THE UNIVERSITY OF HULL

An investigation of the uptake of estradiol in the bivalve *Mytilus spp.* corresponding biological implications and the effect of the addition of Sporopollenin on bioavailability.

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by

Margaret A Harley

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Author's declaration

I hereby certify that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this, or any other university.

Abstract

There has been concern over the past two decades over estrogenic hormones such as 17 α-ethinylestradiol and 17 β-estradiol (E2) in fresh water and marine ecosystems and the risk to aquatic organisms even with low exposure levels. This study is to access whether there is any bioaccumulation in mantle tissue of *Mytilus edulis* exposed to a known concentration of E2 in a controlled experiment and whether the introduction of sporopollenin, exposed to the same concentration of E2, has a similar effect with bioaccumulation in regard to the use of sporopollenin exine capsules (SECs) as a sequestering agent to remove estrogens from water systems.

This study involved an experiment using *M. edulis* exposed over a week with a regular dose of 200 ng/L E2, SECs treated with the 200 ng/L of E2 and with untreated SECs and untreated *Mytilus* as controls. At the end of the experiment, tissue was preserved for histology purposes to ascertain the sex and stage of gametogenesis and to detect any presence of SECs. Portions of the mantle and digestive tract and gills were frozen for chemical analysis to ascertain if either free or conjugated E2 had been bioaccumulated. A portion of the mantle was frozen in RNAlater™ for qPCR determination of mRNA *ER* expression in either of the exposure groups. In parallel, analytical chemistry and extraction methods were optimised to determine the levels of E2 in tissues.

Histological preparations of gonad tissue from all *Mytilus* indicated the presence of SECs in the gonad tissues of the *Mytilus* exposed to SECs only. The water filtrates from the tanks of those animals also indicated the presence of SECs in the water throughout the exposure experiment. Chemical analysis of the water indicated that E2 dissolved in the water was present. The *Mytilus* in the exposure experiment were in the mid to late development stage of gametogenesis. The results of mRNA *ER* 2 gene expression indicated no significant difference in *ER* gene expression in the E2 exposed *Mytilus* compared to the controls but there was a significant difference in *ER* gene expression compared to mussels that were exposed to SECs and SECs plus E2. There was no statistical significance in the *ER* gene expression between the SECs and E2 treated SECs. The chemical analysis of the gonad tissue from the exposure experiment was not undertaken due to time constraints but free E2 was extracted from gonad tissue and from the E2 treated SECs, in a similar experiment. As there is such a notable difference in fold change between both of the SECs treatments and the control, there is an unknown biological influence on the mRNA *ER* gene expression.

In terms of wider implications of this work, the data shows that the SECs are very efficient at adsorbing or absorbing E2 and this property could be applied as a method to remove E2 from

WWTPs. But it is not known if the SECs could be applied for this purpose until there is sufficient evidence of bioavailability.

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Abbreviations

3β HSD	3 β hydroxysteroid dehydrogenase enzyme
5-HT	5-hydroxytriptamine (serotonin)
AG	adipogranular cells
Amu	atomic mass unit
ANOVA	analysis of variance
AOAC	association of official agricultural chemists
AR	androgen receptor
BaP	benzo [a] pyrene
BPA	bisphenol A
BSTFA	N, O-bis(trimethylsilyl)trifluoroacetamide
CAT	catalase
cDNA	complimentary deoxyribonucleic acid
C_T	cycle threshold
d4E1	deuterated estrone (estrone-2,4,16,16-$d_{4)}$
d4E2	deuterated estradiol (17β-Estradiol-$d_{2)}$
DAD	diode array detector
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
DEHP	di(2ethylhexyl) phthalate
DES	diethylstilbestrol or stilboestrol
DHEA	dehydroepiandrosterone
DL	desolvation line
DMABC	4-(Dimethylamino)benzoyl chloride
DNA	deoxyribonucleic acid

DNSC	dansyl chloride (5-(dimethyl amino) naphthalene-1-sulfonyl chloride)
dSPE	dispersive solid phase extraction
E1	estrone or oestrone
E1-GUL	gluconate conjugated estrone
E1-SUL	sulphate conjugated estrone
E2	17β estradiol or 17β oestradiol
E2-GLU	gluconate conjugated estradiol
E2-SUL	sulphate conjugated estradiol
E3	estriol
EDCs	endocrine disrupting compounds
EE	ethinylestradiol
EE2	17α-ethynylestradiol
E & H	eosin and haematoxylin
ELISA	enzyme-linked immunosorbent assay
Em	emission
ER	estrogen receptor
ERR	estrogen related receptors
ESI	electrospray ionisation
Ex	excitation
FLD	fluorescence light detection
FMPTS	2-fluoro-1-methylpyridinium p toluene sulfonate
FSH	follicle stimulating hormone
GAC	granulated activated carbon
GC-MS-MS	gas chromatography tandem mass spectrometry
GLC	gas liquid chromatography

GnRH	gonadotropin releasing hormone
GSH-PX	glutathione peroxidase
GSI	gonad somatic index
GR	glucocorticoid receptor
GR	glutathione reductase
GVF	developing gamete fraction
HBCDD	hexabromocyclodecane
HPLC	high performance liquid chromatography
HP5-MS	(5%-phenyl)-methylpolysiloxane
IPPC	integrated pollution prevention and control directive
IS	internal standard
KDa	kilodalton
LC-MS-MS	liquid chromatography tandem mass spectrometry
LH	luteinising Hormone
LOD	limits of detection
LOQ	limit of quantification
MDA	malondialdehyde
MIQE	minimum Information for publication of quantitative real-time PCR experiments
mL	millilitre
mM	millimolar
µL	microlitres
µm	micrometers
MR	mineralocorticoid receptor
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid (RNA)

MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MTBSTFA	N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide
m/z	mass to charge ratio
ng	nanogram
nm	nanometre
PAC	powdered activated charcoal
PBDEs	polybrominated diphenyl ethers
PBS	phosphate buffered saline
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
PDA	photo diode array detector
PFBBr	pentafluoro benzoyl bromide
PFOS	perfluorooctanesulphonate
pg	picogram
pMol	picomoles
POPs	persistent organic pollutants
PR	progesterone receptor
PSA	primary and secondary amine exchange material
Psi	pounds per square inch
qPCR (or RT-PCR)	quantitative real time polymerase chain reaction
QuEChERS	an acronym “Quick, Easy, Cheap, Effective, Rugged, and Safe”
RNA	ribose nucleic acid
RQ	relative quantification ratio or ($2^{-\Delta C_T}$)
RSD	relative standard deviation
RT	retention time

SD	standard deviation
SIM	single ion mode
SECs	sporopollenin exine capsules
SEM	standard error mean
SOD	superoxide dismutase
SPE	solid phase extraction
StAR	steroidogenic acute regulatory protein
TBBPA	tetrabromobisphenol-A
TBT	tributyl tin
TIC	total ion count
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethyl silyl groups
VCT	vesicular connective tissue cells
VPF	vitellogenin promoting factor
VTG	vitellogenin
WWTP	waste water treatment plant(s)
WFD	water framework directive
YFP	yellow fluorescent protein

Chapter 1. Literature Review

1.1. General background

From 1972, the European Union has engaged an extensive set of regulations and directives to protect the environment and address issues such as acid rain, air quality, the protection of inland surface waters and estuaries, eutrophication, ground-level ozone pollution, industrial and agricultural waste emissions and the reduction of the negative effects of land-fill waste on the environment (CIEEM 2015 EU Environmental Legislation regulations and directives). The Water Framework Directive (WFD) consists of legislation and measures to ensure that aquatic ecosystems meet a required standard. This includes the collection, transport, recovery and disposal of wastes to limit contamination to the environment. The WFD protects the quality of estuaries, coastal waters, ground waters, bathing waters and lakes and reservoirs.

Examples of some of these EU directives are the Groundwater Directive that protects ground waters against pollution and deterioration; the Marine Strategy Framework that protects the marine environment from detrimental effects by human activities; the Integrated Pollution Prevention and Control Directive (IPPC) and Industrial Emission Directive that sets emission limits for pollutants produced by industrial activities in the air, water, groundwater and soil; the Urban Waste Water Treatment Directive that protects the environment from the discharge of waste water into water systems including lakes, streams and reservoirs.

Traditional methods for monitoring air and water quality include chemical analysis by regulatory bodies and researchers, taking samples at regular intervals for laboratory analysis using defined analytical methods. Some end points for investigating the impact of a xenobiotic chemical on an organism are: -

1) Growth development, looking for abnormalities and increased sensitivity to different developmental stages.

2) Reproductive performance, looking for gamete quality and fecundity.

3) Behaviour of the organism, such as changes in feeding, reproductive behaviour and locomotion.

There are two strategies for evaluating potential endocrine disruptors, *in vitro* and *in vivo* system tests. The majority of reports concern laboratory studies using *in vitro* studies. Examples of *In vitro* tests are, the direct assessment of the ability of a compound to bind to an estrogen receptor (Jobling *et al.* 1995); the production of the egg yolk protein, VTG, as evidence of estrogenicity (Jobling and Sumpter,1993); gene transcription assays (Routledge and Sumpter, 1996).

Exposure experiments in controlled laboratory experiments may give some understanding on the main effects of exposure to these new chemicals. Daphnia (*Daphnia magna*), Gammarus (*Gammarus pulex*) and Zebra fish (*Danio rerio*) are examples of organisms that are used in laboratory trials or controlled experiments for the study of the effects of short term or long-term chemical exposure on that organism. These organisms are small and easily maintained in simple aquarium systems and have short generational cycles and develop rapidly into adults. (Lovern *et al.* 2007). The common mussel, *Mytilus edulis*, is widely used as a biosensor of coastal water pollution and has been used to monitor the exposure of a variety of marine contaminants including trace metallic elements, organic pollutants and nanoparticles in bio monitoring programs (Anantharaman and Craft, 2012).

In vivo studies are more challenging because the natural environment has a considerable number of variable parameters to consider such as temperature, tidal fluctuations, pH and diverse chemical composition and these are difficult to replicate in the laboratory. Also, some of the combination of chemicals may have synergistic or antagonistic properties that may need to be anticipated. With the growth of the pharmaceutical Industry and change to more extensive farming methods and the immergence of fish and marine culture as an industry, a new “cocktail” of pollutants has emerged and their impact on the environment has only been researched in the last few decades. This demands a change in monitoring strategies to evaluate the impact on these new materials being developed.

1.2. Endocrine Disrupting Compounds

The World Health Organisation definition of endocrine disruptors and potential endocrine disruptors is as follows:

“An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations” (WHO/PCS/EDC 2002).

Endocrine disruptors are estrogenic chemicals that imitate estrogens by binding to the estrogen receptor and stimulating transcription activity or by acting as anti-estrogens by binding strongly to the estrogen receptor and therefore blocking the effects of endogenous estrogens. Others mimic androgens and thyroid hormones. Endocrine disruptors can occur naturally in the environment or as synthetic xenobiotic pollutants. Naturally occurring phytoestrogens are present in moulds, clovers, fruits and legume plants. For example, the phytoestrogens genistein and daidzein are found in soy products.

Endocrine disruptors interfere with the steroid pathways that produce hormones, some act via the same hormone receptors and multiple receptors. For example, PCBs mimic natural

estrogens and bind with estrogen receptors. They may act at low doses, similar to the naturally occurring hormones but unlike endocrine hormones they may bioaccumulate (Carley *et al* 2014). The production of synthetic organic chemicals immerged after the second world war and increased through the 20th century until the 1960's when concern was raised about synthetic chemicals such as DDT used in agriculture as pesticides were having detrimental effects on the environment. In the early 1990's evidence against a number of organic chemicals was growing and there was an overwhelming consensus that synthetic chemicals could interfere with human health and the well-being of animals in the environment (Colborn *et al.* 1993).

Most of these chemicals, such as the pesticide DDT and industrial polychlorinated biphenyls have now been banned but they have been found to be persistent in the environment. Synthetic endocrine disruptors are found to have profound detrimental effects on the environment as they are not easily broken down, are transported by wind, wave motion and persist in the soil, water, in plants and animals. Studies have shown that they interact with other hormones adversely compromising the immunity, metabolism of wildlife that can be inherited through multiple generations (Guillette, 2006).

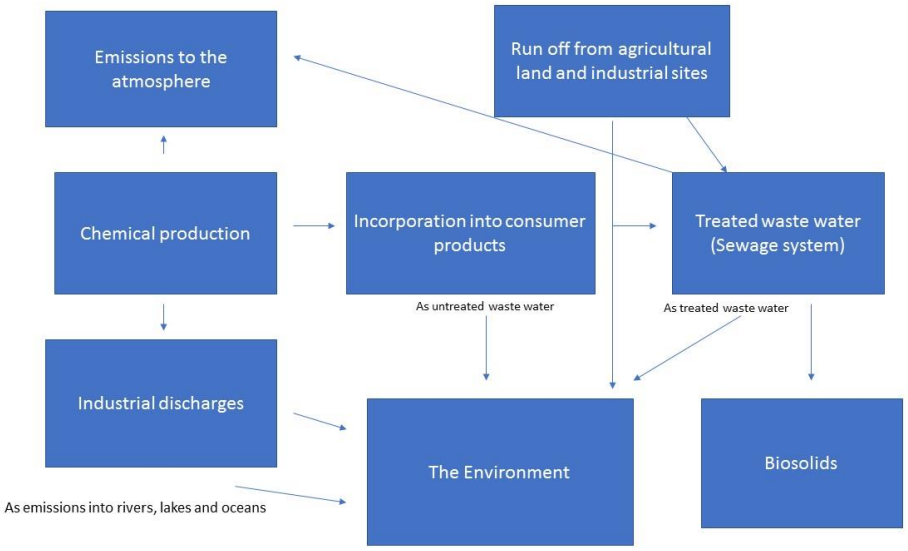


Figure 1.1 Pathways of the distribution of endocrine disruptors into the environment.

Routes of entry into the environment may be due to human activities, the disposal of wastes, deliberate introduction through herbicides and pesticides (Figure 1.1) They can enter by contamination of surface waters, domestic wastes, sewage and industrial wastes. In the case of industrial and sewage waste, their release can be a considerable distance from where the waste was originally treated or discharged. Dilution and chemical degradation may occur so that biological effects are most likely to be seen near locations were the pollutants enter the water systems. There are many different types of endocrine disruptors (Table 1.1).

Chemical Name	Use	Potency	Endocrine Disruption Effects	References
Polychlorinated biphenyls, Dioxins	persistent organic pollutants (POPs) Insulating, heat transfer agents and sealants	Very persistent, accumulate in the food chain	Suppression of thyroid hormone activity in vertebrates. Immune disfunction.	Crinion, 2011
DDT	An insecticide used in agricultural and non-agricultural applications.	Banned in the early 1970s but very persistent	Effects eggshell thinning, educed testosterone in vertebrates.	Lundholm , 1997
Perfluorooctanesulphonate (PFOS)	A surfactant, stain repellent, used in fire-fighting foams	stable to degradation,	Reduced foetal growth, lowered female fecundity.	Washino *et al.* 2009
Polybrominated diphenyl ethers (PBDEs)	Used as flame retardants in textiles, electronic and building materials.	Very persistent in the environment	Egg shell thinning in birds, suppression of thyroid hormones in vertebrates including marine mammals.	Meerts *et al.* 2001
Hexabromocyclodecane (HBCDD), tetrabromobisphenol-A (TBBPA)	Flame retardants in building and insulating materials	Little studied	Have weak estrogenic activity	Canesia *et al.* 2005

Perfluorooctanoic acid	Used as an emulsifier in production of polymers such as Teflon	Effects little studied	Adverse effects on adrenal glands	Kudo *et al.* 2002
DEHP	Used as a plasticizer for polymers and plastics	Bio-transformed to the more potent monoester by enzyme activity	Reduced semen quality and lower testosterone levels and impeded immune function in vertebrates	Davis *et al.* 1994
BaP	A product of incomplete combustion of fossil fuels	Associated with solid particulate matter in the air	Neoplasia in wildlife	Ha *et al.* 1990
Triclosan	An antimicrobial agent, used in personal care products	Readily bio-accumulates in soils and sediments, found in sewage influents and effluents	Disrupts steroidogenic enzymes involved in the production of estrogens and testosterone.	Kumar *et al.* 2009
Bisphenol A (BPA)	An industrial chemical used in the production of polycarbonate plastic and epoxy resins	Readily biodegradable	An environmental estrogen, acting via estrogen receptors in vertebrates	Howdeshell *et al.* 1999
Atrazine	A synthetic herbicide. Banned in the EU but still used in		Intersex in aquatic vertebrates, reduction in immune function in fish	Hayes *et al.* 2002

	many other countries			
Vinclozin	A fungicide used in agriculture		An antagonist at the androgen receptor, causing feminisation of male fish and masculinisation of female fish.	Kelce *et al.* 1994
Fluoxetine	A pharmaceutical used to treat a variety of mental illnesses		Targets serotonergic modulation in aquatic vertebrates and invertebrates.	Stanley *et al.* 2007
Diethylstilboestrol (DES), known formerly as stilboestrol	synthetic steroids used in oral contraceptives		Reduced fecundity in aquatic vertebrates	Batra and Bengtsson, 1978
Cyclic Methyl siloxanes	Products used in cosmetics such as hair and skin products. Used in sealants and defoamers		Little studied, but considered as estrogenic in vertebrates	Wang *et al.* 2013
Phytoestrogens	Naturally occurring plant derived compounds found in soy and other plant products		Shows estrogenic activity	Wuttke *et al.* 2003
Tributyl tin (TBT)	A biocide, banned from use		Imposex in Molluscs	Gibbs and Bryan, 1986

				Matthiessen and Gibbs 1998; Evans, 1999
Nonyl phenol (and other alkyl phenols)	used in manufacturing antioxidants, lubricating oil additives, laundry and dish detergents, emulsifiers, and solubilisers		Shows estrogenic activity	Soares *et al.* 2008

Table 1.1 Probable or known EDCs, listed in IPCS (2002). Global assessment of the state-of-the-science of endocrine disruptors. Geneva, Switzerland, World Health Organization, International Programme on Chemical Safety.

Exposure to EDCs have an impact on both invertebrates and vertebrates. For example, the organometallic compound, tributyl tin (TBT), used as an anti-fouling paint on boats, was found to cause penis development in female snails (*Nucella lapillus*) which blocked the egg releasing duct, producing non-viable eggs (Evans 1999). The presence of alkyl phenols and natural 17β estradiol (E2), natural estrone (E1) and synthetic estrogen, 17α ethinylestradiol (EE2) in water systems were correlated with high incidences of intersex in fish (for example, rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) (Jobling and Sumpter, 1993; Jobling *et al*. 1998 ; Routedge *et al*. 1998; Desbrow *et al* 1998; Jobling *et al*. 2002a; Jobling *et al*. 2002b;). Male fish were producing the egg yolk protein vitellogenin and had feminised genitals (Bortone and Davis, 1994).

An important reproductive process controlled by E2 levels is zona genesis. This is involved in the synthesis of proteins involved in the formation of the eggshell and eggshell membranes. The development of these proteins is critical for the development of the embryo inside the egg. Endocrine disruptors that mimic these hormones can bind to E2 receptors and block this process (Bustnes *et al.* 2001).

Wood pulp emitted from the outfalls of paper mills released plant sterols that were converted by bacterial action to produce male androgens resulted in female fish (*Gambusia holbrooki*) that exhibited male courtship behaviour and masculinised genitals (Jenkins *et al*. 2003). A chemical spill in Florida in 1980, resulted in the exposure of DDT and metabolites to reptiles,

producing health and hormone anomalies common snapping turtle (*Chelydras. serpentina*) (Portellia *et al.* 1999) and alligators (*Alligator mississippiensis*) (Guillette *et al* 1994). They had higher than normal blood and egg estrogen levels. The male juveniles were feminised with small penises and abnormal testes (Guillette *et al*. 1994; Guillette *et al*. 1996).

High pesticide and PCB concentrations were found in the body fat blubber of marine mammals such as polar bears (*Ursus maritimus*), glaucous gulls (*Larus hyperboreus*), walrus (*Odobenus rosmarus*), ring seals (*Phoca vitulina*), bearded seals (*Erignathus barbatus*) and beluga whales (*Delphinapterus leucas*) (Jenssen, 2006). The contamination being passed on through their offspring through consumption of their milk and consumption through the food chain (Anderson et al 2001; Derocher *et al.* 2002). Polar bears (*Ursus maritimus*), animals at the top of the food chain were found to have lower levels of cortisol in the blood, the females having high progesterone and low estrogen levels (Le Bouef *et al.* 2002; Tanabe, 2002; Fossi *et al*. 2003).

1.3. The vertebrate endocrine system

The vertebrate endocrine system consists of groups of ductless glands that regulate the body processes by secreting chemicals called hormones. Hormones act either locally on surrounding organs and tissues or remotely on specific organs and tissues when carried by the bloodstream. There are two types of glands, endocrine and exocrine glands (Encyclopaedia Britannica) (Figure 1.2).

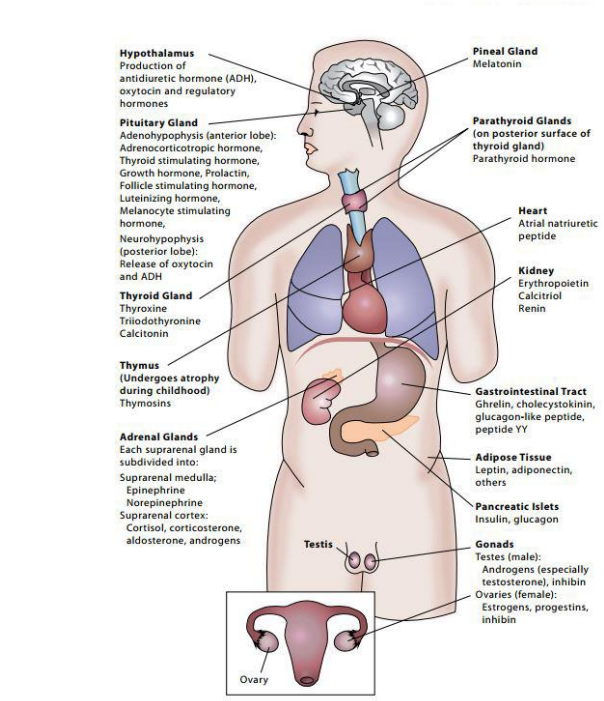


Figure 1.2 Human endocrine system (from State of the Science of Endocrine Disrupting Chemicals - 2012)

Exocrine glands secrete chemicals via ducts onto the internal or external body surface, acting on local tissues near the duct openings, for example the sweat and salivary glands. The endocrine glands, regulated by both the nervous system and the immune system, secrete chemicals that are carried by the blood circulation and exert effects on organs and tissues that can be remote from the site of secretion. When stimulated, specialised nerve cells, called neurosecretory cells, secrete hormones called neurohormones that are released into the blood stream (Encyclopaedia Britannica).

There are two types of hormones, protein hormones, including peptides and modified amino acids, and steroid hormones synthesised from the molecule cholesterol. Protein hormones are water soluble and are readily transported around the body in the blood. They are synthesised as large biologically inactive molecules called prohormones and are broken down to form the active hormones when activated by specific enzymes. Steroid hormones circulate as free hormones in the blood or may be conjugated to proteins. Peptide hormones act on target tissues by binding to and activating molecules on the surface of cells called receptors. Steroid hormones act on receptors within the cytoplasm or nuclei of cells. Specific segments of the hormone molecule have an affinity for specific receptors, so that hormonal actions are specific to selected target cells or tissues. Some hormones have multiple receptors (Encyclopaedia Britannica).

There are six steroid receptors in vertebrates. These are; two estrogen receptors (ER α) and (ER β); a progesterone receptor (PR); an androgen receptor (AR); a glucocorticoid receptor (GR) and a mineralocorticoid receptor (MR). Based on phylogenetic analysis of steroid receptors in basal vertebrates, it is suggested that these receptors were all created from a common ancestral gene. The first steroid receptor was an estrogen receptor, followed by a mineralocorticoid receptor and genome mapping and phylogenetic analysis indicates the full complement of mammalian steroid receptors evolved from these ancient receptors (Thornton, 2001).

The hormone- receptor complex activates chemical responses within cells, for example the activation of enzymes or the secretion of other hormones. These control of important body functions of growth, development and reproduction as they influence the chemical processes within the cell nucleus (Figure 1.3) (Encyclopaedia Britannica; Tulane university e. hormone).

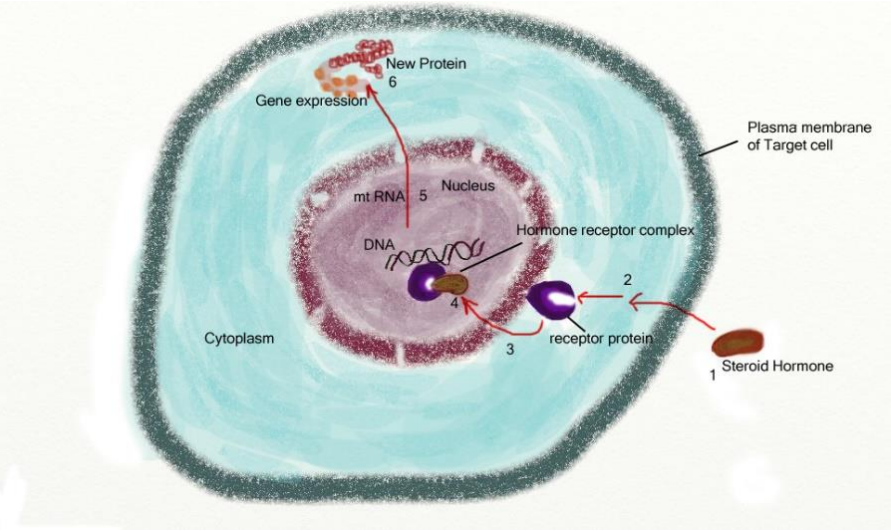


Figure 1.3 Hormone action in the cell. Hormones (1) bind to specific receptors (2) which stimulate protein synthesis (6)

Hormones are active at low concentrations and are usually found bound to serum proteins in the blood with only a small percentage existing as free hormone (Nussey and Whitehead, 2001). There is no bioaccumulation and they exhibit non-linear dose relationships, where high dose effects are not the same as low dose effects (Nussey and Whitehead, 2001). They are tissue specific and exposure during early development stages produce permanent effects (Nussey and Whitehead, 2001).

1.4. Vertebrate sex hormones

These are steroid hormones that are important for the regulation of reproduction, metabolism and body form and weight in vertebrates. The female ovaries produce both ova and hormones necessary for reproduction. Fluid in the oocyte contains estrogens and other steroid hormones such as progesterone, androgens, enzymes and bioactive proteins. The hormone androgen is converted to potent estrogens, E2 (Figure 1.4) and E1 (Figure 1.5) that are carried in the bloodstream. The testes are the male reproductive organ that produces sperm and the hormone androgen that is converted to the hormone testosterone (Figure 1.6). In both sexes, follicular development and ovulation are controlled by gonadotropins, secreted from the pituitary gland, the follicle stimulating hormone (FSH) and luteinising hormone (LH). At puberty, the hypothalamus, secretes gonadotropin releasing hormone (GnRH) which initiates the development of secondary sexual characteristics in both males and females; the maturation of ovarian follicles and the increased production of estrogens in the female and the increased production of androgens and sperm in the male. (Encyclopaedia Britannica).

