Michallet-Ferrier et al. 2004). These reporter gene assays have been used in a large number of studies to investigate the estrogenic activity of marine and freshwater environmental samples and to assess the estrogenicity (expressed as estradiol equivalents or EEQ) of potential EDCs (Murk et al. 2002; Michallet-Ferrier et al. 2004; Soto et al. 2006; van der Burg et al. 2010; Schmitt et al. 2011). All ER-LUC analyses were performed by subcontract analysis at the Institute for Environmental Studies (IVM), The Netherlands as detailed below. Both sediments and biota are suitable for ER-LUC analysis and both matrices formed part of the multi-matrix integrated approach of this thesis.

2.3.2.1 Sediment preparation

Sediment was extracted as per Weiss et al. (2009). 40 g of <63 μm freeze-dried sediment was extracted by accelerated solvent extraction (ASE). Dichloromethane (DCM): acetone (3:1, v/v) was used as extraction solvent. ASE extractions were performed at 50°C, 2000 psi, with three extraction cycles as per Houtman et al. (2006). Extracts were evaporated under a gentle N gas stream until near dryness and reconstituted in 1 mL DCM prior to GPC clean-up.

2.3.2.2 Mussel tissue preparation

Mussel tissue was extracted as per Simon et al. (2010). Briefly, homogenised mussel tissue was mixed with Hydromatrix (calcined and purified, Sigma Aldrich) in a 1:1 wet weight (ww) ratio. The resulting free-flowing powder was left overnight to absorb all water from the sample. This mixture was extracted with DCM: acetone (3: 1 v/v) by pressurised liquid extraction (PLE) on an ASE200 device (Dionex, Sunnyvale, CA) at 70°C, 2000 psi with three extraction cycles. Depending on the amount of dried sample obtained, 22, 66 and 100 mL of PLE cells were used. Extracts were evaporated under a gentle stream of N₂ to a volume of 0.5 mL and transferred to dialysis membranes (polyethylene lay-flat membranes, 6-8 cm length, thickness 50 – 100 µm, Brentwood Plastics, USA) that were pre-cleaned with hexane (Huckins et al, 2000). The dialysis membranes were sealed and left for 24 hours in 15 mL glass tubes filled with hexane to remove interfering lipids. The hexane fraction was collected and this process repeated four times. The final four hexane extracts were pooled, evaporated to near dryness and reconstituted in 1 mL DCM prior to GPC clean-up.

2.3.2.3 GPC clean up

GPC clean-up was performed on two polystyrene – diphenylbenzene columns (PL-Gel, 10 µm, 50 Å, 25 x 300 mm, Polymer Laboratories, Heerlen, The Netherlands) in series, equipped with a precolumn (PL-Gel, 10 µm, 25 x 25 mm, Polymer Laboratories Ltd.) and a 2 mL injection loop. For each sample, 1 mL of the DCM extract together with the sample vial rinsed with 0.5 mL DCM was injected onto the GPC columns, which were eluted in DCM at a flow rate of 10 mL/min. Extracts from the GPC clean-up were evaporated to a volume of 0.5 mL.

2.3.2.4 Estrogen luciferase reporter gene assay

ER-LUC was conducted as per Rogers and Denison (2000). Briefly, BG1Luc4E₂ (BG1) cells were transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) and stable transfectants selected using G418 resistance. For analysis, the BG1Luc4E₂ cell clone resulting from this selection was grown in RPMI 1640 medium. The cells were transferred into flasks containing phenol red-free DMEM media (supplemented with 5% carbon stripped fetal calf serum and G418 sulphate solution), and incubated for four days before harvesting for BG1Luc4E₂ bioassay plating. The cells were then plated in 96 well plates and incubated at 37°C for 24-48 hours prior to dosing with the DMSO extracts. The media solution in each well was removed and 200 µL of phenol red-free

DMEM containing a dilution series of each extract was added to each well. The plate was then incubated for 20 hours before analysis of luciferase activity. Estradiol was used as a positive control to generate calibration curves. An internal reference material was also used.

2.3.2.5 Measurement of luciferase activity

After lysing cells with 100 µL Promega lysis buffer, the luciferase activity was measured in an Anthos Lucy2 Microplate Luminometer, with automatic injection of 50 µL of luciferase enzyme reagent (Promega) into each well. The relative light units (RLUs) measured were compared to that induced by the E2 standard after subtraction of the background activity. The LOD was 17 pg/g EEQ. Results are reported as pg estradiol equivalents per gram dry weight (pg EEQ/g dw). Each reported value is the average of two separate experiments.

2.3.3 Alkali labile phosphate assay

Recent work has identified the presence of vitellin-like (Vn) protein, an egg yolk precursor protein produced in females during oogenesis. It may become elevated in females and produced by males upon exposure to endogenous estrogenic compounds (Gagné and Blaise, 2000). The alkali-labile phosphate assay (ALP) is widely used to indirectly determine Vn-like protein (Blaise et al. 1999; Gagné and Blaise 2000; Marin and Matozzo, 2004). A change in the ALP levels of gonadal tissue of the exposed mussels is used as a measure of endocrine disruption. The assay has been successfully used with a number of marine bivalves, including *Mytilus* spp. (Billinghurst et al. 1998; Li et al. 1998; Pampanin et al. 2005). The Bradford assay (Bradford, 1976) is a method for the determination of microgram quantities of protein. It is used in conjunction with ALP, to determine the total gonadal protein concentration, in order to normalise ALP data. All ALP measurements were conducted by partners in the SeaChange project in Trinity College Dublin.

2.3.3.1 Sample collection for histology, condition factor and the alkali labile phosphate assay.

Mussels for ALP and histology were transported to the laboratory in cold conditions and kept overnight in 80 L tanks with filtered seawater (1 L per mussel). Following this mussels were opened by severance of the adductor muscle and the whole soft tissue weight and shell length was recorded. Firstly, a 2-4 mm cross section of visceral mass containing gonad was sampled and processed as in section 2.3.3.3. The remaining gonad tissue was

then dissected and frozen in liquid nitrogen. Samples for ALP were subsequently stored at -80 °C for further analysis.

2.3.3.2 Condition factor

Fulton's condition factor (CF) was used to define the physiological health of the mussels used in the transplantation study (Chapter 4) and was assessed as per Ernst et al. (1976) using the formula $K = G/L^3 * 100$, where G = soft tissue, wet weight (g) L = shell length (cm).

2.3.3.3 Histological analysis: determination of sex and gonadal stage

Cross sections of 3-4 mm visceral mass were removed from each individual mussel and placed in cassettes in Bakers fixative (calcium chloride 4 g/L, 37-40% formaldehyde solution 100 mL/L, deionised water 900 mL/L) and fixation was allowed to proceed for 24 h before transfer of fixed samples to 70% industrial methylated spirit until further processing. Following this, samples were embedded in paraffin wax and 4-5 µm sections were cut using a microtome and subsequently stained with haematoxylin and eosin. Sex and gonadal stage were determined by microscopic analysis as per Seed (1976), Table 2.7.

Table 2.7: Reproductive status index (Seed, 1976).

RESTING	
S0	Inactive/neuter gonad. May also include mussels that have completed spawning.
DEVELOPING	
D1	Gametogenesis begins. No gametes can yet be seen.
D2	Ripe gametes first appear. Gonad is one-third its final size.
D3	Each follicle contains roughly equal proportions of developing and ripe gametes.
D4	Gonad is two-thirds its final size. Follicles contain mainly ripe gametes.
RIPE	
D5	Spawning commences. Male gonad is distended with morphologically ripe sperm. Female gonad is compacted with polygonal ova.
SPAWNING	
S4	General reduction in sperm density and rounded off ova as pressure is reduced.
S3	The gonad is approximately half empty.
S2	Further reduction in gonad size. Follicles are approximately one-third full of ripe gametes.
S1	Residual gametes remain.

2.3.3.4 Preparation of gonadal mussel homogenate for ALP

Mytilus spp. 5-6 cm in length were sampled to ensure that they were in the same developmental stage and to avoid possible age-or length-confounding factors on the physiological state of the mussels and their levels of Vn-like protein synthesis for ALP

determination. Analysis was based on an adaptation of the procedure of Blaise et al. (1999) modified by Quinn et al. (2004) for zebra mussels. Briefly, the frozen gonad tissue was ground in liquid nitrogen and homogenised in HEPES-NaOH (10 mM) buffer containing 125 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 (24°C) in a ratio of 1:5 (weight: volume) using a Potter Elvehjem homogenizer with PTFE pestle. Gonad homogenate was then centrifuged at 13 000 g for 10 min, the supernatant was removed with 100 µL aliquots stored at -80°C for further analysis. The values for ALP results were normalised against total protein concentration which was determined as per Bradford (1976).

2.3.3.5 Vitellin (Vn)-like protein determination

Aliquots of 100 µL gonad supernatant were added to 54 µL 35% (v/v) acetone. After centrifugation at 10 000 g for 5 min at room temperature, the supernatant was removed. To the remaining pellet 100 µL of 1M NaOH was added and mixed at 60°C for 30 min. Afterwards, 20 µL aliquots were added to a 96-well microplate and read at 815 nm using a spectrophotometer. The amount of Vn was expressed as µg of phosphate per mg of protein. Quality control was ensured by the analysis of one spiked (1 µg phosphate) and one unspiked mussel gonad homogenate samples from a location in Dublin Bay. Bovine Serum Albumin (BSA 1 mg/mL) was used as a standard protein for the Bradford assay of protein.

2.4 Overall statistical assessment

Datadesk version 6.0 for Windows was used to conduct statistical analysis. Assumption tests of normality and equal variance were performed on each data set using the F-Max test. A one-way analysis of variance (ANOVA) was conducted where data was normally distributed, followed by a least significant difference (LSD) post-hoc test. Where data was not normally distributed a Kruskal-Wallis analysis was performed. Non parametric data (gonadal stage) was assessed using a Friedman ANOVA and Kendall coefficient of concordance.

2.5 Discussion

Two LC-MS/MS methods have been developed and validated to detect selected potent EDCs at environmentally and ecotoxicologically relevant levels. For the steroid estrogens in biota, external standardisation using Warfarin (WF) was found to dramatically increase method performance, improving data precision to assess recovery and reproducibility. Both internal (E2-d₂) and external standardisation were trialled. Using WF to quantify sample

concentrations produced higher recoveries and more reproducible results than those calculated using E2-d₂ (88 to 102%, RSD 3.2 to 9% using WF compared with 59 to 70% RSD of 35 to 42% using E2-d₂). WF was then selected as external standard for quantification in this method, to account for ion suppression during analysis, with E2-d₂ used to confirm retention times and to quantify any losses throughout the extraction. Gomes et al. (2006) also found that external standardisation was a more suitable means of quantifying steroid estrogens. They found that signal suppression was different for E2 and E2-d₄. Reproducibility was better using external standard calibration (SD 0.3 to 3.6%) rather than internal standard calibration (SD 7.5-29%).

Filtration of water samples was not found to affect recoveries of E1, E2 and EE2. Analysis of the extracted filter papers were <LOD (range 0.4 to 1.7 ng/g dw) for all compounds. Recoveries of dissolved E1, E2 and EE2 in the filtered extracts were 51, 84 and 51% respectively. The steroid estrogens typically have rapid degradation rates, with half lives reported ranging from hours to days (Andersen et al. 2004; Tarrant et al. 2005; Williams et al. 2008). In a laboratory study on the fate and behaviour of estrogens in river waters, Jürgens et al. (1999) found that the steroid estrogens undergo aerobic degradation and attributed this to bacterial degradation, as no degradation was observed in sterile controls. Labadie and Budzinski (2005) and Jürgens et al. (2002) found that bacterial degradation resulted in half-lives of E1 of 0.2 to 7.2 days. UV sterilization has been demonstrated to remove between 70 and 100 % of E1, E2 and EE2 from wastewater during treatment. The low recoveries of E1, E2 and EE2 after overnight shaking may be due to a combination of bacterial activity, and UV degradation as the jars in this case were not protected from light. Further investigation into the causes of the lower recoveries (potential bacterial activity) was not within the scope of this study. Partitioning of steroids to the particulate phase in water samples was found to be low, thus the LC-MS/MS method was deemed suitable for E2 WFD monitoring purposes.

A variety of concentrations of endogenous steroids have been reported for *Mytilus* spp. A review by Janer and Porte (2007) reported that E2 levels in molluscs are spread over 4 orders of magnitude depending on the species and method used for their detection. E2 has been reported up to 5.42 ng/g dry weight (dw) in *M. galloprovincialis* using ELISA (David et al. 2008), and up to 2.5 ng/g and 0.2 ng/g ww in *M. edulis* gonad tissue and peripheral tissue, respectively, using radio immunoassay (RIA) (Lavado et al. 2006). Detection of steroids at and below these concentrations using MS techniques has proved challenging,

despite the development of methods with sufficiently low detection limits. Steroids were not detected in *Mytilus* spp using GC/MS, despite detection limits of 0.1 to 0.2 ng/g ww (Dévier et al. 2010). Pojana et al. (2007) used LC-MS/MS to analyse E1, E2 and EE2 in mussels taken from the highly urbanised Venetian coastal lagoon. EE2 was detected at 7.2 to 38 ng/g dry weight, but E1 and E2 were not detected, with LODs of 1.5 ng/g dry weight (~0.3 ng/g wet weight assuming 20 % dry matter). LODs attained in this study (0.4 to 1.7 ng/g ww) are similar to those reported by Pojana et al. (2007) and Dévier et al. (2010).

Immunoassay techniques, while generally rapid and inexpensive, are less specific than MS. The specificity of RIA may be limited by cross-reactivity or subjected to interferences from co-extracted matrix components (Gust et al. 2010). Zhu et al. (2003) reported E2 levels of 165 ± 54 ng/g wet weight in *M.edulis* gonad tissue, using Q-TOF-MS, and 854 ng/g using RIA. Further investigation using Q-TOF/MS revealed two E2 immunoreactive materials present in the sample, one corresponding to authentic E2, and the other with a four times higher E2 reactivity, which the authors concluded was an E2 isoform. Endogenous E2 was detected in the gastropod snail *Potamopyrgus antipodarum* by Gust et al. (2010) using RIA, however the authors could not detect E2 using LC-ESI-MS/MS. They concluded that the levels detected by RIA should be used with caution due to the possible presence of interfering substances and cross reactivity. The discrepancy between E2 results by MS and RIA may explain why some sensitive MS techniques are unable to detect E2 at concentrations reported by RIA despite having similar LODs.

The specificity of MS detection as reported herein allows detection of 'authentic' E2 and not the E2 isoform detected using RIA. MS techniques are a powerful tool for the accurate elucidation of the concentration and profiles of endogenous steroids in *Mytilus* spp. Natural and synthetic steroid estrogens have been shown to be biologically active at low concentrations, thus successful analysis requires low detection limits, and highly sensitive and selective analysis. Seasonal fluctuations in endogenous steroids may complicate the interpretation of estrogenic bioassay results, thus their determination will provide invaluable information for use in future studies. The ability to accurately identify endogenous and exogenous estrogens is a major advantage of LC-MS/MS.

NP, OP and the steroid estrogens are often analysed with the same analytical method (Arditsoglou and Voutsas, 2012; Beck et al. 2005; Vigano et al. 2006), due to the similar chemical properties they possess. Initial method development focussed on a multi-residue

method incorporating both the steroid estrogens and APs. Generally when analysing a suite of compounds in a single method, compromises between the optimal instrumental parameters (MS conditions, LC conditions) for each individual compound are required. Method development began by injecting solutions of each individual compound into the MS system and optimising the MS conditions to achieve the greatest response. Standard solutions were prepared in MeOH, 20:80 MeOH: H₂O and 1:1 MeOH: H₂O. Optimal signal intensities for the APs were found for the compounds in MeOH, while for the estrogens, optimal intensities were observed in 20:80 MeOH: H₂O. The optimal MS parameters such as cone voltage, gas pressures and source temperature also differed between the compound groups. The aim of the method development was to achieve very low limits of detection. The optimal conditions for each compound group were such that it was more suitable to develop two methods.

Method development then focused on a method for the steroid estrogens, followed by a method for the APs which built upon the estrogens method with modifications tailored to optimise the analytical performance and extraction efficiency for the APs. The use of a deuterated internal standard (deuterated NP-d₈/NP-d₈) was found to dramatically increase method performance, improving calculation of recoveries and reproducibility, accounting for ion suppression due to matrix effects and confirming retention times. Calibration curves were obtained using fortified tissue samples spiked at different concentrations of NP and OP (for linear range see Table 2.5). The selectivity and sensitivity offered by MS/MS in combination with the recovery ranges and repeatability of extraction as presented were deemed fit for purpose and analysis of samples was completed based on the reported method. NP and OP have been shown to bioaccumulate in exposed organisms (Ferreira-Leach and Hill, 2001). The developed method is capable of detecting NP and OP at trace levels in marine biota and was applied to a number of marine biota: flatfish, oysters and both native and transplanted mussels within the course of this research, generating baseline data within these organisms in an Irish context.

While the developed mass spectrometry method for steroid estrogens is capable of detecting steroids at environmentally and ecotoxicologically relevant concentrations it is clear that their analysis in the marine environment can continue to present analytical challenges, due to high dilution effects, and the variability associated with concentrations detected in spot samples. Although a large sample volume (five L) was selected for water samples due to the low concentrations of estrogens expected to be present in marine waters, passive sampling was also selected as an additional sampling tool to enable

detection of low (sub ng/L) concentrations. Passive sampling by POCIS will allow the detection of trace levels of steroids at sites under investigation accounting for transient or episodic pollution events during deployment.

The alkali labile phosphate assay is a sensitive tool for the assessment of estrogenic ED in exposed organisms, while the estrogen luciferase reporter gene assay provides a measure of the estrogenic potential of a sample. The combination of these techniques can account for the presence of EDCs which have not been selected and detected using LC-MS/MS.

2.6 Conclusions

The techniques developed and described within this chapter provide a comprehensive analytical 'tool box' which were applied in coastal and estuarine locations to investigate the presence and effects of selected EDCs in the marine environment. Targeted chemical analysis using sensitive and selective analytical methods enable the detection of trace levels of selected EDCs, with analysis focussed on some of the most highly potent estrogenic substances. The ER-LUC and ALP assays act as broader non-compound specific methods which can measure ED effects in the environment related to both known and unknown compounds. The application of these techniques to Irish marine locations provides the first comprehensive integrated assessment of estrogenic endocrine disrupting compounds, and endocrine disruption in Irish marine waters. The techniques were applied to the analysis of both transplanted and native shellfish, to native fish, water and sediment from a range of both impacted and non-impacted Irish coastal locations, generating valuable baseline data to fill gaps both nationally and internationally.

Chapter 3: An integrated biological effects and chemical analysis based investigation into short-term exposure effects of 17 α -ethynyl estradiol in *Mytilus* spp. under a simulated sub-tidal regime

3.1. Introduction

Natural and synthetic endocrine disrupting compounds are released into the marine environment via a number of sources, including municipal wastewater effluent. Concern has been raised as to the fate and potentially deleterious effects of these compounds on exposed marine organisms. The synthetic estrogen 17 α -ethynyl estradiol (EE2) is designed to resist degradation in order to be effective as an oral contraceptive with degradation occurring at a lower rate than that of the natural estrogens 17 β -estradiol (E2), estrone (E1) and estriol (E3) (Young et al. 2004), and it has a reduced rate of metabolism in organisms compared to the endogenous steroids (Routledge et al. 1998).

Mytilus spp. are widespread, with a worldwide distribution. Their ubiquity makes them a suitable indicator organism for use in environmental monitoring programs to assess the status and trends of chemical contamination in estuarine and coastal environments, including OSPAR CEMP and in monitoring of the quality of shellfish growing waters via the Shellfish Waters Directive 2006/113/EC (SWD). Knowledge of marine invertebrate endocrinology, including that of *Mytilus* spp., is still very limited.

The alkali labile phosphate assay method monitors endocrine disrupting effects in various organisms by measuring changes in the ALP levels of gonadal tissue of the exposed organisms (Gagné and Blaise, 1998; Blaise et al. 1999; Marin and Matozzo, 2004). These changes are linked to levels of vitellin-like (Vn-like) protein. Histology can be used to measure reproductive stage and metabolic condition, and linked with histopathology; it can be employed to investigate relationships between environmental health and exposure to contaminants (Lowe and Pipe, 1987; Bignell et al. 2008; Bignell et al. 2011). Among the different techniques used for the detection of estrogenic compounds, LC-MS/MS is one of the most commonly used (Croley et al. 2000). It is suitable for the detection of trace EDC concentrations at levels where biological effects may occur, due to the high specificity and selectivity of the tandem MS.

EE2 has been detected at 6.7 ng/L in an Italian coastal lagoon (Pojana et al. 2007), at 1.5 ng/L in a UK estuary (Hibberd et al. 2009), and up to 17 ng/L in a coastal area of the German Baltic Sea (Beck et al. 2005). It has also been detected in marine sediments, with 48 ng/g dry weight reported in Chilean coastal sediments (Bertin et al. 2011), which may render benthic organisms susceptible to exposure. UK receiving waters report EE2 concentrations at 4.6 ng/L (Williams et al. 2003). Direct discharge of untreated wastewater

into surface waters is considered an important source, with EE2 levels up to 13 ng/L reported in untreated wastewater (Baronti et al. 2000).