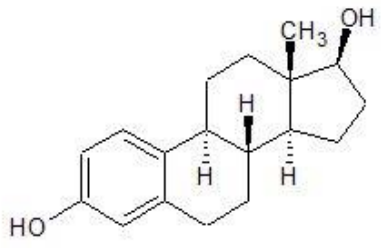


Figure 1.4 17β estradiol (E2) molecular structure

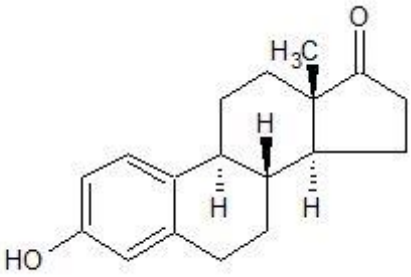


Figure 1.5 Figure estrone (E1) molecular structure

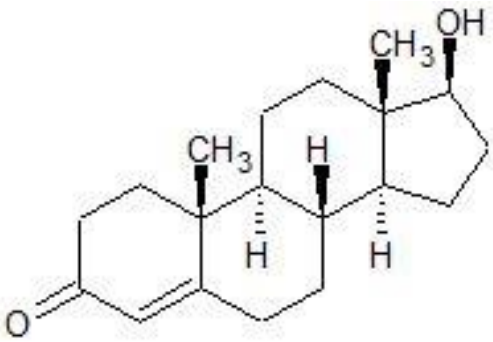


Figure 1.6 testosterone molecular structure

1.5. Steroidogenesis

Steroidogenesis, is the process where cholesterol is converted to dehydroepiandrosterone (DHEA), the primary precursor which is in turn converted to androgens and estrogens (Figure 1.7). Both adrenal and ovarian steroid biosynthesis have the same pathways and use the same enzymes for the initial steps but the final active products are derived by different enzymes in the latter stages.

Cholesterol (Figure 1.8) is the precursor for all steroid hormones and the regulation of steroidogenesis occurs at the level of gene transcription which is regulated by the steroidogenic acute regulatory protein (StAR) (Miller, 2002). This protein facilitates the movement of cholesterol

through the outer mitochondrial membrane where it becomes the substrate for the cholesterol side chain enzyme, a cytochrome P450 enzyme.

There are two types of steroidogenic enzymes, cytochrome P450 enzymes, of which there are two classes, and hydroxysteroid dehydrogenases. These are microsomal enzymes encoded by single genes. Type I cytochrome enzyme is found in the mitochondria, placenta, breasts and extra glandular tissues and type II cytochrome enzyme is found in the endoplasmic reticulum and in the adrenals and gonads (Miller 2002). There are about 20 forms of type II enzymes that participate in the biosynthesis of steroids, sterols and fatty acids and about 15 types of type II enzyme that metabolise xenobiotic agents (Miller, 2002).

Each cytochrome P450 enzyme is encoded by a single gene but has multiple activities. The enzyme P450scc is a quantitative regulator and determines the amount of steroid hormone production whereas the enzyme P450c17 is a qualitative regulator of steroidogenesis and is a key factor in androgen and estrogen synthesis, having two types of activity, catalysing 17α hydroxylase and 17, 20 lyase activities (Miller, 2002).The variation in the two activities selecting which of the steroid hormones is synthesised (Miller, 2002).

The initial step is the scission of the cholesterol side chain and C20α hydroxylation and C22 hydroxylation to give pregnenolone and isocaproic acid (Figure 1.7). Once converted to pregnenolone, there are two possible pathways; P450c17 enzyme converts pregnenolone to 17α hydroxy pregnenolone or the 3β hydroxysteroid dehydrogenase enzyme (3β HSD) converts the hydroxyl groups to ketones and produces an isomer, changing the double bond of the B ring to the A ring to synthesise Progesterone. The scission of the C17,20 carbon bond by action of the enzyme P450c17 on 17α hydroxy pregnenolone synthesises DHEA, the primary precursor of androgens and estrogens. The 17β hydroxysteroid dehydrogenases control the synthesis and metabolism of sex steroids oxidising E2 to E1 and testosterone to androstenedione. The P450 aromatase enzyme catalyses the synthesis of estrogens from androgens by a series of reactions (Figure 1.7) (Miller, 2002).

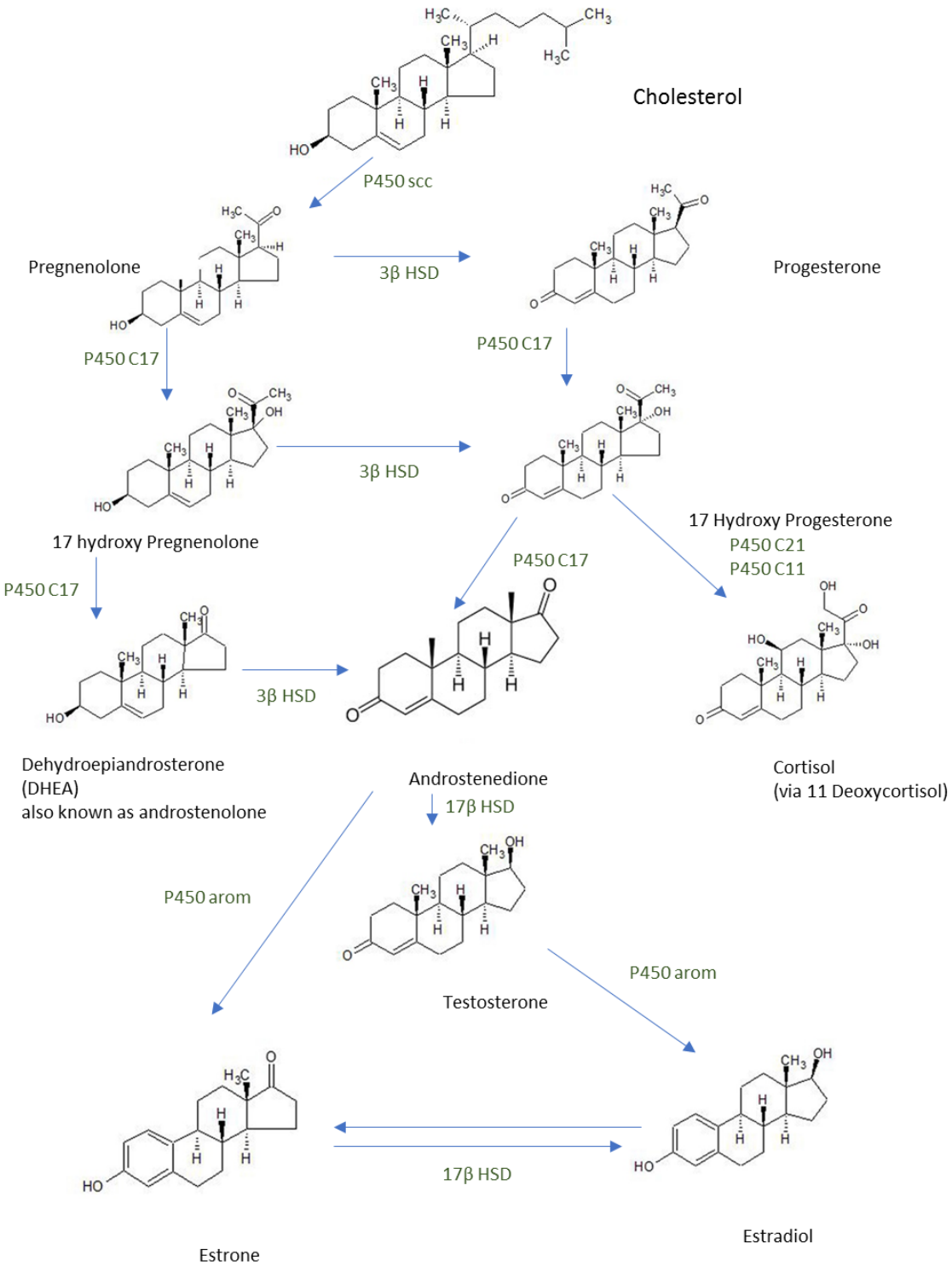


Figure 1.7 Steroid biosynthesis pathway for conversion of the substrate cholesterol to progesterone, androgens and estrogens

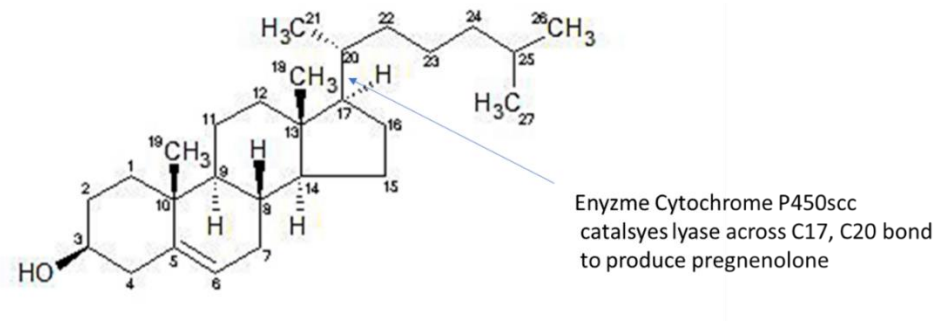


Figure 1.8 cholesterol, the precursor for androgen and E2 synthesis

1.6. The invertebrate endocrine systems through the phyla

All modern taxa of invertebrates utilise neuropeptide signalling mechanisms utilising products of neurosecretory cells to control and co-ordinate physiological and behavioural processes (Pinder *et al.* 1999). These are the prominent effectors for invertebrates but there is ample evidence that non-peptide endocrine messengers present in some groups of invertebrates called ecdysteroids that have important roles (Lafont *et al.* 1997; Pinder *et al.* 1999; Oehlmann *et al*. 2003; Dinan and Lafont, 2006).

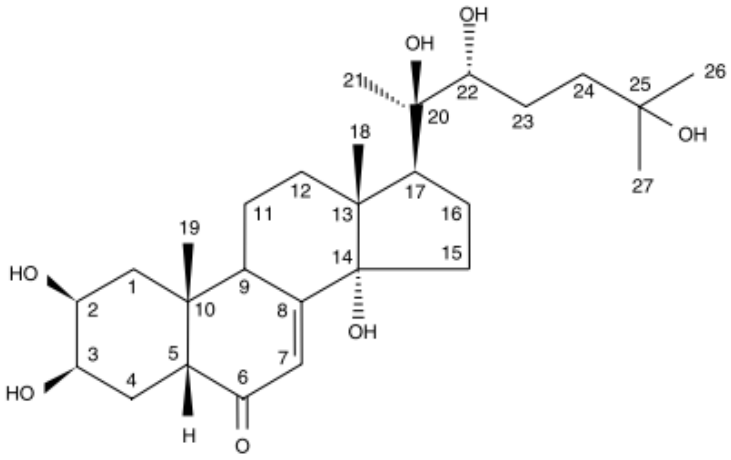


Figure 1.9 Structure of 20-hydroxyecdysone.

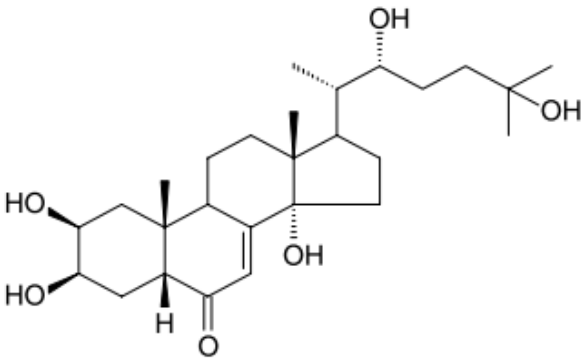


Figure 1.10 Structure of ecdysone

Ecdysteroids, for example 20-hydroxyecdysone (Figure 1.9) and ecdysone (Figure 1.10), are different to vertebrate hormonal steroids in that they are hydrophilic rather than lipophilic molecules and are important complex, multi-functional hormones that regulate the moulting processes in crustacea and insects (Pinder *et al*. 1999). Ecdysteroids are not found in vertebrates (Lafont *et al*. 1997). Animal diversity exploded during the cambrian period. According to nucleotide sequences, all animal species have evolved from a common ancestral colonial protist (Alberts *et al.* 2002). There were two divergent branches of animals containing no true tissues and animals with true tissues (Figure 1.11).

1.6.1. Organisms with no true tissues

Organisms with no true tissues include coelenterate, which include jellyfish, sea anemones, corals and hydroids and porifera, which include sponges. Coelenterate have neurosecretions as primary hormonal co-ordinators but endocrine glands are absent. There is little information on the hormone function in sponges. Vertebrate type steroids and steroid metabolising enzymes have been detected in corals and the release of E2 during spawning has risen to speculation that E2 may have a functional role in regulating the reproductive process (Tarrant, 2005).

Neurosecretory cells are found in the group acoelomata (which include flatworms, tape worms and flukes) and the group aschelminthes (which include rotifers and nematodes). Neurosecretions are released and reach tissues by diffusion as these organisms have no circulatory system, these neurosecretions are possibly steroids but the function is not understood (Pinder *et al.* 1999).

Neuropeptides have been detected in the group annelida (which include segmented worms) but the chemical identity of any hormones has not been established. They possess morphallactic hormones that are involved in the acceleration of growth and re-generation. Ecdosteroids have also been detected (Barker and Rees, 1990). The group protochardata contain neurohormonal peptides, but little is known about their roles (Chang, 1995). Levels of progesterone and testosterone are found in both sexes in the group branchiostoma, while E2 and E1 have been found in elevated levels in females (Chang, 1995).

1.6.2. Organisms with true tissues

There are two classes of arthropods, insects and crustaceans. There is no evidence for the active synthesis of vertebrate like steroids in either insects or crustacea. Vertebrate type hormones and steroids have been found in homogenated insect tissue, for example E2, testosterone, cortisol and progesterone but this may be due to the ingestion of steroid containing food rather than by any biosynthesis of vertebrate steroids from cholesterol (Pinder *et al.* 1999).

The insect endocrine system is complex. Insects use peptide hormones plus juvenile hormone (JH) which are homosesquiterpenoids epoxides and ecdysteroids. The juvenile hormone acts via intracellular receptors in a similar way to steroid hormones but the mechanism is not fully understood (Jones, 1995; Riddiford, 1996). Specialised endocrine glands synthesise and secrete these hormones into the haemolymph and they have vital roles in metamorphosis modulation, including the synthesis of larval and adult proteins and in moulting (Pinder *et al*. 1999).

Crustaceans also utilise peptide hormones and ecdysteroids which are transported unbound in haemolymph and exert their effects by binding to intracellular receptor proteins in target tissues (Pinder *et al.* 1999). Neuropeptides produce substances that are involved in the ionoregulatory processes that inhibit or promote the uptake of water within their bodies. Crustacea also control pigmentation utilising biogenic amines and chromatotrophic hormones controlled by neurotransmitters (Pinder *et al.* 1999) and methyl farnesoate which acts as a hormone controlling their reproductive processes (Laufer *et al*. 1993; Chandra, 2007).

Anthropoids: mollusca are a diverse group that show a wide range of variation in form and habitat and a wide range of reproductive mechanisms. The endocrine system of molluscs is well studied. They utilise a range of peptide hormones but no ecdysteroids have been identified that play any significant roles. Vertebrate type steroid hormones such as progesterone, androgens and estrogens have been reported in three classes, cephalopods, bivalves and gastropods but no functional roles have been determined and it has been suggested that progesterone may be a precursor to other more sex specific steroids (Pinder *et al*. 1999).

Endoderms: These organisms utilise peptide signalling and the purine methyladenine has been identified as being important in the later stages of oocyte development. The presence of vertebrate type steroids has been detected in echinoderms and there is strong evidence that these steroids are synthesised and play a functional role in the control of growth and reproduction (Pinder *et al.* 1999).

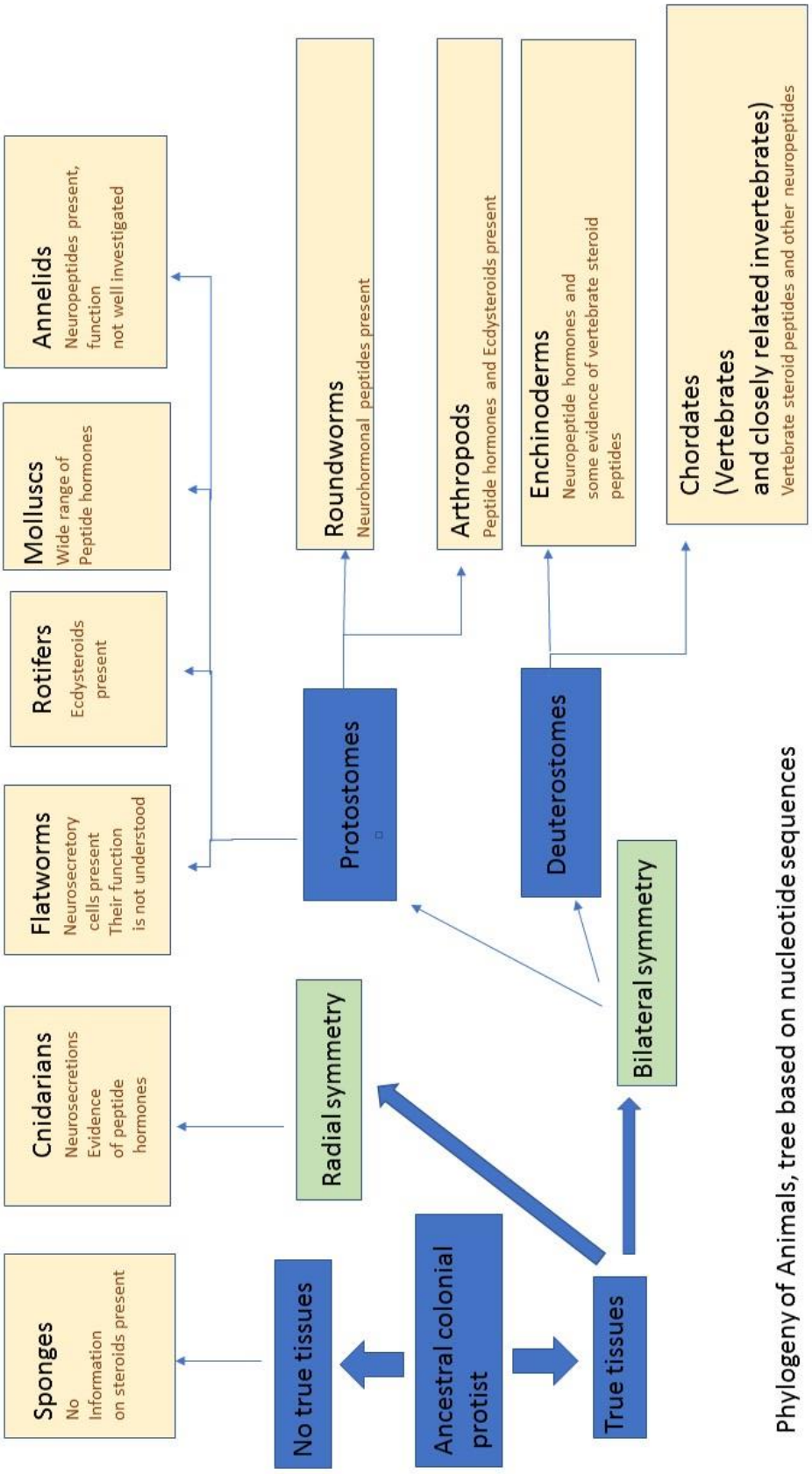


Figure 1.11 Animal and hormone diversity throughout the phyla

1.7. Cytochrome P450 enzymes

The cytochrome P450 enzymes were one of the first modifying enzymes of sterol biosynthesis and most likely have an early eukaryote or bacterial ancestor. Subsequent sterol evolution in microorganisms followed by multicellular organisms has led to varied end products in some organisms and the loss of sterol biosynthesis in others where sterols could be obtained through dietary means (Alberts *et al.* 2002; Kelly *et al*. 2003). Cytochrome P450 enzymes are the largest family of all enzymes. There are at least 481 genes and 22 pseudo genes representing 74 families (Nelson, 1998; Nelson *et al.* 2013).

1.8. Evolution of vertebrate steroid receptors and actions on invertebrates

Estrogen regulation is the most ancient of all modes of steroid receptor control. Based on phylogenetic analysis of steroid receptors of basal vertebrates, the first steroid receptor was an estrogen receptor (Thornton, 2001). These steroid receptors are an ancient class of transcription factors with multiple roles in vertebrates and invertebrates. E2 signalling is thought to have major roles in mollusc physiology but the function of the ER receptors is not understood. The molecular evolution of estrogen receptors (ERs) in vertebrates are known to have multiple ER and estrogen related receptors (ERRs) which are widely distributed in tissue types and are found in diverse species (Nagasawa *et al.* 2015).

The regulation of physiological processes by androgens and corticoids are relatively recent and emerged when vertebrates evolved from non-jawed to jawed vertebrates (Baker, 2004). The advent of corticoid signalling, adding 21 and 18 hydroxylation enzyme pathways allowed the creation of other steroid hormones (Baker, 2004). All of the six steroid receptors in vertebrates are created from a common ancestral gene (Baker, 2004). There have been many studies from several decades ago that show that exposure to vertebrate steroids have various effects on invertebrates (Thornton, 2001).

For example, tissue extracts from echinoderms injected into female rats that have had their ovaries removed, produced epithelial growth suggesting mitogenic estrogens in the extracts. Estrogen and progesterone like compounds were found in the ovaries of *Pisaster ochraceus* and *Strongylocentrus franciscenus* (Hagerman *et al*. 1956; Boticelli *et al.* 1961). Levels of progesterone and estrone in the female *Asterias rubens* were found throughout the annual gametogenetic cycle, indicating an antagonistic relationship in the regulation of vitellogenesis (Voogt and Dieleman, 1984).

It was found that some physiological functions of sex steroids in echinoderms were similar to those of vertebrates (Hines *et al.* 1992a). The presence of sterols, progesterone and E2 in male

and female gonads and pyloric caeca of asteroids were found during the gametogenic cycle (Hines *et al*. 1992a; Hines *et al*. 1992b). Treatment of male lampreys (*Lampetra fluviatilis*) with E2 increased the female gonadal development measurement from 7.8% to 27.7% at the end of their reproductive period (Mewes, *et al.* 2002). The presence of a putative ER in the testis of lampreys, suggests a functional role of oestrogen in testicular regulation (Ho *et al.*1987).

Single ERs have currently been identified in a wide variety of invertebrates, for example in the nematode, *Caenorhabditis elegans* (Mimoto *et al.* 2007); annelids and molluscs (Eick and Thornton, 2011); the fresh water snail *Marisa cornuarietis* (Bannister *et al.* 2007) and *Nereis virens* and *Nereis succinea*. (Nagahama, 1987; Garcia-Alonso and Rebscher, 2005).

A precursor molecule of steroids was reported to be found in *Nereis succinea* with high concentrations of E2 (30 pg/ mL) found in juvenile *Nereis virens* compared to mature males and females. This suggests that the steroid hormone is synthesised at the beginning of vitello genesis and has a role in the hormonal control of vitello genesis in polychaetes. (Nagahama, 1987).

E2 is described as a vitellogenic hormone in invertebrates (Li *et al*. 1998; Varaksina and Varkasin, 1988; Di Cosmo *et al.* 2001; Cosmo *et al.* 2002). A putative ER protein was found in young female *Nereis virens* at the onset of vitello genesis and sites of steroid synthesis were detected in the gut epithelium and eleocytes of *Nereis virens*. Also, the steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD) was found in gut epithelium. This suggests that E2 plays a role in the early stages of oogenesis by activating the expression of vitello genesis in the eleocytes (Garcia-Alonso and Rebscher, 2005).

1.9. Biotransformation

Biotransformation protects the organism by reducing toxicity of xenobiotics but in some exceptions the xenobiotics may be converted to more reactive metabolites. The release of these metabolites from storage may give delayed toxic effects in the long term (Walker *et al.* 2012). These metabolites may have a short half-life but can still cause molecular or cellular damage by binding to DNA. Protective responses include removal of xenobiotics by metabolising them, binding them to proteins and then excreting or storing them and by repairing DNA damage by the release of stress proteins. Exposure to pollutants can cause a change in levels of enzymes, receptors and reactive intermediates and give rise to changes in DNA (Walker *et al.* 2012). These changes may not be toxic but are not beneficial to the organism and these changes carry energy costs (Walker *et al.* 2012). The changes may be specific or non-specific.

Biotransformation has two phases. The first phase, which may have multiple steps, coverts xenobiotics to more polar, hydrophilic, hydroxyl containing metabolites by oxidation, hydrolysis, hydration or reduction (Walker *et al.* 2012). The second, enzyme driven phase involves conjugation

reactions that increases the metabolite's polarity and water solubility which enables them to be passed through membranes and excreted in blood and bile through the liver or hepatopancreas into the digestive tract where they are eventually passed in urine and faeces (Walker *et al.* 2012).

There may be different routes of uptake of xenobiotics, some being more dominant than others and the degree of toxic effect may differ by each route. In vertebrates, the uptake may be orally through the mouth and gut or topically through the skin. For aquatic invertebrates, major uptakes may be through passive diffusion across respiratory surfaces such as gills and into the alimentary tract by ingestion of pollutants suspended in ambient water. For aquatic organisms, uptake from water and food may be by continuous exposure or as a single dose (Walker *et al.* 2012).

1.10. Biomarkers

Exposure to xenobiotics may be detected by selected biomarkers, such as the elevation in levels of enzymes or other proteins that respond to oxidative stress (WHO, 1993). A biomarker is defined as any biological response to a chemical at an individual level demonstrating a departure from normal status (WHO, 1993). These responses may be biochemical, physiological, histological, morphological and behavioural measurements and by mutations in DNA or changes in gene expression (WHO 1993; Strimbu and Strimbu, 2010).

One biomarker is the presence of vitellogenin, (VTG), a female specific protein involved in the production of egg yolk and in controlling the growth of unfertilised reproductive cells (oocytes) (Marin and Matozzo, 2004; Rotchell and Ostrander, 2013). Synthesis of VTG is triggered by gonadotropin hormones in female vertebrates. In vertebrates, the level of VTG produced is monitored by estrogen levels in the liver. In male fish, exposure to estrogens causes an increase in VTG in blood plasma and gene expression of the female gene involved in VTG production (Sumpter and Jobling, 1995). Therefore, the presence of VTG in male vertebrates is a good indicator for exposure to estrogens and other endocrine disruptors that mimic those estrogens (Barucca *et al*. 2006). In a field study by Gagne *et al.* in 2011, VTG protein levels increased in both haemolymph and gonad tissue in both male and female mussels (*M. edulis*) when exposed to municipal effluents of varying dilutions, the VTG response in females being higher than the males. This suggested that the females are more sensitive to estrogens than the males (WHO. Global Assessment of the State-of-the-Science of Endocrine Disruptors 2002).

Cytochrome P450 enzymes are responsible for the metabolism of many endogenous and exogenous substrates. They are found in a large range of organisms from bacteria, insects, and crustacea as well as in vertebrates. The P450 genes are assigned to families and sub families based on their amino acid sequence (Werck-Reichhart and Feyereisen, 2000).

The activity of these enzymes has been demonstrated in numerous marine invertebrates of the phyla Cnidaria, Annelida, Mollusca, Anthropoda and Echinodermata but the understanding of their function in invertebrates is poor. Total P450 protein and enzymatic activities are usually measured in levels ten times lower than in mammals (Synder, 1999). The distribution of P450 enzymes are highest in the organ and tissues involved in digestion and food processing.

The levels of P450 are highest in the digestive gland of molluscs and the enzymes are also found in their haemolymph, gills, foot and gonads (Synder, 1999). They are also found in the heptapancreas of crustaceans and pyloric caeca of echinoderms (Solé and Livingstone, 2005). They possibly play a pivotal role in the biosynthesis of signal molecules, including steroids such as 20 hydroxyecdysone (Masatoshi and Kataoka, 2012). The metabolism of many compounds mediated by P450 enzymes, P450 gene expression and P450 enzyme activity may play a role in the biotransformation of xenobiotics, metabolising a wide range of endogenous compounds such as fatty acids, steroids, eicosanoids and xenobiotics such as plant chemicals (Snyder, 1998a; 1998b).

The CyP4 gene is one of the oldest of the P450 families and probably evolved over 1.25 million years ago, soon after the formation of steroid biosynthetic genes and is related to other cholesterol metabolising enzymes and both may have been involved in maintaining membrane activity in eukaryotes, allowing the transfer of lipophilic compounds across the membrane (Simpson, 1997). This ancient gene may have later evolved for the modulation of other important functions such as sterol synthesis.

P450 enzymes are conserved between species and sometimes phyla to preserve important biosynthetic pathways (Werck-Reichhart and Feyereisen, 2000). They can be selectively expressed in specific tissues or through different levels in various tissues.