An *in vivo* study investigating the effects of EE2 exposure in *Mytilus* spp. was conducted in conjunction with partners in the SeaChange project in TCD and the Shannon Aquatic Toxicity lab (SATL). A concentration of 150 ng/L was selected as the exposure concentration for the current study, which, while higher than generally detected in the environment, may have relevance for point source discharges and large urban population centres, and locations discharging poorly treated effluent. The exposure was conducted at the SATL. Liam Curren from SATL was responsible for administering the dose of EE2 daily and for the maintenance of the tank system including recording temperature and dissolved oxygen measurements. All LC-MS/MS analyses were completed as part of this research with ALP measurements conducted by TCD partners in the Seachange project as detailed in Chapter 2.

The primary aim of this *in vivo* study was to expose mussels to EE2 in a simulated sub-tidal regime under controlled conditions and to monitor short term effects using an integrated battery of biological and chemical analysis based techniques. Control of the possible confounding effects of other chemicals, and abiotic factors, such as varying salinity, dissolved oxygen and temperature was maintained making it possible to directly study EE2 uptake and effects. LC-MS/MS was used to determine tissue concentrations of EE2 following exposure and to examine the short term uptake potential of EE2 in *Mytilus* spp. ALP was used to investigate EE2 related endocrine effects. Gonadal stage was assessed in exposed and control organisms to ensure that observed variations in ALP levels were not due to differing stages of gametogenesis, and to assess the potential effects of EE2 exposure on sexual maturation and gonadal development.

Mussels are adapted to live in intertidal conditions and during low tide they protect themselves from dehydration by keeping their shells tightly closed. In this way they can endure long periods of drought. Mussels take up oxygen through their gills by filtering water, and so their oxygen supply becomes limited during intertidal phases as their water filtration is restricted and their gills are collapsed (Bayne, 1973). During this time, the mussel enters a hypoxic physiological state (i.e. limited oxygen supply). A pilot scale simulated intertidal regime was investigated in order to assess if the limited oxygen supply in a 'low tide' period affected the degree of EE2 related effects.

3.2. Materials and methods

3.2.1 Source of mussels for study

Cultivated long line mussels were obtained from Redbank hatchery in Aughinish Bay, New Quay, Co. Clare, Ireland. EE2 was not detected in the mussels used in this study at time zero (T0). *Mytilus* spp. (5-6 cm in length) were selected to ensure that they were in the same developmental stage and to avoid possible age and/or length confounding factors on the physiological state of the mussels and their levels of Vn-like protein synthesis for ALP determination.

3.2.2 Exposure system

The semi static exposure system consisted of ten tanks of artificial seawater with a salinity of 35‰. This salinity was selected in order to minimise stress on the sampled mussels by mimicking the salinity regime of waters from which they were sourced. Artificial seawater was prepared using 175 g sodium chloride, 2.5 g sodium hydrogen carbonate per five L water. Each tank was populated with 60 mussels with artificial seawater added at a ratio of 1 L per mussel (Figure 3.1). Mussels were unfed throughout the experiment to avoid any confounding effects that a food source may have on the experiment. Mussels were exposed to 150 ng/L EE2 for seven days. EE2 was spiked into tanks from a stock solution prepared in ethanol resulting in a final solvent concentration of 12 mg/L ethanol in each exposure tank. The set up was as follows:

1. Continually submerged (SUB) EE2 exposed mussels (n=3 tanks).
2. Control tanks with artificial seawater (n=3):
 - a. Two control tanks spiked with ethanol (12 mg/L) (SOL),
 - b. One tank containing only mussels and seawater (SW).
3. One tank in which uptake of EE2 was assessed daily (UPT). The volume of water in this tank was reduced by 10 L daily, to normalise the volume of water per mussel over the exposure study period.
4. 'Intertidal mussels' (INT), n=3. A pilot investigation of a simulated 'intertidal' regime was conducted with water removed for four hours per day.

All tanks were drained daily with submerged (SUB, SW, SOL and UPT) tanks refilled immediately with seawater (spiked as appropriate) and INT tanks refilled after 4 hours.



Figure 3.1: Exposure system

3.2.3 Abiotic parameters

Dissolved oxygen, temperature and salinity were recorded daily using a calibrated Sondes multi-parameter probe (YSI Inc. Yellow Springs, Ohio, USA).

3.2.4 Condition of test mussels

The condition of the test mussels over the duration of the study was assessed using condition factor estimates (as per Chapter 2) from 12 mussels per tank and observations of mortalities.

3.2.5 ALP and histological analysis

For ALP and histological analysis, 10-12 mussels were removed from each treatment, and were kept overnight in tanks with filtered seawater (0.7 μm and 1 L per mussel). Mussels were opened by severance of adductor muscle and the whole soft tissue weight and shell length recorded. A 2-4 mm cross section of visceral mass containing gonad was sampled and processed for gender determination and histology as per Chapter 2. The remaining gonad tissue was dissected, frozen in liquid nitrogen, and stored at -80 °C for ALP analysis as per Chapter 2.

3.2.6 LC-MS/MS analysis

Whole body tissue taken from 30 individuals per tank, homogenised using a Waring blender (Waring Commercial, New Hartford, CT, USA); freeze dried and frozen at -30 °C prior to chemical analysis as per Chapter 2. Tissue samples were analysed at time zero (T=0) and after seven days (T=7) to examine EE2 tissue concentrations after seven days (SUB, SW, INT and SOL tanks). Ten mussels from the UPT tank were analysed daily to examine the uptake rate. A 500 mL water sample was collected daily from each tank into individually labelled amber glass bottles, stored at 4°C and processed as per Chapter 2.

Sample extraction was performed as per Chapter 2. As EE2 may be degraded into other metabolites, mussel and water samples were additionally screened for the presence of E1 to evaluate potential for metabolic and other degradation of the parent EE2. Endogenous E2 may complicate interpretation of ALP response, thus samples were also analysed for E2. LC-MS/MS analysis was performed as per Chapter 2. A full analytical quality control program incorporating participation in proficiency studies and use of method blanks, replicates and spiked reference samples was employed throughout the study.

3.2.7 Bioconcentration

Daily bioconcentration factors (BCF) were calculated using tissue and water concentrations from the uptake tank.

3.2.8 Statistics

Assumption tests of normality and homogeneity were performed on each data set using an F-Max test. A one-way analysis of variance (ANOVA) was conducted where data was normally distributed, followed by a LSD post-hoc test. Where data was not normally distributed, a Kruskal-Wallis analysis was performed. Gonadal stage was assessed using a Friedman ANOVA and Kendall coefficient of concordance.

3.3 Results

3.3.1 Environmental control

Temperature, dissolved oxygen (DO) and salinity did not vary between treatments over the duration of the exposure. Dissolved oxygen remained at 12.7 ± 0.8 mg/L, which was deemed to be sufficiently oxygenated to support the survival of the mussels while submerged, although intertidal mussels were at risk of entering an anaerobic state during

the daily four hour 'low tide' period. Temperature was controlled at $8.35 \pm 0.35^{\circ}\text{C}$ and salinity remained at 35‰.

3.3.2 Mussel condition factor and gonadal stage

The condition factor of mussels was calculated for each experimental condition (Table 3.1). The data were normally distributed and statistical analysis was completed using ANOVA followed by a LSD post-hoc test. No statistically significant differences in condition factor between treatment for males ($p=0.18$), females ($p=0.13$) and for males and females pooled per treatment ($p=0.12$) were observed. Furthermore no statistically significant differences in gonadal stage between treatments ($p=0.87$, Friedman ANOVA and Kendall co-efficient of concordance) were found. Gonadal stages are presented in Table 3.1.

3.3.3 Concentrations of ALP and protein in exposed mussels

ALP results were not normally distributed, so they were assessed using a Kruskal Wallis analysis. There were no statistically significant differences in ALP concentrations between males and females in each individual treatment (T0 mussels prior to exposure $p=0.78$, SW $p=0.83$, SOL $p=0.26$ INT $p=0.87$, SUB $p=0.81$), however differences were observed between treatments (Figure 3.2). Significant differences in ALP levels between males in INT tanks and SOL, SW and SUB males ($p=0.01$, 0.016 and 0.015 , respectively) were observed. Elevations in ALP in SUB mussels were only statistically significant relative to SOL mussels ($p=0.015$). ALP levels in INT females displayed a significant two fold increase in ALP relative to T0 control mussels ($p=0.004$) and were 240 and 170% higher than SOL and SW ($p=0.004$ and 0.001 , respectively), see Table 3.1. ALP was 32% higher in INT females compared to SUB females; however this difference was not statistically significant ($p=0.36$). Increases in SUB female mussels were not statistically significant relative to any other treatment ($p=0.31$, 0.07 and 0.46 compared with T0, SOL and SW, respectively). No significant difference in protein content between test regimes over the exposure period was found ($p=0.95$, Table 3.1), thus ALP levels, normalised against protein concentration, were not affected by differences in protein concentration between treatments.

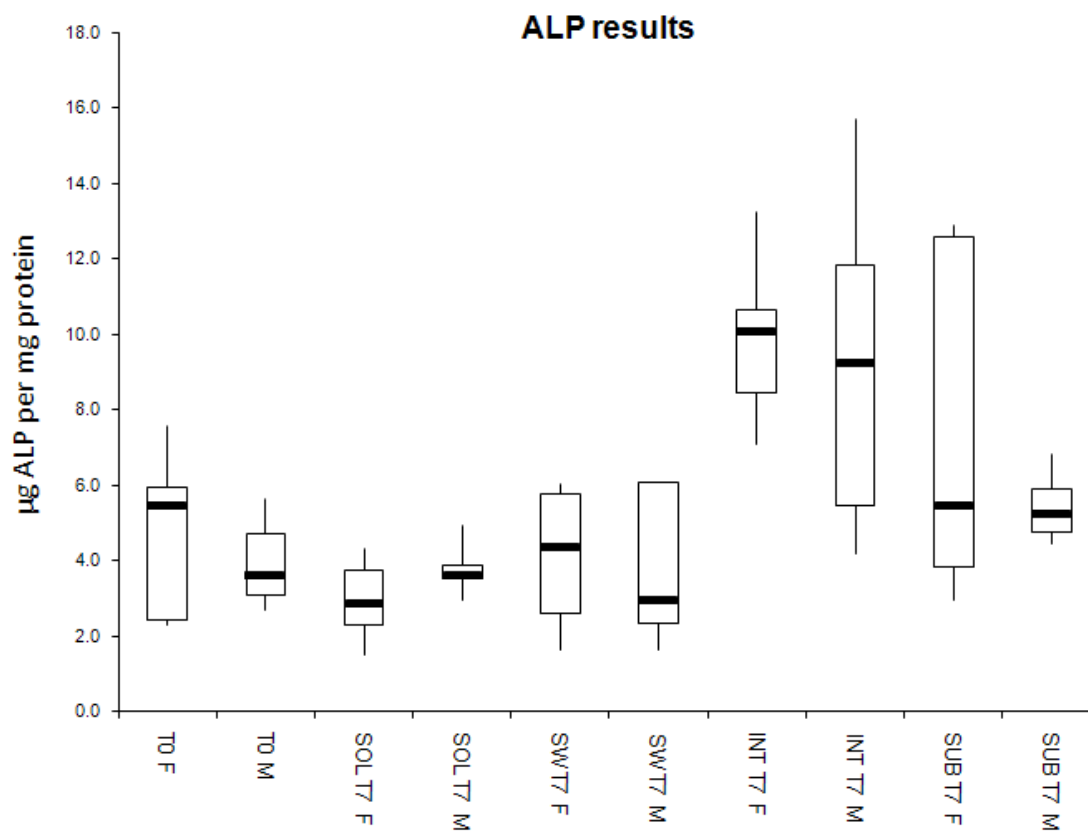


Figure 3.2: Box plots of alkali-labile phosphate concentration ($\mu\text{g ALP/mg protein}$) in each treatment.

T0 = time zero (mussels tested prior to exposure, T7 = results after 7 days (n=12 per treatment). Depicted are the median (= line), upper and lower quartiles (= box) and the mean standard error (=bars).

Table 3.1: Condition factor (wet weight: cm³), ALP (µg ALP/mg protein), gonadal stage (number at each stage per treatment) and protein content (mg/mL)

Treatment	Time 0 (T=0)	SOL	SW	INT	SUB
Males (average value ± RSD %)					
Condition factor (wet weight:cm ³)	3.0 ± 7.7%	2.7 ± 9.8%	3.2 ± 8.5%	3.1 ± 18.7%	2.7 ± 14.2%
Protein content (mg /L)	11 ± 21.4%	10.0 ± 36.7%	13.2 ± 26.6%	10.2 ± 34.2%	10.5 ± 54.2%
ALP (µg ALP/mg protein)	4.0 ± 41.6%	3.8 ± 22.0%	3.5 ± 53.9%	9.4 ± 51.8%	5.4 ± 18.0%
Gonadal stage 2 (n=0)	0	0	0	0	0
Gonadal stage 3 (n=19)	3	3	6	4	3
Gonadal stage 4 (n=8)	0	2	0	2	4
n=	3	6*	6	6	7
Females (average value ± RSD %)					
Condition factor (wet weight:cm ³)	2.6 ± 11.8%	2.9 ± 11.2%	3.0 ± 18.1%	3.0 ± 7.3%	3.1 ± 5.5%
Protein content (mg/mL)	16.2 ± 25.4%	16.4 ± 26.6%	16.4 ± 19.9%	18.1 ± 19.8%	19.2 ± 22.6%
ALP (µg ALP/mg protein)	4.7 ± 45.8%	2.9 ± 40.0%	4.1 ± 55.0%	10.0 ± 25.2%	7.5 ± 65.2%
Gonadal stage 2 (n=3)	2	1	0	0	0
Gonadal stage 3 (n=22)	7	3	2	6	4
Gonadal stage 4 (n=5)	0	2	2	0	1
n=	9	6	6	6	5

*gonadal stage in one male in SOL T7 could not be defined

3.3.4 Concentrations of E1, E2 and EE2 in water and mussels

EE2 in the individual exposure tanks was determined after each 24 hour period and an average of 40.0 ± 27.0 ng/L was measured (Table 3.2). Highest EE2 water concentrations were determined in all exposed tanks after 48 hours. EE2 was not detected in seawater from any of the control tanks on any of the seven days of the exposure study (Table 3.2) (limit of detection-LOD 0.11 ng/L). Comparison of daily EE2 water concentrations in test tanks using a one way ANOVA with a LSD post-hoc test revealed no statistically significant difference in EE2 concentrations between the tanks ($p=0.97$). The BCF steadily increased over the duration of exposure from day 2, and did not differ between INT and SUB tanks (BCF = 0.03, assessed on day 7), although this BCF was slightly lower than that of the uptake tank on the final day (BCF = 0.05). Tissue concentrations were observed to increase within the first 48 hours and remained relatively consistent for the remainder of the exposure. Concentrations in the exposed mussel tissues did not exceed 1.23 ng/g wet weight (ww) (day 5, uptake tank). EE2 was not detected (LOD 0.3 ng/g ww) in mussel tissue prior to the beginning of the exposure study ($T=0$), or in the solvent and seawater controls at $T=7$. EE2 increased to an average of $0.84 (\pm 0.06)$ ng/g ww, and $0.56 (\pm 0.44)$ ng/g ww, in INT and SUB mussels respectively after the seven day exposure period. A Kruskal-Wallis test revealed no significant difference in EE2 concentrations between INT and SUB test regimes ($p=0.564$). E1 and E2 were not detected in any mussel or water samples.

Table 3.2: EE2 concentrations in daily water (ng/L) and in tissue (ng/g wet weight) samples

Tank conditions	Water concentration ng/L [^]							Tissue concentration ng/g ww
	Day 1 (T=1)	Day 2 (T=2)	Day 3 (T=3)	Day 4 (T=4)	Day 5 (T=5)	Day 6 (T=6)	Day 7 (T=7)	Mussel Tissue T=7
1. Seawater control (SW)	nd	nd	nd	nd	nd	nd	nd	0
2. Solvent Control (SOL)	nd	nd	nd	nd	nd	nd	nd	0
3. Solvent Control (SOL)	nd	nd	nd	nd	nd	nd	nd	0
4. EE2 intertidal (INT)	58.0 ± 0.01	119 ± 0.01	43.1 ± 0.01	25.8 ± 0.01	29.8 ± 0.01	18.0 ± 0.01	25.2 ± 0.01	0.79 ± 0.02
5. EE2 intertidal (INT)	74.1 ± 0.01	97.0 ± 0.01	25.1 ± 0.01	20.1 ± 0.01	29 ± 0.01	22.6 ± 0.01	x	nd (0.30)
6. EE2 intertidal (INT)	45.5 ± 0.01	96.7 ± 0.01	29.8 ± 0.01	28.6 ± 0.01	19.8 ± 0.01	21.4 ± 0.01	24.7 ± 0.01	0.88 ± 0.02
7. EE2 submerged (SUB)	69.7 ± 0.01	102 ± 0.01	16.0 ± 0.01	29.5 ± 0.01	30.3 ± 0.01	26.4 ± 0.01	x	<0.60*
8. EE2 submerged (SUB)	65.3 ± 0.01	80.7 ± 0.01	21.5 ± 0.01	21.1 ± 0.01	15.8 ± 0.01	x	19.7 ± 0.01	1.07 ± 0.02
9. EE2 submerged (SUB)	77.3 ± 0.01	55 ± 0.01	35.5 ± 0.01	27.2 ± 0.01	27.5 ± 0.01	23.1 ± 0.01	19.1 ± 0.01	<0.60*
Average concentration exposed tanks	65.0 ± 0.01	91.7 ± 0.01	28.5 ± 0.01	25.4 ± 0.01	25.4 ± 0.01	22.3 ± 0.01	22.2 ± 0.01	0.77 ± 0.02
RSD %	18.0	23.6	34.5	15.5	23.9	13.7	14.6	39.1

x = sample not available

* 1/2 LOQ used in generation of summary statistics

nd: LOD 0.11 ng/L in water samples.

3.4 Discussion

Elevations in ALP levels were observed in *Mytilus* spp. after seven days exposure to EE2. The health status of mussels in the current study remained high, despite being maintained under starving conditions, with no statistically significant difference in condition factor after seven days ($p > 0.05$). Ethanol was used as a dispersion solvent at a concentration of 12 mg or 16.7 $\mu\text{g/L}$, which is below the OECD recommended maximum solvent concentration of ethanol of 78.9 mg/L (OECD, 2000). Recently, the use of organic solvents and their concentration limits have been debated, as they become confounding effects in aquatic toxicity tests. Hutchinson et al. (2006) recommend concentrations not exceeding 20 $\mu\text{L/L}$. The ALP results of the solvent control mussels confirm no recordable estrogenic effect of the carrier solvent ethanol.

Increases in ALP in EE2 exposed mussels confirmed that endocrine disruption occurs under the set experimental conditions. In the current study no statistically significant difference was noted in gonadal stage between treatments, thus observed increases in ALP were not attributed to the influence of differing stages of gonadal development. A recent study by Schmidt et al. (2012) found no statistically significant differences in ALP levels comparing male cultivated fully submerged mussels to male wild mussels taken from a number of positions on the intertidal rocky shore. The farmed mussels used in the study by Schmidt et al. (2012) and the current study were obtained from the same aquaculture farm. For this reason all control tanks remained fully submerged for the duration of the experiment, however a more detailed study is recommended involving full INT and SUB controls, and an extended 'low water' duration replicated in both exposed and control tanks.

Purdom et al. (1994) reported that effect-levels for E2 and EE2 in sensitive species are in the range 0.1-10 ng/L. A concentration of 150 ng/L EE2 may be considered high; however significant increases in ALP levels in submerged male mussels were only noted relative to a solvent control group. ALP in female submerged mussels did not significantly increase relative to any control group. EE2 exposed mussels in the submerged condition may have been expected to have the highest ALP values as a consequence of continual exposure. Interestingly, the intertidal (INT) mussels actually showed the highest and most significantly elevated ALP levels, with the most pronounced differences observed in males. ALP levels were significantly 148 and 170% higher than the SOL and SW controls

and 73% higher than EE2 exposed SUB males. ALP levels in INT females were also considerably elevated, with a statistically significant two fold increase in ALP relative to T0 control mussels, and a 240 and 170% higher ALP level relative to SOL and SW controls, respectively. No significant difference in exposure concentration or EE2 in mussel tissue was observed between individual tank treatments, thus observed differences in ALP response between treatments were not considered to be due to differences in water concentrations. From these data it appears that the intertidal condition may have exacerbated the effects of EE2, particularly in males, a finding which requires further research in order to fully test the hypothesis.

Elevated vitellogenin levels have been reported in both male and female Pacific Rock oysters (*Saccostrea glomerata*) when exposed to 50 ng/L EE2 for eight weeks with a number of individuals showing the presence of intersex (ovotestis) when examined using histology, highlighting the value of vitellogenin induction as a marker of biological effects. The potency of EE2 was further demonstrated in males in the same study, who were at an earlier stage of spermatogenesis than control groups after 8 weeks exposure to 5 ng/L EE2 (Andrew et al. 2008). Organisms may be more susceptible to the effects of EE2 depending on the stage of gametogenesis. Ciocan et al. (2010) found a significant increase in VTG and ER2 mRNA expression in *M. edulis* exposed to estrogens E2 and EE2 at the early stage of gametogenesis, whereas mature mussels displayed no statistically significant changes.

The mussels in the current study were approaching sexual maturity with developing gonads and were predominantly in stage 3 (78%), with 3 % in stage 2 and 18 % in stage 4. INT males exhibited the greatest range of ALP response (9.4 ± 4.9 μ g ALP/mg protein), with SUB females exhibiting the next highest range (4.9 ± 4.9 μ g ALP/mg protein). Mussels in these treatments were predominantly in stage 3 of gametogenesis (66-80%) with a number in stage 4 (20-33%). It is possible that those in stage 3 were more susceptible to ED effects, whereas those in stage 4 were not. This could have resulted in a wider range of ALP levels in these mussels, than in treatments where the stages were more closely related. For example, SUB males, most of whom were at a more advanced gametogenic stage (43% stage 3, 57% stage 4), did not exhibit such a wide range in ALP levels. Although mussels were selected at a similar size range in order to reduce the impact of different stages of development on ALP levels, and there was no statistically significant difference in gametogenic stage in each treatment, a range of stages (from stage 2 to 5, Table 3.1) were

encountered. The sample size used in this study (n=12 per treatment, 3-8 males per treatment) does not permit a detailed statistical evaluation of the effects of gametogenic stage on the EE2 in exposed mussels subject to reduced oxygen conditions, as mussels could not be staged or sexed before exposure. It is recommended that future studies are conducted using a greater sample size (for example n=30 per treatment) to increase the number of both males and females in each treatment, and increase the power of the statistical evaluation.

Mussels were exposed via spiking to a nominal concentration of 150 ng/L EE2 in artificial seawater, which reduced to a mean level of 40.0 ± 27.0 ng/L after 24 hours. No degradation of parent EE2 was determined in water and/or mussel tissue. A similar reduction in concentration after 24 hours was noted in studies by Ciocan et al. (2010) and Van den Belt et al. (2004) who found that EE2 water concentrations reduced to between 25% and 73 % of the nominal spiking concentration after 24 hours. In the current study, EE2 did accumulate in the mussel tissue, and when normalised relative to the EE2 water concentration the BCF suggests an initial lag phase in uptake by the mussels.

A wide range of bioconversion and detoxification enzymes are needed for the metabolism of contaminants in mussels (Gosling, 1992) and oxygen is a main coupling factor for many of these enzymatic reactions. Schmidt et al. (2012) found differences in lipid peroxidation and DNA damage between long line submerged and low shore intertidal mussels, suggesting that mussel location has an influence at the level of oxidative stress. Detoxification processes rely on oxygen for the serial reactions of oxygenation required for the metabolism, detoxification, degradation and excretion of contaminants. For example, the cytochrome P-450 system, the mixed-function oxygenase system (MFO) and other oxygenases and reductases require inputs of oxygen to carry out molecular detoxification and degradation of contaminants such as EE2 (Kirchin et al. 1992), in this respect, the detoxification processes might not be as efficient in the hypoxic intertidal mussels, which could lead to increased retention of EE2. De Zwaan, (1977) calculated that anaerobic energy efficiency is just 5 to 10% of that of aerobic processes. This is supported by Durand et al. (2002), who observed that after exposure to benzo-a-pyrene (BaP), intertidal mussels showed a decrease of BaP depuration compared to submerged mussels as measured by wet weight tissue concentrations. They suggested that the reduced contact to seawater, and thus reduced oxygenation, experienced by intertidal mussels caused them to have a higher body-burden of BaP. It is also suggested that metabolites might be retained longer in the

mussel tissue because of decelerated excretion induced by the general decrease of the metabolic activity during intertidal periods (Bayne 1973). Janer et al. (2004) recorded a down-regulation of the cytochrome P-450 aromates in *Mytilus galloprovincialis* exposed to high levels of E2 in a seven days *in vivo* study. This could suggest that the detoxification processes in intertidal mussels exposed to high levels of EE2 are disrupted at two levels: (1) by the insufficient supply of physiologically available oxygen and (2) by the reduced levels of metabolising enzymes capable of carrying out the reactions. A reduced detoxification capacity could cause an increased level of EE2-mediated endocrine disruption in the gonadal tissue of intertidal mussels, eliciting an endocrine response in the mussels that would lead to increased synthesis of vitellin-like proteins.

Alternatively, EE2 might be degraded into other metabolites, e.g. to estrone (E1), which while “less” active would still harbour endocrine potential (Lee et al. 2003; Janer et al. 2004). E1 was not detected in any of the mussel samples. Endogenous 17 β -estradiol (E2) has been detected in *Mytilus* spp. (Zhu et al. 2003), and may also complicate the interpretation of ALP response. E1 and E2 were not detected in any water or mussel samples by LC-MS/MS (data not presented), but E3, glucuronate and sulphate metabolites were not included in screening. It has been suggested that metabolites might be retained longer in the mussel tissue because of decelerated excretion induced by the general decrease of the metabolic activity during intertidal periods (Bayne, 1973). This may explain why elevated ALP levels were observed in the INT T7 mussels, when EE2 concentrations did not differ between treatments, although further analysis of metabolite levels is required to support this hypothesis, and is recommended in future studies. The ability to detect metabolites and endogenous E2 allows greater confidence in attributing the observed elevations in ALP response to EE2 exposure. The detection is made possible using selective and sensitive chemical analysis by LC-MS/MS, and would not be possible using ALP measurements alone.

3.5 Conclusions

This present study has demonstrated the estrogenic effects of EE2 in *Mytilus* spp. Increases in ALP after seven days confirmed the endocrine disruptive effects of EE2 on Vn-like protein levels by significantly elevating levels of this protein in both intertidal and submerged mussels compared to unexposed individuals. EE2 accumulated in exposed mussel tissue; however the concentrations were not influenced by the different exposure regimes. Intertidal conditions appear to have increased the negative effects of EE2 in

exposed mussels as measured by ALP. The observed differences were most pronounced in males, suggesting that they are more sensitive to the ED effects of EE2 *in vivo*. The physiologically hypoxic state entered by intertidal mussels during periods of low tide may enhance the effects of EE2 by reducing their detoxification capacity. It is possible that subtidal mussels, being continuously in contact with seawater, are not forced into a physiologically hypoxic state thus enabling their detoxification of EE2 to be more rapid, or uninhibited. The information derived from this study adds to the current understanding of the effects of EE2 in *Mytilus* spp., however further work is required to understand these processes in more detail, particularly the modes of action and chronic effects of EE2 and EDC exposure in *Mytilus* spp. and the differences in susceptibility to ED effects related to the position of the organisms in the marine environment. A longer ‘intertidal’ period, the use of intertidal control tanks and an increased number of mussels per treatment are all recommended in order to increase the power of the statistical evaluation of EE2 effects and reduced oxygen supply at different gametogenic stages. Analysis of E2 metabolites is also recommended. Combining sensitive and selective chemical analysis by LC-MS/MS and sensitive biological effects measurements will greatly assist in understanding the complex processes at work in the endocrine system of *Mytilus* spp. both naturally, and upon exposure to EDCs.

**Chapter 4: An integrated approach to investigating
endocrine disruption in marine waters using caged
Mytilus spp.**

4.1 Introduction

Release of EDCs into the marine environment may arise via a number of sources, including municipal and industrial wastewater effluent, agricultural and terrestrial run off, and accidental release (Desbrow et al. 1998; Terzic et al. 2008; Liu et al 2011). Research has primarily focussed on freshwater environments and studies often exclusively focus on chemical or biological based disciplines with little data existing on integrated approaches, especially for the marine environment. The detrimental effects of EDCs to exposed organisms have been demonstrated in a number of studies (Pandian and Sheela, 1995; Allen et al. 1999; Livingstone et al. 2000; Quinn et al. 2004; Peck et al. 2007; Canesi et al. 2008). Data generated using biological effects techniques provides valuable information regarding the qualitative effects aspects of EDCs, including estrogenic, synergistic, or anti-estrogenic properties of a sample (Chang et al. 2009). Targeted chemical analysis enables the identification of the causative chemicals, as a specific response to a single compound may not yet be defined.

Integrating chemical analysis with biological effects monitoring has been recommended within the WFD, the ICES/OSPAR Study Group on Integrated Monitoring of Contaminants and their effects in Coastal and Open Sea Areas (SGIMC) and the Working Group on Biological Effects of Contaminants (WGBEC) in order to more comprehensively assess the effects of pollutants within the environment. Further to this WGBEC recommends combining PS and “bioanalyses” as an important link between WFD and the EU Marine Strategy Framework Directive (MSFD), (WGBEC 2007).

Mytilus spp. are sessile filter feeding bivalves who filter large amounts of water and bioaccumulate many compounds, making them suitable for use in monitoring studies (e.g. Oslo Paris Commission (OSPAR) or the WFD) to assess the status and trends of chemical contamination in estuarine and coastal environments. Caging marine bivalves is an emerging technique enabling the time-integrated assessment of the effects of pollution to be determined. Besse et al. (2012) reviewed the literature relating to biomonitoring studies and found that biomonitoring for WFD purposes using transplanted organisms was more suitable than passive approaches (using indigenous species), as they implement reproducible strategies, control biotic confounding factors and provide robust, comparable results. Caging has been applied in a number of international studies to measure the endocrine response of freshwater bivalves placed downstream of wastewater effluent using biomarkers (Gagné et al. 2004), to assess responses to pollution (Martel et al. 2003;

Tsangaris et al. 2010), to examine contaminant uptake and bioavailability using chemical techniques (Gagnon et al. 2006) and to measure trends in coastal water quality (Hunt and Slone, 2010).

The detection of these EDCs at and below the existing and proposed total water EQS values using traditional spot water samples and MS methods has proved challenging due to issues with instrument sensitivities. Data derived from spot sampling may also be unrepresentative due to the dynamic nature of marine environments. Episodic pollution events may not be captured. Conducting a sufficiently comprehensive spot sampling programme would be costly and time consuming. Passive sampling technologies have the potential to provide a cost effective means of detecting trace levels of pollutants in water. Their application has been suggested as being a suitable complementary method for use in WFD surveillance, operational and investigative monitoring (EC, 2009) and they may be used to corroborate or contradict spot sampling data, or as screening tools to identify problem and non problematic areas. The polar organic chemical integrative sampler (Alvarez et al. 2004) has been shown to concentrate E1, E2 and EE2 from the water phase (Alvarez et al. 2007; Morin et al. 2012). While calibration data and sampling rates for POCIS have been reported and reviewed for many EDC groups (Morin et al. 2012), sampling rates which consider all the environmental variables present in the field are yet to be defined. POCIS is generally accepted for use as an EDC screening/surveillance device.

The two main aims of this study were

1. The first application of an integrated chemical and biological effects based approach to the monitoring of selected EDCs at three Irish marine sites with varying degrees of anthropogenic input.
2. The investigation of transplantation of bioindicators as a technique to enable clearer interpretation of differences in biomarker responses observed between sites.

Transplanted *Mytilus* spp. were deployed for three months, to three Irish coastal sites, two of which were potentially impacted by WWTP effluent and a reference site with little to no anthropogenic input. Steroid estrogens provided the focus for the highly sensitive LC-MS/MS and PS analyses due to their legislative relevance, high potency and potential for effects in exposed organisms. Spot water samples were analysed for E1, E2 and EE2 while POCIS samplers were used as a sensitive screening tool to identify E1, E2 and EE2 in the dissolved phase. Biological responses were assessed using ER-LUC and ALP as a measure

of total estrogenicity and estrogenic effects and these were further supported by mussel histology and condition factor estimate. This study was a collaboration with partners in the SeaChange project, who conducted ALP, condition factor and histology analyses. These biological effects assays were chosen in order to account for the potential of effects due to other estrogenic compounds not identified using PS and/or LC-MS/MS. Complementarities between biological and chemical approaches and suitability in terms of ease of use and sensitivity of results were investigated. The study presents the first Irish data on the presence and effects of selected estrogenic EDCs in three Irish marine sites and is the first to use an integrated biological and chemical assessment approach for the assessment of selected estrogenic EDCs and ED in Irish marine waters.

4.2 Materials and methods

4.2.1 Site selection

Three coastal sites were selected for this study, with varying degrees of anthropogenic input. Particular focus was placed on sites with WWTP inputs. The sites are shown in Figure 4.1, with a map presented in Figure 4.2

Site 1, the North Bank Lighthouse (NBL) is situated in the estuarine area of Dublin Bay (N 53°20'41''; W 06°11'35'') in the vicinity of Dublin Port, Ireland's premier port, which handles ~50% of all of Ireland's imports and exports. NBL, Dublin is positioned 700 m downstream of Ringsend WWTP, which serves 1, 640,000 PE with tertiary treatment using UV sterilisation (Monaghan et al. 2009).

Site 2, Mutton Island (MI), is situated in Galway Bay, on the Atlantic coast of Ireland (N 53°15'13''; W 09°03'17''). Cages were placed in the vicinity of the diffuser pipe of Mutton Island WWTP, which serves 91,600 PE from Galway City and surrounds, providing secondary treatment (activated sludge) to effluents (Monaghan et al. 2009).

Site 3, Omev Island (OI) is a remote island off the West coast of Ireland, with little to no anthropogenic input (N53°31'58''; W 10°09'52''). It is only accessible at low tide and was selected as a control site.



Figure 4.1: Caging locations. Depicted are (1) North Bank Lighthouse, Dublin and (2) Mutton Island (cages attached to existing structures) and (3) Omey Island (cages attached to marker buoys and mooring).

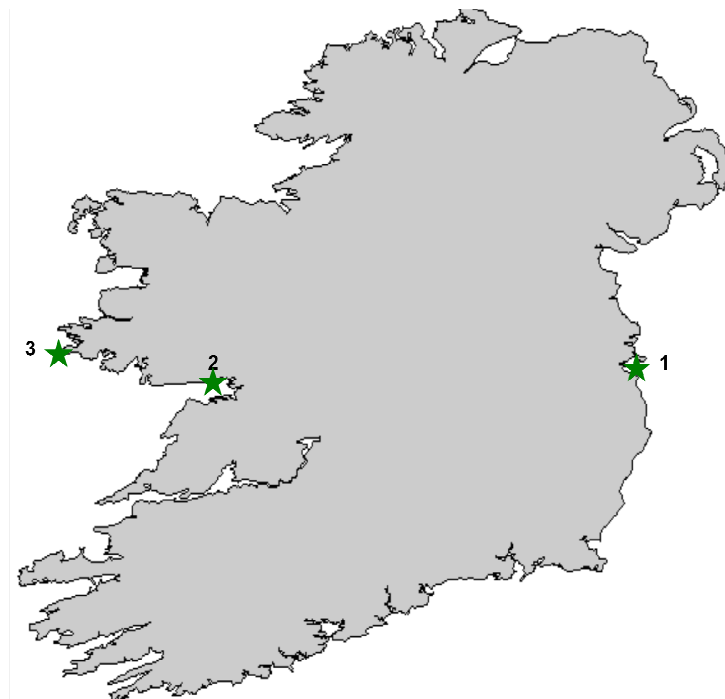


Figure 4.2: Map displaying deployment locations (1) North Bank Lighthouse, (2) Mutton Island and (3) Omey Island.

4.2.2 Preparation and deployment

In June 2010 mussels from New Quay Co. Clare (N 53°09'27.28; W 09°04'03.84) measuring between 4 and 6 cm were placed in oyster bags with 14 mm mesh size (250 mussels per bag, 2 bags per site) and acclimatised for 24 hours in artificial seawater (Figure 4.3).

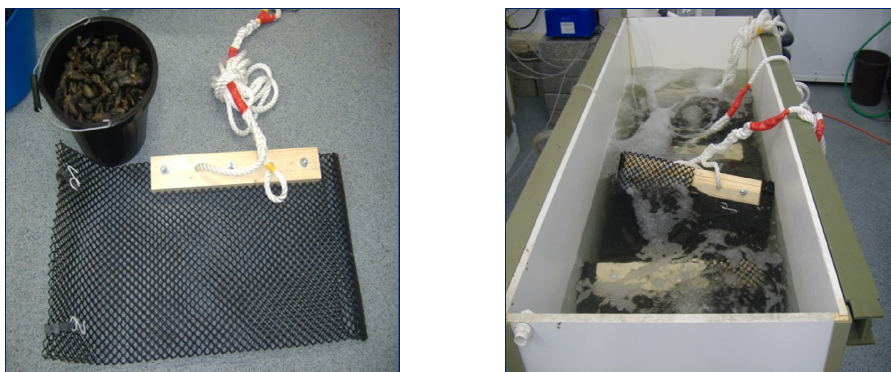


Figure 4.3: (a) Oyster bag and mussels and (b) acclimatisation tank.

Bags were attached to existing structures (MI and NBL) or attached to marker buoys (OI) and deployed 1 m below the water surface. Mussels remained fully submerged for the three month exposure. The Irish coastal zone contains a mixture of pure, hybrid and introgressed individuals (Coghlan and Gosling, 2007), and since there is no single morphological characteristic that can be reliably used to separate this mixed population, exact classification of the *Mytilus* species used in this study was not possible. At each site, a POCIS passive sampling device containing three discs was attached to the mussel cages and replaced on a monthly basis.

4.2.3 Sample collection

At monthly intervals each canister containing three POCIS PS discs was removed and replaced at each site, the POCIS disks and corresponding field blank were then stored at -30°C prior to EDC analysis at the National Laboratory Service, Environment Agency, UK. 30 mussels per site were collected monthly, whole body tissue homogenised using a Waring blender (Waring Commercial, New Hartford, CT, USA); freeze dried and frozen at -30°C prior to chemical analysis as per Chapter 2. Monthly water samples (five L) taken at 1 m depth were collected in pre-washed amber glass bottles, filtered through 0.45 µm glass fibre filters (Whatman GF-F, General Electric, US) and 50 mL of Formalin (1% v/v) was added to prevent bacterial growth. Water samples were stored in the dark at 4°C prior to

LC-MS/MS analysis (Chapter 2). Salinity and temperature were recorded monthly to a depth of 2 m, at 0.5 m intervals at each sampling event. At time zero (T=0) and after three months (T=3) samples were collected for ALP, histology, condition factor (CF) and ER-LUC and prepared as per Chapter 2.

4.2.4 Sample analysis

Sample analysis was conducted as per Chapter 2. There were no deviations from the methods as described. Experimental design, sample collection and chemical analysis of water and mussel samples were completed as part of this research thesis. ALP, condition factor and histology were completed by partners in the SeaChange project. ER-LUC and POCIS analyses were completed under subcontract to Institute for Environmental Studies (IVM), The Netherlands, and the National Laboratory Service, Environment Agency, UK, respectively, with methods detailed in Chapter 2.

4.2.5 Availability of assessment criteria

With the exception of EU derived EQS, ecotoxicological focused assessment criteria are generally still in development (e.g. via ICES WGBEC and or SGIMC) for the range of biological analysis completed during this study.

4.2.6 Statistical analysis

Assumption tests of normality and equal variance were performed on each data set (F-Max test). A one-way analysis of variance (ANOVA) followed by a LSD post-hoc test were conducted where data was normally distributed. Where data was not normally distributed a Kruskal-Wallis analysis was performed. Gonadal stage was assessed using a Friedman ANOVA and Kendall coefficient of concordance.

4.3. Results

4.3.1 Physicochemical parameter monitoring (salinity and temperature)

Salinity was highest and most stable at Omev Island ($33.3 \pm 2.8\text{‰}$). NBL had the lowest and most variable salinity, reflecting the estuarine nature of the site ($26 \pm 5.8\text{‰}$), and salinity at Mutton Island was ($29.6 \pm 4\text{‰}$). Temperature was highest in July and August, and displayed a greater degree of variation at NBL and Omev Island ($16.8 \pm 2.1^{\circ}\text{C}$ and

16.2 ± 2°C respectively). Temperature was 16.7 ± 0.8°C at Mutton Island over the duration of the study.

4.3.2 Mussel condition factor and protein content

No significant difference in condition factor (CF) was observed between males and females in each individual site (i.e. males vs. females at Omey Island, males vs. females at NBL etc), so the assessment of the differences between locations was performed on pooled males and females per site (Figure 4.4). Data was normally distributed, so an ANOVA was completed followed by a LSD post-hoc test. Results are presented in Table 4.1. CF was significantly higher at Mutton Island than at Omey Island ($p = 0.08 \times 10^{-18}$) and NBL ($p = 0.08 \times 10^{-10}$). It was also significantly higher at NBL than at Omey Island ($p = 0.03 \times 10^{-3}$). In general, protein content in females was higher than males (Table 4.1). Protein content in females at Mutton Island was significantly higher than that of males at Mutton Island ($p = 0.02$), males at NBL ($p = 0.01$) and males at Omey Island ($p = 0.01$). Significant decreases in protein content were noted in NBL males ($p = 0.04$), and Omey Island males ($p = 0.047$) relative to T0.