1.11. Bivalves, history and *Mytilus* anatomy

Bivalves are a member of the mollusca phylum which is classified into six classes. These are: monoplacophora, which are the ancestral form of molluscs from which all modern-day molluscs are derived; gastropoda, which include spiral shelled molluscs such as whelks; cephalopoda, which include soft bodied molluscs such as the octopus; polyplacophora which include chitons; scaphopoda, which include tusk shells and finally bivalvia, which include mussels, clams and scallops (Gosling, 2003). Bivalves are forms of mollusc where there are two hard shelled valves joined by a dorsal hinge that fully encloses the internal soft body parts.

Bivalves have been on the earth in the present form from the mesozoic period (Gosling, 2003). They originated in warm, shallow coastal waters and gradually invaded estuaries and brackish water systems. They are a large and diverse species and are found in abundance

throughout the world and are an important source of food and they are economically important in aquaculture and fisheries industry (Dame, 1996; Gosling, 2003). They are adaptable to changing temperatures and salinity which is variable due to wave action in intertidal zones, preferring a salinity of about 35% and a temperature range of between -3° C and 44° C (Dame, 1996). Their protective shells are a major attribute to survival, closing firmly when the tide recedes. They have a planktonic larval stage found dispersed in the oceans and the adult form are semi-sessile, benthic, burrowing in sediments or anchored to rocks in dense communities (Gosling, 2003). They are filter feeders, removing phytoplankton from tidal waters by means of ciliary action. Although bivalves have diverse forms and habits, they share a similar body plan (Figure 1.12; Figure 1.13; Figure 1.14).

Figure 1.12 *M. edulis* external view

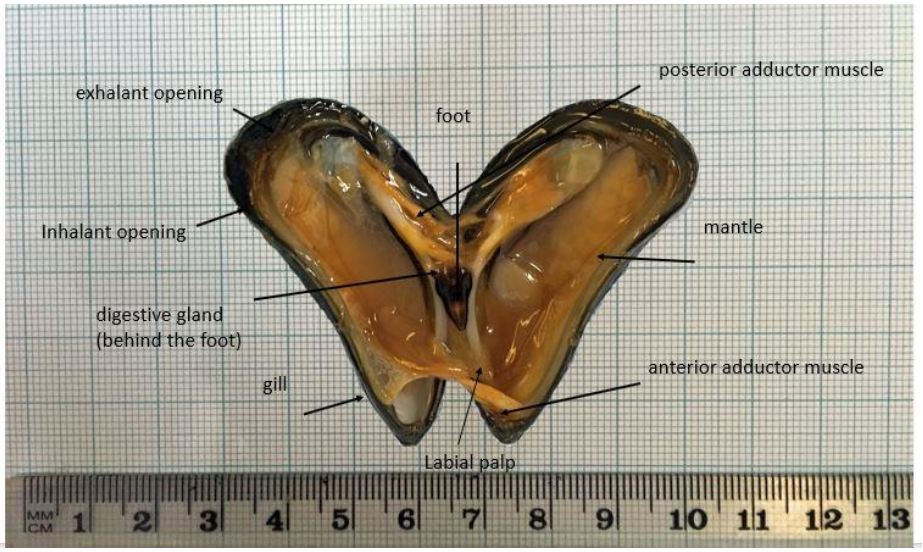


Figure 1.13 *Mytilus* anatomy

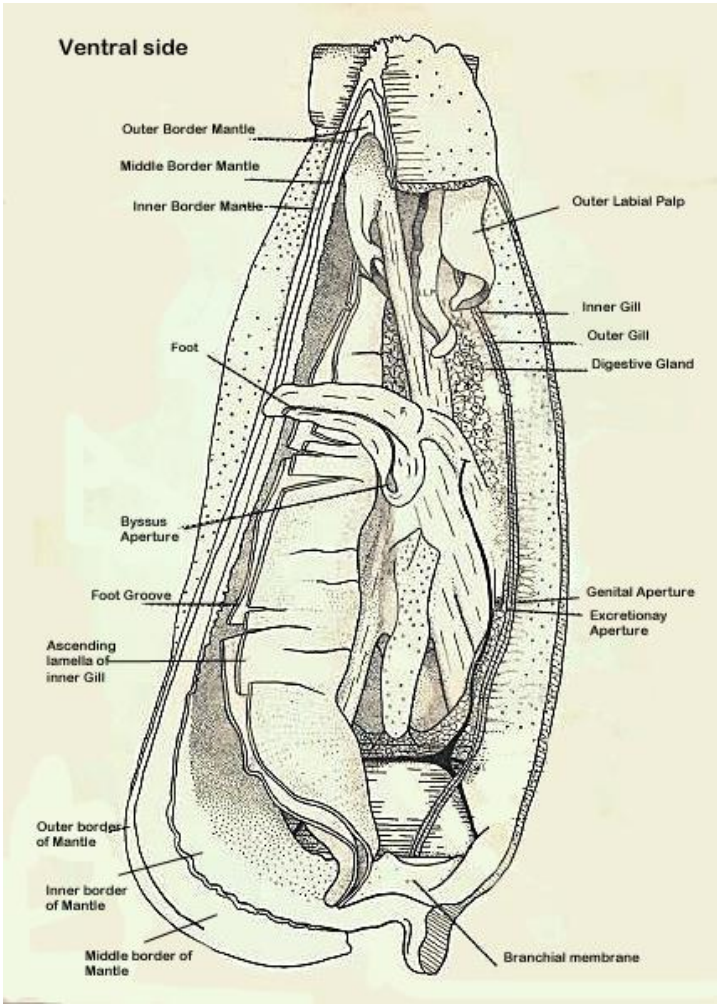


Figure 1.14 Ventral dissection of *M. edulis* (White 1937)

Mussels are bivalves of the *Mytilus* species. Their shell consists of three layers of hard shell consisting of an outer protective layer made from calcium carbonate and a matrix of proteins called conchiolin (Gosling, 2003; White, 1937). A middle layer made of calcite and an inner smooth calcareous layer. They are two shelled valves of similar size, hinged at the anterior side by a ligament. Inside the shell, two retractor muscles, a larger posterior adductor muscle and a smaller anterior adductor muscle control the opening and closing of the shell and control movement of a muscular foot which is used for locomotion. This foot secretes a proteinous substance called byssus, which anchors the mussel to rocks or other mussels (White, 1937). Their shell colour can vary between species, being genetically determined and their shape and size is variable depending on the density of the mussel community. *M. edulis* has a shell length of between 100 to 130 mm in length in optimal conditions but can be as small as 20 to 30 mm in length in less favourable conditions (White, 1937; Gosling, 2003). Inside the outer shells there is a mantle which is comprised of two lobes of soft tissue (White, 1937).

1.12. Bivalve feeding processes

Mytilus are suspension feeders, consuming diatoms, dinoflagellates, bacteria, proto-zooplankton, general detritus and dissolved organic matter, the particulate size being less than 110 microns (Newall *et al* 1989; Jorgensen, 1996; Davenport *et al* 2000). *Mytilus* species are lamellibranch molluscs belonging to the order Filibranchia (White, 1937). The group of Filibranchia have specialised gill structures that play an important part in food collection. The gills are elongated into filaments composed of ascending and descending regions whose tips remain in contact with one another medially and with the mantle laterally (Figure 1.15) (Newall, 1979). There are well developed sorting mechanisms on both the gills and labial palps retaining particles greater than 3 to 6 microns and these particles are swept across pass to the mouth across ridges in the labial palps. The inner surfaces of the palps are ridged with three types of ciliary tracts (Newall, 1979).

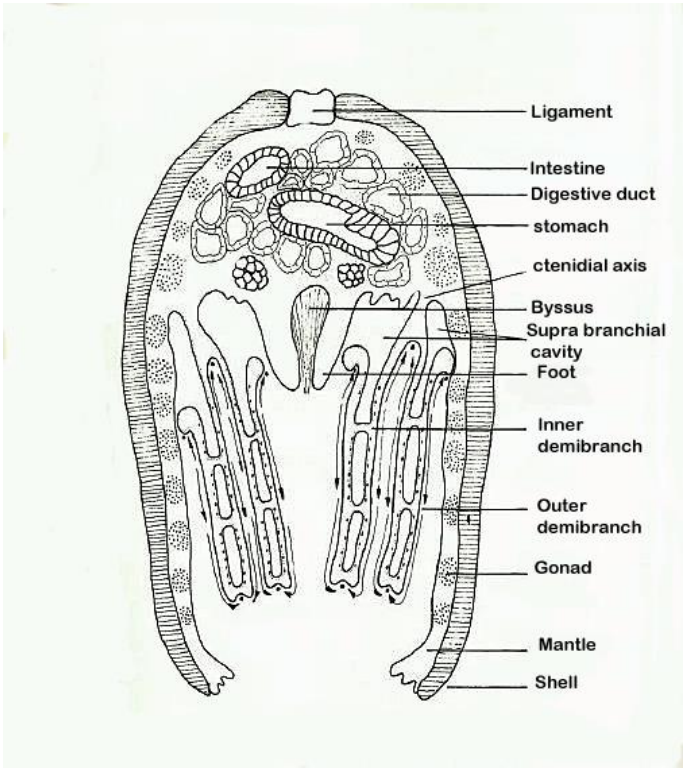


Figure 1.15 Diagrammatic transverse section through *M. edulis* to show the form of the gills and the direction of the main ciliary currents, represented as arrows (Bayne, 1976)

The mantle contains the gonads and is the site of gametogenesis. It is also the main site for storage of nutrient reserves, especially glycogen. There is a cavity between the mantle and the internal organs which consist of connective tissue with haemolymph or blood vessels, nerves and muscles. The inner surface of the mantle contains specialised feather like cilia which play an important role in directing food particles into the gills and deflecting unwanted heavier particles

towards the inhalant opening where they are rejected by a sudden, forceful closing of the shell valves (White, 1937; Gosling, 2003).

The mantle margins consist of three folds of which the outer fold is responsible for shell secretion, the middle fold for sensory function and the inner fold is responsible for controlling water flow through the mantle cavity. The exhalant opening consists of a siphon which is a small, smooth conical structure with a wider aperture fringed by sensory papillae. Muscles in the inner mantle fold control siphon retraction and siphon extension is controlled by haemolymph and water pressure in the mantle cavity (White, 1937; Gosling, 2003).

There are different sets of cilia within the *Mytilus* body. Some draw water into the mantle cavity which pass through gill filaments, the gills having both an important role in respiration and feeding. Gills have a large surface area, rich in haemolymph and dissolved oxygen is taken up by gas exchange. The heart lies in the mid dorsal region, close to the hinge of the shell and consists of a single muscular ventricle with two thin walled auricles. There is a well-developed circulatory pathway through the mantle where the haemolymph has many roles, including gas exchange, osmoregulation, nutrient distribution, waste elimination and provides organ rigidity (White, 1937; Gosling, 2003).

Haemolymph circulates through hollow filaments, gas exchange taking place across the thin filament walls. Each gill filament has a branch of vein that carries deoxygenated haemolymph from the kidneys to the gills. Oxygenated haemolymph from each filament gill vein travels to the kidney and the heart. Movement of the cilia is under nervous control; each gill axis being supplied with branchial nerves from the visceral ganglion. As the alimentary canal lacks a muscular wall, food material is propelled by ciliary motion and directed to the stomach which consists of an oval shaped organ embedded in the digestive gland (White, 1937; Gosling, 2003).

The digestive gland is the major site of intracellular digestion and consists of two types of cells, digestive and secretary. In a two-way process, material from the stomach is digested and absorbed and waste materials are passed as faecal pellets through the exhalant opening to the anus. The digestive gland is also an important area of storage of metabolic reserves and is an important source of energy during gametogenesis and in periods of stress (White, 1937; Gosling, 2003).

Bivalves have no distinct head but instead have sense organs distributed at the edges of the mantle. Stimulus of these nerves produces a reflex action that contracts the adductor muscles to close the valves. The cerebral ganglia consist of different types of neurosecretory cells which produce peptides that are released into the circulatory system. Some of these peptides have important roles in the regulation of metabolism, growth regulation and gametogenesis (Gosling, 2003).

These are: -

- Ventrally beating rejection tracts in the base of the grooves into which dense particles fall.
- Acceptance tracts occurring on the ridges, which beat across the direction of the folds that sweep particles into the mouth.
- Resorting tracts which redistribute particles which fall into the groves (Newall, 1979).

Particles are selected and retained on a quantitative rather than a qualitative basis, filtration efficiency approaching up to 100% for particles of a diameter greater than 6 microns, the retention declining for particles less than 4 microns (Dral, 1967; Newall, 1979). Material of a non-nutritive value, such as silt, is removed without ingestion (Davenport *et al.* 2000). Water pumping and filtration are autonomous processes and are not subject to physiological regulation, the mechanisms being of a fluid mechanical nature (Jorgensen, 1996).

Generally, the filtration rate and ingestion rate are linked to food concentration in a manner that the amount of food ingested remains constant over a wide range of particle concentrations. The filtration rate also depends on body size due to differences in metabolic rates, the smaller animals having a greater rate than larger animals. The rate of ingestion increases with an increasing concentration of suspended material until the ingestion system is saturated and the excess material is ejected as pseudo faeces. Higher concentrations of suspended particles elicit the secretion of mucus that may play an important role in the normal feeding process and the binding and conversion of unwanted particles as pseudo faeces, but its purpose is not fully understood (Atkins, 1936, 1937a, 1937b; White, 1937; Jorgensen, 1996; Newall, 1979).

The rate of ingestion is also regulated by the homogeneity of suspended food material, the rate of gravitational settling and the pumping or filtration rate. The optimum filtration rate is at a temperature between 5 and 15° C and is significantly depressed at 25° C (Widdows, 1978a).

1.13. Reproduction and Gametogenesis in Bivalves

Bivalves possess a pair of gonads, which are on either side of the mantle of the *Mytilus* species. They have the potential to develop as either females or males but they are physically indistinct. Their sex can be determined by histological investigation at various stages of development. They have an annual reproductive cycle which involves a period of gametogenesis, i.e. egg and sperm development, followed by a single extended or several spawning events followed by a period of reconstitution. The seasonal cycle consists of nutrient storage in summer,

gametogenesis in autumn and winter and spawning in spring. Frequency and seasonality of spawning varies according to the geographical distribution of populations. *Mytilus* species in northern areas around the British coast spawn once a year in late spring but those in the milder southern areas spawn twice a year in spring and late summer (Seed, 1976; Lowe *et al.* 1982).

The gonad has two groups of primordial germ cells that multiply to give either spermatogonia or oogonia cells. Multiplication of these cells allows gonad growth and restoration. There are many stages of gametogenesis, an undifferentiating stage, where the cells for both ovaries and testis are the same; an early differentiating stage; a growing stage; a mature stage; a spawning stage; a degenerating stage and a post spawning stage (Seed, 1976; Lowe *et al.* 1982; Barnabe, 1994; Gosling, 2003).

Cytology is used as a descriptive tool for investigating developing gametes. There are various cell types that form mantle tissues. These are gametes, blood cells, adipogranular cells (AG) and vesicular connective tissue cells (VCT) plus a blood circulatory network consisting of vessels, ducts and sinuses. AG and VCT cells are comprised of intrafollicular connective tissue, with the gametes they have a seasonal occurrence. This gamete cycle and spawning activity is well documented (Lowe *et al.* 1982).

Energy sources for the gametogenesis cycle are stored in the adipogranular and vesicular connective tissue cells of the mantle, this is accompanied by the synthesis of glycogen and lipids. Glycogen is utilised in gametogenesis and is replaced in summer when food is more abundant (Fearman *et al.* 2009).

There are no apparent external signs of sexual dimorphism in *Mytilus*. During the breeding season there is an internal visually determinant feature in the colour of the gonads, females tend to be a pink, orange colour whereas the male gonads tend to be creamy white in colour. There are various methods to establish macroscopic development of the gonads. The gonad somatic index (GSI) (Gosling, 2003) is one example, where the weight of gonad is expressed as a percentage of soft tissue, as dry weights.

Histological preparations of gonad tissue are the best determinant of sex during the breeding season when genital tissue is found in the whole of the body tissues with the exception of the pericardium, foot and gills. *Mytilus* are sexually mature at about 6 to 7 mm in length, however sexual maturity is a function of age and not size (Seed, 1969).

Based on qualitative morphological characteristics these stages can be classified into four main stages A to D, stages B (Developing) and D (Spawning) are split into a further 4 sub divisions (Seed, 1969) (Figure 1.16; Table 1.2).

A Resting spent gonad July/August	
Stage 0	Inactive. This stage includes animals that have completed spawning and recently mature animals that have never spawned.
B Developing gonad September/October	
stage 1	Gametogenesis begins with no ripe growth is visible. The volume of AG cells is about 50 to 60%.
Stage 2	Ripe gametes first appear. The gonad is one third of its eventual final size. The AG volume is unchanged but there is a measurable quantity of developing gametes present but the sex is not yet apparent. The gonad is larger and thicker and more coloured. There is less free water and the follicles are larger and closer together. There is little connective tissue. In the males, spermatozoa are arranged radially in the follicles; half grown oocytes are attached to the follicle wall in the lumen of the females.
Stage 3	There is a general increase in gonad mass to half the fully ripe condition. The area is ripe and developing gametes are equal. The AG volume fraction decreases and the developing gamete fraction (GVF) increases. The gonad is larger and thicker and contains very little free water. The follicles are becoming packed together. The lumina is becoming packed with either spermatozoa or fully-grown oocytes. The connective tissue is reduced.
Stage 4	The gonad is two thirds or more to the final size. Gametogenesis is progressing and the follicles contain mainly ripe gametes
C Ripe Gonads February/March	
Stage 5	The gametes are fully ripe. The gonad has gained its maximum size and contains no free water. The follicles are closely packed and coloured, the ovaries being orange to red in colour and the testis being cream coloured. The male follicle walls are extremely

	thin and the follicles are packed with is distended with morphologically ripe spermatozoa. The ova are compacted into polygonal or hexagonal configurations. The male gonad reduction in the developing GVF is reduced with minimal volume. The ripe GVT increases reaching 70 to 80% of the volume fraction prior to spawning.
D Spawning April/May	
Stage 4	There is active emission and a reduction in sperm density. The ova are rounded in shape due to the reduction in pressure of the follicles. The tissue is dull, containing free water.
Stage 3	The gonad is half empty. Following spawning the total GVT is zero with 70 to 80% of the volume being empty follicles.
Stage 2	The follicles are a third full of ripe gametes. There is a reduction of area occupied by the gonads. In some sections, ova or sperm may be seen in ciliated ducts and there may be zones containing unspawned follicles containing ripe germ cells at stage 4 of development.
Stage 1	This is the first recognisable stage after spawning. The gonad is growing containing a lot of free water. The follicles become larger and denser with connective tissue between them. The lumina are filling with growing oocytes. The genital ducts are losing their circular configuration. There are residual gametes. The volume diminishes post spawning due to tissue resorption.

Table 1.2 Stages of gametogenesis (Seed, 1969)

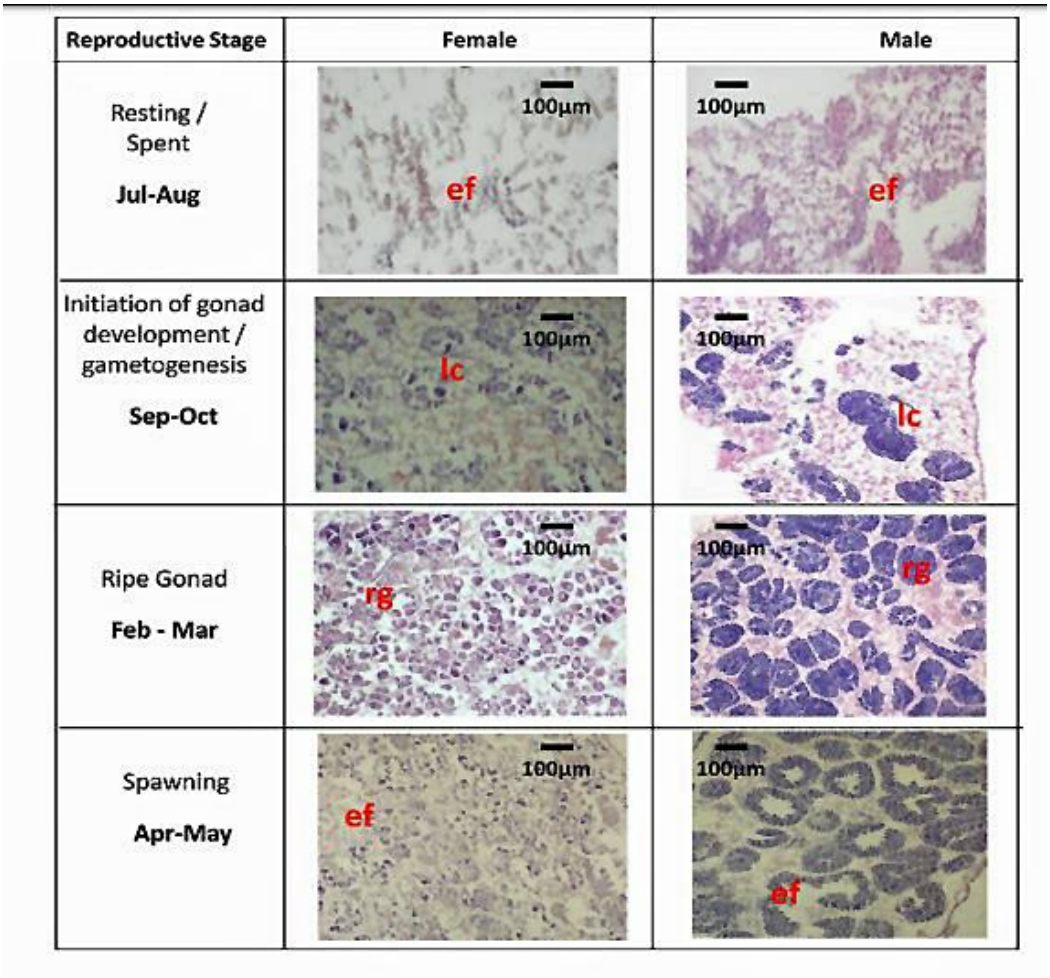


Figure 1.16 Representative images of the H&E-stained mantle at 20x magnification, from various stages of gametogenesis in mussels (Anantharaman and Craft, 2012).

Abbreviations ef - empty follicle or tubule; lc - Leydig cells; rg - ripe gametes

1.14. Factors regulating gametogenesis

There are both exogenous and endogenous factors than have an influence on reproduction. The most important exogenous factors are temperature, salinity, light quality and nutrition. One of the most important exogenous factors that can cause interruption or cessation of gametogenesis is temperature. The temperature range for optimum gametogenesis is species specific, for *M. edulis* this is in the range of 10 to 12° C. Lower temperatures increase mortality and higher temperatures cause anomalies in gonad development. The rate of gametogenesis is directly proportional to the rate of temperature change, in day degrees. Temperature influences the initiation of oocyte growth by regulating the transfer of nutrient reserves to the gonad. Gametes released into the water column by ciliary movement and contraction of the gonoducts are initiated by temperature (Seed, 1976; Barber *et al.* 2006).

Nutrition is important as the quality and abundance of food supply is an important factor in forming sufficient storage of energy reserves. There is little information on whether tidal motion due

to the lunar phases and changes in salinity effect gametogenesis. Physical stimulation can also stimulate spawning (Seed, 1976).

Once spawning has been initiated, the presence of gametes in the water provide chemical stimulus to other ripe individuals and therefore enhances the chances of fertilisation. Fertilisation is achieved by the simultaneous release of eggs as dense clouds and sperm which is broadcast as a steady stream through the exhalant (Helm *et al*. 2004).

Endogenous regulation of spawning is chemically regulated by environmental chemical cues and by internal chemical mediators. The chemical serotonin, 5-hydroxytriptamine (5-HT), acts as a major promotional factor in oocyte maturation, sperm motility and spawning the scallop, *Patinopecten yessoensis*, (Yuan *et al.* 2012). The reproductive cycle is controlled by interactions between the cerebral ganglion of the central nervous system, neurosecretory cells and steroids (Seed, 1976; Barnabe, 1994). The neurosecretory cells secrete neuropeptides that exert direct physiological effects on the gonad, stimulating mitosis. The peptide hormone, gonadotropin releasing hormone (GnRH) can be compared to the equivalent human follicle stimulating hormone (FSH) or luteinising hormone (LH) from the pituitary gland.

GnRH peptides have origins in ancient non-bilateral ancestors and the presence of GNRH like peptides have been found in bivalves and cephalopod molluscs. These molluscan GnRHs have a conserved structure found in other animals (Osada and Treen, 2013). Full cDNA sequences of the GnRH cloned from the scallop, (*Patinopecten yessoensis),* and the oyster, (*Crassostrea gigas)*, have been found to have a high similarity to GnRH of the octopus (*Octopus vulgaris*). A GnRH peptide of 10^{-5} M concentration extracted from octopus, (*Octopus vulgaris*), induced the release of LH from vertebrate pituitary cells confirming that this non-vertebrate GnRH peptide produced the same actions as a vertebrate GnRH peptide (Osada and Treen, 2013). This peptide also was found to be capable of inducing steroidogenesis of testosterone, progesterone and E2 (Kanda *et al.* 2006). It is suggested that GnRH is involved in spermatogonial proliferation by mediating gonadal steroidogenesis (Osada and Treen, 2013). There is a correlation between the neurosecretory cycle and the gamogenetic cycle. Secretory material is accumulated in the cerebral ganglia during gametogenesis and evacuated when the gametes are fully mature (Bayne *et al.*1976).

1.15. The effects of endocrine disruptors on invertebrates

The physiological and endocrine processes that are affected by endocrine disruptors are not as well understood as for many vertebrate species. The susceptibility of invertebrates to endocrine disruptors varies between taxa and their life stages.

There are varying degrees of understanding of the endocrine type processes and limited reports of steroid synthesis in the primary groups Coelenterata, Porifera, Acoelomata, Aschelminthes and Annelida (Pinder *et al*. 1999).

The marine anti fouling compound tributyl tin (TBT) is the most well-known endocrine disruptor in invertebrates. It is believed to inhibit the P450 dependent enzymes which converts testosterone to E2. Its most noted effect is the exhibition of imposex in molluscs which has been reported to occur in about 70 species (Pinder *et al*. 1999). For example, in the species *Nucella lapillus*, deformations of the female sex tract have resulted in infertility (Gibbs *et al*. 1988). However, there is no known apparent effect of TBT exposure in crustaceans.

Heavy metals such as cadmium and selenium interfere with the function of ecdysteroids in the crustacean reproductive system that have a major influence on the moulting cycle (Bodar *et al.* 1990). The synthetic estrogen, diethylstilboestrol, influences and reduces the moulting frequency in *Daphnia magna* but the mechanism is not understood (Pinder *et al.* 1999). The xenobiotic nonylphenol has shown to produce the accumulation of androgenic products in *Daphnia magna* resulting in external masculinisation and malformation of the carapace (Gerritsen *et al*.1998).

1.16. The influence of sex steroids on marine invertebrates

The presence of vertebrate sex steroids has been demonstrated in gonadal tissues of marine invertebrates including molluscs, crustaceans and echinoderms (Schoenmakers, 1979; Osada *et al*. 2004). It has been suggested that the estrogens detected in marine invertebrates are possibly synthesised by P450 mediated aromatase activity in estrogenic cells of the gonads (Osada *et al.* 2004). It is still to be established whether marine invertebrates synthesise sex steroids or obtain precursors from their diet. Classical biochemical approaches have established that marine invertebrates contain P450 enzymes that catalyse a variety of different reactions; however, there is no verification of any biosynthetic pathways or the identification of any specific P450 enzymes that may be involved (Santos *et al*. 2005).

Osada *et al.* (2004) proposed that VTG in bivalves is under the control of E2 at a transcriptional level and VPF from the central nervous system at a translational level and mRNA is stabilised by endogenous E2 resulting in retained high amounts of *VTG* mRNA at the spawning stage (Osada *et al.* 2004). This residual *VTG* mRNA is not translated into VTG protein. A clone containing partial cDNA *VTG* sequence was isolated from the cDNA of the Scallop (*Patinopecten yessensis*) to identify the *VTG* mRNA expressing cell and analyse the VTG regulation and expression of *VTG* mRNA (Osada *et al*. 2004).