Table 4.1: ALP ($\mu\text{g ALP/mg protein}$), protein content (mg/mL), condition factor (wet weight: cm^3) and ER-LUC results ($\text{ng estradiol equivalents (EEQ)/g dw}$)

Result	T=0	NBL Dublin T = 3	Mutton Island T = 3	Omey Island T = 3
Female				
ALP $\mu\text{g P/mg protein}$ ($\pm \text{RSD } \%$)	15.7*	14.2 ± 46.6	19.1 ± 27.8	11.18 ± 59.6
Protein, mg/mL ($\pm \text{RSD } \%$)	8.79*	7.2 ± 11.8	7.7 ± 10.8	6.8 ± 9.0
Condition factor ($\pm \text{RSD } \%$)	x	3.5 ± 18.6	5.0 ± 14.6	2.8 ± 16.9
n=	1	15	9	6
Male				
ALP $\mu\text{g P/mg protein}$ ($\pm \text{RSD } \%$)	15.3 ± 35.8	15.3 ± 138.4	11.3 ± 61.4	9.9 ± 61.6
Protein, mg/mL ($\pm \text{RSD } \%$)	7.3 ± 12.9	6.1 ± 26.6	6.6 ± 18.2	6.4 ± 21.2
Condition factor ($\pm \text{RSD } \%$)	x	3.5 ± 21.2	4.8 ± 14.6	2.8 ± 13.9
n=	10	15	15	23
Sexes pooled				
ER-LUC ng EEQ/g dw	0.23	0.34	0.13	0.09
n=	30	30	30	30

x=CF was not assessed at T=0

* Only one individual available

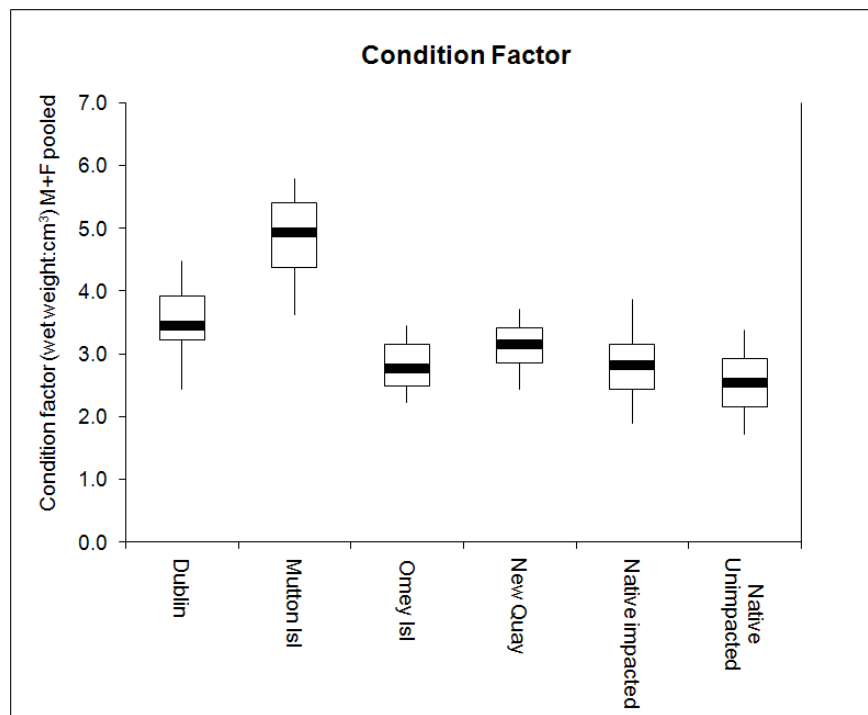


Figure 4.4: Box plots of condition factor results.

Depicted are the median (= line), upper and lower quartiles (= box) and the mean standard error (=bars). CF results from native impacted and un-impacted sites included for reference. Dublin = NBL.

4.3.3 ALP and gonadal stage

ALP data was not normally distributed so a Kruskal Wallis analysis was performed. A significant decrease in ALP levels was detected in male mussels at Omey Island after exposure (T=3) ($p=0.02$), compared with T0 mussels from New Quay (Figure 4.5 and Table 4.1). Levels of ALP were significantly higher in females at Mutton Island and females at Omey Island and NBL ($p=0.01$ and 0.04 , respectively). Significantly higher ALP levels were measured in females at Mutton Island relative to males at Mutton Island and Omey Island ($p=0.01$ and 0.01 , respectively).

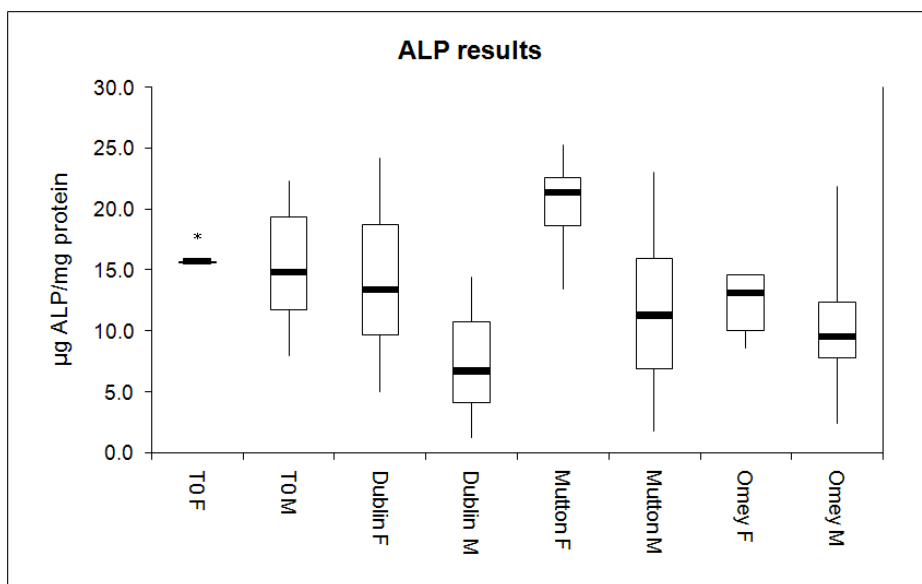


Figure 4.5: Box plots of alkali-labile phosphate concentration ($\mu\text{g ALP/mg protein}$) per site and sex.

Depicted are the median (= line), upper and lower quartiles (= box) and the mean standard error (=bars). * = Only one value for this site. M denotes males and F denotes females. Dublin = NBL.

When ALP results were pooled per site and analysed independently of sex, a significant decrease was observed after exposure (T=3) in mussels at Omey Island and an increase observed in mussels from Mutton Island ($p=0.01$ and 0.03 respectively), see Figure 4.6.

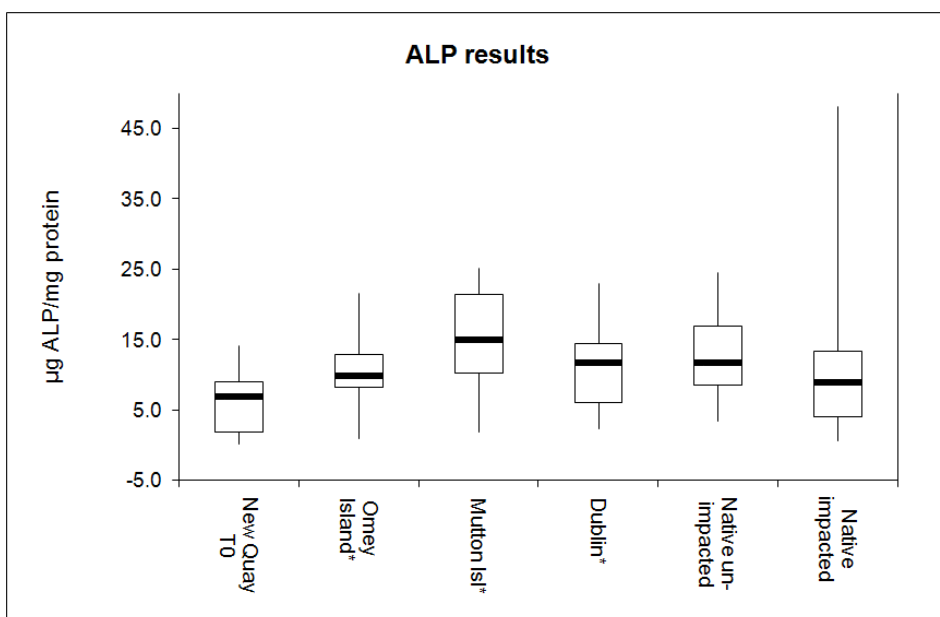


Figure 4.6: Box plots of pooled male and female alkali-labile phosphate concentration ($\mu\text{g ALP/mg protein}$) at test sites.

Native ALP results ($\mu\text{g ALP/mg protein}$) included for reference. Depicted are the median (= line), upper and lower quartiles (= box) and the mean standard error (=bars). * = sites where mussels were transplanted. Dublin = NBL.

Gonadal stage data were non-parametric measurements and were assessed using a Friedman ANOVA and Kendall co-efficient of concordance. There were no statistically significant differences between gonadal stages between sites thus differences in ALP were not related to gonadal stage. There were no statistically significant differences between gonadal stage in sexes pooled ($p=0.38$), males and males ($p=0.23$), females and females ($p=0.29$) and males and females ($p=0.25$) between sites. Gonadal stages are presented in Table 4.2. A description of stages as per Seed (1976) is presented in Table 2.7, Chapter 2.

Table 4.2: Gonadal stage per site measured as per Chapter 2.

Stage	NBL F	Mutton Isl F	Omey Isl F	New Quay F	NBL M	Mutton Isl M	Omey Isl M	New Quay M
D1	3	0	0	0	1	0	2	1
D2	3	0	3	1	1	0	4	0
D3	3	6	1	0	5	1	5	0
D4	3	2	1	0	5	10	4	2
D5	2	0	0	0	0	2	1	0
S0	0	0	0	0	0	0	1	0
S1	0	0	1	0	0	0	1	3
S2	0	0	0	0	0	0	3	4
S3	1	1	0	0	0	0	2	0
S4	0	0	0	0	3	2	0	0
n=	15	9	6	1	15	15	23	10

F denotes females, M denotes males

4.3.4 Estrogen luciferase reporter gene assay

ER-LUC results, expressed as ng/g estradiol equivalents (EEQ) dry weight, increased from T=0, (0.23 ng/g dw), to 0.34 ng/g dw at T=3 at NBL. Decreases in ER-LUC levels were observed from T=0 New Quay mussels (0.23 ng/g dw) to 0.13 and 0.09 ng/g dw at Mutton Island and Omey Island, respectively. By way of comparison native mussels from NBL were also collected at T0 and T=3 and native mussels from Mutton Island which were collected in March 2009 were also assessed using ER-LUC. Levels were found to be higher than those observed in the caged mussels, 0.81 ng/g dw at Mutton Island in March, and 0.72 and 0.24 ng/g dw in NBL in July and October respectively.

4.3.5 Concentrations of EDCs in water and mussels by LC-MS/MS

LC-MS/MS results presented in Table 4.3 show that E1 was detected at T=0 and T=3 at NBL, ranging from 0.14 to 1.11 ng/L. E2 was detected on a single occasion only, (NBL (T=3) at 0.13 ng/L). E1 and E2 were not detected (LOD= 0.07 ng/L) at Omey or Mutton Islands. EE2 was not detected (LOD= 0.11 ng/L) at any site. E1, E2 and EE2 were not

detected in mussels from any site at any of the sampling timeframes (LOD= 0.7, 0.9 and 0.3 ng/g ww, respectively).

Table 4.3: E1, E2 and EE2 in water samples (ng/L) and biota (ng/g ww) by LC-MS/MS, and in POCIS (ng/device)

Location	E1 Water	E1 POCIS	E1 Mussel tissue	E2 Water	E2 POCIS	E2 Mussel tissue	EE2 Water	EE2 POCIS	EE2 Mussel tissue
NBL T0	0.14 ± 0.01	*	nd	nd	*	nd	nd	nd	nd
NBL T1	nd	6.3	nd	nd	2.9	nd	nd	nd	nd
NBL T2	nd	4.7	nd	nd	1.1	nd	nd	nd	nd
NBL T3	1.11 ± 0.1	3.6	nd	0.13 ± 0.01	0.7	nd	nd	nd	nd
MI T0	nd	*	nd	nd	*	nd	nd	nd	nd
MI T1	nd	6.9	nd	nd	2.1	nd	nd	nd	nd
MI T2	nd	7.6	nd	nd	1.2	nd	nd	nd	nd
MI T3	nd	N/A	nd	nd	N/A	nd	nd	nd	nd
OI T0	nd	*	nd	nd	*	nd	nd	nd	nd
OI T1	nd	N/A	nd	nd	N/A	nd	nd	nd	nd
OI T2	nd	N/A	nd	nd	N/A	nd	nd	nd	nd
OI T3	nd	3.01	nd	nd	0.48	nd	nd	nd	nd

LOD water: 0.07 ng/L E1 and E2, 0.11 ng/L EE2

LOD tissue: 0.4, 0.9 and 0.3 ng/g ww for E1, E2 and EE2.*Sample not available for T0 due to required equilibration time required (1 month).

N/A Device detached from mooring or damaged.

NBL-North Bank Lighthouse, MI-Mutton Island, OI- Omei Island

4.3.6 POCIS by LC-MS/TOF

POCIS required one month to accumulate the compounds, thus there are no values for T0. Indicative surveillance POCIS testing prior to transplantation of mussels revealed the presence of E1 and E2 in NBL and Mutton Island (E1 at 8.5 and 15.3 ng/device and E2 at 3.8 and 3.9 ng/device in NBL and Mutton Island, respectively). Following transplantation E1 was detected at all sites, with highest values of 6.9 ng/POCIS and 7.6 ng/POCIS observed in July (T=1) and August (T=2) at Mutton Island (Table 4.3). E1 was detected at 6.3 ng/device in NBL in July, and decreased over the study period. E2 was also detected at each site, and showed a similar pattern of decreasing concentration over time at both Mutton Island and NBL. EE2 was not detected at any site (LOD 0.1 ng/device). E2 concentrations were consistently lower than E1, with a similar ratio of E1 to E2 detected in NBL in water by LC-MS/MS and POCIS samples when both analytes were present in the sample. Although Omei Island was a control site, E1 and E2 were detected in devices (Table 4.3). The highest E1 concentration was detected in POCIS in July (T=1), however it was not detected in water samples taken at the same time. The highest E1 water

concentration was detected in October (T=3), which corresponded to the lowest E1 concentration in POCIS at that site. This was also the case for E2 at T=3, where the only positive water sample was collected, while the E2 concentration was the lowest observed at that site during the study.

4.4 Discussion

The presence and effects of EDCs were assessed at three marine sites using a suite of biological analysis, chemical analysis and passive sampling technologies. Overall low level presence of steroidal EDCs in water and in mussels and low biological effects of EDCs in caged (and in native mussels) were detected at the three marine sites.

Omey Island exhibited the highest and most stable salinity values reflecting the open coastal nature of the site. Salinity was lower and had a higher degree of variability in the other two sites, particularly in the estuarine NBL. Mutton Island, while in a coastal location, is subject to freshwater input from the River Corrib to the East. The average temperature was similar among sites, although there was a greater degree of variability at NBL and Omey Island. Mussel condition factor estimates varied greatly between sites and were highest at Mutton Island, where the cages were in close proximity to the wastewater diffuser pipe which may provide a potential nutrient source. The higher CF values observed at Mutton Island and NBL may be due to the greater nutrient loadings in these industrial sites. CF estimates were lower at NBL, 700 m downstream of the wastewater input, and lowest at the control site, where there is little to no anthropogenic input to influence the condition of mussels. While results from the bioassays do not suggest endocrine disrupting effects at any of the sites tested, the potential for condition factor based impacts in more heavily impacted sites cannot be discounted and thus can be a drawback of using transplanted organisms. Many anthropogenic and environmental factors may influence the condition of an organism, including food availability, stress, stage of sexual development and abiotic factors such as temperature changes and salinity (Gosling, 1992; Gardner and Thompson, 2001). Fluctuations in salinity have been shown to reduce filtration rates and growth (Bøhle, 1972). It is essential to consider these variables when using a transplanted bioindicator organism. Overall condition factor estimates and physicochemical parameters measured suggest that environmental conditions were such that the organisms were not negatively affected by transplantation.

A wide range of gonadal stages were present at each site. Mussels at each site were predominantly in the early stages of gonad development. Mussels at Mutton Island were slightly more advanced than those at NBL and Omey Island, which may explain the significantly elevated ALP in females at Mutton Island; however the differences in gonadal stage were not statistically significant. Observations in the field also suggest that mussels at Mutton Island spawned earlier in the study, due to the presence of large numbers of juveniles within the cages observed after two months deployment. This was not observed at NBL or Omey Island. The range of natural variability in ALP levels in native mussels from impacted and un-impacted sites are presented in Figure 4.6 for reference. Levels observed in transplanted mussels were not significantly elevated relative to natural levels in native mussels, thus ALP was not deemed to have been induced at any of the three study sites, and they were deemed to be of low risk of estrogenic ED as measured with ALP.

It is clear that mussels have a marked seasonality, therefore seasonal changes in biomarker response need to be fully understood in order to enable accurate risk assessment to be completed (Blaise et al. 2002; Ortiz-Zarragoitia and Cajaraville, 2006). A variety of concentrations of endogenous steroids have been reported for *Mytilus* spp. E2 has been reported up to 5.42 ng/g dry weight (dw) in *M. galloprovincialis* using ELISA (David et al. 2008), and up to 2.5 ng/g and 0.2 ng/g ww in *M. edulis* gonad tissue and peripheral tissue respectively, using radio immunoassay (RIA) (Lavado et al. 2006). These values are slightly higher than the range of EEQ levels measured with ER-LUC in the transplanted mussel stock, both naturally and after transplantation. Observed variations in ER-LUC response were within the natural range of response recorded in the transplanted stock (0.06 to 0.34 ng/g dw depending on stage in the gametogenic cycle, n=4 samples taken over one year) and the natural EEQ responses (0.08 to 0.47 ng/g dw) from other native Irish mussels (discussed in detail in Chapter 5), thus increases and decreases observed after exposure, although different at each site, were not deemed to have been altered due to exposure or transplantation. Mussels from NBL had a higher EEQ value than Mutton and Omey Islands; however no significant difference in gonadal stage was determined.

EEQ in native mussels from NBL and Mutton Island were higher than those observed in the transplanted mussel stock at 0.24 to 0.72 ng/g dw at NBL, and 0.81 ng/g dw at Mutton Island (discussed in Chapter 5) which could suggest a greater variation in EEQ in these native populations, or may suggest that native populations in these locations may be experiencing some effects of exposure to EDCs in their environment. In the absence of

peer reviewed ecotoxicological risk assessment criteria it is unclear whether the elevated responses observed in native mussels from NBL and Mutton Island suggest a greater natural variation in EEQ or whether results are reflective of actual effects of exposure to EDCs in their environment. NBL and Mutton Island transplanted samples were more elevated than those from Omey Island therefore it may also be possible that the three month transplantation time may be insufficient to elicit the same level of response observed in continually exposed native organisms.

Research to accurately determine steroid levels in *Mytilus* spp. is ongoing, and the presence of endogenous steroids is a limitation in using bioindicators to assess ED in the field. Using a well characterised indicator organism and monitoring developmental stage reduced some of the uncertainties related to endogenous steroids. While results from the bioassays used do not suggest endocrine disrupting effects at each site, the impact of reduced condition may influence results in more heavily impacted sites, and is one of the drawbacks of using transplanted organisms.

Overall EDC levels in all waters tested were low. E1 was detected in water samples from NBL only (T=0 and T-3), with E2 detected only once, again at NBL (T=3), this value exceeding the proposed total water WFD EQS for E2. The three other samples taken in the course of the study were <LOD (0.07 ng/L). When these results are combined the AA value at this site is <0.08 ng/L, thus E2 levels were not deemed to be of concern. Particulate loadings in seawater are typically low (<15 mg/L) (Anderson and Meyer, 1986; Dobrynin et al. 2010) reaching up to 70 mg/L occurring in certain areas, such as river plumes (Ferraria et al. 2003; Dobrynin et al. 2010). Lopez de Alda and Barceló (2001) and validation of the LC-MS/MS method completed in Chapter 2 have demonstrated that filtration does not lead to significant losses of steroids from water samples, thus analysis of filtered seawater was deemed suitable for monitoring of E2, however WFD EQS values are set for total water concentrations. Further monitoring is required to strengthen the information with respect to E2 WFD compliance at this site. E1 and E2 were not detected at either Omey Island or Mutton Island while EE2 was not detected at any site. Steroids are generally detected in the low ng/L range, thus levels detected in this study are in line with those detected in many other international studies.

E1 and E2 were detected at low ng/device concentrations at each site using POCIS with E1 levels consistently higher than E2 at all sites. Where measurable, E1/E2 ratios in water

samples concurred with those derived from POCIS devices in NBL at T=3. Interestingly, the pattern of concentrations (e.g. highest to lowest measured values) did not concur between POCIS and water samples containing E1 and E2. Levels of E1 and E2 in POCIS deployed in NBL, Dublin were highest at T=1, and decreased over the duration of the study. In the aquatic environment, E2 is rapidly biodegraded into E1 (Jürgens et al. 1999) thus E1 being the only detected steroid, or detected at higher concentrations than E2 in this study concurs with a number of other studies (Noppe et al. 2007; Liscio et al. 2009; Saravanabhavan et al. 2009; Grover et al. 2011a).

E1 was only detected in NBL, Dublin in June and October, with highest concentration collected in October, corresponding to the lowest E1 concentration in POCIS. For both E1 and E2 in NBL, Dublin in July and August, the analytes were detected in POCIS but not in water samples, despite the levels in POCIS being among the highest detected over the course of the study. The variability of spot water samples has been previously demonstrated (Williams et al. 2003), and may explain the differences observed between POCIS and water samples. The site at NBL is estuarine and subject to tidal influences. Samples were collected approximately two hours before or after low tide depending on the time of the tide, as the cages were not accessible at high tide. Effluent from the Ringsend WWTP is discharged upstream of the site. The highest concentrations of steroid estrogens were detected in POCIS in Mutton Island in both T=1 and T=2, although E1 and E2 were not detected in water samples. Due to the low concentrations of steroids expected in the marine environment, a water sample volume of five litres was selected in order to enhance the detection capacity of the LC-MS/MS method. Despite this, E1 and E2 were not detected in most of the water samples collected despite being detected in POCIS. EE2 was not detected with either sampling method. Sampling rates for POCIS have been reported at between 0.02 and 0.85 litres per day for E1, E2 and EE2 (Morin et al. 2012). Using the highest value suggests that up to 25.5 L of water may have been sampled by POCIS during deployment, which may explain detection of E1 and E2 by POCIS when it was not detected in water. A larger water sample volume than five L may be necessary in order to detect lower level E1 and E2 in the marine environment until such a time as accurate POCIS sampling rates are defined.

Using an average estimated uptake rate of 0.42 L/day from values reported in the literature (Morin et al. 2012), it could be estimated that the approximately 12.6 L of water passed through the samplers during deployment (based on a 30 day deployment). Using this

estimate on POCIS results from this study, the highest E1 and E2 levels detected would be 0.11 and 0.04 ng/L respectively. These values are below the LC-MS/MS LOQ for E1 (0.12 ng/L) and below the LOD for E2 (0.07 ng/L). This may explain why E1 and E2 were not detected in water samples from Mutton and Omey Islands, while they were detected in POCIS. E1 and E2 were detected in water samples in NBL, Dublin above these POCIS estimates. Spot samples provide only a snapshot of the conditions within what is generally a dynamic environment. A wide variance in E1 concentrations measured in spot samples has been demonstrated by Williams et al. (2003), thus this may explain the difference in reported and calculated results. This estimated uptake rate is for illustrative purposes only; sampling rates for POCIS in the field are yet to be accurately and robustly determined. Zhang et al. (2008) found that field derived sampling rates for E1 and E2 in optimised POCIS were significantly higher than those derived in the laboratory. Considerable research is still needed to accurately determine POCIS uptake rates in the field. For the purposes of this study, the POCIS devices are used as a screening device only.

Adverse weather conditions at Omey Island resulted in retrieval of only one POCIS PS device and while Omey Island was classed as a relatively un-impacted site, E1 and E2 were detected in September (T=3, Table 4.3). Clear anthropogenic sources for these compounds are unclear. Steroids may be produced within mussels at very low ng/g concentrations. Reis-Henriquez et al. (1990) identified vertebrate like steroids, such as E2 in mussels while David et al. (2008) studied concentrations of E2 over the reproductive cycle in *Mytilus galloprovincialis*. Concentrations ranged 2.05 to 5.42 ng/g dw for males, 2.06 to 5.11 ng/g dw for females and from 1.71 to 4.94 ng/g dw in the “indifferent” stage where it was not possible to determine the sex of the individuals. In a further study by Liscio et al. (2009), the uptake of a number of EDCs was compared between PS and the freshwater mussel *Unio pictorum* exposed to WWTP influent and effluent. E2 was detected in the control tanks at 6 ng/POCIS, and the authors suggested that this may be due to naturally occurring E2 released from the mussels. E1, E2 and EE2 were not detected in mussels from any site at any of the sampling timeframes. The sample analysed using LC-MS/MS was a pooled extract from 30 mussels. The POCIS devices were attached to the bags of mussels. There were approximately 500 mussels from this study at T=0, which was reduced by 60 mussels at each sampling event. Two bags containing an additional 500 mussels were also deployed next to these bags and were used for a study completed by researchers from the Galway-Mayo Institute of Technology. It is possible that endogenous E1 and E2 were released from these mussels into the surrounding water at very low

concentrations. POCIS has the capacity to sample larger water volumes than the 5 L collected for the LC-MS/MS method, thus E1 and E2 may have been concentrated to a level in POCIS which is within the range of detection of the LC-MS/TOF method, but below the detection limit of the LC-MS/MS method detailed in Chapter 2. This may explain the detection of E1 and E2 in POCIS at Omey Island as the devices were in close proximity to the caged mussels. The concentrations in POCIS decreased over the duration of the study, as did the number of mussels in close proximity to the devices (a reduction of 120 mussels per sampling event). Continued research is therefore merited in order to accurately quantify endogenous EDC levels in test species in order to accurately inform the ongoing process of the derivation of biota based “ecotoxicological or assessment criteria”. It is recommended that future studies using this approach should position POCIS at a suitable distance from caged organisms.

In this study chemical analysis of a water sample by LC-MS/MS detected E2 in NBL, Dublin Bay at 0.13 ng/L at T=3. It could be interpreted from this value that organisms at this site may be at risk of endocrine disruption. Spot samples only provide a snapshot of the environment in question, by using a multi-parameter approach, a more comprehensive assessment of the risks at this site was possible. Passive sampling data obtained in the same month revealed the lowest recorded E2 values by PS at that site over the duration of the study. Combined with low EEQ present in the caged mussels as detected with ER-LUC, and low ALP levels relative to controls, it was concluded that this site was not at high risk from the selected EDCs. LC-MS/MS and POCIS permitted the detection of the most potent estrogenic compounds, the steroid estrogens. Many compounds may interfere with the endocrine system. Using non-compound specific techniques (ALP and ER-LUC) broadened the scope of the study, to include other estrogenic compounds which may have been present.

4.5 Conclusions

This study reports the first data on selected EDC levels in Irish marine waters and the first use of an integrated chemical and biological effects approach to assess the presence and effects of selected EDCs at three Irish coastal sites. The combined multi-compartment, multi-parameter approach in this study enabled the presence of estrogenic EDCs, and EDC associated effects to be assessed at three Irish coastal sites. Results demonstrated that levels of selected EDCs detected by LC-MS/MS in water and mussel tissue, and PS were low. No conclusive evidence of ED was detected using biomarker responses assessed with ALP and

ER-LUC, however further research into seasonal variations in native mussels is required to better understand biomarker responses observed in the field. The use of *Mytilus* spp. transplanted from a site with a previously characterised biomarker response allowed for a clearer interpretation of biomarker responses observed at each site following transplantation. Histology differences were also found to contribute to observed differences in ALP response. Caging was found to be a valuable tool enabling the control of biotic confounding factors and providing robust, comparable results.

PS was found to be a useful screening tool for monitoring EDCs at these sites, but may be subject to complications as a result of the potential presence of biomarker derived steroids. Future studies combining biomarkers and PS should take into account the presence and release of naturally occurring steroids and deployment of PS should be completed at a suitable distance from caged indicator species. Although one water sample analysed by LC-MS/MS detected E2 in NBL, Dublin Bay was above the proposed WFD EQS, the AA EQS determined using three other samples collected in this study determined that overall this E2 at this site was below WFD EQS for E2. The measured E2 concentrations are in the dissolved phase only and while filtration was not deemed to result in analyte losses during validation of the LC-MS/MS method, further monitoring is recommended to investigate the E2 concentrations at this site in order to fully comply with WFD EQS ‘total’ water requirements. Results generally agree that water and mussel tissue EDC levels were low as were ED effects as measured with ALP and ER-LUC. The utilisation of a multi-parameter integrated approach as detailed in this study ensured that a more comprehensive assessment of the risks at the three sites was possible and that none of the three sites were deemed to be at a high risk from the selected EDCs.

Chapter 5: A summary assessment of the presence and effects of EDCs in Irish marine waters

5.1 Introduction

Directive 2000/60/EC requires that surface water bodies in all Member States achieve good status by 2015. In addition, pollution from priority substances has to progressively be reduced and emissions of hazardous substances ceased or phased out. Once a water body has been classified, a further requirement of the WFD is that there should be no deterioration in water quality. E2, EE2, NP and OP have been included in the WFD priority substances list, with corresponding environmental quality standards (EQS). While not specifically included in the SWD, the analysis of EDC levels and effects in shellfish further enhances consumer confidence and the health status of the animals themselves. Further to this it is clear that integrated assessment approaches (including data on endocrine disruption as presented herein) will be fundamental to completion of future MSFD reporting obligations. Currently there is no data available on the presence of these compounds in Irish coastal waters.

It is widely accepted that a single analysis type, such as chemical monitoring, is insufficient to assess pollution impacts in the marine environment. Integrating chemical analysis with biological effects monitoring has been recommended within the WFD, by OSPAR, WKIMON, and WGBEC. *Mytilus* spp. are used within the OSPAR CEMP to assess the status and trends of chemical contamination in estuarine and coastal environments. *Limanda limanda* (dab), *Pleuronectes platessa* (plaice) and *Platichthys flesus* (flounder) are among the species of fish which are used as biomonitoring species within WGBEC, ICES, OSPAR and WKIMON.

Issues have arisen with detection of some compounds at and below EQS values and at biologically relevant levels using traditional spot water sampling, particularly in marine environments, where dilution can be high. Passive sampling devices have been suggested as complementary methods which can be used in WFD surveillance, operational and investigative monitoring in a 2009 Guidance on Surface Water Chemical Monitoring under the WFD (EC, 2009). They may be used to corroborate or contradict spot sampling data, or as screening tools to identify problem and non problematic areas.

This chapter presents an assessment of the presence and effects of selected EDCs in Irish coastal waters, conducted using a range of techniques, including chemical analysis of indigenous shellfish and fish, passive sampling and biological measurements of shellfish. This study presents the first spatially comprehensive integrated marine based dataset on the

occurrence and effects of EDCs in Irish waters and in resident marine species. Analytical data are compared within the confines of available assessment criteria. Site and parameter based commentary is presented and the needs for continued research and monitoring are highlighted.

5.2 Site and sample selection

This EDC study was completed in part fulfilment of a project supported by the Marine Institute (under the Sea Change strategy) and the Environmental Protection Agency (under the STRIVE Programme); and financed by the Irish Government under the National Development Plan 2007–2013. The project entitled “*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*” primarily investigated the potential for contaminants based effects in resident species in Irish marine waters.

Four key datasets were compiled to complete this assessment:

1. **Tier I locations:** Nine sites were selected, and analysed for the following: E1, E2, EE2, NP and OP in *Mytilus* spp. by LC-MS/MS. Vn-like protein content determined by measurement of ALP levels was also conducted in *Mytilus* collected at these sites. The estrogenicity of mussel tissues in estradiol equivalents (EEQ) using ER-LUC was determined. Estrogenicity of sediment from four sites in EEQ was also determined by ER-LUC.
2. **Tier II locations:** Four sites were selected from Tier I analyses for the completion of further “integrated” based study. Samples were analysed for the following: E1, E2, EE2, NP and OP in muscle and liver samples of *Limanda limanda*, *Pleuronectes platessa* and *Platichthys flesus* by LC-MS/MS. POCIS devices were also deployed at these sites for the detection of E1, E2 and EE2.
3. **SWD designated locations:** Shellfish (mussels and oysters) from 15 of the 60+ designated shellfish waters bodies included for SWD monitoring were analysed for E1, E2, EE2, NP and OP by LC-MS/MS. Additionally, native mussels collected from Galway and Dublin Bays were included in the assessment.
4. **Caging study (Chapter 4):** The three sites studied in Chapter Four were included in the assessment. Samples from these sites include water samples which were analysed for the presence of E1, E2 and EE2 by LC-MS/MS and POCIS devices which were screened for E1, E2 and EE2. E1, E2, EE2, NP and OP in native

Mytilus tissue and transplanted *Mytilus* were also determined using LC-MS/MS. Samples from these sites were assessed for ED effects and estrogenicity by ALP and ER-LUC. The relative merits of caging versus native species monitoring are discussed.

All sites were selected on the basis of historical data, proximity to anthropogenic input, particularly WWTP effluent inputs from population centres of varying sizes, and agricultural inputs. SWD sites were selected in order to cover a wide spatial range. Potentially impacted and un-impacted sites were chosen, with details of potential impacts at each site presented in Table 5.1. Maps showing the sampling locations are presented in Figures 5.1 and 5.2. Full details of the individual sampling programs are presented throughout this thesis and are briefly described below.

Table 5.1: Sampling sites and potential pressures^{1,2}

Location	County	Dataset	Potential pressures	Nearest agglomeration (PE)	Treatment	Plant PE
Donegal Bay	Donegal	SWD	WWTP inputs from Donegal Town, possible agricultural run-off	5400	Secondary	12,000
Sligo Harbour	Sligo	SWD	WWTP inputs from Strandhill and Sligo town, possible input from domestic sources	1728	Secondary Strandhill, Secondary with nutrient reduction (nr) Sligo	Strandhill: 1500. Sligo town: 50,000
Killala Bay	Mayo	SWD	Untreated waste from Killala (1,500PE), downstream of Ballina (16,000PE)	16,000	Secondary	20,000
Inislaughill (Clew Bay)	Mayo	SWD	WWTP inputs from Westport and Castlebar, agricultural run-off, inputs of untreated waste	29,905	Secondary with nr	35,000
Omey Island	Galway	Caging, Tier II	Little to no anthropogenic input, possible agricultural run-off and input of untreated waste from domestic sources	<10 Omey Island, <1000 Claddaghduff	none	n/a
Mutton Island (Galway Bay)	Galway	Caging+ Tier II	WWTP inputs, agricultural run-off from river Corrib and others, inputs of groundwater	91,600	Secondary	91,600
Kinvara	Galway	Tier I	Untreated waste, possible agricultural run-off, input of groundwater	850	none	n/a
New Quay	Clare	Tier I+II	Little anthropogenic input, possible agricultural run-off and input of untreated waste from domestic sources	<1000	none	n/a
Shannon	Clare	Tier I+II	River Shannon drains a large catchment with urban, agricultural and industrially derived inputs. Sampling area is near Carrigaholt and outer Shannon Estuary	582 (Carrigaholt)	none	n/a
Cromane	Kerry	SWD	Little to no anthropogenic input, possible source of treated and untreated waste from nearby towns, agricultural run-off	7,717 (Killorglin)	Secondary	5,000 (Killorglin)

Continued overleaf

Location	County	Dataset	Potential pressures	Nearest agglomeration (PE)	Treatment	Plant PE
Tralee	Kerry	Tier I	WWTP inputs from Tralee, possible agricultural run-off and untreated waste from domestic sources.	33,483 (Tralee)	Secondary	42,000 (Tralee)
Kilmackilloge	Kerry	SWD	Little anthropogenic input, possible agricultural run-off and input of untreated waste from domestic sources. Input of secondary treated waste from nearby Kenmare	6768 (Kenmare)	Secondary	3,500
League point (Bantry Bay)	Cork	SWD	Untreated wastewater	2700	none	n/a
Castletownbere	Cork	SWD	Untreated wastewater	1,100	none	n/a
Dunmanus Inner	Kerry	SWD	Little to no anthropogenic input, possible input of untreated waste from domestic sources, possible agricultural run-off	334 (Durrus)	none	n/a
Roaring Water Bay	Cork	SWD	Untreated wastewater	3,500 (Skibbereen)	none	n/a
Kinsale	Cork	SWD	WWTP inputs, upstream inputs in River Bandon (secondary effluent from 8000 PE)	5,000	Preliminary only	n/a ¹
Cork Great Is. Nth Channel	Cork	SWD	WWTP inputs from Cork city and surrounds, industrial inputs, agricultural run-off	333,000	Secondary (Cork city) Secondary with nr (Middleton)	423,000
Cork		Tier II				
Dungarvan	Waterford	SWD	WWTP inputs	13,000	Secondary	20,000
Woodstown	Waterford	SWD	WWTP inputs upstream from Waterford, agricultural run off	146,500 (Waterford)	Secondary	190,6002
Wexford Harbour Inner	Wexford	Tier I+II, SWD	WWTP inputs, agricultural run off	17,000	Secondary with nr	30,000
North Bank Lighthouse	Dublin	Caging	WWTP inputs from Dublin city and surrounds, industrial inputs, agricultural run-off	2,770,537	Ringsend 1,640,000 tertiary, Fingal (141,640 secondary, 3,700 primary and 25,800 none), Dun Laoghaire 65,700 preliminary only. Coliemore 1000, no treatment ¹	
Tolka		Tier I				
Dublin Bay		Tier II				

PE = Population Equivalents. Plant PE = Capacity of WWTP in PE.

¹Monaghan et al. 2009, ² Environmental Protection Agency, Ireland: www.epa.ie

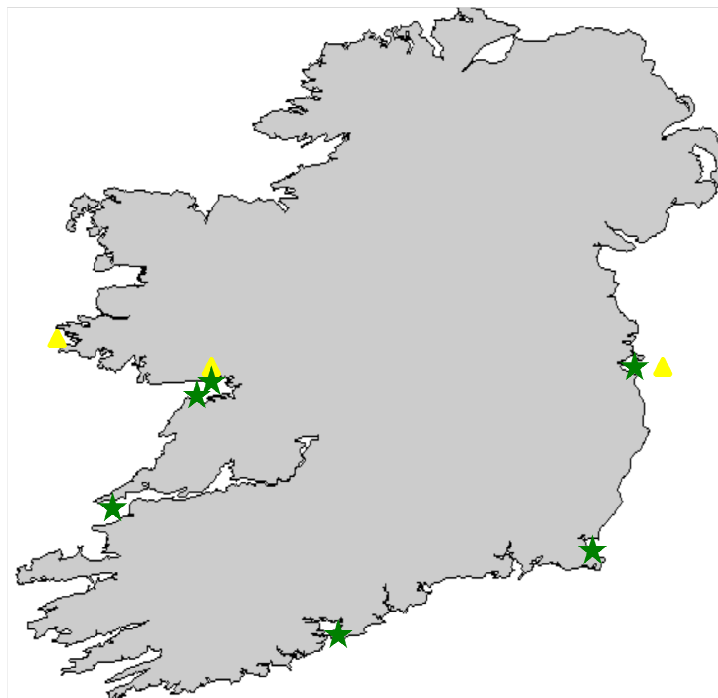


Figure 5.1: Sampling locations for Tier II and caging.

Depicted in yellow are transplantation sites

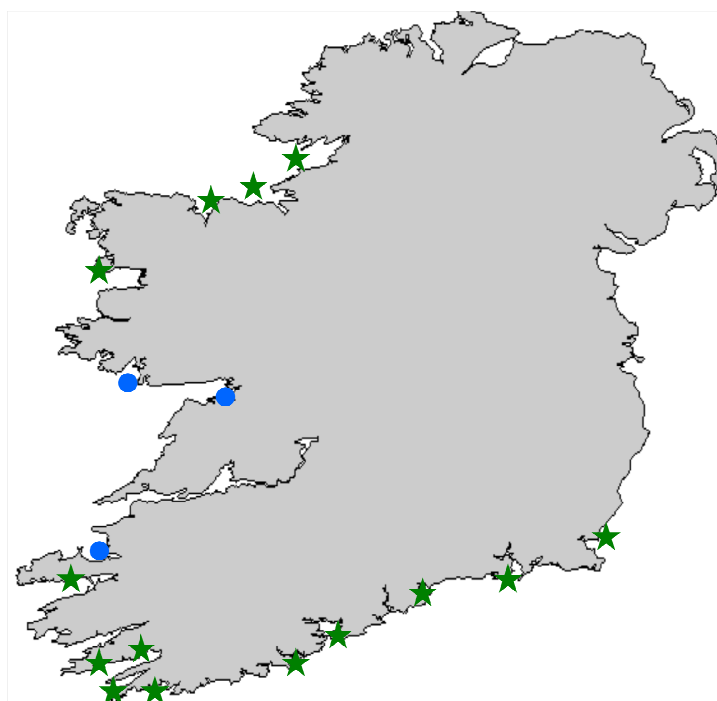


Figure 5.2: SWD and shellfish sampling map.