This sequence exhibited a comparative match of 20-35% with the sequence from the *VTG* from the ovarian tissue of oviparous vertebrates. Using *in situ* hybridisation and cultured *VTG*

mRNA, the conclusion was that VTG was synthesised in the auxillary cells closely associated with growing oocytes, thus indicating that VTG is incorporated directly into the oocytes through a synthetic pathway without mediation through the blood circulatory system (Osada *et al.* 2004). Work on the *VTG* mRNA of the same species above of oysters and scallops found a sequence with a 35% match (Matsumoto *et al.* 1997).

1.17. Exposure of Estrogens to *Mytilus spp*.

It has been observed that estrogenic compounds are present in mollusc tissue (De Longcamp *et al.* 1974; Reis-Henriques *et al.* 1990; Hines *et al.* 1996; Zhu *et al.* 2003; Jobling *et al.* 2004; Ciocan *et al*. 2010; Zabrzańska *et al.* 2015) but the origin and their involvement, if any, in hormonal regulation is not understood as for vertebrates. Various experiments have been conducted to observe the effects of exposure of estrogens, E1, E2 and E3 on various species of mussels (Janer *et al*. 2005; Puinean *et al*. 2006; Ciocan *et al.* 2010; Zabrzańska *et al.* 2015). The results gave strong evidence that molluscs have the ability to absorb steroids from the water, even at low concentrations of 20 ng/L to 200 ng/L and conjugate them to fatty acid esters (Janer *et al*. 2005; Peck *et al.* 2007). In the case of E2 exposure, it was found that the concentration of conjugated E2 was highest in the gills, implying that the accumulation of E2 is exogenous in origin (Zabrzańska *et al.* 2015). There was more E2 in the testes of male molluscs than in the ovaries of female molluscs, but there was no disturbance in gametogenesis and in the sex ratio (Zabrzańska *et al.* 2015).

The uptake of E2 from water by mussels was studied by exposing them to tritiated E2 and the consequent radioactivity in their tissue was measured. This was found as estratrione 3, 17 β diol 3 sulphate and hydrolysis of the tissue released intact E2. No radioactivity was found in the shell or water within the bodies of the mussels. This gives strong evidence that mussels display an ability for rapid and high capacity uptake of the vertebrate steroid E2 from water (Schwarz *et al.* 2016).

Exposure of low concentrations of E2 in mussels over 10 days, led to a 15-fold concentration of esterified tissue but not in free E2. No change in the mRNA levels of *ER* or *VTG* genes were recorded, suggesting that the E2 is conjugated to esterified products by means of an unknown regulatory mechanism that maintains low levels of free E2 (Puinean *et al*. 2006). Levels of E2 in bivalves was shown to vary seasonally and was synchronised with variations of oocyte diameter and GSI and that E2 is involved in the regulation of reproductive processes such as vitello genesis (Puinean *et al.* 2006; Ciocan *et al.* 2010). However, this role has not been elucidated. The effects of low levels such as 50 ng/L of estrogen exposure over 10 days at different stages of gametogenesis in mussels was observed to determine if there was any effect on *VTG* and *ER* gene expression and it was found that there was a significant increase in *VTG* and *ER*

gene expression at the early stage of gametogenesis, but no change for mature mussels (Ciocan *et al.* 2010).This suggested that the reproductive physiology of molluscs is susceptible to damage by environmental estrogens at certain early stages of the gametogenesis process (Ciocan *et al.* 2010).

A similar exposure experiment showed that the levels of free E2 remained low but the level of total E2 sharply increased in a dose dependent manner (Janer *et al.* 2005). There was no change in testosterone levels, implying any testosterone synthesis pathways remain unchanged by exposure to E2 (Janer *et al.* 2005). The activity of enzymes 17β-HSD, P450 aromatase and estradiol sulphotransferases were investigated in microsomal digestive gland and cytosolic fractions and it was found that the activity of the P450 aromatase was depressed for low levels of E2 exposure but higher than normal activity for high levels of E2 exposure. Histology of gonad tissue showed that low levels of E2 doses induced gametogenesis (Janer *et al.* 2005). The authors suggested that low concentrations of E2 (20 ng/L) behave as an endogenous steroid in mussels and regulates some physiological functions, inhibit P450 aromatase activity and stimulate gamete maturation; whereas high concentrations of E2 significantly increases the activity of acyl transferases as a mechanism to inactivate excess free E2 and increases the synthesis of P450 aromatase activity and that the conjugation of E2 by fatty acid esterification plays a role in the above mechanisms (Janer *et al.* 2005).

In a study by Canesi *et al.* in 2007, different amounts of E2 (5, 25 and 100 pmol) were injected into the adductor muscle of *Mytilus galloprovincialis* and the tissues were examined 24 hours after the injection .The authors selected genes whose expression are modulated by estrogens in vertebrate systems to investigate if they are also possible targets for action by E2 in invertebrates. Further work suggested that there may be genomic and non-genomic modes of action involving ER like receptors and receptor independent mechanisms that participate in mediating the effects of E2 exposure (Canesi *et al*. 2007).

Results demonstrate that the digestive gland in mussels is a target of action for E2 and the hormone may modulate lysomal function as well as lipid and glucose metabolism in the digestive gland (Canesi *et al*. 2007). Exposure to E2 may also alter oxidative stress conditions in digestive tissue by transcription of genes that play a role in anti-oxidant defences (Canesi *et al*. 2007).

There is accumulated evidence that mollusc ERs are independently regulated transcription factors (Bridgham *et al.* 2014). There is evidence that one *ER* auto-regulates expression in gonadal cells in *Mytilus* species and this gene is responsive to E2 and other estrogenic compounds. An *ER* receptor and *ER* related receptor was found in *M. edulis* and *M. galloprovincialis* gonads by phylogenetic analysis of cDNA sequences. These sequences had a strong similarity to known *ER* sequences found in vertebrates. Gene expression analysis showed both receptor mRNAs localised in oocytes and follicle cells in the ovaries, testes and gills.

The presence of an *ER* and *ER* related receptors may explain why there are observed responses by *Mytilus spp*. when exposed to vertebrate steroids and endocrine disrupting compounds (Nagasawa *et al.* 2015). E2 exposure of *M. edulis* and *M. galloprovincialis* at early stages of gametogenesis showed signal increasing in *VTG* and *ER2* transcripts but not at mature stages (Puinean *et al.* 2006; Ciocan *et al.* 2010).

Although there is strong evidence that molluscs do have an ability to uptake estrogens into their tissue as conjugated esters (Janer *et al.* 2005; Schwarz *et al*. 2016), the fact that this may be an endogenous mechanism has been questioned by some researchers (Scott, 2012; Scott, 2013).

For instance, it is suggested that E2 triggers rapid lysomal membrane breakdown in haemocytes and that this is a typical inflammatory response and it is not proof that E2 is acting as a hormone in this case (Scott, 2013). Steroid hormones in vertebrates have been assayed by in a number of studies using immunoassay methods and these methods are prone to matrix effects and cross reactions, therefore over estimating levels of steroids extracted from water or tissue samples. Also, these assays do not characterise which steroids are present (Scott, 2012).

There is the possibility of steroids being taken up by steroids present in the environment by human contamination or by the presence of algae and bacteria in the water (Scott, 2012). Many of the studies investigate transformations in biosynthetic pathways that are known vertebrate steroids and these are mainly the steroids at the end of the pathways, such as estrogens and androgens rather than the precursors of the steroid pathway (Scott, 2012).

1.18. Pathway of estrogens in a UK water treatment sewage system

The most common treatment of waste sewage water in the UK was established from 1913 and involves a process of converting aqueous organic compounds into a biomass that can be separated from its aqueous phase by settlement. About 30,000 tons of waste water is treated per hour with a treatment time of 4 to 14 hours. This produces an effluent of a standard that meets a quality that protects the general aquatic environment but is not of a potable standard (Johnson and Sumpter, 2001).

The treatments consist of a primary stage where the waste is filtered to remove debris, macerated and degreased. Sedimentation may be assisted by the use of chemicals. The secondary stages consist of aeration by mechanical means using a trickling system, injection of oxygen and the use of biological degradation with adsorption onto bio-films, followed by a tertiary stage involving settlement in lagoons or irrigation over grasslands or constructed wetlands or root zone treatment. Extra treatments include physiochemical and biological methods and extra filtration including nitrifying filters and micro strainers (Khanal *et al.* 2006; Johnson *et al.* 2007).

It has been estimated that over 700 emerging pollutants, metabolites and transformation products are listed as present in the European aquatic environment, potentially contaminating the aquatic ecosystem (Lamastra *et al.* 2016). These new emerging pollutants are not dealt with, due to a lack of knowledge of their pathways, properties and limited detection due to limitations of analytical techniques (Lamastra *et al.* 2016). However, advances in analytical chemistry are now detecting these chemicals in water and sediments at low concentrations (Lamastra *et al.* 2016). These pollutants are present as a complex mixture of potentially toxic compounds which may be leading to synergistic and additive effects (Petri *et al.* 2015).

The European directive 2013/39/EU has introduced twelve new substances including diclofenac, E2 and EE2 as priority substances for monitoring. This selection is based on the exposure hazard, risk, lack of monitoring data at European levels (Lamastra *et al.* 2016).

The treatment of waste in waste water treatment plants (WWTP) was not originally designed for the elimination of xenobiotics and there are few studies on the removal of these emerging pollutants. (Lamastra *et al.* 2016). The WWTP is considered as the" gatekeeper", playing a major role in mediating the extent of EDC content, especially steroid removal efficiency, in receiving water systems (Johnson *et al.* 2007).

There have been some studies on the fate of estrogens in engineered waste treatment systems. Studies by Routledge in 1998 and Johnson in 2007 measured the efficiency of E2 removal and estimated that the eventual discharge in UK systems of E2 is 2 ng/L and E1 is 8 ng/L (Johnson *et al.* 2007). Using grab samples from WWTPs and analysis using GC-MS-MS or LC-MS-MS analysis, the efficiency of estrogen removal was calculated as 80-90% with discharge concentrations of 2.4 ng/L of E2 and 7.8 ng/L of E1, with more variation of E1 concentrations than E2 (Desbrow *et al.* 1998). The artificial steroid EE2 was also detected at low concentrations but is considered a greater threat to the environment due to its potency (Desbrow *et al.* 1998; Routledge *et al.* 1998; Williams *et al.* 2003; Johnson and Williams, 2004).

A study by Baronti *et al.* (2000) gives a limit of detection (LOD) of steroid estrogens as less than 1 ng/L using modern chemical analysis techniques with recovery rates of between 86 and 91% with 95% of E3 removal, 87% of E2 removal, 85% of EE2 removal and 61% of E1 removal. The median concentration of estrogens leaving the outlets is estimated at 0.3 to 1 ng/L of E2 and 0.45 ng/L of EE2 (Baronti *et al.* 2000; Johnson *et al.* 2000; Fawell *et al.* 2001; Hamid and Eskicioglu 2012). These low concentrations are still significant as 1-10 ng/L of E2 and 0.1 ng/L of EE2 have been found to provoke feminisation in fish, namely, rainbow trout (*O. mykiss*) and adult roach (*R. rutilus*) (Desbrow *et al.* 1998). It has been estimated that 10-100 µg of E2, EE2, E1 and E3 are excreted daily by menstrual woman. Pregnant women may be excreting up to 30 mg of E3 per day (Baronti *et al.* 2000).

The most dominant form of steroid estrogens are excreted as conjugated steroids in influent but detected by analytical methods as de-conjugated free estrogens (Andreolini *et al.* 1987; Routledge *et al.* 1998; Guengerich, 1998; Gomes *et al.* 2009). De-conjugation occurs as a biotic process, mediated by enzymes and occurs mainly in the secondary stage of waste treatment, free estrogen and bound estrogens passing through the primary stage of treatment. Steroid estrogens are not volatile, so the main removal processes are by biotic degradation by mainly aquatic bacteria, such as the faecal bacteria *E Coli*; photolysis and absorption as free estrogens as they readily partition from the aqueous phase into the solid sludge due to their hydrophobicity (Ternes *et al.* 1999; Khanal *et al.* 2006; Celiz *et al.* 2009). The de-conjugation of estrogens increases their total estrogenic potency (Khanal *et al.* 2006; Ternes *et al*. 1999). The presence of antibiotics in influent is most likely to have an antagonistic effect on biotic degradation (Khanal *et al*. 2006).

The order of biodegradation rate by biotic processes is: -

E2-GLU > E2 > E2-SUL >E1-GUL >> E1> E1-SUL (Khanal *et al.* 2006).

Sulphate conjugates are less likely to degrade as they are more resistant to enzyme cleavage, so as E1 is more likely to exist as a sulphate conjugate than other conjugates it makes it a more persistent steroid than E2 and E3 (Gomes *et al.* 2009). EE2 is excreted as the glucuronide conjugate and is a potent active estrogen found in the WWTP. It is highly stable and persistent and due to having poor aqueous solubility and is found as a precipitate in the activated sludge with no detectable degradation (Gomes *et al.* 2009).

Other processes have been introduced as part of the WWTP in order to remove xenobiotics. For example, granulated activated carbon (GAC) will readily absorb E2 but has a disadvantage in that the absorption process competes with other soluble organics and has a reduced efficiency in heavily contaminated waste (Khanal *et al.* 2006). Powdered activated charcoal (PAC) removes over 90% of E2 but requires a continuous supply of media and is therefore expensive and is usually only applied as a temporary or seasonal measure (Khanal *et al.* 2006). A nitrification stage as part of the secondary stage treatment will increase the rate of degradation significantly (Khanal *et al.* 2006). Other treatments such as chemical oxidation, ozone treatment and photo degradation, microfiltration, ultra-filtration using membrane bioreactors have proven effective by removing estrogen bound colloids but they are costly to implement (Johnson and Sumpter, 2001; Liu *et al.* 2015).

1.19. Estrogens and their interactions with sediments

A study by Garcia-Reyero *et al*. in 2005 suggested that dissolved estrogens discharged into the aquatic environment may rapidly become adsorbed, on contact, to suspended solids and that other hydrophobic chemicals such as PAHs will compete for those adsorption sites. Therefore, estrogens have a tendency to partition to organic carbon rich sediments and other organic matter. The adsorption, on contact to suspended solids was rapid and occurred within the first thirty minutes of contact. There is a correlation of adsorption of estrogens and carbon content of sediments, and there are differences in the amount of adsorption in clay mineralogy and with other sediment coating phases such as iron and manganese oxides (Lai *et al.* 2000, Langston *et al* 2005).

The adsorption of estrogens by sediment was also observed to increase with an increase in salinity. Therefore, estrogens are expected to exhibit greater removal from estuarine or marine environments than in freshwaters. Oxygenation is most critical factor influencing degradation of estrogens with low degradation rates in anoxic conditions. The adsorption onto sediments acts as a sink for estrogens and impedes their dilution and natural degradation (Lai *et al.* 2000, Langston *et al* 2005). Therefore, precipitated estrogens may be held in anaerobic sediment layers for a considerable time without the potential of degradation by oxidation or biological degradation.

In this study SECs were also selected and studied as a result of a parallel study at the University of Hull where they were being developed as a means to absorb or adsorb contaminants from the environment with a long-term aim of trying to mitigate sediment pollution problems and water clean-up.

1.20. Sporopollenin

Sporopollenin is the resistant non-soluble material that is left after acetolysis of spores of land plants, whose chemical nature depends on the source of the material (Mackenzie *et al.* 2015). The most common, commercially available material to produce sporopollenin are the spores of the *Lycopodium* species, usually *Lycopodium clavatum*, or club moss.

These spores are about 25 μm in diameter and are regular in size. The spores are the mobile reproductive particles of plants and consist of an outer membrane and inner contents which include fats, vitamins, proteins and carbohydrates. The layered wall has a robust outer layer, the exine, and an inner layer or intine which is composed of cellulose and polysaccharides (Figure 1.17).

The outer shell, or exine called sporopollenin is a resistant organic material whose structure is has been investigated by infra-red spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and the analysis of the by-products of chemical reactions (Barrier, 2008). There is a

large presence of aromatic and olefinic groups that contribute to the lipophilic character of the SECs. There are also more polar functional groups such as hydroxyl groups, carboxylic acids and phenols which imparts some amphiphilicity. These lipophilic and amphiphilic characters of SECs impart properties such that the sporopollenin exines can act as microcapsules capable of loading a range of materials with different polarities (Barrier *et al*., 2011; Mackenzie *et al.* 2015).

This natural organic material is highly resistant to strong acid and bases, physical, biological and oxidative aggressions and is insoluble in most common solvents, however sporopollenin can be degraded biologically by the action of plant and other biological enzymes (Barrier *et al*. 2011; Diego-Taboada *et al.* 2014; Punt *et al.* 1999; Barrier, 2008).

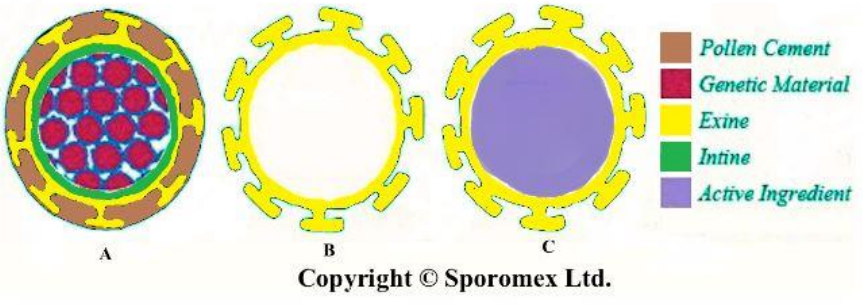


Figure 1.17 Sporopollenin structure, (Diego-Taboada *et al*. 2014).

Sporopollenin are consistent in size within the species and show high mono dispersity. The outer wall or exine is tunnelled with an array of nano sized channels. When the inner contents of the sporopollenin is extracted, the residue or shell left is called the exine. Extraction of the inner contents, or sporoplasm, is by several different methods, most involving hydrolysis with very strong acid. The exines are considered as empty, permeable closed capsules and because of the network of channels within the wall of the exine, liquids can enter passively by simple contact or driven inside by capillary action or other forced methods. This process is called encapsulation which can be rapid with small molecules such as alcohols or amino acids.

The extracted sporopollenin exines are called sporopollenin exine capsules (SECs). Compounds less than 1 KDa to 464 KDa have been successfully treated, including both polar and non-polar products. The loading capacity of exines can be high, especially with saturated solutions, due to their large inner cavity (Punt *et al.* 1999; Barrier, 2008; Barrier *et al*. 2011; Diego-Taboada *et al.* 2014).

Presently, microencapsulation is used as a tool in the food, cosmetic and pharmaceutical industry to deliver food supplements and drugs by ingestion or topically, especially for chemicals that are liable to oxidation. As sporopollenin is derived from plant tissue it is a renewable, bio compatible and biodegradable material that is plentiful in supply. SECs, float on water when empty and are barely wetted as they have hydrophobic properties but sink progressively as they absorb

water. They will readily soak up lipophilic compounds, especially when dissolved in alcohols or other organic solvents, producing a suspension that is highly mono dispersed (Punt *et al.* 1999; Barrier, 2008; Barrier *et al*. 2011; Diego-Taboada *et al.* 2014).

1.21. Literature review summary

Estrogens have been known to be hormone disruptors in aquatic vertebrates for several decades and *in vitro* and *in vivo* studies on aquatic invertebrates have demonstrated that estrogens also have effects on aquatic vertebrates but the mechanism of how they affect invertebrates is not understood. It is widely accepted that molluscs bioaccumulate estrogens and that E2 has been found at high levels in the digestive glands, gills and gonads of *Mytilus spp.* as conjugated E2 when exposed to low levels of E2 in the water. This may be explained that the organisms are removing free E2 from their systems by conjugating them prior to biotransformation and excretion.

However, several studies have found that exposure to E2 has had effects similar to the effects of exposure on vertebrates, namely vitellogenesis and has been seen in *Mytilus* male gonads. Gene expression for genes that are normally associated with vertebrate steroidogenesis have been in non-invertebrates such as lampreys (*Lampetra fluviatilis*), (Mewes *et al.,* 2002), sea stars (*Asterias vulgaris*), (Hines *et al.,* 1992) and starfish (*Asterias rubens*), (Schöenmakers and Dieleman, 1981). Also, the effects are mainly seen in the early stages of gametogenesis, whereas mature mussels are unaffected. If free E2, a vertebrate steroid, is affecting hormonal regulation in *Mytilus,* a species whose hormone regulation is normally regulated by neuropeptide hormones, the mechanism of how estrogens are interfering with this mechanism is not understood.

SECs have been demonstrated that they are excellent vehicles to absorb and adsorb small biomolecules, especially lipophilic molecules, from solutions. There is no published work on encapsulation of estrogens by SECs and the capacity of encapsulation. It is not known if there is total or partial encapsulation with a combination of adsorption on the outer exine wall or whether there is only adsorption on the outer exine wall when SECs are added to water.

There is no information on whether the adsorption or absorption of E2 on SECs from an aqueous environment is reversible. As E2 is lipophilic it has the tendency to readily precipitate out of water and stick to sediments, especially carbon rich sediments. When estrogens are trapped in sediment layers in anoxic conditions, the natural degradation by oxidation is compromised, a similar possibility may occur if SECs treated with E2 sink down and become embedded in sediment layers.

Although the SECs are hydrophobic and tend to float on water, they quickly sink when they absorb water or other molecules and this implies that estrogenic SECs would be in contact with

benthic species such as *Mytilus*, if introduced into the water system. There is no published research on the exposure of *Mytilus* to SECs and whether the SECs are transported to the digestive system or ejected by *Mytilus* as pseudo faeces or any published investigations as to whether there is any inflammatory reaction when passing through their gills. There is a possibility that conjugated estrogens as sulphates and glucuronides, when treated with SECs may be prevented from enzymatic hydrolysis or degradation into the more potent free estrogens.

Therefore, before SECs are to be used as remediation technology to remove estrogens from waste water sources, it important to understand if there would be a negative impact by the addition of SECs to the water environment. For example, if there was an increase in bioavailability of estrogens by the prevention of oxidation or degradation or by ingestion and enzymatic degradation of the exine layers of the SECs, thus releasing any absorbed estrogens.

1.22. Aims and objectives

The main aim of this work was to determine the uptake and biological impacts of estrogens in the mussel gonadal, digestive and gill tissues. A second aim was to determine whether estrogens bound to sporopollenin are bioavailable once experimentally exposed to mussels.

These aims were arranged into a series of work tasks set out below.

- To optimise an efficient and reliable extraction protocol to extract E2 from water samples and *M. edulis* gonad tissues.
- To optimise an efficient, reliable and sensitive analytical method, using either GC-MS; GC-MS-MS or LC MS-MS to quantify E2 in the above extracts, using a deuterated internal standard.
- To produce external and internal calibration curves, using the practice of standard addition using a series of diluted standards of E2 and the addition of deuterated internal standards in order to quantify any E2 found in the tissue and water extracts.
- To analyse, through histological techniques, the effects of *M. edulis* mantle, digestive tract and gill tissue when exposed to SECs and the passage, if any, of the SECs through the animals using in controlled laboratory experiments and the above extraction and analysis techniques.
- To establish whether *M. edulis* take up E2 at low exposure levels of 200 ng/L and whether this is conjugated as fatty acid esters and stored in their mantle tissue or is bioavailable as free E2.
- To establish whether the E2 adsorbed or absorbed in SECs at the same low concentrations is absorbed through the digestion tract and/ or gills and whether this is also conjugated as fatty acid esters and stored in their mantle tissue or is bio-available as free E2.
- Using PCR and qPCR methods of *Mytilus* estrogen receptor *2 MER* (Ciocan *et al.* 2010; Ciocan *et al.* 2011) gene sequences to investigate if there is any increase in gene expression in the E2 and SECs/E2 exposed *M. edulis* compared to the untreated controls

1.23. Hypothesis

- H1 1 E2 is absorbed by *M. edulis* and this free E2 is conjugated into fatty acid esters and stored mainly in mantle tissue.
- H1 0 E2 is absorbed by *M. edulis* and this free E2 is not conjugated into fatty acid esters and stored mainly in mantle tissue.
- H2 1 E2 in the water column exerts an estrogenic effect increases *MER* gene expression relative to the control group.
- H2 0 E2 has no effect upon *MER* expression in either of the exposure groups relative to the control group.
- H3 1 SECs are absorbed by *M. edulis* and the free E2 is bio available and thus conjugated as fatty acid esters.
- H3 2 SECs are absorbed by *M. edulis* but the free E2 is not bio-available and thus not conjugated into the fatty acid esters.
- H3 0 SECs are not absorbed by *M. edulis* and are passed through their gills and excreted as pseudo faeces

Chapter 2. Extraction of estrogens from water and tissue and development of analytical chemistry methods for their subsequent analysis

2.1. Extraction of E2 from water

There are established protocols available to extract estrogens from river and sea waters (Cunningham *et al*. 2014; Zhai and Fu 2016, Tavazzi *et al.* 2016). The predominant method is using the technique of solid phase extraction (SPE) which involves passing the water sample slowly, under a slight vacuum, through a plastic cartridge packed with a specialised coated silica (called the stationary phase) (Phenomenex, 2017). The coated silica adsorbs the estrogens in the water sample whilst allowing the water, dissolved salts and interferents to pass through. Before passing the water through the cartridge, the coated silica is pre-wetted or conditioned by passing methanol through the cartridge. The methanol is then washed out with water before the cartridge is "loaded" with the water sample. After the sample has passed through the cartridge, the cartridge is washed with water to remove any residual unwanted salts and dried by drawing air through the cartridge. The estrogens adhering to the stationary phase are then eluted by passing methanol through the cartridge at a slow rate, the eluent being collected and evaporated down to give a concentrated sample ready for analysis by LC-MS-MS. Several samples can be processed at the same time with the use of a manifold connected to a vacuum pump (Figure 2.1).

Figure 2.1 SPE manifold with SPE cartridges.

The protocol used to analyse the water samples from the exposure experiment was adapted from Cubero-Leon *et al.* (2012) with some minor modifications. All of the samples were extracted within 24 hours of collection having been stored in the dark at 5° C. The protocol is given in short as follows.

- The water sample was filtered through a 0.45 µm cellulose acetate filter and 250 mL of the sample was acidified with 500 µl of glacial acetic acid to give a 0.2% v/v acidified solution.
- A C18U 3 mL volume cartridge (Phenomenex) was conditioned with 2 x 3 mL of methanol and washed with 2 x 3 mL of acidified water (made by adding 2 mL of glacial acetic acid to one litre of milliQ water).
- All of the water sample was passed through the cartridge at a rate of about 1 mL a minute. The bottle containing the water sample was rinsed with 6 mL of acidified water and passed through the cartridge.
- The column was then washed with 2 x 3 mL of milliQ water and air dried for 20 minutes by drawing air through the cartridge under vacuum.
- About 0.5 g of anhydrous sodium sulphate was added to the top of the dried cartridge. The bottle was then rinsed with 6 mL of methanol and this was added to the cartridge and the methanol eluent was collected. Another 3 mL of methanol was added to the top of the cartridge and collected in a test tube to ensure all of the adsorbed estrogens were removed from the cartridge.
- The methanol was evaporated to about 1 mL in volume using a rotary evaporator and this concentrated extract was returned to the test tube and the contents evaporated to dryness under a gentle flow of nitrogen and stored at -20° C until analysis.

In order to produce a calibration curve for quantification purposes, the same sea water was used in the exposure experiment was filtered through 0.45 µm cellulose acetate membranes and 250 mL of this water was spiked with a range of E2 from 25 ng to 250 ng. These samples were then extracted using the above protocol and the dried extracts were stored at -20° C until derivatisation and analysis.

2.2. Extraction from mussel gonad tissue

2.2.1. Introduction

As estrogens are lipophilic and may be present as free and conjugated estrogens it is important to use a solvent or series of solvents that dissolve all of these steroids. The majority of extraction protocols involve the homogenisation of the tissue followed by extraction with methanol, dichloromethane or acetonitrile or a mixture of these solvents. A portion of the extract is hydrolysed in a strong potassium hydroxide / methanol solution to convert any conjugated steroids to the free

steroids. Other methods use an enzyme hydrolysis protocol in order to convert any conjugated steroids back to the free form.