Depicted in green are SWD sites. Depicted in blue are Tier I sites.

5.3 Collection of samples

Full details of sampling methods and analytical techniques are presented throughout this thesis. The sampling programme is briefly described below.

5.3.1 *Collection of shellfish samples*

Mussels measuring 4-6 cm and/or commercially sized oysters were collected at each site and transported to the lab in cold conditions. Samples were dissected, the whole soft tissue weight recorded and shell length measured to the nearest 0.1 mm using a callipers. Tissue was homogenised, freeze dried and frozen at -30°C prior to analysis. Mussels measuring 5-6 cm were collected for ALP analysis and processed as detailed in Chapter 2.

5.3.2 *Collection of fish samples*

Samples of dab, flounder and plaice were collected at four locations. Fish were sacrificed, the weight and length measured. Muscle and liver samples were collected and pooled from between 20-50 individuals. All biota were collected where present in accordance with OSPAR CEMP sampling protocols (OSPAR, 1999). Tissue was homogenised, freeze dried and frozen at -30 °C prior to analysis.

5.3.3 *Passive sampler deployment*

POCIS devices containing three discs per sampler were deployed at selected sites for a period of one month. A field blank per sampling event was exposed to the environment during deployment and retrieval. The POCIS and corresponding field blank were transported to the laboratory in cool conditions in an airtight metal canister, and stored at -30°C prior to analysis at the National Laboratory Service, Environment Agency, UK.

5.3.4 *Collection of water samples*

Five L water samples (taken at 1 m depth) were filtered through 0.45 µm glass fibre filters (Whatman GF-F, General Electric, US) and 50 mL of Formalin (1% v/v) was added to prevent bacterial growth. Water samples were stored in the dark at 4°C prior to LC-MS/MS analysis.

5.3.5 *Collection of sediment samples*

Sediment was collected from four locations using a Van Veen grab. Samples were sieved to <63µm, freeze-dried and stored at -30 °C prior to ER-LUC analysis.

5.3.6 Analysis

A wide ranging suite of analytical tools was used to perform this assessment. Method details and associated validation and QC procedures are documented throughout this thesis. LC-MS/MS analysis of water and tissue samples were performed in the Marine Institute using methods developed for this thesis. Analysis of POCIS devices was completed by subcontract analysis at the National Laboratory Service, Environment Agency, UK. ER-LUC samples were analysed by subcontract analysis at the Institute for Environmental Studies (IVM), The Netherlands. Measurement of ALP was conducted by research partners in the Zoology Department, Trinity College Dublin. Full quality assurance procedures in accordance with best international practice were employed during all analyses.

5.4 Data integration and assessment.

Many different systems exist for integration of data with most models generally comparing analytically derived concentration/effect data to a reference/consensus value or to a legislative standard. To enable greater communication of assessment outcomes colour coding systems are usually applied to denote whether the results pass or fail available assessment criteria.

One relevant example in the context of this thesis is the OSPAR MON regional assessment tool which derives individual assessments of determinands (contaminant concentrations or biological responses) in specific matrices at individual sites and assesses them against defined assessment criteria (OSPAR, 2008). The results are tabulated and colour coded to give a visual snapshot of the status of each test site. In general for organic compounds colour coding is completed as follows: Initial comparisons determine whether the determinand and site combinations are < the background assessment criteria (BAC, marked in blue), between the BAC and environmental assessment criteria (EAC), marked green or > EAC (red colour). Missing data are generally marked grey. The OSPAR system currently does not include assessment criteria for E2 and EE2 in water or biota however reference values are available in the form of WFD EQS for dissolved water only. No legislative thresholds and/or assessment criteria are yet defined for ALP, POCIS derived steroid concentrations and/or ER-LUC.

This current assessment has utilised a variety of reference values collated from currently relevant thresholds in addition to literature derived assessment criteria, details are

presented in Table 5.2. Where defined, WFD assessment criteria such as environmental quality standards (EQS) for other surface waters and quality standards (QS) for concentrations in biota used in the derivation of the EQS values were used. Where maximum allowable concentration (MAC) values have not been defined for a compound, the annual average (AA) EQS values are considered protective against short-term pollution peaks in continuous discharges since they are significantly lower than the values derived on the basis of acute toxicity.

No legislative criteria were available for E1 however its potency relative to that of E2 has been determined as 0.5 using the yeast estrogen screen (YES) (Routledge and Sumpter, 1997), so an AA EQS of 0.16 ng/L was calculated for E1 and used in this assessment to give an indication of the potential risks associated with E1 at each site. Errors associated with analytical quantification of EDCs in water samples are 3.1% for E2 and 4.3% for EE2. Errors have been applied to all water results included in this assessment.

For E2, where analytical data was <95% of the EQS the results were shaded green. Where results were within $\pm 5\%$ of the EQS value they were shaded orange while results >105% of the EQS were shaded red.

It was not possible to attain a LOD for EE2 in water below the current WFD EQS. For the purposes of this assessment, where EE2 is detected, this was shaded red, while “non-detects” were shaded orange striped to indicate the uncertainty of the measurement with respect to the capability to detect EE2 at sufficiently low levels.

For E1, where results were <95% of the EQS they were shaded green. Where results were $\pm 5\%$ of the EQS value or above, they were shaded orange. As definitive EQS are not available for E1, red was not used to highlight results above the calculated E1 EQS.

Definitive pass/fail criteria are not yet available for E1, E2, EE2, NP and OP in biota in the form of EQS. QS limits to prevent secondary poisoning of predators and to protect human health via consumption of fishery products were used in the derivation of WFD EQS for total water. These QS were used as guideline assessment criteria in the current assessment. As per water data method based upper-bound analytical errors were applied to analytical data.

POCIS was used as a screening device within this research, with concentrations reported as ng of analyte per device. Non detection was deemed to correspond to ‘good’ status (coloured green) while detected analytes were coloured orange (striped) to denote presence in the environment but no assessment criteria available’.

A wide range of POCIS sampling rates as determined in laboratory studies have been reported at between 0.02 to 0.85 litres per day for E1, E2 and EE2 (Morin et al. 2012). The average estimated sampling rate was thus determined from these values as 0.42 L/day. Using this average value it was estimated that approximately 12.6 L of water was sampled during a 30 day deployment (ranging from 0.6 L to 25.5 L in 30 days using the lowest and highest sampling rates reported, respectively).

In order to estimate EDC water concentrations the sampling rate of 0.42 L/day was applied to E1 and E2 values detected in POCIS and these were then compared against WFD EQS. Green striped equated to the derived water concentration being <95% EQS, with values above this shaded orange striped to indicate a potential exceedance of the EQS but not a definitive failure due to the errors associated with the sampling rates. In instances where multiple POCIS results were available and the dataset was found to have instances of both EQS “compliance” and “exceedance” the dataset was shaded orange striped with the range of results additionally presented. Where the derived concentrations were all found to “exceed” the EQS these were shaded orange striped to indicate possible exceedances of the EQS. It must be noted that such data are estimations of water concentrations only and are based on laboratory derived sampling rates that do not account for field based conditions. Derived water concentrations must be treated as estimates only and must not be quoted as non compliant, given the range of sampling rates available, the fact that they are laboratory derived for particular batches of POCIS devices and cannot definitively be linked to field based influences. Therefore the inclusion of estimated steroid water concentrations based on POCIS results are for illustration purposes only in order to aid the assessment.

Definitive pass/fail criteria are not available for ER-LUC and ALP as natural variability in response may occur under different local and regional conditions, differences in impacts, adaptability of native species to local conditions and due to differences in organism gonadal stage. The results of these measurements are included in Table 5.3 for reference and are not assigned a colour. Updated associated assessment criteria will become

available as more focused marine based data emerge, therefore the assessment criteria listed will remain “live”.

For the purpose of this assessment absolute upper-bound analytical data were then directly compared to the collated assessment criteria. No further statistical techniques were employed primarily given the concerns related to assessment criteria detailed above.

Table 5.2: Assessment criteria and other relevant parameters

Compound/criteria	E1	E2	EE2	NP	OP	ALP	EEQ	Transition criteria	Colour transition	Comments
POCIS (ng/device)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Presence/ Absence	 	Only denotes presence/ absence, not quantitative
AA EQS other surface waters WFD ng/L	n/a	0.08	0.007	0.3 µg/L	0.012 µg/L	n/a	n/a	Pass <95% EQS = green, 95-105% EQS = orange, Fail >105% EQS = red	  	Note WFD EQS are on a total water basis
MAC EQS other surface waters WFD ng/L (µg/L)	n/a	not derived	not derived	2 µg/L	0.13 µg/L	n/a	n/a		  	
Relative potency to E2 by YES (converted concentration)	0.5 (0.16 ng/L) ¹	1	1.19 ³	0.00014 ²	0.007 - 0.001 ¹	n/a	n/a	Pass <95% EQS = green /Potential risk >95% EQS = orange/ orange striped	 	EQS is calculated on total water basis as E2 EQS multiplied by relative potency of E1 to E2
AA EQS other surface waters ng/L used for assessment of POCIS results based on average sampling rate of 0.42 L/day.	0.16	0.08	0.007	0.3 µg/L	0.012 µg/L	n/a	n/a		 	Note the POCIS derived concentrations are estimated and results above EQS represent potential risk only. Confirmatory analysis with water samples is required.
WFD QS Biota used in assessment -limit to prevent secondary poisoning of predators	n/a	0.7 ng/g	0.07 ng/g	10 µg/g	10 µg/g	n/a	n/a	Pass <95% EQS = green, 95-105% EQS = Orange, Fail >105% EQS Red	  	
WFD QS Biota used in assessment -human health via consumption of fishery products	n/a	3 ng/g	0.06 ng/g	8.7 µg/g	8.7 µg/g	n/a	n/a		  	
BCF mussels	not defined	not defined	not defined	2000-3000	not defined	n/a	n/a	BCF> 100 to trigger derivation of a quality standard referring to the protection of top predators from secondary poisoning		
BCF fish	51	6.5	610	1280	634	n/a	n/a			
EEQ sediment data: non-impacted Irish sites	n/a	n/a	n/a	n/a	n/a	n/a	0.32 [*]	Represents average values from non-impacted Irish sites * EEQ in ng/g for sediment, **µg ALP/mg protein *** EEQ in ng/g dw in biota		
ALP/EEQ biota data: non-impacted Irish sites	n/a	n/a	n/a	n/a	n/a	11.46 ^{**}	0.24 ^{***}			

¹Routledge and Sumpter, 1997, ²Routledge and Sumpter, 1996, ³Rutishauser et al. 2004

5.5 Results and discussion by parameter

An overall assessment of the presence and effects of selected EDCs in Irish marine waters was conducted. Each site was analysed using a range of techniques. A full assessment table containing results for Tier II sites is presented in Table 5.3. Criteria described above are applied to the results to indicate sites at potential risk of ED (orange and red results).

Table 5.3: Results and assessment

Site Location /Bay	Matrix overall	Matrix/species	E1 POCIS ng/device	E2 POCIS ng/device	EE2 POCIS ng/device	E1 POCIS ng/L estimated**	E2 POCIS ng/L estimated**	EE2 POCIS ng/L estimated**	E1 Water ng/L	E2 Water ng/L	EE2 Water ng/L	E1 Tissue ng/g	E2 Tissue ng/g	EE2 Tissue ng/g	NP Tissue ng/g	OP Tissue ng/g	ng EEQ/g dw;	ALP µg ALP/mg protein
Cork	Shellfish (n=2)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.08	12.5
	POCIS (n=2)	POCIS	4.12- 5.18	0.12- 0.49	<0.40	0.33- 0.41	0.01- 0.04	nd	*	*	*	*	*	*	*	*	*	*
	Fish (n=6)~	Dab	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	*	*
	Sediment	Sediment	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.1	*
Dublin Bay	Shellfish (n=12)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	*	*
	Shellfish	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.24 - 0.72	7.5 - 14.8 ¹
	POCIS (n=4)	POCIS	3.59- 8.45	0.69- 3.8	<0.10	0.28- 0.67	0.05- 0.30	nd	*	*	*	*	*	*	*	*	*	*
	Water (n=5)	Water	*	*	*	*	*	*	0.76- 1.11	nd- 0.13	nd	*	*	*	*	*	*	*
	Sediment	Sediment	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.29	*
	Fish (n=4)	Dab, plaice	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	*	*
Shannon	Shellfish (n=1)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.38	12.8
	POCIS (n=1)	POCIS	<1.0	<0.30	<0.10	<1.0	<0.30	nd	*	*	*	*	*	*	*	*	*	*
	Fish (n=3)~	Dab	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	*	*
Wexford	Shellfish (n=2)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.10 ²	40.2 ²
	POCIS	POCIS	6.5	1.61	<0.40	0.52	0.13	nd	*	*	*	*	*	*	*	*	*	*
	Fish (n=2)~	Dab	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	*	*
	Sediment	Sediment	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.21	*

Continued overleaf

Site Location /Bay	Matrix overall	Matrix/species	E1 POCIS ng/device	E2 POCIS ng/device	EE2 POCIS ng/device	E1 POCIS ng/L estimated**	E2 POCIS ng/L estimated**	EE2 POCIS ng/L estimated**	E1 Water ng/L	E2 Water ng/L	EE2 Water ng/L	E1 Tissue ng/g	E2 Tissue ng/g	EE2 Tissue ng/g	NP Tissue ng/g	OP Tissue ng/g	ng EEQ/g dw;	ALP µg ALP per mg protein
Mutton Island Galway Bay	Shellfish (n=10)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.13 - 0.81	15.2 ²
	POCIS (n=3)	POCIS	6.94 - 15.3	1.22 - 3.9	<0.10	0.55-1.21	0.1-0.31	nd	*	*	*	*	*	*	*	*	*	*
	Water (n=5)	Water	*	*	*	*	*	*	nd	nd	nd	*	*	*	*	*	*	*
New Quay	Shellfish (n=6)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.06 - 0.34 ³	6.2- 15.5 ¹
	Sediment (n=1)	Sediment	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.32	*
Omey Island	Shellfish (transplanted n=7)	<i>Mytilus spp</i>	*	*	*	*	*	*	*	*	*	nd	nd	nd	nd	nd	0.09	10.5
	POCIS (n=2)	POCIS	3.01 - 6.35	0.48-2.1	<0.10	0.24-0.5	0.04-0.17	nd	*	*	*	*	*	*	*	*	*	*
	Water (n=5)	Water	*	*	*	*	*	*	nd	nd	nd	*	*	*	*	*	*	*

¹ n=2 samples ² n=1 sample,

³ Fish species tested dab and plaice.

Chemical analysis conducted on muscle and liver tissue.

** based on an average sampling rate from the literature of 0.42 L/day. *POCIS derived water concentrations are for illustration purposes only.*

5.5.1. Water concentrations of E1, E2 and EE2 by LC-MS/MS

Water samples from Dublin Bay, Mutton Island, Galway Bay and Omey Island were analysed for the presence of E1, E2 and EE2. E1 was detected in water samples in June, July and October 2010 in Dublin Bay, with concentrations ranging from 0.14 to 1.11 ng/L. E2 was only detected at Dublin Bay in October 2010 at 0.13 ng/L. E1, E2 and EE2 were not detected at Omey Island or Mutton Island, Galway Bay. Although E2 in one sample from Dublin Bay is reported at 0.13 ng/L, the WFD criteria for E2 are based on an annual average concentration. Four other samples taken in the same year were <LOD (0.08 ng/L). When these results are combined the AA value at this site is <0.08 ng/L, thus these waters were deemed to be of low risk. The measured E2 concentrations are in the dissolved phase only and while filtration was not deemed to result in analyte losses during validation of the LC-MS/MS method; further monitoring is recommended to investigate the E2 concentrations at this site in order to fully comply with WFD EQS 'total' water requirements. EE2 was not detected at any site. It should be noted that such spot samples provide only a snapshot of the conditions within the sampling environment. Factors such as drought, rainfall, position within a tidal cycle and the movement of water currents can all affect analyte concentrations, thus other "time integrated" mechanisms such as passive sampling may be a more cost effective and scientifically more robust means of deriving environmental status information.

5.5.2 POCIS results

Passive samplers were deployed at six locations in order to assess their suitability for use in surveillance monitoring/screening and in support of biological effects based measurements. E1 and E2 were detected in samplers deployed at 5 of the 6 sites studied (Table 5.3). E1 and E2 were not detected in Shannon. The highest E1 value, 15.3 ng/device, was detected at Mutton Island, Galway Bay in June 2010. When detected, E1 ranged from 3.1 to 15.3 ng/device, with an average of 6.49 ± 3.2 ng/device. E2 uptake was consistently lower than E1. The highest E2 value of 3.9 ng/device was also detected at Mutton Island, Galway Bay in June 2010. When detected, E2 ranged from 0.48 to 3.9 ng/device, with an average of 1.71 ± 1.3 ng/device. Sites with a higher level of anthropogenic input exhibited the highest concentrations per device. EE2 was not detected at any site. The ratio of E1:E2 differed at each site, with highest E1:E2 ratios observed in Cork (97:3 and 91:9, in May 2011). The nature of these differences was outside the scope of this thesis and requires further monitoring to be completed. The ratio of E1:E2 was approximately 80:20 at most sites. Samplers were deployed for four consecutive months in

Dublin. Concentrations of E1 and E2 showed a general decrease between June and October 2010, although the ratio of E1:E2 showed an increase, from 68:32 in June, to 82:18 in October. A similar pattern was noted at Omev Island from samplers deployed in June and October 2010, with overall concentrations decreasing, but the ratio of E1:E2 increasing.

PS was found to be a useful screening tool for monitoring EDCs at these sites, but may be subject to complications as a result of the potential presence of biomarker derived steroids. Future studies combining biomarkers and PS should take into account the presence and release of naturally occurring steroids and deployment of PS should be completed at a suitable distance from caged indicator species

Levels reported in the literature for steroids in POCIS have been represented as ng/L values, based on an estimation of sampling rates by the devices (Arditsoglou and Voutsas, 2008; Zhang et al. 2008), and in EEQ based on measurement of the estrogenicity of POCIS extracts (Liscio et al. 2009). It has proven extremely difficult to accurately assess sampling rates which consider all the environmental factors in a sampling site (Morin et al. 2012). Estimated water concentrations have been calculated for each of the study sites using the average uptake rate (0.42 L/day) from studies reviewed in the paper by Morin et al. (2012), and are presented in Table 5.3. This sampling rate however, could be as low as 0.6 L or as high as 25.5 L sampled in 30 days if using the lowest and highest sampling rates reported in the literature, respectively. This has major implications for the assessment of POCIS derived water concentrations, as for example, using the lowest sampling rate on Cork POCIS E2 concentrations there would be an estimated concentration of 0.2 to 0.8 ng/L E2, above the EQS whereas using the highest sampling rate would result in estimated water concentrations of 0.01 and 0.02 ng/L, below the EQS. Field derived sampling rates for E1 and E2 in optimised POCIS have also been found to be significantly higher than those derived in the laboratory (Zhang et al. (2008). Further work is required to improve the performance of POCIS as a complementary technique to spot sampling. Overall POCIS has been deemed to be a good screening/surveillance tool and has been assessed as such in this report. Until such time that definitive sampling rate information are obtainable in the field the use of POCIS will be as a screening tool and not for the purposes of quantitative assessment.

5.5.3 E1, E2, EE2, NP and OP in shellfish samples by LC-MS/MS

Native samples of *Crassostrea gigas* and *Mytilus* spp. from nineteen sites were analysed for the presence of E1, E2, EE2, NP and OP. The selected EDCs were not detected in any biota sample, despite the proximity of some locations to WWTP effluent inputs, and the detection of E1 and E2 in some nearby water samples and POCIS devices. It is important to screen for EE2, NP and OP in biota as their presence could have negative impacts by affecting gonadal development and reproduction, as could elevated levels of E1 and E2 from exogenous sources. Current results demonstrate that E1, E2, EE2, NP and OP are not present in shellfish as sampled from 15 SWD designated water bodies, and four additional sampling sites. Based on the analytical results and currently available ecotoxicological criteria the risk of ED related to these compounds is deemed low at these sites.

5.5.4 E1, E2, EE2, NP and OP in fish samples by LC-MS/MS

Muscle and liver samples from fish (from plaice, flounder and dab) collected from four sites (Cork, Dublin, Wexford and Shannon) were analysed. E1, E2, EE2, NP and OP were not detected in any sample. NP has been detected in flounder muscle at 10 ng/g wet weight (Vethaak et al. 2005). NP was not detected in plaice collected in offshore UK marine sites (LOD 0.1 to 0.5 ng/g), but was detected at 0.2, 0.6 and 0.8 µg/g in muscle tissue taken from chub, roach and gudgeon samples respectively, taken from the River Aire (Blackburn et al. 1999). E1, E2, EE2, NP and OP were detected in muscle, gill and liver tissues in silvery minnow, carp and crucian carp, at concentrations up to 10.7, 11.5, 18.7 14.7 and 67.5, ng/g dw respectively with highest concentrations up to 10-15 times higher in the liver than in muscle (Liu et al. 2011). Based on currently available ecotoxicological criteria the risk of ED related to these compounds is deemed to be low at the studied sites.