2.2.2. Preparation, E2 extraction and hydrolysis steps prior to analysis

After extraction and hydrolysis, E2 extracts are passed through at least two types of SPE cartridges to remove unwanted artefacts that interfere with the analysis. These include a cartridge that uses an ion exchange mechanism to remove the steroids from solution, followed by a silica cartridge to remove unwanted lipids. The protocol of Cubero-Leon *et al.* (2012) uses gonad tissue that is homogenised in methanol that is ultrasonicated in 5 mL of dichloromethane, followed by 3 mL of dichloromethane, 1 mL of methanol and finally 500 µL of methanol.

The pooled solvents are combined, evaporated under a flow of nitrogen and re-suspended in 1.5 mL of methanol. This solution is split into two parts, 500 µL is used to analyse total estrogens and is hydrolysed by heating with 2.2 mL of water, 300 µL of 3 molar potassium hydroxide solution at 80° C for one hour. The cooled solution is then neutralised with 700 µL of 1 molar hydrochloric acid. Water is added to this solution so that the total volume of the extract is 7 mL and the methanol content is less than 10% v/v. Likewise, the other 1 mL of extract (free estrogen) is evaporated down to almost dryness under nitrogen and diluted down to 7 mL of water, ready for the SPE step.

2.2.3. SPE clean up steps

This involves a series of steps as follows.

- A 200 mg, 3 mL, Strata X-AW SPE cartridge (Phenonmenex) is conditioned with methanol and washed with 2 x 3 mL of 0.05 M sodium acetate buffer pH 7.0.
- The diluted extract is passed through the cartridge at a rate of about 1 mL/minute, washed with 2 x 3 mL of the above buffer, followed by 2 x 3 mL of milliQ water, dried and eluted with 6 mL of ethyl acetate.
- The sample is evaporated to dryness under nitrogen and re suspended in 50 µL of ethyl acetate and 950 µL of cyclohexane.
- A 500 g Sep Pak® silica cartridge (Waters Ltd., Elstree, UK) is conditioned with cyclohexane and the dissolved extract is passed through this cartridge and eluted with 6 mL of cyclohexane / ethyl acetate mixture 60-40 v/v.
- The collected extract is evaporated to dryness and stored at -20° C until analysis.

2.3. Alternative tissue extraction methods used for optimisation and comparison

An Agilent application method (Fu and Zhai, 2009) homogenises 200 mg of fish tissue in 5 mL of methanol and this mixture is ultrasonicated for 10 minutes on ice. The supernatant is removed, evaporated to dryness under nitrogen and reconstituted in 5 mL of 5% of aqueous methanol. This diluted extract passed through a conditioned SampliQ OPT SPE cartridge and eluted with 6 mL of methanol. The extract is evaporated to dryness and reconstituted in aqueous methanol for HPLC analysis.

A similar extraction method (Long *et al.* 2007) has been used to analyse steroids from fish and shellfish. In this method, 5 g of the tissue is homogenised in sodium acetate buffer with an internal standard added and this mixture is ultrasonicated in 10 mL of acetonitrile and the contents are then centrifuged. The pellet is extracted with a further 10 mL of acetonitrile. The supernatants are combined and extracted with 2 mL of hexane to remove unwanted dissolved fats and then evaporated to dryness on a rotary evaporator. The residue is dissolved in 2 mL of acetonitrile, filtered with a 0.45 µm filter and water is added so that the total volume is 10 mL. This extract is then passed through a C18 SPE cartridge as for the previous described protocols. This SPE step removes unwanted phospholipids and glycoproteins that are not eliminated by the previous hexane extraction. The acetonitrile is evaporated to dryness prior to analysis by GC-MS. In order to analyse total E2, the tissue is hydrolysed using an enzymatic hydrolysis protocol that cleaves the steroid gluronide / sulphate conjugates.

A method to extract steroids from meat tissue, (Blasco *et al.* 2007), uses enzymatic digestion, using a protease called subtilisin, followed by extraction with methanol. The extract was cleaned up by two SPE steps using C18 and NH2 columns.

Another validated method to extract pesticides from fruit and vegetables named QuEChERS (Anastassiades *et al.* 2003) has been adapted to extract similar lipophilic compounds from a variety of tissues. The QuEChERS method is a streamlined approach that makes it easier and less expensive for analytical chemists to examine pesticide residues in food. The name is a portmanteau word formed from "Quick, Easy, Cheap Effective, Rugged, and Safe" (Restek, 2007). This method involves adding acetonitrile to a homogenised mixture followed by a small amount of water and, in the case of lipid rich material, a small amount of hexane. A sachet of a mixture of salts are added to this mixture and the whole mixture is vigorously shaken for two minutes. The pesticides are hydrophobic, so readily partition out of the salt saturated solution into the upper acetonitrile layer and the unwanted lipids partition into the top hexane layer. Proteins are precipitated by the strong salt solution and remain in the bottom salt pellet. The acetonitrile layer is removed to a new tube and a 1 mL portion of this is added to a 2 mL tube containing a dispersive

reagent which is a mixture of resins and salts that remove unwanted residual artefacts from the acetonitrile. After vortexing and centrifugation the extract is ready for derivatisation and analysis.

2.3.1. Enzyme digestion to facilitate E2 extraction from tissue

The *Mytilus* tissue was pre-digested using the enzyme subtilisin (P 5380, Merck, Darmstadt, Germany) in the following procedure. A working solution was made by dissolving 5 mg of the enzyme in 50 mL of 10 mM sodium acetate plus 5 mM calcium acetate buffer, pH 7.5, to give sufficient enzyme for 50 samples (this solution keeps up to 2 days if refrigerated). To 250 mg of wet homogenised tissue in a 1.5 mL Eppendorf tube, 1 mL of the above solution was added and the mixture was incubated in a water bath at 37° C for 2 hours, with periodic vortexing. The digest was transferred to a 10 mL test tube and diluted to a total volume of 3 mL with 100 mM sodium acetate buffer pH. 4.5. A 1 mL portion of the digest was retained in the Eppendorf tube for further enzymatic hydrolysis using the enzyme β glucuronidase/arylsulfatase (10127060001 Merck, Darmstadt, Germany) to convert any conjugated E2 in the extract into free E2. The other 2 mL of the digest was transferred to a 50 mL centrifuge tube for QuEChERS extraction of free E2.

2.3.2. Enzyme hydrolysis to convert residual conjugated E2 back to the free E2 for total E2 estimation

To prepare a working solution of glucuronidase, 5 µL of the above concentrated solution was added to 3075 µL of 100 mM sodium acetate buffer pH 4.5, this was sufficient solution for 12 samples (aliquots of the diluted enzyme can be frozen at -20° C). Then 250 µL of the above working solution of β glucuronidase/arylsulfatase enzyme was added to the 1 mL digest remaining in the test tube and the mixture was vortexed and incubated in a water bath for 2 hours at 37° C. This digest was then transferred to a 50 mL centrifuge tube for E2 extraction. Both free and total extracts are treated using the same QuEChERS extraction protocol.

2.4. QuEChERS methods used for E2 extraction from tissue.

2.4.1. Introduction

As estrogens are also small lipophilic/hydrophobic molecules there is a possibility that a QuEChERS method would be a suitable replacement for the SPE steps used to extract estrogens from tissue. Acetonitrile is a very good solvent for estrogens as it readily precipitates proteins, has

a lower solubility of lipids and readily penetrates homogenised tissue. The addition of sodium acetate and acidification reduces analytical interferents (Long *et al*. 2007).

2.4.2. Published QuEChERS protocols

There is a published paper that uses the QuEChERS method to extract a variety of chemicals including pharmaceuticals and estrogens from worm tissue (Berge and Vulliet, 2015). They extracted a variety of steroids using a two-step QuEChERS method using 250 mg of homogenised worm tissue using the following protocol.

- 10 mL of acetonitrile, 6 mL of water and an internal standard is added to 250 mg of homogenised tissue and the mixture is vortexed for 20 seconds.
- 3 mL of hexane is added and the mixture is vortexed for 40 seconds.
- A sachet of acetate buffer salts is rapidly added (AOAC salts containing 6 g of anhydrous magnesium sulphate and 1.5 g of anhydrous sodium acetate), the tube is manually shaken for 20 seconds followed by mechanically shaking at 1250 rpm for 2 minutes.
- The mixture is centrifuged for 2 minutes at 5000 rpm and 6 mL of the acetonitrile layer is transferred to a second 12 mL tube containing a dispersive absorbent (950 mg anhydrous magnesium sulphate, 150 mg of PSA and 150 mg of C18). This dispersive solid phase extraction powder (dSPE) removes residual interferents such as pigments that are co-extracted into the acetonitrile phase.
- The mixture is manually shaken for 20 seconds and vortexed for 40 seconds and centrifuged for 2 minutes at 5000 rpm for 2 minutes.
- 4 mL of the supernatant are removed and evaporated to dryness and stored at -20° C until analysis.

There is a published application paper (Syljohn *et al.,* 2016; Phenomenex, TN-0096) that uses a QuEChERS technique to extract steroid hormones from sediments. This is similar to the above method, but uses acetonitrile acidified with 1% glacial acetic acid and freezes the acetonitrile/water/salt extract after centrifugation to enable an easier removal of the supernatant at the end of the first step.

2.5. Optimising published QuEChERS extraction protocols for the extraction of E2 from Mytilus tissue

Information from the published protocols above was applied to produce a new protocol specifically for the extraction of E2 from *Mytilus* gonad, gill and digestive tract tissue.

It has been reported that gonad tissue contains carotenoids, such as β, β carotene, lutein and lycopene (Maoka, 2011) both being strong yellow pigments. These pigments are the metabolic products of digested food such as algae (Maoka, 2011). A thin layer chromatography (TLC) plate spotted with the QuEChERS acetonitrile extract and petroleum ether extracts of carrot and tomato juice showed that the yellow pigment may contain similar carotenoids as the bands for the mussel gonad extract travel at similar rate as the carrot extract (Figure 2.2) Carotenoids are chemically unstable and degrade readily by light, heat and acids (Butnariu, 2016). The yellow interferent band on the TLC plate also quickly fades, implying that it is a carotenoid. These pigments were not removed by the dSPE step.

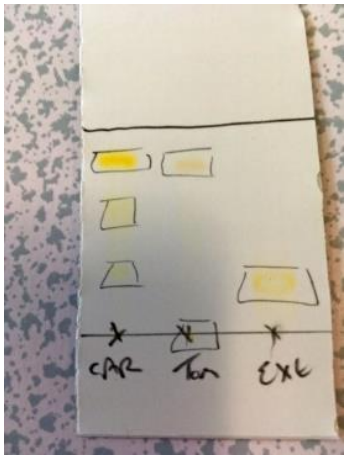


Figure 2.2 TLC of crude gonad extract (far right). The yellow pigment in the extract travels a similar distance as the carotenoid or lycopene bands from the carrot and tomato extracts (carrot (left) and tomato extracts(middle)

As this yellow pigment is retained on a C18 HPLC column and is only eluted at high acetonitrile concentrations, the extracts in 60% aqueous acetonitrile were passed through Waters C18 Sep Pak® cartridges. The E2 and internal standard, Diuron (a pesticide with the synonym: 3-(3,4-Dichlorophenyl)-1,1-dimethylurea), are not absorbed on the C18 cartridge at this concentration but the yellow interferent is. The method was optimised using E2 and Diuron standards and it was found that there was 100% recovery, by passing the extract through dissolved in 5 mL of 60% acetonitrile, followed by a further 5 mL of 70% aqueous acetonitrile to ensure all of the E2 and Diuron is washed through the cartridge. The yellow pigment can only be removed by back flushing the cartridge with 5 mL of absolute methanol.

Therefore, the QuEChERS method was amended and the second dispersive SPE step, normally found in QuEChERS protocols, was replaced by an SPE step using Waters C18 Sep Pak® cartridges. There were also some other small amendments to increase extraction efficiency.

The final extraction protocol was as follows.

- The internal standard used is the pesticide called Diuron. 10 µL of a stock of 1 mg/mL Diuron in acetonitrile is added to the 250 mg of homogenised tissue in a 50 mL centrifuge tube. At this stage the tissue may be spiked with E2 in acetonitrile, using a 10 ng/µL stock solution for the calibration curve. The tissue can be enzyme digested tissue or fresh gonad tissue
  The amount of Diuron as an internal standard can be reduced to adding 10 µL of a more dilute solution of 0.05 mg/mL Diuron for HPLC-FLD analysis and reduced further for LC-MS-MS analysis to give a resulting concentration of 1 pg/mL of extracted sample. However, as some samples were already extracted with the higher concentration of Diuron, the amount of Diuron was kept at 10 µL of 1 mg/mL solution.
- 5 or 6 mL of water is added and the contents are vortexed for 20 seconds. (If fresh tissue is used 6 mL of water is added, if the tissue is enzyme digested tissue, 5 mL of water is added as the digested tissue is already in 1 mL of buffer).
- 10 mL of acetonitrile acidified with 1% acetic acid is added and the mixture is vortexed for 20 seconds.
- 3 mL of hexane is added and the mixture is vortexed for 40 seconds
- A packet of the AOAC salt is added and the mixture is hand shaken for 20 seconds, vortexed for 40 seconds and put on a mechanical shaker (Heidoplh Reax, Schwabach, Germany) at 1250 rpm for 2 minutes.
- The mixture is centrifuged at 5000 rpm for 5 minutes and the aqueous layers are poured into a 15 mL centrifuge tube and centrifuged again for 5 minutes at 5000 rpm. The bottom salt pellet is discarded (Figure 2.3)
- The top hexane layer is discarded. The tubes are put in the -20° C freezer for at least one hour (the bottom aqueous salt layer freezes).
- The middle acetonitrile layer is transferred to a 50 mL pear shaped rotary evaporator flask and most of the solvent is removed on a rotary evaporator with the water bath at 40° C, leaving about 0.5 mL of liquid left.

The crude extract is cleaned up by passing through a Waters C18 Sep Pak® cartridge. The yellow pigment is retained on the cartridge and the E2 and Diuron passes through the cartridge (Figure 2.4).

The cartridge is conditioned by passing 5 mL of methanol through it.

- The cartridge is washed with 5 mL of 60% aqueous acetonitrile.

- The crude extract is removed from the rotary evaporator flask and put in a 10 mL beaker. 0.5 mL of 60% aqueous acetonitrile is added to the rotary evaporator flask and swirled around to remove any residual extract. The washings are placed into the beaker with the extract. This process is repeated 2 more times so the total volume of the diluted extract in the beaker is now about 2 mL.
- All of the extract is drawn up by a 5 mL syringe and the syringe is connected to the Sep Pak® cartridge and the extract is slowly passed through the cartridge and the eluent is collected into the washed out 50 mL rotary evaporator flask.
- 5 mL of 60% acetonitrile is added to the empty 10 mL beaker and the contents are drawn up into the 5 mL syringe and passed through the Sep Pak® cartridge and the eluent is collected into the washed out 50 mL rotary evaporator flask and combined with the previous eluent.
- 5 mL of 70% aqueous acetonitrile is added to the beaker and this is passed through the cartridge and collected as before.
- This process is repeated with another 2 mL of 70% acetonitrile. The total volume in the flask is now 14 mL.

All of the solvent is removed on the rotary evaporator. 1mL of 60% acetonitrile is added to the dry extract and the flask is swirled around to ensure all of the extract is dissolved. The dissolved extract is added to a 2 mL vial for injection on the HPLC. The final optimised method is described in Appendix 2 (2.1; page IX).

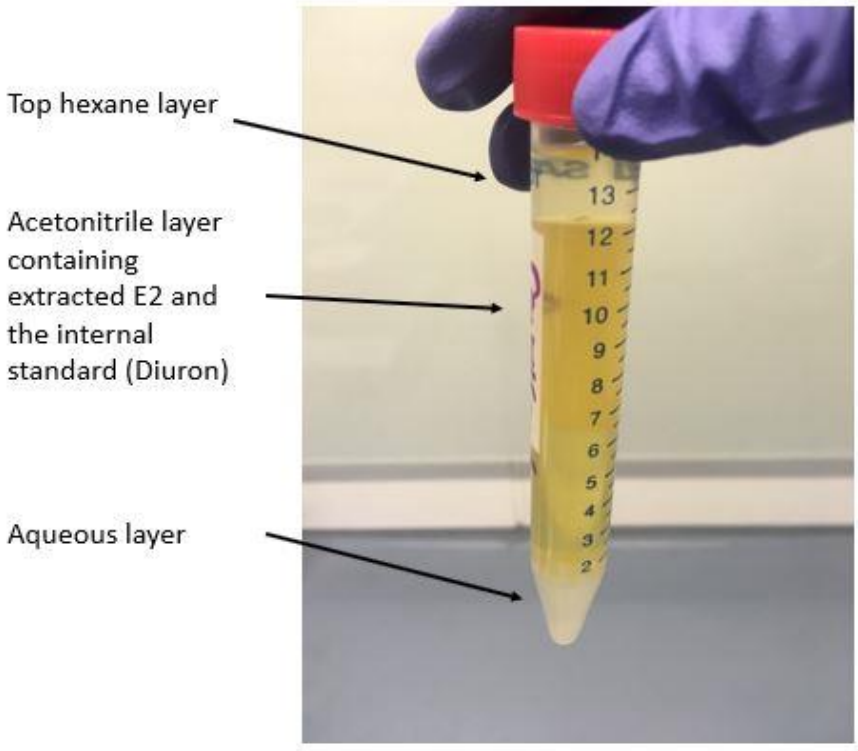


Figure 2.3 QuEChERS extraction, showing the three layers produced

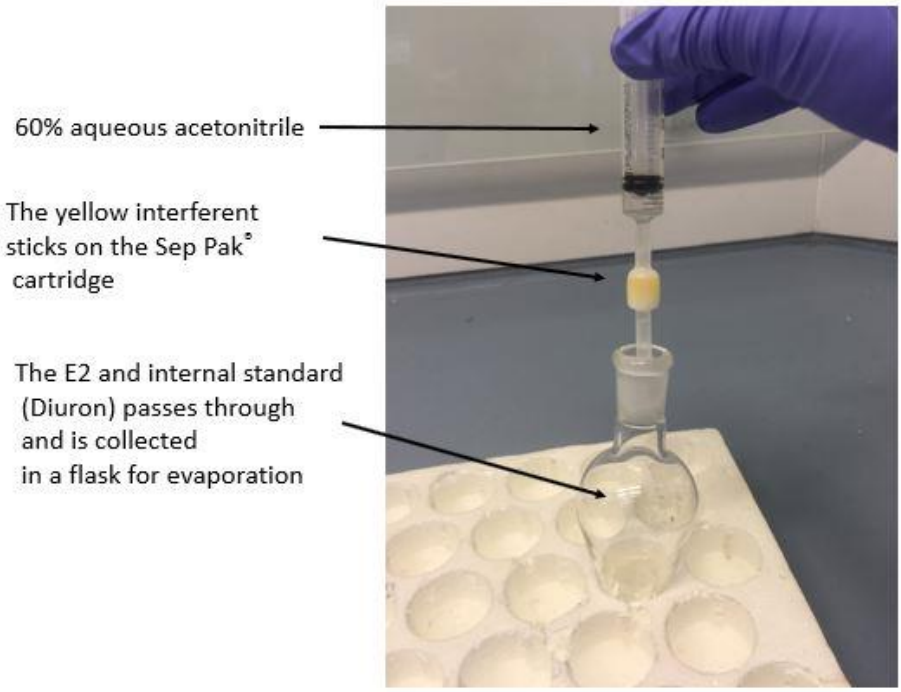


Figure 2.4 Second clean up stage using Waters Sep Pak® cartridges.

2.6. Chemical analysis methods for estrogen identification

2.6.1. Introduction

Extracted estrogens can be analysed and quantified by using Gas Liquid Chromatography (GLC), high performance liquid chromatography (HPLC) with UV and fluorescence light detection (FLD), HPLC with mass spectrometry detection (LC-MS and LC-MS-MS) and by immunoassay methods. Both GC and HPLC instruments use the principle of chromatography to separate the sample into its separate constituents.

2.6.2. Analysis of E2 by gas chromatography with mass spectrometry detection (GC-MS)

Figure 2.5 An Agilent GC-MS instrument used in the analysis of derivatised E2 extracts

In the case of GC analysis, the sample in a volatile solvent is injected into a hot inlet, through which a flow of an inert gas, usually helium, passes (the mobile phase). This sample and solvent are vaporised and pass onto the head of a long (30 metres) glass capillary column coated with (5%-phenyl)-methylpolysiloxane (HP5-MS) (stationary phase) that is in a temperature-controlled oven. Initially the temperature of the oven is relatively low, so that the sample condenses at the head of the column. This focuses the sample into a concentrated band at the head of the column. The oven is then heated gradually to a high temperature so that the sample is then vaporised again and carried through the column.

The components of the sample are retained by the stationary phase coating the column by adsorption and partitioning onto the stationary phase of the column, the degree of interaction depending on the polarity of each of the components. Adsorption is affected by both temperature and pressure with a decrease in adsorption with increasing temperature and pressure. Therefore, the components are separated out as they pass through the column, the components with the least interaction passing through first, followed by the components that have the most interaction (Grob, 1995).

When each of the component molecules exits the column, it passes into the detector. The detector used in this case is a mass spectrometer, so the instrument used is a gas chromatogram, mass spectrometer (GC-MS) (Figure 2.5). As the molecule enters the source of the detector it bombarded with a beam of electrons in a high vacuum and is fragmented into a stream of gaseous ions in a pattern that is unique to that molecule. These charged ions are deflected through a charged reflector disk and series of lenses into a quadrupole that by continually changing its electromagnetic field selects ions of a particular charge to mass ratio through a range over a few

milliseconds in scan mode and at a set range of charge to mass ratio in selected ion mode (SIM) (Grob, 1995).

The selected ions that pass through the quadrupole hit an electron multiplier at its end and are converted into an electronic signal that is converted by the computer software into a value called a total ion count (TIC), and recorded electronically or photographically as a spectrum. The time that the molecule takes to emerge from the detector is called the retention time (RT) and the area of that peak is proportional to the amount of the component (Grob, 1995) (Figure 2.6). As each molecule has a unique fragment pattern, it is possible to compare the fragment pattern with those of standards in a library for confirmation. Also, the retention time of each component of interest can be compared by injecting a range of standards of a known concentration for confirmation and quantification (Grob, 1995) (Figure 2.7).

Usually the GC-MS is run in scan mode for identification of components in an unknown mixture for qualitative purposes and run in SIM mode for quantification of already identified molecules in the mixture (target analysis). Running the GC-MS in SIM mode increases the sensitivity of the machine as only a few ions of interest are selected for the mass spectrometer to scan at those mass to charge values. Usually one of the fragment ions is selected that is unique to that molecule in the mixture (target ion) and one or two other ions (qualifying ions) (Grob, 1995). As the GC-MS instrument needs the sample to pass through the column as a vapour, the sample mixture must be sufficiently volatile in order to pass through the system, this limits analysis to relatively non-polar molecules of a size of about a molecular weight of 700 or less (Grob, 1995).

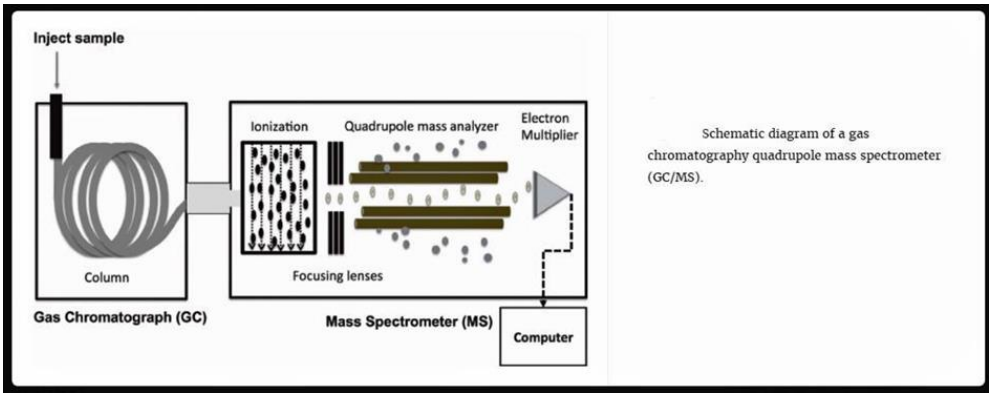


Figure 2.6 Diagram of a GC-MS with single quadrupole detector

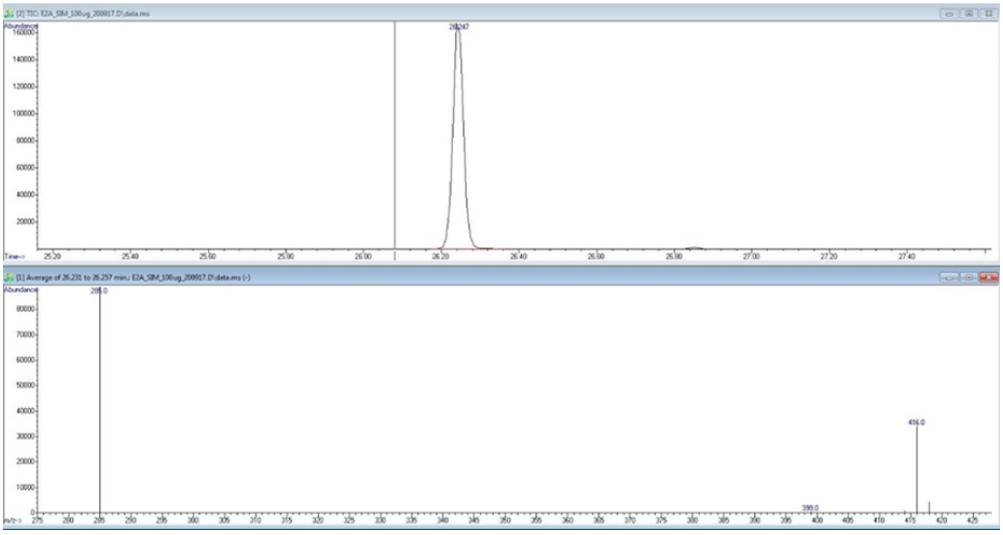


Figure 2.7 Example of a chromatogram of E2 and, below, Spectra (In SIM mode)

2.6.3. Analysis of E2 by High Performance Liquid Chromatography (HPLC)

The separation of a component mixture by HPLC is similar to that of GC but in this case the mobile phase is a liquid and the stationary phase is a coated silica with a small particle size packed in a short metal column (Figure 2.8).

Separation is performed by selective absorption of the components of the mixture through the stationary phase as it passes through the column. The column is kept at a constant temperature, so selectivity is controlled by changing the components in the mobile phase and by ionising or charging the components by using a buffer as the aqueous mobile phase. Initially the mobile phase has a low organic solvent and high aqueous solvent and the amount of organic phase is increased with time. As the molecules are not initially vaporised, HPLC is used as an alternative to GC in the case of larger or more polar molecules (Ardrey *et al.* 2003).

The most common detectors for HPLC instruments are UV detectors such as diode array detectors (DAD) and fluorescence detectors such as fluorescence light detectors (FLD). However, E2 is a weak chromophore and therefore these detectors are not sufficiently sensitive to detect E2 at low concentrations (Ardrey *et al.* 2013). These detectors are useful for method optimisation where tissue can be spiked at larger concentrations, usually in the range 25 µg to 200 µg.

A mass spectrometer can be used as a detector with HPLC separation but as the mobile phase is a mixture of liquids, it must be evaporated off before the entrance of the detector. This is done by passing the mobile phase through a nebuliser with a flow of drying gas (nitrogen). As the molecules must be ionised before entering the detector, if a buffer is not incorporated as part of the mobile phase, ionisation is produced by the addition of 0.1% formic acid into both the aqueous and

organic phase. The mass spectrometer for a HPLC works in the same principle as for a GC-MS and a HPLC coupled with a mass spectrometer is called a liquid chromatography mass spectrometer (LC-MS) (Ardrey *et al.* 2013).