5.5.5 ALP results: mussels

ALP results are presented in Figure 5.3. ALP results were highest and most variable at Wexford (40.2 ± 70.4 µg ALP per mg protein), and averaged 10.2 ± 9.3 µg ALP per mg protein at the remaining eight sites. While no definitive assessment criteria are available for ALP in marine species and the range of values reported is consistent with low relative potential for anthropogenic impact. ALP has been reported up to approximately 80 µg ALP per mg protein in *M. galloprovincialis* from an un-impacted site, and up to 100 µg ALP per mg protein in impacted sites in Venice (Pampanin et al. 2005). ALP of approximately 30 and 150 µg ALP per mg protein have been reported as in *M. galloprovincialis* from a control site (de los Ríos et al. 2012), and some authors consider a value of 100 µg ALP per

mg protein to be a background level (Ortiz-Zarragoitia and Cajaraville, 2006). Characterisation of ALP levels from un-impacted Irish sites revealed a range of 6.2 to 15.5 μg ALP per mg protein. ALP in Wexford, while elevated relative to the other sites studied, is still within background levels reported in literature. Further investigation may be merited at this location to elucidate whether natural or anthropogenic inputs are a source of the measured variation.

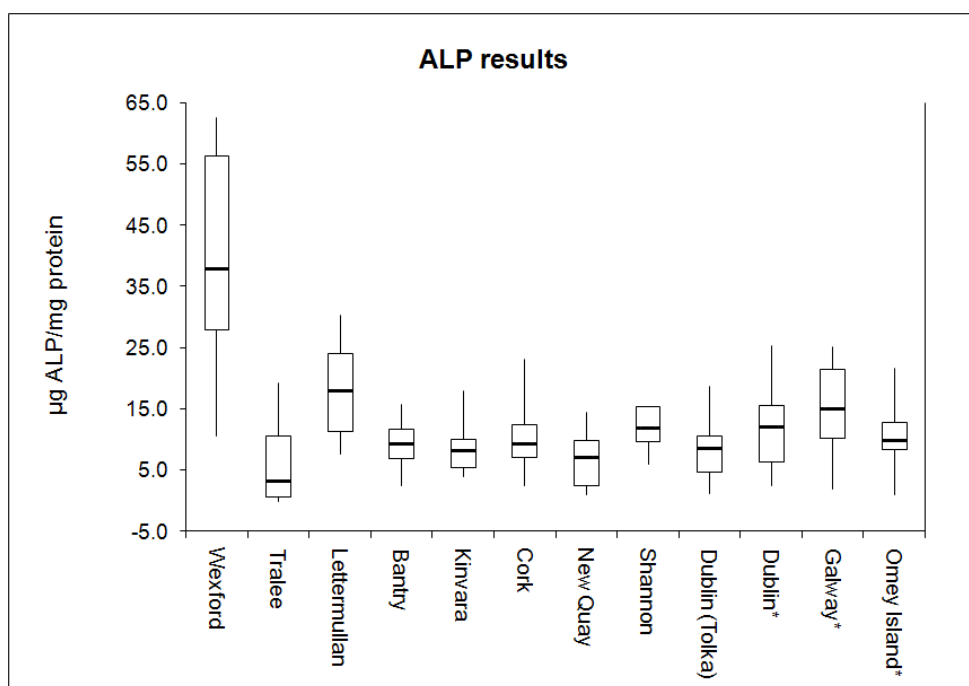


Figure 5.3: Alkali-labile phosphate results.

Box and Whisker plot depicts the median, upper and lower quartiles (= box) and the mean standard error (= bars). * Indicates results from transplanted mussel stock.

5.5.6 ER-LUC results

Fifteen shellfish samples were analysed using ER-LUC. One sample of *C. gigas* from Kinsale was <LOD. EEQ in ng/g dw were detected in the remaining samples, all of which were *Mytilus* spp. Concentrations ranged from 0.06 to 0.81 ng/g EEQ dw, with an average of 0.29 ± 0.24 ng/g EEQ dw. Results are presented in Table 5.3. The highest value was detected in native mussels from Mutton Island, Galway Bay (0.81 ng/g EEQ dw). EEQ levels were measured in mussels from New Quay, an un-impacted site collected at three month intervals (n=4 samples) and ranged from 0.06 to 0.34 ng/g EEQ dw.

EEQ in native mussels from NBL and Mutton Island, Galway Bay were found to be more elevated than those observed in the transplanted mussel stock, ranging from 0.24 to 0.72 ng/g dw at NBL, Dublin Bay and 0.81 pg/g dw at Mutton Island, Galway Bay, relative to EEQ responses of 0.09 to 0.34 pg/g dw in mussels transplanted to these sites, and 0.07 to 0.47 ng/g dw in other native Irish mussels. Mussels have a marked seasonality, therefore seasonal changes in biomarker response need to be fully understood in order to enable accurate risk assessment to be completed (Blaise et al. 2002; Ortiz-Zarragoitia and Cajaraville, 2006).

In the absence of peer reviewed ecotoxicological risk assessment criteria it is unclear whether the elevated responses observed in native mussels from NBL, Dublin Bay and Mutton Island, Galway Bay, suggest a greater natural variation in EEQ or whether results are reflective of actual effects of exposure to EDCs in their environment. NBL and Mutton Island transplanted samples were more elevated than those from Omey Island therefore it may also be possible that the three month transplantation time may have been insufficient to elicit the same level of response observed in continually exposed native organisms.

Average EEQ in sediments collected from Cork, Dublin, Wexford and Shannon was 0.28 ± 0.16 ng/g. The highest values were detected in Dublin Bay (0.49 ng/g EEQ), with the lowest detected in Cork (0.1 ng/g EEQ). EEQ in sediments have been reported between 12 and 155 ng/g in Italian coastal sediments (Pojana et al. 2007). Houtman et al. (2007) reported EEQ of 0.004 ng/g in sediment from a marine national park, and 0.47 ng/g in Zierikzee harbour, in the Netherlands. EEQ in Dublin are comparable with the value from Zierikzee harbour. E1 and E2 were found to be the main contributors to the estrogenicity in Zierikzee harbour (Houtman et al. 2006). EEQ values in this study are considerably lower than those detected by Pojana et al. (2007). The relative risk of ED related to sediment estrogenicity is therefore deemed to be low for most sites, but potentially of concern in Dublin Bay.

5.6 Site based assessment

For the purposes of this assessment the level of commentary is dictated by both the number and scope of analyses completed and on the relevance of available assessment criteria. As such this study presents a summary assessment of EDCs in shellfish from designated SWD

sites and a more detailed multi-parameter, multi-matrix based assessment for the Cork, Galway (Mutton Island), Dublin, New Quay, Omev and Wexford locations.

5.6.1 SWD and other selected locations.

Levels of E1, E2, EE2, NP and OP were found to be below the LOD at all of the 15 locations tested. It must be noted that in the case of these locations biota were collected from nationally designated sampling sites within the relevant water body. This exercise was completed as a surveillance/screening programme and not as a wider spatial study. While levels of the compounds are below LOD the spatial power of the assessment is restricted to the area where the sample originated. Given the limited sampling programme further spatial extrapolation is not recommended beyond the sampling point reported.

While each of the sampled locations is subject to different degrees of anthropogenic pressures ranging from domestic waste to larger scale treatment plants (see Table 5.1) the non-detection of these compounds in shellfish at these sites and based on currently available ecotoxicological criteria, the risk of ED related effects due to these compounds is likely to be low.

5.6.2 Tier II and other selected locations

Tier II locations (Dublin, Wexford, Cork and Shannon) in addition to Omev Island, Mutton Island Galway and New Quay Co. Clare were subjected to a wider regime of EDC related levels and effects monitoring. Individual site specific aspects are discussed below.

5.6.2.1 Omev Island

Omev Island is a remote island off the West coast of Ireland, with little to no anthropogenic input. It has very few urban surroundings and no nearby industrial areas. It is only accessible at low tide and was selected as a control site. Water sample results were <LOD for E1, E2 and EE2. E1 and E2 were detected in POCIS deployed in June and October 2010. E1, E2, EE2, NP and OP were not detected in mussels transplanted to this location for four months. The potential for endogenous EDC production is discussed elsewhere and must be considered when completing POCIS based studies in tandem with mussel studies. ALP and ER-LUC results were not significantly different relative to other locations. The combination of this dataset suggests that the presence and effects of selected EDCs at this site are low.

5.6.2.2 Mutton Island, Galway

Galway Bay is a large bay on the Atlantic coast. Native mussel samples were collected from the shores of Mutton Island. Mutton Island WWTP serves 91,600 PE from Galway City and surrounds, providing secondary treatment to effluents. Storm water overflow vents were situated next to this sampling site. ER-LUC measurements of native mussels were higher than that of mussels transplanted to this location for four months (0.81 vs. 0.13 ng/g EEQ dw in native vs. transplanted), suggesting that native populations may be subject to the effects of WWTP inputs at this site, however natural variability in EEQ levels may also explain these differences. E1, E2 and EE2 were not detected in water samples at this site. E1 and E2 were detected in POCIS, at the highest levels detected in this study. POCIS were deployed in close proximity to the effluent diffuser pipe, which may explain the levels observed at this site. ALP levels in females were significantly higher than males at this site, although this may be explained by natural differences in males and females as previously described. ALP levels at this site were similar to those detected at other locations. Although this site is in close proximity to WWTP inputs, levels and effects of EDCs as measured with the current techniques suggest that this site is at low risk, though mussels in close proximity to stormwater overflow vents may be at increased risk of ED.

5.6.2.3 New Quay

Aughinish Bay, New Quay is a designated SWD water body. Mussels from this site were used for transplantation and exposure studies described in detail in Chapters 3 and 4. In order to provide baseline data on the variability in biomarker response and tissue concentrations of selected EDCs, EEQ and concentrations of E1, E2, EE2, NP and OP in *Mytilus* samples from New Quay were assessed in four samples taken over a season (Sept 2009 to July 2010). EEQ ranged from 0.06 to 0.34 ng/g dw. E1, E2, EE2, NP and OP were not detected in mussel tissues. ALP levels in samples collected in September 2009 and July 2010 ranged from 6.2 to 15.5 µg ALP/mg protein. EEQ in sediment was close to the average sediment concentration of all sample sites. The presence and effects of EDCs at this site were deemed to be low.

5.6.2.4 Shannon

The Shannon International River Basin District (SIRBD) is Ireland's largest river system, with an average discharge of 206 m³/s, controlled by Ardnacrusha hydroelectricity station (OSPAR, 2006). The catchment area includes agricultural, urban and industrial areas, many discharging wastewater into the river system. While Kelly et al. (2010) found

significantly elevated VTG levels in feral brown trout in three out of 11 sites studied in SIRBD, specifically in the River Hind, Nenagh and Little Brosna (Birr), these sites were approximately 1-2 km downstream of discharge points of either WWTP, industrialized or agricultural sites. Estrogenicity was also detected using YES with estradiol equivalency factors (EEF) up to 2.67 ng/L. Water and sediment were sampled for phthalates and nonylphenol. NP, DBP, DEHP, DINP and DIDP were detected in river water up to 5.32, 11.52, 73.13, 1.89 and 1.81 µg/L respectively, and in sediment at 0.82, 19.44, 25.27, 6.16, and 10.26 mg/kg (Kelly et al. 2010).

The site sampled in this current study was at the mouth of the Shannon Estuary relatively distant from the freshwater locations studied by Kelly et al. (2010). EEQ in mussels from this site were close to the average value of all sites studied. ALP levels were found to be significantly higher than in Kinvara, New Quay, Bantry, Tralee and Dublin. E1, E2 and EE2 were not detected in POCIS, fish or mussel tissues. NP and OP were not detected in mussels or fish. When non-detection of selected EDCs, particularly the steroid estrogens, which are among the most potent estrogenic compounds, and ER-LUC levels are taken into account, it is likely that elevated ALP levels relative to other sites are due to natural variability in ALP levels, rather than the presence of EDCs. ALP levels are lower than those reported in a number of international studies. Non detection of EDCs in fish tissues also indicate a low risk of ED related to tissue levels of selected EDCs. The site as selected was deemed to exhibit low ED risk.

5.6.2.5 Cork

Cork Harbour is an estuarine area with varying salinity (Lyons et al. 2003). It is an industrialised natural harbour (100km² surface), which opens southwards to the Celtic Sea (Touzer et al. 2010). It has been suggested that stress on mussels along the South Irish Coast revealed by scope for growth measurements may be due to untreated wastewater from population centres such as Cork City, which flows into Cork Harbour (Widdows et al. 2002). Secondary treated waste enters Cork Harbour from Cork City (333,000PE) (Monaghan et al. 2009). Other potential sources of EDCs include industrial waste and riverine inputs of agricultural run-off and untreated waste from the River Lee catchment. POCIS were deployed at two locations in Cork Harbour. E1 and E2 were detected, with a dominance of E1 in the samplers (> 90%). E1, E2, EE2, NP and OP were not detected in mussel or fish tissues, indicating a low risk of ED related to tissue levels of selected EDCs. EEQ in mussels and sediment were low relative to most other sites. ALP levels were

consistent with levels detected at other coastal locations. The combined dataset suggests that the presence and effects of EDCs in Cork are low.

5.6.2.6 Wexford

Wexford Harbour is situated on the South East coast of Ireland. ALP levels at this site displayed the greatest range in concentration and the highest average ALP content (40.2 µg ALP/mg protein compared with averages of between 6.2 and 15.5 µg ALP/mg protein at the other study sites). ER-LUC results in mussels were lower than at many other sites, with EEQ in sediment slightly lower than the average sediment concentration. E1 and E2 levels detected in POCIS devices deployed in Wexford Harbour were close to average values for all sites (E1 Wexford 6.5 ng/device, average all sites 6.49 ng/device, E2 Wexford 1.61 ng/device, average all sites 1.71 ng/device). E1, E2, EE2, NP and OP were not detected in mussels or fish taken from Wexford indicating a low risk of ED related to tissue levels of selected EDCs. Reduction in oxygen supply can affect the detoxification capacity of mussels (Bayne, 1973). It is possible that elevations in ALP levels are a result of reduced detoxification due to the potentially eutrophic status of Wexford Harbour, as measured by the EPA (EPA, 2009).

5.6.2.7 Dublin Bay

E2 in one sample from Dublin Bay was 0.13 ng/L, above the WFD AA EQS of 0.08 ng/L, however the WFD criteria for E2 is based on an annual average concentration. Four other samples taken in the same year were <LOD (0.08 ng/L). When these results are combined the AA value at this site is <0.08 ng/L, thus these waters were deemed to be of low risk with respect to E2. WFD EQS are set for total water, and while filtration was not deemed to have resulted in analyte losses during method development, classification of this site with respect to 'total water' WFD EQS levels requires further monitoring. An estimated EQS of 0.16 ng/L was calculated for E1 based on the potency of E1 relative to E2 as measured by YES according to Routledge and Sumpter (1997). E1 was detected in three of five water samples, two of which were above this value, and the average concentration was 0.40 ng/L. It is important to note that the estimated EQS is based on relative potency using YES, and not from *in vivo* studies, thus the inference of risk at this site related to exceedance of this calculated EQS is tentative. A national risk assessment investigating the risk of intersex in fish arising from steroid estrogens was conducted in the UK in 2008 (Williams et al. 2008). The authors set a PNEC of 1 ng/L E2 equivalents. Using this value, resident fish populations in Dublin Bay are deemed to be at low risk from endocrine

disruption related to the E1 and E2 concentrations detected in water. Potential sources of E1 and E2 detected in this study may include via Ringsend WWTP effluent (1,640,000 PE), and/or other effluent or agricultural run-off entering upstream in the River Liffey catchment. The concentrations detected are consistent with values reported in surveys of receiving waters and coastal waters, with estrogens generally reported in the range of <1 to 10 ng/L in the UK and Canada, for example (Williams et al. 2003; Saravanabhavan et al. 2009).

E1 and E2 were detected in POCIS from June to October 2010. The concentrations decreased over these months. The estrogenicity of effluent and receiving waters from the Ringsend WWTP has previously been determined using the yeast estrogen screen, and was conducted prior to the addition of tertiary UV treatment at the plant (Tarrant et al. 2005). Estrogenicity, expressed as estradiol equivalents, was 16 ng/L in effluent but was not detected in receiving waters. EEQ in sediment taken in Dublin Bay ranged from 0.29 to 0.49 ng/g, with the higher concentration detected at NBL, which is situated in Dublin Port, downstream of Ringsend WWTP. EEQ of 0.49 ng/g may be considered to be of concern, and further investigation at NBL and the Liffey estuary is suggested.

ALP levels in native mussels from this site were higher than levels detected in mussels transplanted to this site for four months. EEQ in native mussels were also higher than transplanted mussels, which may suggest, in a similar manner to Mutton Island, Galway Bay, that native populations may be more susceptible to ED effects in these environments. E1, E2, EE2, NP and OP were not detected in fish sampled from outer Dublin Harbour. Elevated VTG levels have been recorded in native male rainbow trout sampled upstream in the River Liffey catchment, downstream of Osberstown WWTP (68,000 PE), near Naas, Co. Kildare (Tarrant et al. 2005). Although a considerable distance from Dublin Bay, inputs at this site may increase ED pressures downstream. The AA E2 water concentration in filtered water was below the proposed WFD AA EQS 'total water' value, although E1 levels may be of concern. Resident fish species situated in the Liffey estuary may be susceptible to ED effects due to the presence of steroid estrogens and WWTP inputs. There is potential for concern at this site however further monitoring of steroid and EDC levels in water and ED effects in resident species is recommended.

5.7. Linking biological effects measurements with chemistry results-overall site assessments

No distinct relationship was determined between chemistry data and biological effects measurements. It must be noted that the determined overall levels and effects are generally low in Irish marine waters and as such clear correlations between derived concentrations and effects would be subject to greater variability at the detection capabilities of the methods.

Some of the highest steroid levels reported in this study were detected in POCIS samples collected from Dublin Bay. Chemical analysis of water also confirmed the presence of E1 and E2 in a number of water samples although clear correlations are not evident. Transplanted *Mytilus* at this site did not display elevations in ALP relative to controls, although the ALP levels and EEQ measurements were higher at this site relative to other sites studied. The higher ALP and ER-LUC results combined with detection of E1 and E2 in both water samples and POCIS suggest a potential risk of estrogenic ED at this site (Table 5.3).

Mutton Island, Galway exhibited the highest POCIS E1 and E2 concentrations, along with the highest EEQ in native mussels, but ALP levels were similar to those reported at the other sites studies (including New Quay, the site used to source mussels for transplantation). The steroids were not detected in water samples taken at this site, however the given the detection of E1 and E2 in POCIS deployed near the WWTP diffuser pipe at Mutton Island, and high EEQ in native mussels collected at the storm water overflow vents of the WWTP, it is likely that greater potential for exposure and effects would be expected at such high population equivalent sites, close to point source discharges.

E1, E2 and EE2 were not detected in POCIS deployed in Shannon, but the ER-LUC and ALP results from mussels at this site were higher than at sites (Omey Island and Cork) where E1 and E2 were detected at concentrations of up to 5.2 and 0.5 ng/device, respectively. While Cork and Wexford had similar E1 and E2 POCIS concentrations and EEQ in mussel samples, the ALP response differed substantially (12.5 compared with 40.2 µg ALP/mg protein in Cork and Wexford, respectively). This highlights one of the issues with using biomarkers as an indicator of estrogenic contamination. Seasonal variations in response and between site variability (for example, due to differences in the stage of

gametogenesis) can impact the interpretation of a biomarker response. Using a battery of biomarkers with well characterised seasonal responses is one way to mitigate these effects. E2 levels in POCIS were higher in Wexford than in Cork (Table 5.3) however combined with the low EEQ values at both sites, these sites were deemed to be at low risk of estrogenic ED.

5.8 Conclusions

This study presents the first spatially comprehensive integrated marine based dataset for Irish coastal waters and resident marine species. More elevated EDC levels and effects were found at locations with greater anthropogenic pressures, particularly Dublin Bay. Mussels located near point source discharges at sites with higher population equivalents may consequently also be at risk. Significant differences in ALP levels were noted between sites. No direct link between proximity to potential sources of EDCs and ALP levels were found. E2 water levels at three sites studied (NBL Dublin, Mutton Island Galway and Omev Island) were below WFD AA EQS total water values, thus in using the available criteria, the sites were considered to have lower ecological risk status in terms of E2. Further monitoring with respect to WFD EQS compliance is required in order to classify the study sites. Initial levels and effects data presented indicate that the levels and associated effects are generally low in the marine waters studied, with a low risk of estrogenic ED to resident species; however a few exceedances of selected assessment criteria are reported for Dublin Bay.