Unlike a GC-MS, the fragmentation of molecules does not produce a unique pattern. This is because the fragmentation pattern is influenced by several parameters such as the buffer used and the type of column selected. However, the structure of the molecule can still be confirmed by its spectra and retention time and the molecule can be quantified in the same way as for GC-MS. The restrictions with LC-MS are that if buffers are to be used, they should also be sufficiently volatile to be evaporated by the nebuliser and any non-volatile inorganic salts must be removed from the sample in the extraction protocol. Therefore, the buffers used are salts of formates, carbonates or acetates and other buffers such as phosphates should be avoided.

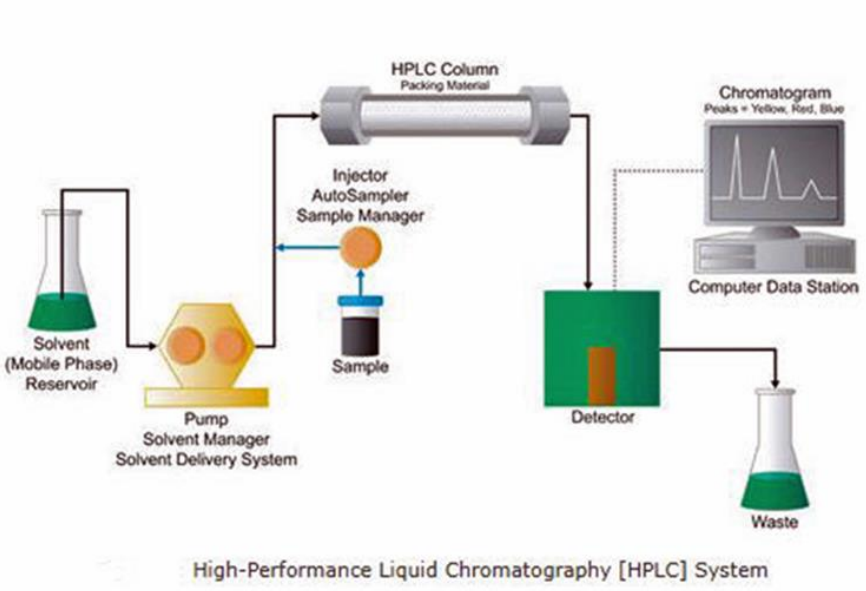


Figure 2.8 Diagram of a typical HPLC system (Waters)

2.6.4. Analysis of E2 by GC-MS-MS and LC MS-MS

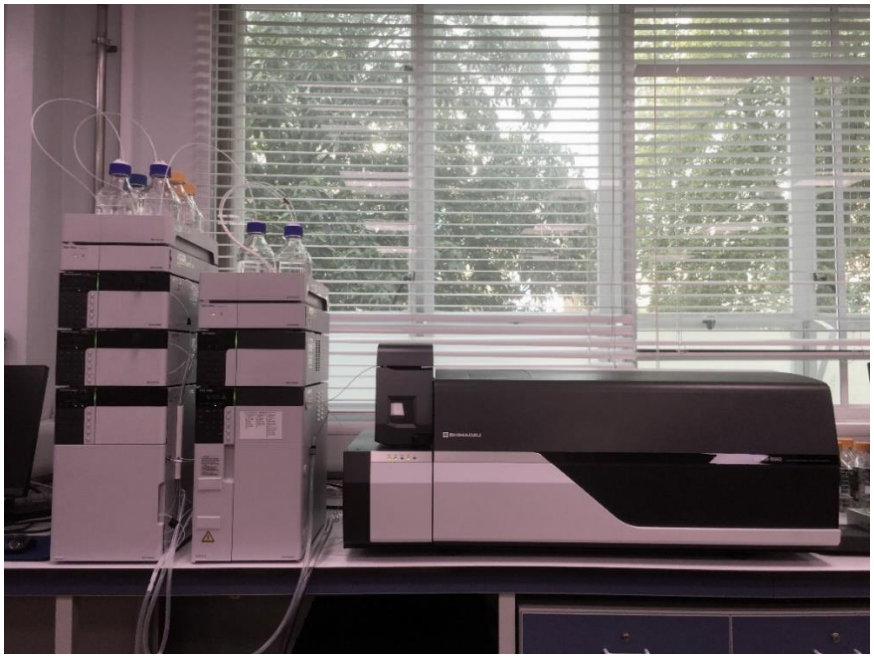


Figure 2.9 Shimadzu LCMS-8060 liquid chromatography mass spectrometer

LC-MS-MS (liquid chromatography tandem mass spectrometry) instruments are the same as above LC-MS instruments with the exception that the detector has more than one quadrupole in series (Figure 2.9). This makes the machines even more sensitive as the selectivity of the molecules of interest is enhanced. The first quadrupole selects ions of a particular size (a parent ion), this parent ion is then broken down into more fragments (qualifier ions) in the second quadrupole, using a beam of small molecules of a gas such as ammonia, rather than a beam of electrons (Ardrey *et al.* 2003).

These smaller fragments then pass through a final quadrupole that selects fragments from the second quadrupole that are unique to the parent ion of interest. This eliminates the background noise of ions common to those of the analyte that pass through a single quadrupole system. Other mass spectrometers such as orbital traps operate in the same way as for quadrupole systems. The fragmentation of the parent ion to the qualifier ions are called transformations (Ardrey *et al.* 2003).

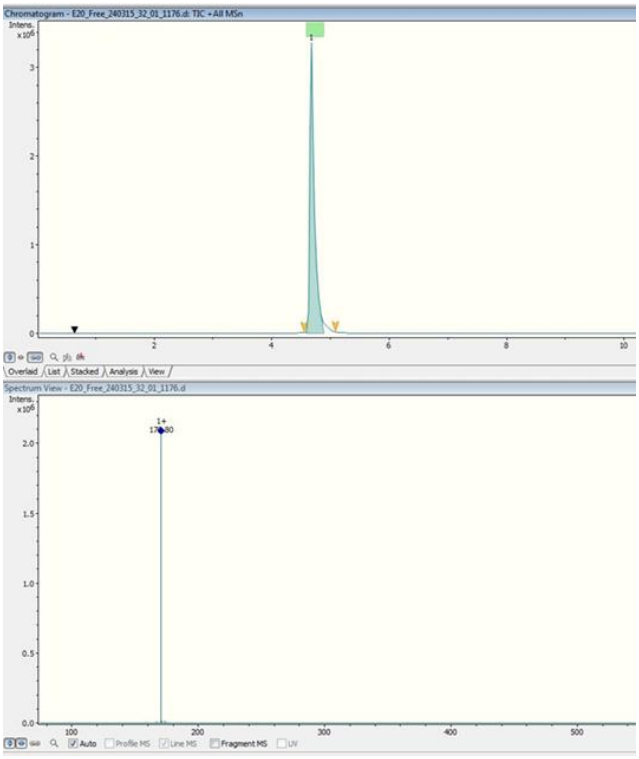


Figure 2.10 Chromatogram of an E2 peak and spectra in SIM mode using a Bruker LC-MS-MS

2.6.5. Consideration of other analysis methods

Immunoassay methods can be used when there is no access to the instruments above as they can be done with general laboratory equipment, the measurement of E2 using a plate reader or spectrophotometer. However, these methods are unsuitable for applications that require a low detection limit as they lack sensitivity and are subject to interference by cross reacting substances (Thienpont and De-Leenheer, 1998; Sinicco *et al.* 2000). Also, they can be slow, labour intensive and may involve the use of radioisotopes. For these reasons they were not considered to be suitable for this project.

2.7. Derivatisation to enhance E2 chemical analysis

2.7.1. Introduction

Using GC-MS and LC-MS analysis, separation and detection can be assisted by the technique of derivatisation. Chemical derivatization is a technique which modifies substances with a low UV absorption into highly sensitive products. The chemical compound is transformed into a product of similar chemical structure, called a derivative. This can be done before column separation (pre-column) or after column separation but before the sample enters the detector (post column) In pre-column mode, the reaction is performed manually before injection. The derivatives

have properties more amenable to a particular analytical method, give better peak shapes, greater chemically stability and increased sensitivity.

2.7.2. GC-MS derivatisation to reduce polarity, increase volatility and thermal stability of E2 during analysis

Compounds that have poor volatility, poor thermal stability, or that can be adsorbed in the injector will exhibit non-reproducible peak areas, heights, and shapes. Silylating reagents react with compounds containing active hydrogens; these reagents are the most common type used for GC analysis. Such silylating agents are BSTFA (N, O-bis(trimethylsilyl)trifluoroacetamide), MTBSTFA (N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide) and MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide). These reagents convert the estrogen by replacing the hydrogen of the hydroxyl groups in the estradiol molecule with trimethyl silyl groups (TMS). For moderately hindered or slowly reacting compounds, a catalyst may be added to the BSTFA or MSTFA such as TMCS (trimethylchlorosilane) or sodium iodide in mercaptoethanol (Figure 2.11). It is recommended to add the silylating reagent in excess and because the derivatization reactions are sensitive to water, all moisture should be removed from the extract that is to be derivatized. Without derivatization, estrogenic compounds show little or no response by GC analysis. With the addition of the TMS group, these analytes show great peak shape and response (Sellers 2010; Sigma Aldrich 2018).

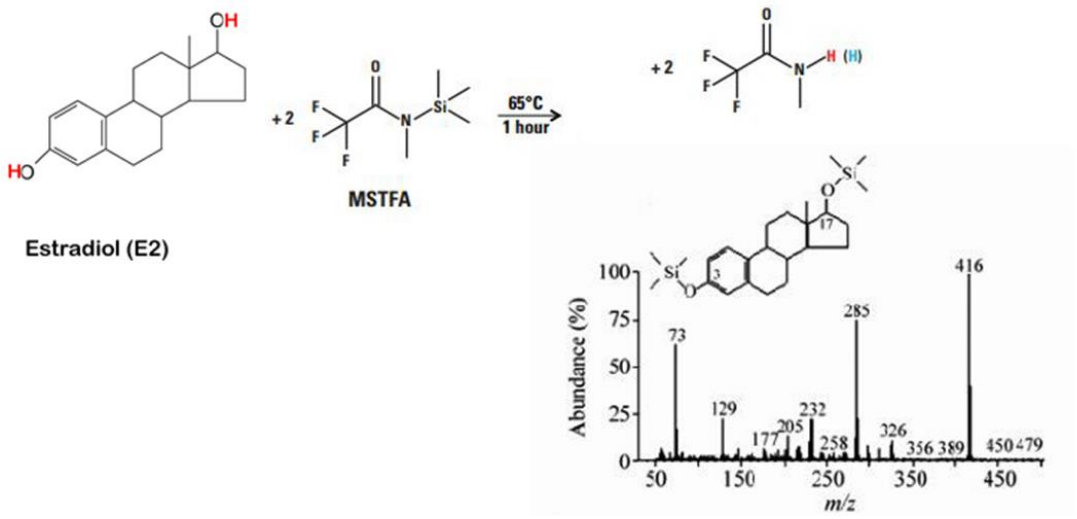


Figure 2.11 Diagram of the derivatisation of E2 using silyation with MSTFA and the resulting spectra in scan mode

2.7.3. Derivatisation for LC-MS and LC-MS-MS analysis

It is necessary to modify the structure of estrogens so that their ionization efficiency is increased, making them more detectable by LC-MS analysis and therefore increasing sensitivity. Appropriate derivatization reagents are dansyl chloride (DNSC), 2-fluoro-1-methylpyridinium p toluene sulfonate (FMPTS) and pentafluoro benzoyl bromide (PFBBr) (Domnica Briciu *et al.* 2009).

Derivatisation by dansyl chloride (Synonym: 5-(dimethyl amino) naphthalene-1-sulfonyl chloride) replaces a hydroxyl group on the E2 molecule with a dansyl molecule and produces a parent ion of size 506 amu (atomic mass unit). This fragments to a qualifying ion of size 171 amu. This fragment corresponds to 5 dimethyl amino naphthalene moieties of the dansyl molecule formed by cleavage of the C-S bond of the derivatised estrogen (Nelson *et al.* 2004; Kohling, 2011) (Figure 2.10; Figure 2.12; Figure 2.13) The general protocol for dansyl derivatisation is as follows.100 µL of 100 mM sodium bicarbonate buffer pH 9 to pH 10.5 and 100 µL of 1 mg/mL dansyl chloride in acetone is added to the dried extract and the mixture is vortexed and heated at 60° C on a heating block for 4 minutes. The cooled mixture is then injected into the machine (Nelson *et al.* 2004; Xu *et al.* 2007; Kohling, 2011; Yang *et al.* 2016).

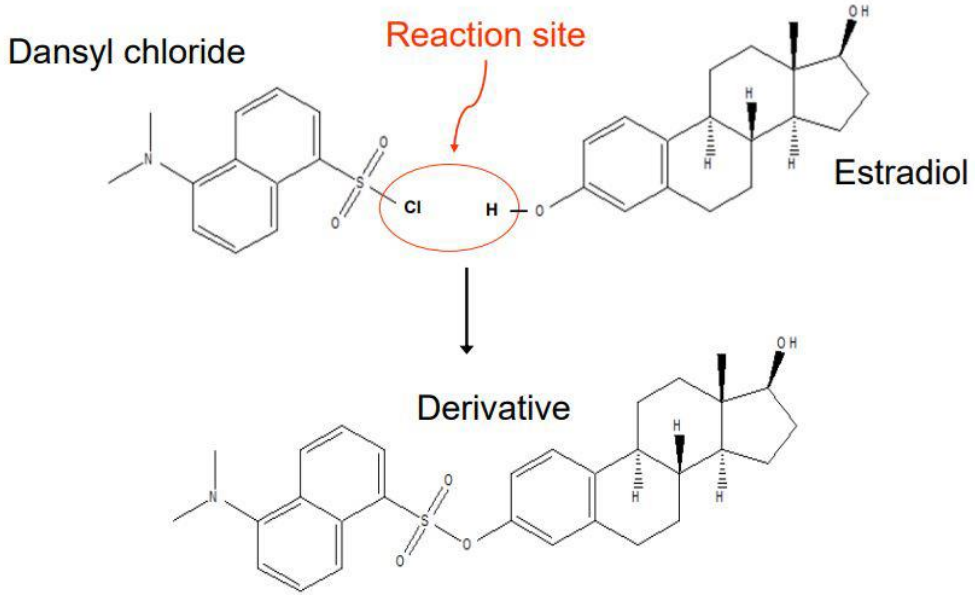


Figure 2.12 Diagram of the fragmentation of E2 with dansyl chloride, producing the parent ion

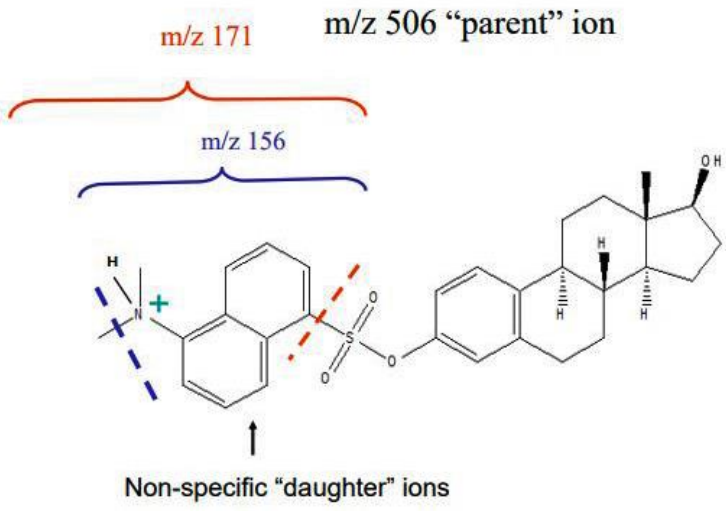


Figure 2.13 Diagram of how the parent ion (506) fragments into the daughter ions, the ion size 171 amu is the selected qualifying ion

2.8. Analysis by HPLC using Fluorescence Light detection

2.8.1. Introduction

E2 is a weak chromophore and measurement using UV detection is not sensitive, however it does fluoresce and detection by FLD is more selective and sensitive. Although detection using FLD is not sufficiently sensitive for analysing the small amount of E2 expected in the tissue samples (about 25 ng per 250 mg of tissue is expected according to the results from other research papers (Cubero-Leon *et al.* 2012). The sensitivity is sufficient for measuring tissue spiked with E2 for method optimisation.

2.8.2. Method optimisation using an Agilent 1100 HPLC with DAD and FLD detectors

The QuEChERS method was optimised and validated using an Agilent 1100 HPLC with FLD detection as there were problems with the MSTFA derivatisation using the GC-MS and dansyl chloride derivatisation using the LC-MS-MS. In the case of GC-MS, there were problems with incomplete derivation using MSTFA and isotope scrambling of the deuterated internal standard. In the case of the LC-MS-MS there was a loss of ions with time. A build-up of salts appeared in the detector, possibly from the bicarbonate buffer in the derivatisation reaction which may be causing ion suppression.

Figure 2.14 Agilent 1100 HPLC system with FLD and DAD detection

2.8.3. QuEChERS extraction with a SEP PAK® clean up step using E2 spiked gonad tissue

Commercial *Mytilus* gonad tissue was spiked with 10 µg of E2 standard and extracted using the optimised QuEChERS protocol, scaled down by 5, to estimate the recovery rate and reproducibility of the optimised protocol. The protocol was scaled down in order to use an adapted vortex genie machine to shake the extracts. This machine could only accommodate tubes of a maximum volume of 10 mL as the shaking machine that could accommodate 15 mL and 50 mL tubes eventually used for QuEChERs extraction was not available then.

The commercial gonad tissue was homogenised and 50 mg of the wet tissue was weighed out into 10 mL glass centrifuge tubes, 10 µg of an E2 standard was spiked into the homogenate and vortexed for 30 seconds. The mixture was digested by adding 2 mL of subtilisin enzyme. The resulting liquid was split into two with 1 mL of the digest being extracted by the QuEChERS protocol for free E2 analysis and the other 1 mL portion being hydrolysed by enzyme hydrolysis for total E2 analysis. Also, un-spiked 50 mg of tissue, tubes with 10 µg of E2 with no tissue and tubes with no tissue or spiked E2 were also extracted in the same way as above as positive and negative controls. Initially the analysis was by GC-MS, using d4E2 as the internal standard but the results of these extractions and consequent GC-MS analysis could not be used as the d4E2 internal

standard, added to all of the tubes was reverting back into E2, presumed to be due to isotope scrambling. The extractions were repeated without adding an internal standard, using HPLC analysis with FLD detection.

The results using HPLC with FLD analysis, showed that all of the extracts with E2 added gave peaks of similar areas and there were no peaks for E2 in the negative controls. (Figure 2.15; Figure 2.16; Figure 2.17).

There was slightly less E2 recovered when the spiked tissue was hydrolysed. This reduced recovery may be due to the extra manipulations for total E2 extraction compared to free E2 extraction. The recovery rates of E2 varied from about 82% to 135% and were similar in amount to the positive controls (Table 2.1). The mean recovery rate of free E2 extracted from tissue is 110.60% with a standard deviation of 15.02% and the mean recovery rate of total E2 from tissue is 90.28% with a standard deviation of 4.23% (Table 2.2).

There is variation in the values of all of the extracts because there has been no internal standard added to compensate for any sample loss. Also, there may be errors introduced when splitting the sample into parts. This may be due to pipetting the enzyme digest which is a colloidal viscous liquid. There may also be a contribution to the amount of E2 recovered by any E2 in the tissue used for spiking.

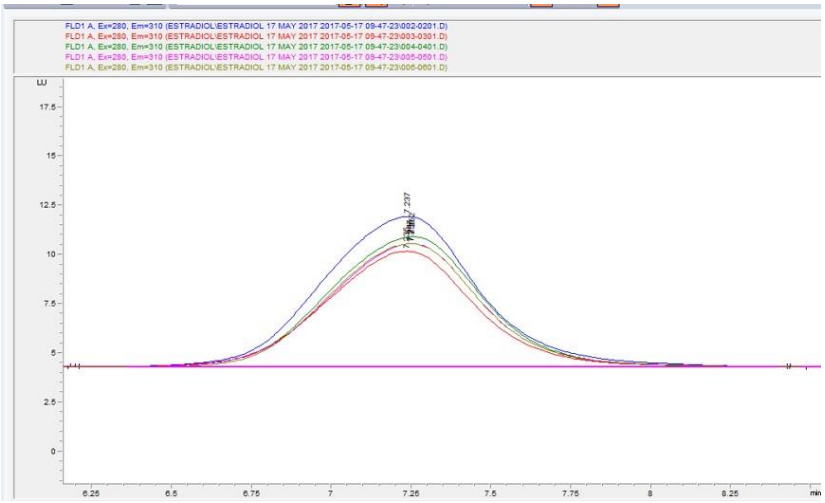


Figure 2.15 Overlaid HPLC chromatograms of several QuEChERS extractions of 50 mg gonad tissue spiked with 10 µg E2. The green peak is the 10 µg E2 standard.

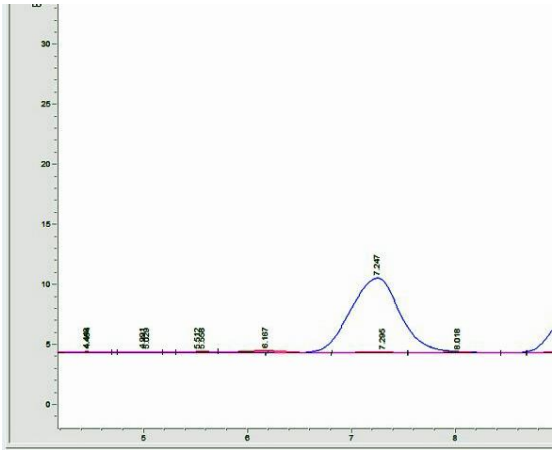


Figure 2.16 HPLC Chromatogram of the QuEChERS extract spiked with 10 µg of E2 and the negative control (red)

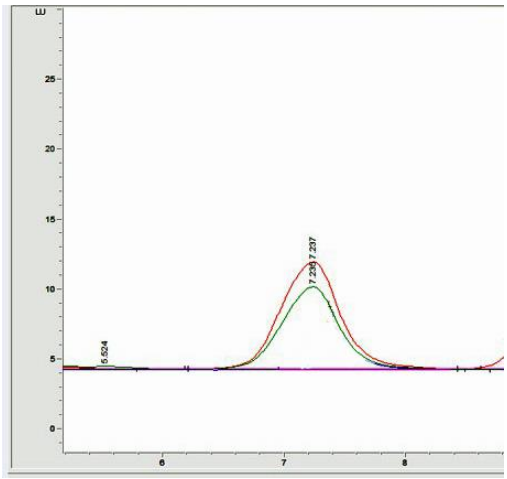


Figure 2.17 Chromatogram of a free E2 QuEChERS extract (red) overlaid with the corresponding total E2 extract (green)

Recovery of free and total E2 from 50 mg of gonad tissue spiked with 10 µg of E2, using HPLC-FLD analysis		
Sample	Amount Extracted E2 (µg)	% recovery
No tissue, positive control	5.85	116.90
Q1 free E2 extract	4.86	97.17
Q2 free E2 extract	4.86	97.28

Q3 free E2 extract	6.19	123.83
Q5 free E2 extract	4.85	97.05
Q6 free E2 extract	5.62	112.49
Q7 free E2 extract	6.79	135.79
Q4 free E2, no tissue	4.93	98.55
E2 standard positive control	5.24	104.79
Mean value	5.47	109.32
SD	0.66	13.16
RSD (%)	12.04	12.04
Q1 total E2 extract	4.65	93.07
Q2 total E2 extract	4.14	82.17
Q3 total E2 extract	4.78	95.54
Q5 total E2 extract	4.55	91.02
Q6 total E2 extract	4.61	92.23
Q7 total E2 extract	4.36	87.10
Q4 total E2, no tissue	4.22	84.45
E2 standard positive control	4.35	87.09
Mean value	4.46	89.15
SD	0.21	4.20
RSD (%)	4.71	4.71

Table 2.1 Percentage recovery of free and total E2 from 50mg of gonad tissue spiked with 10µg E2 with positive and negative controls (HPLC-FLD)

Recovery of E2 from spiked tissue using GC-MS analysis		
Sample	Amount Extracted E2 (µg)	% recovery
Q1 free E2 extract	4.86	97.17
Q2 free E2 extract	4.86	97.28
Q3 free E2 extract	6.19	123.83
Q5 free E2 extract	4.85	97.05
Q6 free E2 extract	5.62	112.49
Q7 free E2 extract	6.79	135.79
Mean value	5.53	110.60
SD	0.75	15.02
RSD (%)	13.58	13.58
Q1 total E2 extract	4.65	93.07
Q2 total E2 extract	4.14	82.71
Q3 total E2 extract	4.78	95.54
Q5 total E2 extract	4.55	91.02
Q6 total E2 extract	4.61	92.23
Q7 total E2 extract	4.36	87.10
Mean value	4.51	90.28
SD	0.21	4.23
RSD (%)	4.68	4.68

Table 2.2 Percentage recovery of free and total E2 from 50mg of gonad tissue spiked with 10µg E2

2.8.4. Selecting an internal standard for use by both HPLC and FLD detection and LC-MS-MS

A possible internal standard was tried to replace deuterated internal standards used in LC-MS-MS. In this case the pesticide Diuron was chosen. Diuron has similar chemical properties to E2, other than it is slightly more hydrophilic than E2. Diuron co elutes at the same time as E2 on the C18 HPLC column used but unlike E2 it absorbs UV but does not fluoresce, so both chemicals can be measured during the same run using DAD and FLD detectors in series. Diuron also has the advantage in that it can be used by LC-MS-MS as well, although the amount of Diuron added as an internal standard would need to be reduced for LC-MS-MS analysis due to the extra sensitivity of the detector.

An external calibration graph was produced using a diluted series of E2 standards to estimate the sensitivity of E2 using FLD detection and therefore the amount of E2 that could be used to spike tissue samples for a standard addition curve (Figure 2.18).

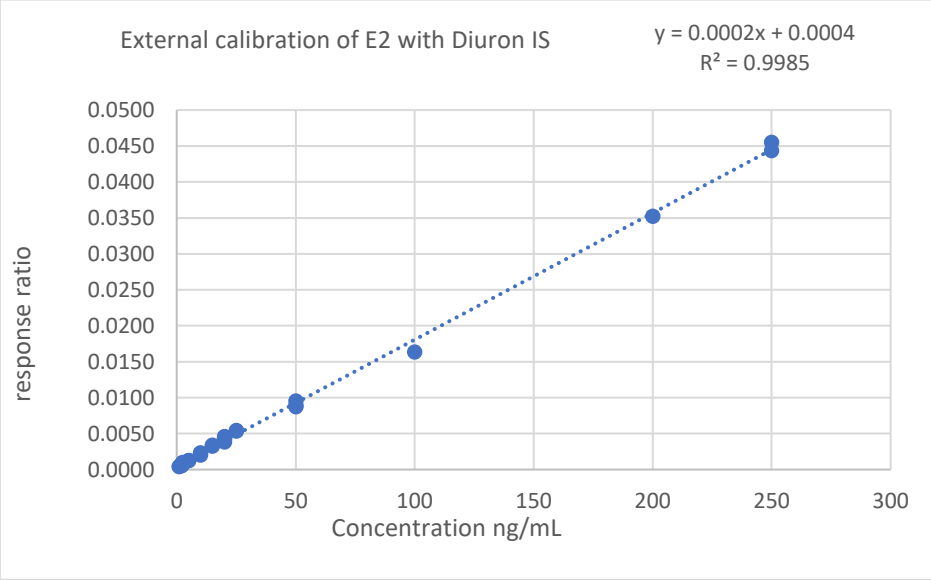


Figure 2.18 External calibration of E2 standards with Diuron as an internal standard

(SEM used for error bars, n=4)

The HPLC used was an Agilent 1100 quaternary system with DAD and FLD detectors. The column was a C18 Phenomenex Luna column 150 mm long, 4.6 mm diameter, 5 µm particle size with a C18 cartridge. The mobile phase was for pump A, 45 % aqueous acetonitrile, pump C 100% acetonitrile using the following gradient with a constant flow of 1 mL/minute at a column temperature of 40° C

	Mobile phase A	Mobile Phase C

0-10 minutes	100%	0%
10-15 minutes	75%	25%
15-25 minutes	20%	80%
25-30 minutes	20%	80%

With a post run re-equilibration time of 10 minutes back to 45% at A

The injection volume was 50 µL.

Diuron was dissolved in acetonitrile to give a 1 mg/mL stock solution and 10 µL of this solution was added as an internal standard for each sample. The E2 was detected using FLD detection with excitation 280 nm and emission 310 nm. Diuron was detected at 254 nm using DAD detection. Both chemicals have the same retention time of about 7 minutes, with the Diuron having a time lag of a few seconds as the sample passes through the DAD first before the FLD detector. Standards of E2 and Diuron were run separately and the E2 standard had no absorption at 254 nm and the Diuron had no signal for the FLD detector. (Figure 2.19; Figure 2.20).

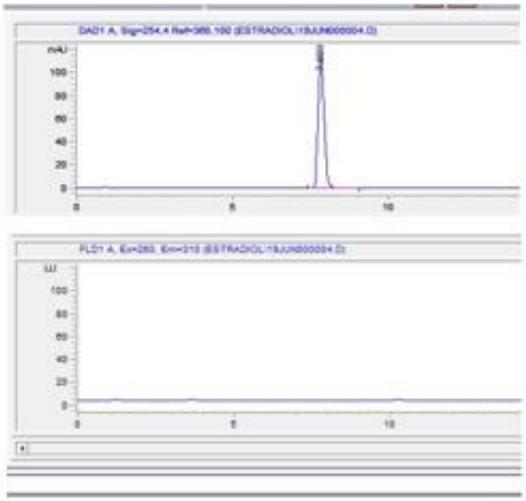
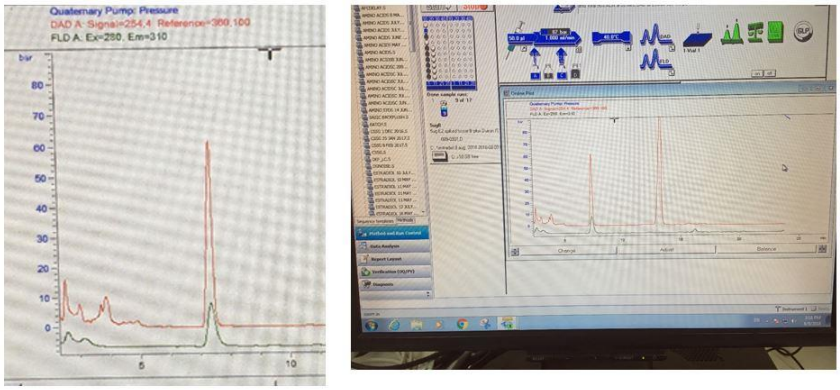


Figure 2.19 Chromatogram of Diuron, DAD signal at 254 nm showing a peak for Diuron but no FLD signal



Diuron (red DAD signal)and E2 peaks (green FLD signal)

Figure 2.20 Screen capture showing the Diuron and E2 peaks that co-elute

2.8.5. Calibration of E2 by standard addition, using E2 spiked gonad tissue

The QuEChERS protocol above was compared to that of the published method by Cubero-Leon *et al.* (2012) by spiking the same amount of homogenised, commercial mussel gonad tissue with the same range of E2 standards and analysed using HPLC with FLD detection and Diuron as the internal standard.

In order to produce a standard addition curve, fresh mussels were purchased from a supermarket and the gonads, gills and digestive tracts were removed into separate glass jars, homogenised and quickly frozen. For the QuEChERS protocol, the homogenised gonad tissue was weighed into 250 mg portions and digested with subtilisin and frozen at -20° C for later extraction.

Some aliquots were frozen without digestion to compare the extraction efficiency of enzyme digested and fresh homogenised tissue. Aliquots of 250 mg of homogenised gonad tissue were also freeze dried for extraction using the protocol from Cubero-Leon *et al.* (2012) to compare both extraction protocols. Standard addition curves using the protocol from Cubero-Leon *et al.* (2012) and the optimised QuEChERS protocol were produced by spiking the 250 mg of either freeze-dried tissue or the enzyme digested tissue with 10 µL of Diuron internal standard and concentration levels of from 1µg to 25 µg in duplicate (Figure 2.21; Figure 2.22).

The two standard addition curves were also plotted alongside each other for comparison and both methods gave similar results, the calibration curves having similar gradients and intercepts (Figure 2.23). The Kolmogorov-Smirnov test, comparing both Y ordinates for the same X ordinates gives a dissimilarity value of 0.27043 and the Kruskal-Wallis test for the same variables gives a p-value of 0.65015. The result is not significant at $p < .05$ (The H statistic is 0.2057 (1, N = 20)). Therefore, both regression curves are similar.

QuEChERS extractions of undigested wet tissue and the same amount of freeze-dried tissue spiked with 10 µg of E2 gave the same results, likewise for the enzyme digested tissue. Therefore, if only free E2 analysis is required, either freeze drying or digesting the tissue is not necessary. Digestion of the tissue or freeze drying and pre-extraction with methanol/dichloromethane is only required to produce a homogenous solution that can be easily partitioned for parallel hydrolysis for the estimation of both total and free E2 from the same extract.

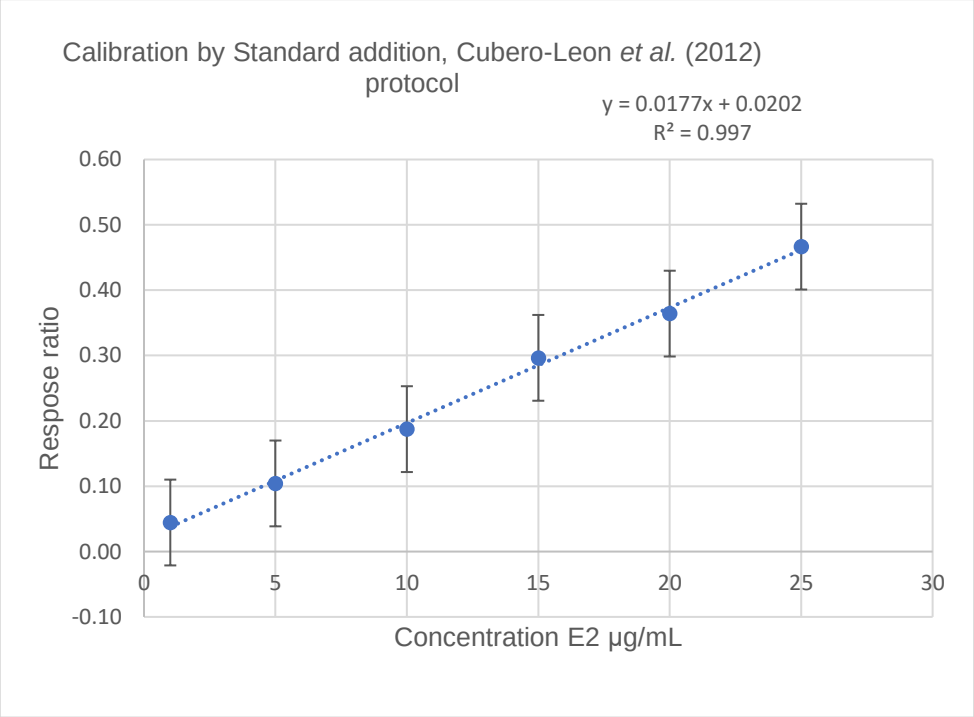


Figure 2.21 Calibration curve using the method of standard addition and the protocol by Cubero-Leon *et al.* (2012).

(SEM used for error bars, n=2)

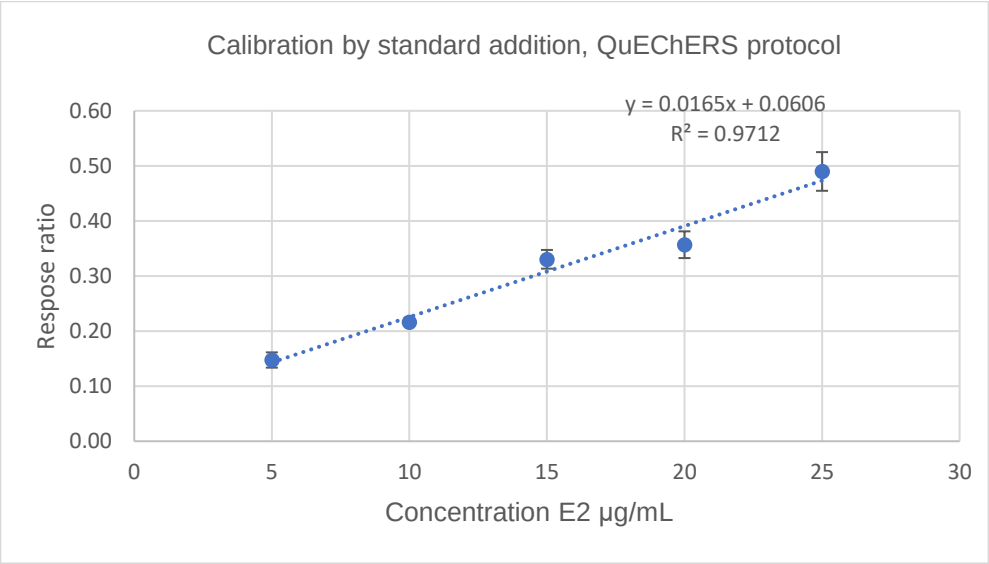


Figure 2.22 Calibration curve using the method of standard addition and the optimised QuEChERS protocol

(SEM used for error bars, n=2)

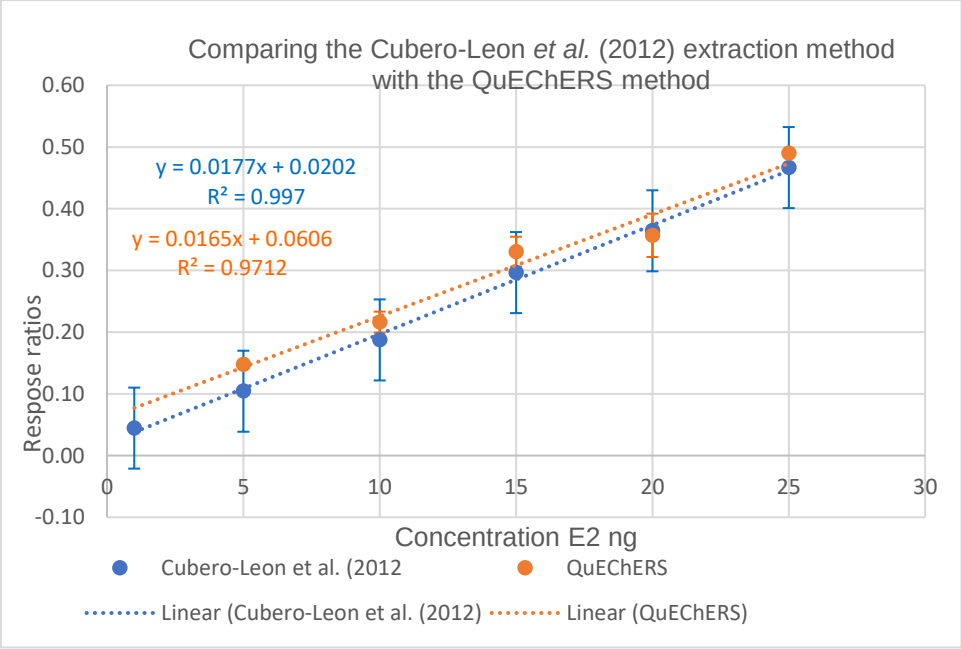


Figure 2.23 Comparing the QuEChERS method with the previously used published method (Cubero-Leon *et al.* 2012).

(SEM used for error bars, n=2)

2.8.6. Limit of detection and recovery rates for the QuEChERS protocol using spiked mussel gonad tissue and SEP PAK® clean up protocol using both HPLC and GC-MS analysis

In order to determine the LOD of E2, the E2 standards were progressively diluted and injected on the HPLC system until the signal to noise ratio was less than 3 in value. The signal to noise ratio being determined by using the Chemstation analysis software (Chemstation, version B.04.03-SP1, Agilent Technologies, Cheadle, UK). Injections of 15 ng/mL E2 solution gave an average signal to noise ratio of 2.65 and injections of 20 ng/mL E2 solution gave an average signal to noise ratio of 3.6, so the LOD of E2 for FLD analysis is somewhere in the region of these two values, an estimation being about 17 ng/mL (Figure 2.24).

The limit of quantification (LOQ) was estimated from E2 spiked gonad tissue using the QuEChERs extraction protocol. The mean peak areas of E2 were equivalent to about 5 µg/mL concentration of spiked gonad tissue having a mean signal to noise area of 30.

The LOD for GC-MS analysis was not determined but peaks for derivatised E2 are visible above the baseline noise at injections of 10 ng of MSTFA derivatised E2. The recovery rates for the E2 extractions using the QuEChERS protocol and HPLC-FLD analysis are tabulated (Table 2.3).

The recovery rates for SEP PAK® extractions of a 10 µg E2 loaded solution in 60% aqueous acetonitrile, with HPLC-FLD analysis are tabulated (Table 2.4). The recovery rates for the E2 extractions using the QuEChERS protocol and GC-MS analysis are tabulated (Table 2.5).

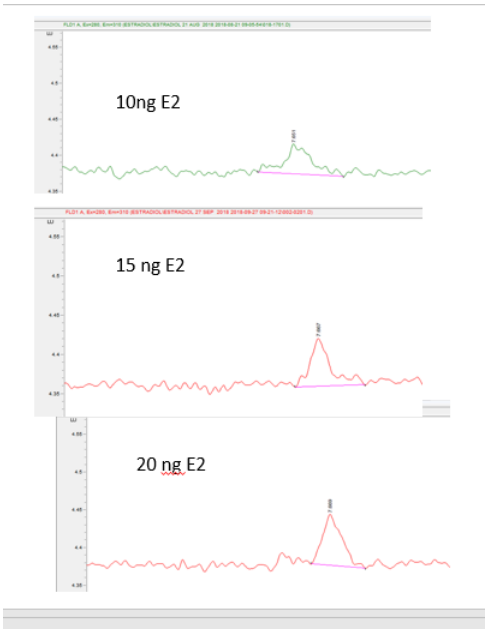


Figure 2.24 FLD Signals of E2 near LOD

Recovery of E2 from spiked digested and undigested gonad tissue using HPLC analysis				
Tissue (50 mg)	E2 added (µg)	E2 recovered (µg)	% recovery	RSD (%)
Digested wet tissue	10	9.72	97.15	0.55
Digested wet tissue	10	9.82	98.22	
Undigested wet tissue	10	9.62	96.15	2.90
Undigested wet tissue	10	10.19	101.91	
Freeze dried tissue	10	8.85	88.52	4.09
Freeze dried tissue	10	9.61	96.07	
Mean value		9.63	96.34	
SD		0.40		
RSD (%)		4.16		

Table 2.3 Recovery rates for E2 extracted using the QuEChERS method from gonad spiked tissue

Recovery of E2 from tissue extracts using a SEP PAK clean up protocol			
	E2 added (µg)	E2 recovered (µg)	% recovery
SEP PAK® extraction, no tissue	10	10.04	100.45

SEP PAK® extraction, no tissue	10	9.85	98.47
Mean value		9.95	
SD		0.10	
RSD (%)		0.99	

Table 2.4 Recovery rates for E2 extracted using SEP PAK® cartridges

Recovery of E2 from E2 spiked tissue using GC-MS analysis			
Sample	E2 Spike per 50 mg tissue (ng)	E2 recovered (µg)	% recovery
QuEChERS extraction	200	215.29	107.65
QuEChERS extraction	200	194.96	97.48
QuEChERS extraction	200	203.80	101.90
QuEChERS extraction	200	202.83	101.02
Mean value		204.02	102.01
SD		7.30	
RSD (%)		3.58	
QuEChERS extraction free E2	100	86.17	86.17
QuEChERS extraction free E2	100	82.62	82.62
Mean value		84.39	
SD		1.78	
RSD (%)		2.10	

QuEChERS extraction total E2	100	74.04	74.04
QuEChERS extraction total E2	100	73.90	73.90
Mean value		73.97	
SD		0.07	
RSD (%)		0.10	

Table 2.5 Recovery rates for free E2 extracted using the QuEChERS method from gonad spiked tissue and hydrolysed tissue

The Sep Pak® clean up protocol is very efficient with almost a full recovery from E2 in an aqueous acetonitrile solution. The best recovery of E2 from tissue is from using wet tissue without any pre-treatment and the least efficient is from freeze dried tissue, but usually freeze-dried tissue need not be used, unless it is pre-extracted with solvent for partition into parts for free and total E2 extraction from the same tissue sample. The GC-MS analysis of E2 extracted tissue did not have any internal standards added, so the results are not as accurate as for HPLC-FLD analysis. The extraction of E2 from spiked tissue that has been hydrolysed, that is for total E2, had a lower percentage recovery than for the free E2 extraction only.

2.9. Extraction of tissues from mussels exposed to E2 and SECs treated with E2

2.9.1. Introduction

In order to access the amount of tissue needed to be extracted per sample for the analysis of the tissue samples from the exposure experiment in January 2018, duplicate exposure experiments were performed using mussels collected at the same time as the exposure experiment in early January 2018 and in August 2017 in order to provide pooled tissue for method optimisation of the extraction protocol and chemical analysis.

2.9.2. Details of the methods used and results

For the January 2018 experiment samples consisted of gonad tissue collected from one control tank and gonad tissue collected from two tanks containing mussels exposed to 200 ng/L of E2. The gill and digestive tissue of mussels from the controls and SECs treatments used in the January exposure experiment was also collected and pooled. Each of the samples of pooled, homogenised tissue was frozen in 50 mL glass jars for extraction and analysis. For the August

2017 experiment, the exposure experiment was replicated using one tank each of the control, SECs plus 100 ng/L E2, water plus 100 ng/L E2 and SECs loaded with 100 ng E2 treatments. In this case, the gonad tissue was removed, homogenised and freeze dried.

About 250 mg or 500 mg of wet tissue from each of the samples was weighed to four decimal places in10 mL glass test tubes and digested with subtilisin, 10 µL of Diuron internal standard was added and extracted using the optimised QuEChERS protocol. The final extracts were dissolved in 1 mL of 60% aqueous acetonitrile and analysed with the Agilent 1100 HPLC with FLD and DAD detection. About 50 mg of the freeze-dried tissue from 2015 was weighed to four decimal places, 10 µL of Diuron internal standard was added and 1 mL of milliQ water. The tubes were left for one hour at 5° C to allow the freeze-dried tissue to be hydrated, before being digested with subtilisin and then extracted using the optimised QuEChERS protocol.

Gonad, gill and digestive tract tissue from an exposure experiment performed in August 2018 were extracted using the optimised QuEChERS protocol in case there was sufficient extracted E2 to be detected. The control tissue, showed no peaks for E2 but there were small peaks for the E2 exposed gonad tissue (Figure 2.25; Figure 2.26). however, the peaks were difficult to integrate as they were too close to the limits of detection.

Likewise, the gill and digestive tract tissue exposed to 200 ng E2 treated SECs had a peak at the same retention time as expected for E2 but was too close to the limits of detection to accurately quantify, also the E2 peak that elutes at 7.55 minutes co elutes with a peak that elutes after the E2 peak (Figure 2.27).

The results of the above tissue using HPLC with FLD analysis indicates that using FLD detection is not sufficiently sensitive as an analytical method for the quantification of E2 within the tissue for the present exposure experiment but is a useful method for extraction optimisation methods for E2 in mussel tissue other media, where the quantity of E2 is above 20 ng/mL of extract.

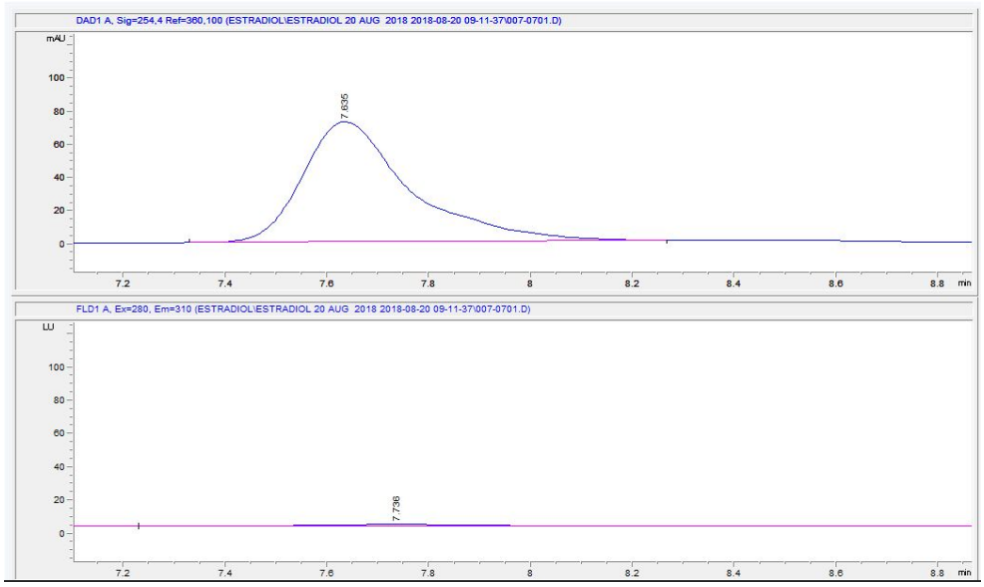


Figure 2.25 DAD signal of Diuron, top window and FLD signal of gonad tissue exposed to E2, bottom window

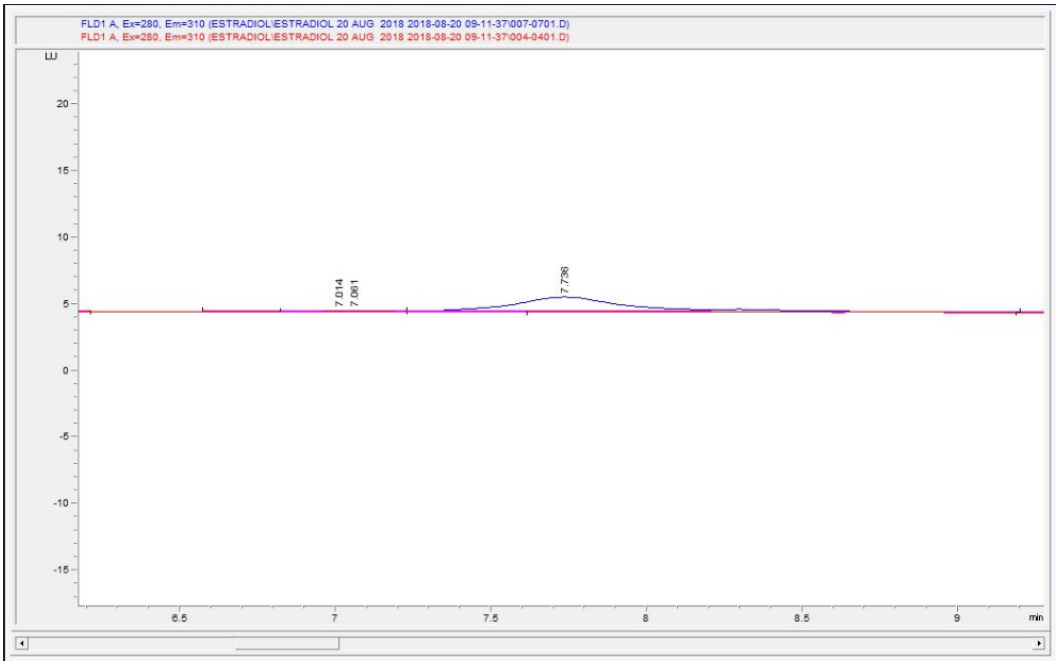


Figure 2.26 Extract of gonad control tissue (red) overlaid with gonad tissue exposed to E2 (blue)

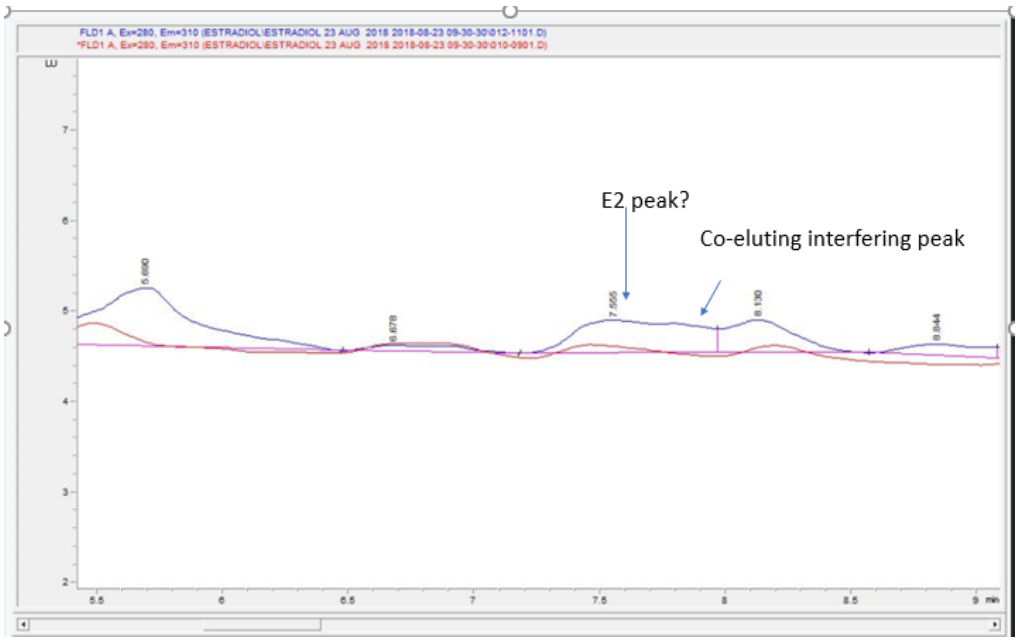


Figure 2.27 Chromatogram of an extract of tissue exposed to SECs treated with 200 ng E2 (blue) overlaid with an extract of tissue exposed to empty SECs (red).

2.9.3. Hydrolysis of the tissue extract for the estimation of total E2

A second set of tissue samples were weighed to four decimal places as 250 mg and 500 mg aliquots and hydrolysed by both the chemical and enzyme hydrolysis protocols from 2.2.2 (page 46) and 2.3.2 (page 48). Aliquots of 250 mg tissue were also spiked with E2 sulphate and hydrolysed and extracted as well as positive controls of E2 sulphate standard. As one mole of conjugated E2 is equivalent to one mole of free E2, the amount of conjugate that is equivalent to100 ng or 200 ng of E2 can be calculated. 100 ng of E2 sulphate is equivalent to 137.44 ng of the conjugated E2 sulphate and 100 ng of E2 is equivalent to 172.73 ng of the conjugated E2 glucuronate.

Both digests gave similar results on extraction and analysis (Figure 2.8). The enzyme digested tissue gave a colloidal solution after digestion, whereas the chemically digested tissue gave a clear solution that was easier to pipette into portions. For 50 mg of tissue, 172.73 ng of conjugated E2 sulphate was added and the tissue was hydrolysed by enzyme action. Using QuEChERS extraction and HPLC-FLD analysis, 109.10 ng of E2 was recovered, which was about 9 ng more than the expected recovery of 100 ng for 100% hydrolysis. However, the extra E2 recovered may be explained by the hydrolysis of E2 already conjugated within the tissue used for spiking.