While quantifiable correlation between chemical and biological analyses were not determined, the combination of techniques were generally found to deliver complimentary responses and thus are considered as valuable tools for the investigation of EDCs in the marine environment. The techniques used provided a cost effective means of assessment, with a range of analyses applied to a single sample. Continued lowering of analytical detection limits in order to keep with proposed EQS values in water and potential future EDC EQS in biota is recommended. As quantitative analysis of polar compounds by PS improves there is an obvious role for the technique in the provision of time integrated analytical data. Future studies combining biomarkers and PS should take into account the presence and release of naturally occurring steroids and deployment of PS should be completed at a suitable distance from caged indicator species. One limitation with using *Mytilus* spp. as bioindicators is the marked seasonality in response and sensitivity to

variations in abiotic factors, such as temperature and salinity. Despite these limitations, biomarkers were found to be valuable tools for assessing ED effects in the environment, accounting for the effects of many compounds not selected for by targeted chemical analysis. Further work is needed to elucidate the biological pathways and responses, and with respect to seasonal responses in Irish *Mytilus* spp. in order to enhance the application of biomarkers in the field of pollutant monitoring and enable accurate risk assessment to be completed. The requirement for “integrated” datasets as presented herein and the ongoing development of appropriate assessment criteria is vital to further empower environmental assessment.

Chapter 6: Conclusions and future recommendations

6.1 Conclusions

This thesis details the first comprehensive assessment of estrogenic endocrine disrupting compounds and endocrine disruption in Irish marine waters. A range of techniques were trialled, including ‘traditional’ chemical analysis methods, and more recently developed passive sampling technologies. Specific analytes with potent estrogenic activity were selected for targeted chemical analysis, while biomarker techniques measuring overall estrogenic activity and estrogenic effects were utilised in order to account for the presence and effects of unknown estrogenic compounds.

The following research questions were addressed within this thesis:

Question 1: What are the presence and effects of selected endocrine disrupting compounds in the Irish Marine Environment?

Question 2: What is a suitable means of detecting EDCs and assessing their impacts?

Question 3: What are the cause and effect relationships of these compounds?

Following a detailed literature review (Chapter 1) the steroid estrogens and alkylphenols were selected for study based on their documented estrogenic effects, estrogenic potency, and relevance within the legislative context of the WFD. A specific and selective LC-MS/MS method capable of detecting steroid estrogens in water and biota was developed and validated, with the capacity to detect E2 at proposed WFD AA EQS levels and the capacity to detect steroid estrogens in biota at biologically relevant levels. A second LC-MS/MS method for the detection of NP and OP in biota was developed and validated. Both methods were successfully applied to the analysis of both transplanted and native shellfish, and to native fish from a range of both impacted and non-impacted Irish coastal locations. The WFD EQS is set for ‘total water’, that is the total concentration of the analyte reflecting both the dissolved and particulate bound concentrations (EC, 2009). An important finding in this research is that although water samples were filtered, filtration was not deemed to have an effect on E2 concentrations (Chapter 2).

Water samples were collected monthly between June and October 2010 at the North Bank Lighthouse (NBL) in Dublin, Mutton Island in Galway Bay, and Omey Island. E1 was detected in three water samples from Dublin Bay at 0.14, 0.76 and 1.11 ng/L. E2 was

detected in one water sample from this site was 0.13 ng/L, which is above the EQS value of 0.08 ng/L. The EQS is the annual average (AA) concentration. Four other samples at this site were <LOD, thus when all of the results are combined, the AA value at this site is below the WFD AA EQS, indicating a low risk at this site with respect to E2. Jobling et al. (1995) reported effects on vitellogenin production and testicular growth in sexually maturing rainbow trout exposed to 2 ng/L E2 for three weeks. The lowest effect level for vitellogenin production reported in the literature is 0.3 ng/L E2 in immature male rainbow trout after 18 weeks of dosing (Sheahan et al. 1994). Effects have also been detected at 10 ng/L E2 in male rainbow roach exposed for three weeks in flow-through aquaria (Routledge et al. 1998). The concentrations of E2 detected in water samples collected in this study are below those reported to induce effects in these studies. Additionally, using the PNEC of 1 ng/L E2 equivalents set by Williams et al. (2008), Dublin Bay, Omev Island and Mutton Island Galway Bay are deemed to be at low risk for intersex in fish. Negative effects on spermatogenic development have been noted in male rock oysters (*Saccostrea glomerata*) exposed to 5 ng/L EE2 over 8 weeks (Andrew et al. 2010). Kidd et al. (2007) found elevated VTG levels in both male and female fathead minnows (*Pimephales promelas*) subject to chronic low level EE2 exposure (5-6 ng/L). They also found intersex, decreased gonadosomatic indices and the collapse of the fish population after seven years exposure. EE2 was not detected in any water samples or POCIS devices in the current study. The risks to resident populations related to EE2 exposure are deemed to be low.

POCIS devices were also assessed as an alternative/supporting means of water sampling. E1 and E2 were detected up to 15.3 and 3.9 ng/device, respectively, in POCIS with the highest levels detected in devices placed in close proximity to a WWTP diffuser pipe in Galway, followed by devices deployed in Dublin Bay. It is difficult to ascertain the potential risks to resident species as a result of these concentrations. There is a wide variation in the range of sampling rates reported for POCIS in the literature (Morin et al. 2012). Reported sampling rates vary between 0.02 L/day (Rujiralai et al. 2011) and 0.85 L/day (Li et al. 2010), suggesting that between 0.6 L and 25.5 L may be sampled in 30 days deployment if using the lowest and highest sampling rates reported in the literature, respectively. This has major implications for the assessment of POCIS derived water concentrations. For example, the 1.61 ng/device E2 detected in POCIS at Wexford (Table 5.3) could have an estimated water concentration of anywhere between 0.06 and 2.68 ng/L depending on which sampling rate is used. If POCIS is to be used for future E2 WFD monitoring purposes (setting aside the issue of total vs. dissolved water concentrations),

accurate sampling rates are essential, as in this example, the higher sampling rate results in a water concentration of 0.06 ng/L, below the EQS, however using the lower sampling rate, the site appears to fail to meet the WFD EQS, with a derived water concentration of 2.68 ng/L.

E1 and E2 were also detected in POCIS deployed at a site which was considered a reference site (Omey Island). The devices were attached to cages containing transplanted mussels. The concentrations of E1 and E2 in POCIS decreased over the duration of the study, as did the number of mussels in close proximity to the devices (a reduction of 120 mussels per sampling event). It is feasible that endogenous E1 and E2 may have been released by these mussels into the surrounding water at concentrations which were below the detection limit of the LC-MS/MS method, but which were concentrated to a sufficient level in POCIS to be within the range of detection of the LC-MS/TOF method used. This finding has not yet been reported for field studies reported in the literature. It is thus recommended that future studies combining EDC biomarkers and PS should incorporate chemical based techniques in order to take into account of the presence and release of naturally occurring steroids and that deployment of PS should be completed at a suitable distance from caged indicator species.

Few data studying the uptake and effects of EDCs in marine species are available. A study examining the effects of 17 α -ethynyl estradiol in *Mytilus* spp. *in vivo* (Chapter 3) revealed elevated estrogenic effects as measured with ALP, and acted as a positive control for further studies into effects in the environment. Within this study, an increase in ALP response was noted in mussels which underwent a simulated intertidal regime. As the only difference between sub-tidal and simulated intertidal regimes was a daily 4 hr period where the mussels were left exposed, the reduced detoxification capacity induced by exposure to air during the 'low tide' period of the intertidal regime is hypothesised to have influenced the increase in ALP levels observed. The findings of this pilot study have a number of implications with respect to environmental monitoring and in the derivation of toxicity and biological effects criteria, much of which has come from laboratory studies conducted in a 'fully submerged' setting. Further comprehensive research into the effects of EDCs in an 'intertidal versus sub-tidal' study is recommended to address potential differences in response related to tidal effects.

Caging of bioindicator species in tandem with a variety of other screening and biological effects based methodologies is readily becoming relevant in the provision of water quality information, especially given the documented limitations of traditional spot water sampling and analysis. Chapter 4 details a multi-parameter approach used to investigate the presence and effects of selected EDCs using caged *Mytilus* spp. at three coastal locations with varying degrees of anthropogenic input (NBL Dublin, Mutton Island Galway and Omev Island). Targeted analysis was completed using passive sampling and LC-MS/MS, while ALP and ER-LUC monitored overall estrogenic effects at each site. Test organisms for the caging study were taken from a well characterised site and biotic factors (e.g. condition factor and organism gonadal stage) which can also influence biomarker response were accounted for thus ensuring that biological effects responses observed after exposure could be better interpreted. Despite this, significant differences in CF were noted between sites after exposure, with CF highest in Mutton Island and lowest at Omev Island. There were also significant decreases in protein content in the exposed male mussels at NBL and Omev Island relative to T0, while protein content increased at Mutton Island relative to T0. ALP levels in male mussels at Omev Island were significantly lower than male mussels at T0, although they were not significantly different to male mussels deployed at NBL and Mutton Island. The ALP levels detected in this study were low compared with values reported in the literature, so the organisms were not deemed to be at risk of ED at these sites as measured with ALP. Higher ALP levels were detected in this study in native mussels than in transplanted mussels. Further examination is merited on overall effects of chronic exposure and additionally to determine whether native animals can rapidly adapt to the nature and level of contamination in their environment and to what extent successive generations of a species may be affected.

A detailed integrated assessment of all EDC related data generated within (and external to) the scope of this research was conducted in Chapter 5. Based on this expansive multi-parameter multi matrix pilot study it appears that currently the majority of Irish coastal sites studied are at relatively low risk of estrogenic ED. Concentrations of selected EDCs in water and biota were generally low, as were observed biomarker responses, even at sites subject to high levels of anthropogenic input. E2 water concentrations, measured at three coastal locations, were below proposed WFD AA EQS values, however further monitoring is required in order to classify these water bodies with respect to E2 in total water for WFD purposes. Dublin Bay contained the highest sediment EEQ (0.49 ng EEQ/g) and high EEQ (0.72 ng EEQ/g dw) in native mussels relative to other sites in this study. E1 and E2 were

also detected in water and POCIS. ALP levels however, were within the normal range of native Irish mussels and were not deemed to be elevated. Given the higher population equivalents, higher sediment and mussel EEQ, detection of E1 and E2 in water and POCIS the sites in Dublin Bay were highlighted as at potential risk of estrogenic ED compared to the other sites studied.

Further temporal and spatial research to monitor effects in resident species is merited at a number of sites including e.g. the Liffey estuary. Mussels situated in close proximity to WWTP (e.g. as measured at Mutton Island) may also be at risk of estrogenic ED as EEQ in native mussels were the highest detected of the sites studied (0.81 ng EEQ/g dw) and the highest E1 and E2 levels were detected in POCIS deployed at this site. Water analysis however, did not statistically correlate with these findings, thus further research is merited. The remainder of the sites studied within this research were deemed to be at low risk of ED, however ALP levels in native mussels from Wexford relative to the other study sites may warrant more detailed investigation.

Both POCIS and water samples were valuable tools for detection E1 and E2 in the marine environment; however discrepancies were noted between sampling methods. At NBL, Dublin Bay, the concentrations of E1 and E2 decreased in POCIS between July and October 2010, while water based concentrations of E1 increased in this period. E2 was also only detected in water collected in October, while the E2 level in POCIS at this time was the lowest recorded at this site. Higher E1 and E2 concentrations were recorded at Mutton Island, Galway, however E1 and E2 were not detected in any water samples from this site. Waterborne concentrations determined using spot samples may vary due to the 'spot' nature of sampling, tidal dilution effects, drought, rainfall and pollution events. POCIS devices provide a time-integrated means of sampling. The differences observed between spot samples and POCIS may be due to any of these factors, but tidal effects are likely to have a strong influence in marine systems. It is recommended that water samples are taken more frequently in future studies perhaps at different stages of the tidal cycle where feasible in order to mitigate some of these effects.

Of the biological effects tools used in this study the ER-LUC analysis of sediment was the most robust measurement, less subject to the influence of biotic factors. Levels in sediment ranged from 0.1 to 0.49 ng EEQ/g, with the highest concentration recorded in sediment from Dublin Bay, where E1 and E2 were detected in both water and POCIS. The

measurement of EEQ in mussels was also valuable, varying between sites, with the highest concentration detected in native mussels from Mutton Island Galway close to a point source discharge of WWTPE (0.81 ng EEQ/g dw), followed by native mussels from Dublin, the site with the highest level of anthropogenic pressures (0.72 ng EEQ/g dw). The implications of these concentrations for resident species are unclear.

Although variations were noted between sites, a direct link between ALP levels and EDC concentrations could not be made. The influence of the natural seasonal ALP response in native mussels is a factor which currently limits the value of ALP as a marker of estrogenicity. ALP of approximately 30 and 150 μg ALP per mg protein have been reported in *M. galloprovincialis* from a control site (de los Ríos et al. 2012), and some authors consider a value of 100 μg ALP per mg protein to be a background level (Ortiz-Zarragoitia and Cajaraville, 2006). Levels of ALP determined in the current study were below values considered to be background, thus the sites were not deemed to be at risk of estrogenic ED as measured using this biomarker. A more sensitive biomarker, such as the YES or ELISA may be a valuable addition in future studies of estrogenic ED in marine environments.

Based on the techniques evaluated throughout the course of this research, future integrated studies should consider chemical analysis of specific EDCs using both spot water samples (>five L depending on analytical sensitivity) and PS for marine waters, in combination with the ER-LUC and another sensitive bioassay, such as the YES. While POCIS was found to be valuable as a screening tool, PS devices for which accurate sampling rates have been defined are the preferred choice over POCIS unless limited by the log K_{ow} of the target analyte.

6.2 Recommendations

Overall chemical and biological analyses were found to be complimentary tools for the investigation of EDCs in the marine environment, and are recommended for future monitoring purposes. Passive sampling using POCIS was a valuable screening tool for EDCs in this study. POCIS are easy to manage and deploy and are not complicated by biotic factors experienced while using biomarkers, however as reported in this thesis care should be taken to ensure that the potential for endogenous EDC production is accounted for during analysis. The capacity of POCIS based methodologies to support environmental

legislative objectives continues to be problematic given that WFD EQS are currently set for ‘total water’ concentrations while PS measures the dissolved phase only, and that quantitative analysis is restricted by a lack of performance reference compounds suitable for low log K_{ow} sampling rate determination. Suspended particulate matter in seawater is typically low (Anderson and Meyer, 1986; Dobrynin et al. 2010) and was not found to affect recovery of steroid estrogens (López de Alda and Barceló, 2001) this latter research being confirmed during validation of the LC-MS/MS method developed in this thesis. Derivation of accurate uptake rates will enhance the suitability and value of POCIS as a cost effective method for WFD monitoring purposes and until such work is reported POCIS is suggested for use as an initial screening device and/or for the purposes of toxicity identification evaluation (TIE) approaches on POCIS extracts, thus identifying target compounds for more detailed investigation.

Biomarkers were found to be valuable tools for assessing ED effects in the environment; however knowledge of the invertebrate endocrine system is still limited. Further research should focus on further elucidation at a cellular level and key EDC uptake and degradation pathways in order to enhance the application of biomarkers in the field of pollutant monitoring. Baseline and limited seasonal data on the natural variability of ALP levels in indigenous *Mytilus* spp. have been determined within the wider SeaChange project and in the absence of internationally accepted assessment criteria for ALP, have been utilised to support an assessment of EDC effects in Irish species. ALP and EDC concentrations with full supporting abiotic and biotic co-factor information as reported in this present study provide valuable information in an area where data are limited. Additional wider internationally driven research is required to further determine more detailed information on temporal and seasonal variations in order for ALP to be incorporated into a routine bio-effects monitoring programme.

The predominant endocrine disrupting effects observed in the environment have been estrogenic in nature, and accordingly, research has focussed on estrogenic EDCs. Many other compounds affect the endocrine system. Future research is required to investigate androgens and anti androgens, progestins and anti-progestins, aryl hydrocarbon receptor agonists and thyroid hormone disruptors in the Irish aquatic environment. Synergistic and additive effects of compounds present in WWTP effluent and the environment should also be considered, as well as anti-agonistic effects.

Irish coastal and transitional waters have generally ‘good’ status in terms of WFD dangerous substances in WFD coastal and transitional waters (McGovern et al. 2011). EDC and ED data generated within this thesis for E2 in water at three coastal locations further adds to the information available on priority pollutant levels for WFD and shellfish waters assessment purposes. The current WFD priority and hazardous pollutant listings contain other compounds which have known estrogenic ED effects however little is known in relation to the presence of these compounds in Irish marine waters, thus future research should further endeavour to determine the spatial and temporal extent of these compounds in coastal and transitional waters. Continued lowering of detection limits (analytical and biological effects) is required in order to comply with legislative obligations but more importantly in order to be able to accurately detect subtle ED effects as a consequence of trace pollutant levels.

Concurrent development of species-specific “marine baseline” EDC effects and assessment criteria are merited, it is only through purpose built programs capable of detection of ultra-trace contaminant levels and effects that a true understanding of the potential for EDC related effects in the marine environment can be determined.

Ongoing development of the integrated chemical and biological effects toolkit as reported in this thesis is recommended. The proposed toolkit should consist of a range of specific EDC and combined ED effects and be capable of measurement of effects and concentration levels across multiple trophic levels. It should also be capable of detailing effects from a sub-cellular to population level and be measured in a variety of matrices from water to biota. Temporal (and spatial) integrated trend monitoring is merited especially in areas where anthropogenic impact may be expected (e.g. high PE and/or no treatment of wastes).

The combination of PS, chemical analysis and biological effects measurements provide a detailed in depth assessment of the current status of the studied sites, and has highlighted future monitoring and research needs. This thesis has on occasion documented the low level presence of a number of highly potent EDCs in Irish marine waters. It is the first study to utilise a combined chemical and biological effects approach to assess these compounds in the Irish marine environment. Information derived from this integrated approach is of value to researchers and environmental managers worldwide, and highlights strengths and limitations of the approaches used. The research is also valuable in terms of

derivation of future “criteria” to support future broader ecosystem based assessments such as under the MSFD.

The extent of the work presented in this thesis, and supported by partners in the ‘*Biological Effects and Chemical Measurements for the Assessment of Pollution in Irish Marine Waters*’ project has enhanced Ireland’s capacity for integrated monitoring of EDCs in the marine environment. The information gathered using the techniques developed has contributed to the current knowledge of the presence and effects of selected EDCs in the Irish marine environment.

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Appendix A: Associated and Proposed publications

1. Ronan, J. and McHugh, B. A sensitive liquid chromatography/tandem mass spectrometry method for the determination of natural and synthetic steroid estrogens in seawater and marine biota, with a focus on Water Framework Directive Environmental Quality Standards. *Rapid Communications in Mass Spectrometry*, 2013, 27, pp. 738-746.
2. Giltrap, M., Ronan, J., Hardenberg, S., Parkes, G., McHugh, B., McGovern, E., Wilson, J.G. Assessment of biomarkers in *Mytilus edulis* to determine Good Environmental Status for implementation of MSFD in Ireland Marine Pollution Bulletin 2013. Article in press.
3. An integrated assessment of endocrine disruption in marine waters using caged *Mytilus* spp, passive sampling, chemical and biological analysis and histology (in draft).
Authors: Jenny Ronan^a, Brendan McHugh^b, Michelle Giltrap^a, Heather Rochford^a, Ronan Mag Aoidh^{b,c}, James Wilson^a and Evin McGovern^b
^a School of Natural Sciences, University of Dublin, Trinity College, Dublin 2, Ireland
^b The Marine Institute, Rinvilla, Oranmore, Co. Galway, Ireland
^c University College Dublin, Belfield, Dublin 4, Ireland
4. An integrated assessment of the presence and effects of endocrine disrupting compounds in Irish marine waters (in draft).
Authors: Jenny Ronan^a, Brendan McHugh^b, Michelle Giltrap^a, Heather Rochford^a, Ronan Mag Aoidh^{b,c}, James Wilson^a and Evin McGovern^b
^a School of Natural Sciences, University of Dublin, Trinity College, Dublin 2, Ireland
^b The Marine Institute, Rinvilla, Oranmore, Co. Galway, Ireland
^c University College Dublin, Belfield, Dublin 4, Ireland
5. Acute exposure effects of 17 α -ethynyl estradiol in *Mytilus* spp. (in draft).
Authors: Jenny Ronan^a, Andrea Lenderink^{a,c}, Liam Curran^d, Michelle Giltrap^a, Silvia Hardenberg^a, Ronan Mag Aoidh^{b,e}, James Wilson^a, Evin McGovern^b and Brendan McHugh^b
^a School of Natural Sciences, University of Dublin, Trinity College, Dublin 2, Ireland
^b The Marine Institute, Rinvilla, Oranmore, Co. Galway, Ireland
^c Department of Biology, University of Bremen, Bremen, Germany
^d Shannon Aquatic Toxicity Laboratory, Enterprise Ireland, Shannon, Co. Limerick, Ireland
^e University College Dublin, Belfield, Dublin 4, Ireland