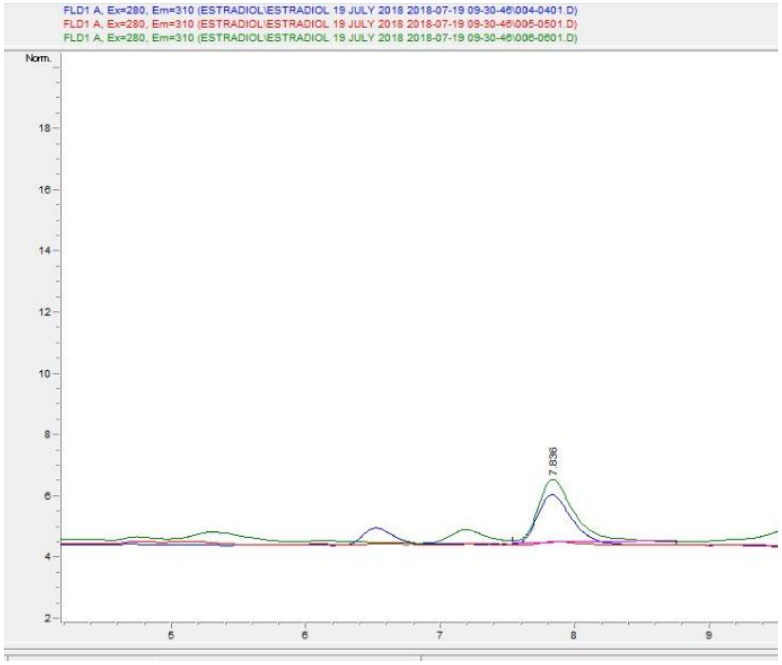


Figure 2.28 Enzyme digest of E2 sulphate overlaid with a chemical digest of E2 sulphate

2.10. FLD detection of Dansylated E2

2.10.1. Introduction

As there were problems when injecting the dansylated E2 reaction mixture due to the presence of the sodium bicarbonate buffer, an alternative buffer was tried. The sodium bicarbonate buffer was substituted with 100 mM ammonium bicarbonate buffer, buffered to pH 10.5 with ammonium hydroxide solution. The use of this alternative buffer was investigated as a substitute for the non-volatile sodium buffer.

2.10.2. Methods and results

On injection of a 10 ng E2 standard, derivatised using the normal protocol but with the ammonia buffer instead of the sodium buffer and injected into Shimadzu LC-MS though a restriction capillary (The Shimadzu LC-MS was model CBM-20Alite using Shimadzu LabSolutions software and an Advion mass spectrometer in scan mode), the parent ion (506) or fragments (171, 156), nor any adducts could be seen, implying that the derivatisation had not been successful. The specifications for the Shimadzu LC-MS-are:

The Degasser was model DGU-70A

The autosampler was model SIL-20A

Binary Gradient pumps, both models LC-20AD

With mobile phases of Water with 0.1% formic acid at A and Acetonitrile with 0.1% formic acid at C with constant flow of flow 0.3 mL/minute.

The gradient was as follows

0.01 minutes	60% C
4.00 minutes	95% C
6.00 minutes	95% C
8.00 minutes	99% C
14.00 minutes	99% C
14.50 minutes	60% B
20.00 minutes	stop

The column (when used) was an Ascentis Express 2.7 µm, 10 cm x 2.1 mm (58323-U, Supelco, Merck,Darmstadt, Germany), with column oven model CTO-20A at 35° C

The detectors were:

A Mass Spectrometer Advion expression^{L}

Acquisition MRM, polarity positive with scan parameters for the target compound dansyl E2 of mass 506.20 m/z (mass to charge ratio) with a dwell time 100. There was also a UV detector in series, model SPD-20A with a deuterium lamp at wavelengths 280 nm and 254 nm.

When dansylated E2 was run using the same column, flow rate and mobile phase gradient conditions for LC-MS analysis, there was no peak around the expected retention time of about 5 minutes using the FLD parameters for underivatized E2.There are no published methods that use FLD detection to analyse dansylated E2 although the dansylated E2 molecule should be a good candidate for FLD detection as it has been labelled with dansyl chloride which is a strong chromophore and should be fluorescent.

In order to try to find the most sensitive parameters to detect the dansylated E2, the same reaction mixture, diluted by 10^6 with water, was placed in a 3 mL quartz glass cuvette and scanned using a Perkin Elmer LS 55 fluorescence spectrophotometer using a fixed emission wavelength of 210 nm, scanning the excitation wavelength from 220 nm to 700 nm. This was repeated, using a fixed excitation wavelength of 500 nm with an emission wavelength range of 220 nm to 700 nm. The scans were also repeated using the same 10 ng E2 standard and the derivatisation protocol

using sodium bicarbonate buffer. The dansylated product was run on the Agilent HPLC, using a method that had been previously optimised for dansylated E2 samples on an Agilent 1100 binary HPLC system with a Bruker orbital trap mass spectrometer. The method parameters were as follows.

Mobile Phase A; Acetonitrile and 0.1% formic acid

Mobile Phase B: Water and 0.1% formic acid

Flow rate 0.35 mL/minute at 35° C

Gradient

Minutes	%A	%B
0	60	40
4	95	5
6	95	5
8	99	1
14	99	1
14.5	60	40
20	60	40

The column used was an Ascentis Express 2.7 µm, 10 cm x 2.1 mm (Supelco 58323-U, Merck, Darmstadt, Germany), and the Injection volume is 20 µL.

There are some published papers that use spectrofluorometric methods to measure the concentration of dansylated E2. The excitation (Ex) and emission (Em) parameters are dependent on the choice of mobile phase used (Roos, 1978; Lamparczyk, 1992) as can certain phases can quench the fluorescence of the molecule of interest. Most earlier papers use normal phase HPLC and the parameters for dansylated E2 in chloroform are Ex 298 nm, Em 545 nm with an increase in Em as polarity of solvent increases. The selection of solvent polarity and pH are important as they change the charge status of the chromophore. Schmidt *et al.* (1978) measured dansylated E2 analytes with a fluorescence detector using excitation and emission wavelengths of 350 and 540 nm, respectively. Some of the published excitation and emission parameters were used to try to measure the dansylated E2 by FLD detection.

The results were the same for both ammonium and sodium buffers, with the optimum emission wavelength being 504.34 nm. The excitation spectrum was multimodal with optimum wavelengths at 360.80, 419.00, 484.22 and 528.72 nm.

From previous results using LC-MS analysis with the same column and method conditions, the expected retention time of the dansylated E2 for this method is about five minutes. However, using the optimised parameters obtained from the scanning fluorimeter, there was no apparent peak at the expected retention time. There was a very large peak at a retention time of about two minutes, which implies that this is the excess dansyl chloride that is not retained on the column and that the optimised parameters are for the excess dansyl chloride, rather than the dansylated E2 product and the dansyl derivatised product is not being detected at these selected excitation and emission parameters.

Alternative combinations of FLD parameters also tried. For the parameters Ex 362 with Em 504.there were no peaks at expected retention time but there were large peaks eluted early on at about a retention time of two minutes. The same results were obtained with a dansyl chloride and sodium carbonate buffer mixture on its own without any E2 present, so the earlier peaks at two minutes were probably only excess dansyl chloride. Also, the parameters Ex 350 with Em 540 (Schmidt *et al*. 1978) were tried without success.

The same mixture was run using the same method and column as for above but with the FLD parameters of Ex 280 nm and Em 310 nm used to detect underivatized E2 and there was a peak indicating that E2 was present in the mixture with the ammonia buffer but not in the sodium buffer. This again, indicates that the derivatisation using the alternative ammonia buffer is not successful whereas the sodium buffer derivatisation is successful but the product does not appear to be detectable using any of the FLD Ex or Em parameters applied.

2.11. LC-MS-MS Analysis of extracted E2 from water and tissue samples

2.11.1. Introduction

The previous analytical instruments used were not sufficiently sensitive to detect the small amount of E2 extracted from the sample. Due to having three quadrupole mass selectors, an LC-MS-MS instrument is more sensitive than its single quadrupole counterpart. The analysis was performed on a Shimadzu LC-MS-MS system comprising of a binary pump system with two Shimadzu LC-30AD pumps, a Shimadzu SIL-30AC autosampler and a Shimadzu CTO-20AC column oven. Both the mobile phase lines and the autosampler lines are degassed with a Shimadzu DGU-20A degassers. The detectors are a Shimadzu SPD-M20A diode array detector in series with a Shimadzu LCMS-8060 liquid chromatography mass spectrometer.

2.11.2. Method and parameters used for analysis by LC-MS-MS

The method and parameters for LC-MS-MS analysis are as follows,

The LC-MS-MS instrument used was a Shimadzu model CBM-20Alite, using Shimadzu LabSolutions software

The binary pumps were both model LC-30AD

The mobile phase was LC-MS quality water with 0.1% formic acid at channel A and LC-MS quality acetonitrile with 0.1% formic acid at channel B at a constant flow of 0.5mLs/ minute

The gradient was: -

0.1 minutes	50% B
4.50 minutes	95% B
5.50 minutes	95% B
6.0 minutes	50% B
10 minutes	50% B

The column was a Shimadzu G155-HP 3 µm C18 3 µm x 100 mm

The column oven was model CTO-20AC at constant temperature of 40° C

The autosampler was model SIL-30AC and the injection volume was 10 µL

The acquisition was MRM with positive polarity

The target compound was dansyl E2, precursor 506.20 and product 171.20 with a dwell time of 100 at quadrupole 1 with a retention time of E2 at about 4.65 minutes

The Interface was ESI (electrospray ionisation) at temperature 300° C

The DL (desolvation line) temperature was 250° C

The nebulizer gas flow was 3.00 L/Min

The Heating gas flow was10.00 L/min

The drying gas flow was 10.00 L/min

The PDA (photo diode array) Detector was model SPD-M20A, with a deuterium lamp and tungsten lamp

Set at 254 nm 4 nm slit width, scanning at wavelength range 190 nm to 800 nm

2.11.3. Using derivatisation with dansyl chloride for LC-MS-MS detection of E2.

It was observed that directly injecting the dansylated E2 extracts was causing problems by salt build up on the needle and cone of the LC-MS system, resulting in ion suppression and sample loss. There is no mention or references in any of the papers about salt deposition, or any information of how they overcame this problem, but it became evident that some sort of desalting method must have been used. Precipitating the excess salt by the addition of methanol was unsuccessful so an alternative method of desalting using an SPE process was investigated. As the reaction mixture is only 200 µL, rather than using an SPE cartridge, a specialised 10 µL or 100 µL filter tip containing a C18 sorbent in the tip (Thermofisher, Pierce, Waltham, MA, USA) was used to desalt the reaction mixture.

As the LC-MS-MS is very sensitive, samples injected into the system must be of a concentration in the order of 1 pg/µL. In order to inject a sample of 1pg/µL, the following protocol was used. 20 µL of a 1 ng/µL E2 solution in acetonitrile was added to a test tube and the solvent evaporated under a flow of Nitrogen. This solution was derivatised by the protocol (2.7.3; page 61) to give a resulting 200 µL of derivatised mixture.

- The C18 tip was conditioned by drawing up acetonitrile through the tip and discarding the solvent ten times.
- The tip was then washed with water by drawing milliQ water through the tip and discarding another ten times.
- 10 µL of the reaction mixture was pipetted into a 200 µL Eppendorf tube. The volume on the pipettor was increased to 15 µL and the reaction mixture was slowly drawn up from the 200 µL Eppendorf and replaced back into the Eppendorf 20 times. Increasing the volume on the pipettor ensures that all of the mixture is drawn up through the C18 sorbent at the end of the tip.
- The tip is washed with water by drawing up milliQ water as before, the washings being discarded.
- The dansylated E2 is eluted from the tip by drawing 1 mL of 95% aqueous acetonitrile through the tip and replacing back again. The final acetonitrile solution is vortexed and placed in a 2 mL autosampler vial for analysis.

The residual reaction mixture is stored at -20° C.

2.11.4. External calibration for analysis of Dansylated E2 using LC-MS-MS

In order to optimise the LC-MS-MS detection parameters a 10 pg/µL solution of dansylated E2 was prepared by derivatising 20 ng of E2 and desalting 10 µL of the resulting 200 µL reaction mixture using the protocol in (2.11.3; page 85) and eluting the product into 1 mL of acetonitrile. An injection of 25 µL of this standard solution using a restriction column was used to optimise the parameters for the triple quadrupole mass spectrometer detector to select the optimum conditions for the 506 m/z parent ion and 171 m/z collision fragment. These parameters were then saved to the method as described in (2.11.2; page 84).

An eight-level external calibration curve was obtained by diluting a stock solution of 100 pg/µL desalted dansylated E2 standard in 60% aqueous acetonitrile in the concentration range from 1 pg/µL to 100 pg/µL with an injection volume of 1 µL. This stock solution was also serially diluted down in 60% aqueous acetonitrile from 10 pg/µL down to 0.01 pg/µL to obtain an estimation of the LOD for dansylated E2 (Figure 2.29).

A series of E2 standards in the same range as for the above serially diluted derivatised standard was derivatised and desalted individually to produce an external calibration graph (Figure 2.30; Figure 2.31). Although the external calibration curve is linear, without the addition of an internal standard, there is considerably more scatter than for the previous E2 calibrations using the FLD detector without derivatisation and a desalting step and for the derivatised, desalted standard that was serially diluted down. This implies that there is some sample loss in either the DNSC derivatisation or in the final desalting step, which cannot be compensated for due to the lack of an internal standard.

One of each of the calibration levels of the individually derivatised and desalted standards were left on the sample tray over the weekend and were re-run using the same method (Figure 2.32). The results show an amount of degradation by being left a 10° C for two days, therefore it is important to run samples in small batches, to minimise the amount of time left in the autosampler tray.

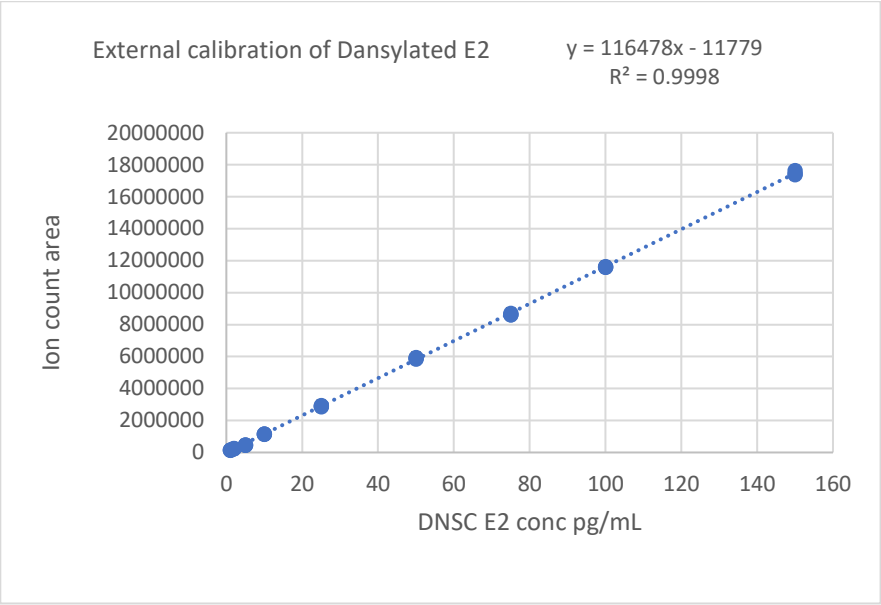


Figure 2.29 External calibration graph produced by serial dilution of a desalted dansyl chloride derivatised 20 ng E2 standard.

(SEM used for error bars, n=8)

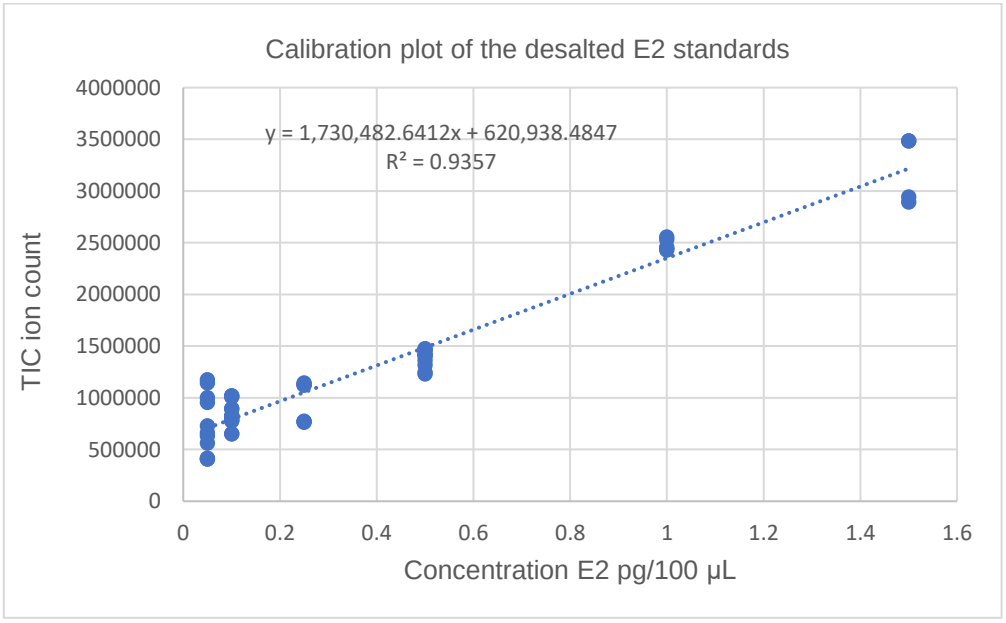


Figure 2.30 External calibration graph of E2 standards, individually derivatised and desalted, in the same range as for the above external calibration range, showing increased scatter at the lower concentrations of E2

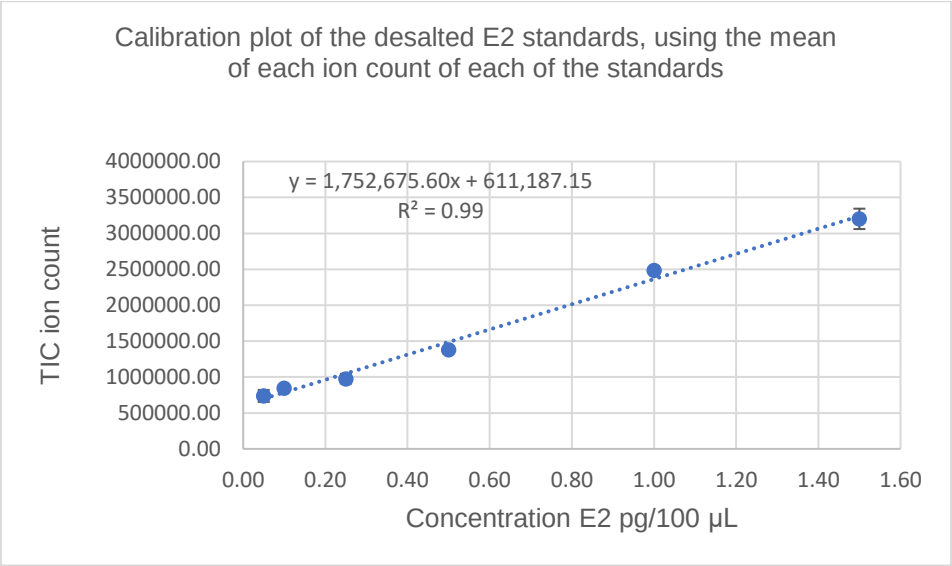


Figure 2.31 External calibration graph of E2 standards, individually derivatised and desalted, in the same range as for the above external calibration range, using the mean of each of the standard concentrations

(SEM used for error bars, n=8)

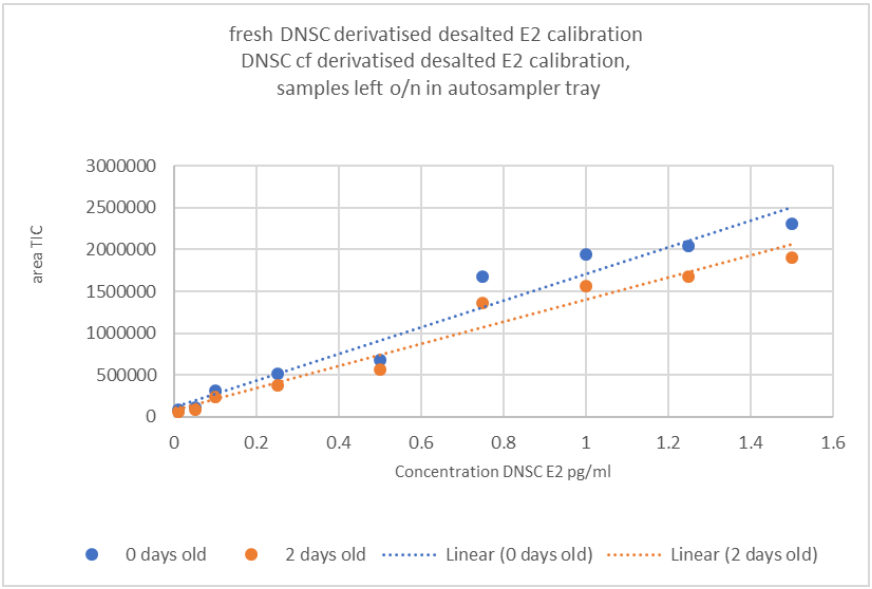


Figure 2.32 External calibration curves for DNSC derivatised and desalted E2 standards overlaid with a curve for the same samples left on the autosampler tray, showing some degradation.

(SEM used for error bars, n=8)

2.11.5. An External calibration with dansylated E2 with Diuron as an internal standard

An external calibration plot was produced derivatising a range of E2 concentrations from 1 ng to 30 ng, adding 20 µL of a 1 ng/µL Diuron per sample. The method from (2.11.2; page 84) was modified to include the internal standard Diuron, the extra multiple reaction monitoring (MRM) parameters being: -

Diuron precursor 233.20 m/z, product 72.05 m/z dwell time 100

precursor 233.20 m/z, product 160.05 m/z dwell time 100

After derivatisation, 10 µL of the resulting 200 µL reaction mixture was desalted using the protocol from 2.11.3 and eluted into 1 mL of acetonitrile to give a calibration range of 1.5 pg/µL to 0.05 pg/µL with 1 pg/µL of Diuron as an internal standard. As the Diuron is less soluble in concentrated acetonitrile some of the standards were also eluted into a 60% aqueous acetonitrile solution, but the recovery rate of the DNSC derivatised E2 was significantly reduced.

The recovered dansylated E2 and dansylated Diuron is normalised by dividing the total ion area of the E2 by the area of the total ion count of the Diuron for the same desalted extract. The results were disappointing as there appears to be no linearity for the calibration plot (Figure 2.33). The areas for replicate extracts are not consistent for both the dansylated E2 and the Diuron internal standard.

Although the calibration graph produced by serially diluting a derivatised standard at a higher concentration is linear (Figure 2.30) and the calibration plot produced by desalting dansylated E2 is also linear but with a reduced correlation (Figure 2.31) incorporating Diuron into the reaction mixture as an internal standard is not effective. One explanation is that this may be due to the difference in solubilities or a difference in retention on the C18 tip between Diuron and dansylated E2.

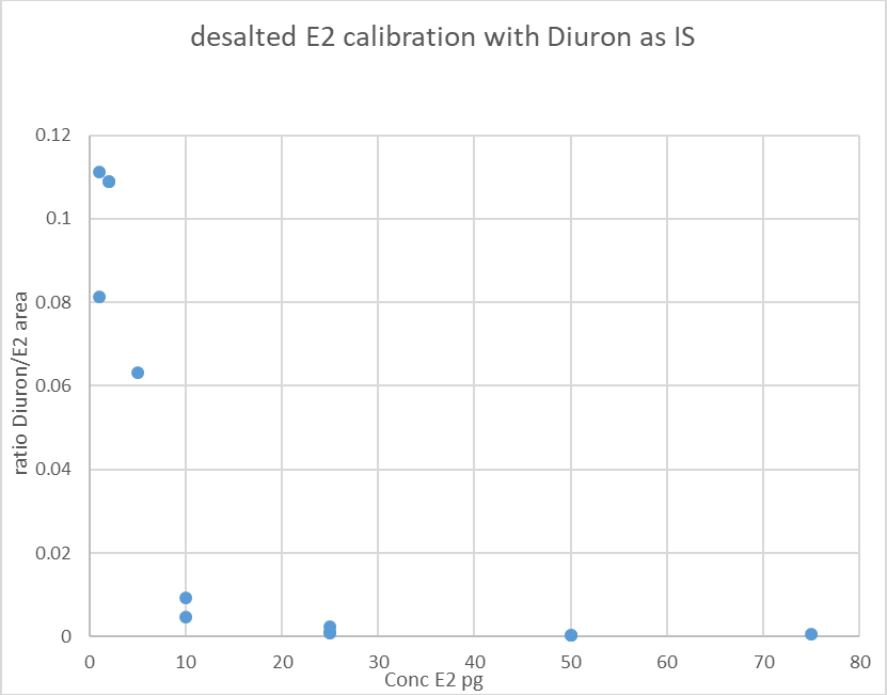


Figure 2.33 Scatter plot of dansylated E2, normalised by adding Diuron as an internal standard.

2.11.6. Water Analysis from the exposure experiment, methods and initial attempts at analysis

Water samples were taken from tanks for the four treatments, the tanks being selected randomly. Before the water was changed, another sample of the water was taken from randomly selected tanks that had been treated with either E2 or SECs plus E2 to estimate the amount of residual E2 was in the water after three days.

The water samples from the exposure experiment were extracted using SPE with a C18 cartridge following the protocol from (2.1; page 44) and eluted using methanol and stored at -20° C until analysis by LC-MS-MS.

The methanol extracts were placed in 5 mL Pyrex test tubes with Teflon lined screw tops and the contents evaporated to dryness at 40° C under a flow of nitrogen. The dried extract was derivatised using dansyl chloride using the protocol from (2.7.3; page 61) and the reaction mixture was placed in a 200 µL glass insert in a 2 mL autosampler vial and stored at -20° C until analysis.

When the water samples were initially collected, they were spiked with the internal standard DES with the intention of analysing them using the GC-MS, however this was postponed until a more sensitive LC-MS-MS instrument became available. The samples were run on a Bruker LC-MS-MS system with dansyl chloride derivatisation without desalting, however the nebuliser on the

machine gradually became compromised by salt deposition. The analysis was abandoned without producing a calibration curve and the dansylated extracts were stored at -20° C until an alternate method of an analysis was determined.

For LC-MS-MS analysis using the Shimadzu LCMS-8060 liquid chromatography mass spectrometer, 10 µL of the derivatised reaction mixture was withdrawn from the autosampler vial and desalted using the protocol from (2.11.3; page 85) and eluted in 1 mL of acetonitrile and 1 µL was injected for analysis using the method from (2.11.2; page 84).

2.11.7. Gonad tissue extract analysis from a 100ng E2 exposure experiment

2.11.7.1. Introduction

E2 was extracted from 0.025 g of homogenised freeze-dried gonad tissue from four treatments in an exposure experiment in August 2017. The extractions were done in multiples of four, of which a set of three were retained for LC-MS-MS analysis and one set for HPLC FLD analysis.

The following treatments were: -

- Control Mussels with no treatment
- Mussels exposed to empty SECs
- Mussels exposed to 100 ng/L of E2
- Mussels exposed to SECs treated with 100 ng of E2

Also, blank extractions were done with no tissue added.

2.11.7.2. Methods

Initially, the tissue was extracted with DCM and methanol using the protocol from Cubero-Leon *et al. (*2012). This method was used in preference to the enzyme digested method as the resulting solution is more amenable to pipetting an exact volume than the for the more colloidal enzyme digest. The solvent was evaporated from the extract over a flow of nitrogen at 35° C and then dissolved in 1 mL of acetonitrile and the resulting solution was split into two parts of 750 µL and 250 µL. The 750 µL portion was then used to extract E2 using the QuEChERS protocol and estimate the amount of free E2 in the tissue. The 250 µL portion was chemically hydrolysed using the protocol from Cubero-Leon *et al.* (2012) and extracted using the same QuEChERS protocol as

for the estimation of total E2. The internal standard Diuron (1 pg/µL) was added to both portions, after the chemical hydrolysis step but before the QuEChERS extraction was performed.

The QuEChERS extraction protocol from (2.5; page 50) was reduced to half scale, using 15 mL centrifuge tubes instead of 50 mL tubes.

- For the extraction of free E2 fraction, 3 mL water and 4.25 mL of acetonitrile and 1.5 mL hexane and half an AOAC sachet (3.75 g) was used.
- For the extraction of the total E2 fraction 5 mL of acetonitrile and 1.5 mL hexane and half a sachet of AOAC salts (3.75 g) was used.

After centrifugation, the tubes were frozen for about 2 hours and 5 mL of the middle acetonitrile layer was removed to a new set of tubes and evaporated to dryness over a flow of Nitrogen. The resulting dry extract was dissolved in 2 mL of 60% acetonitrile and passed through a Waters C18 Sep Pak® cartridge following the protocol from 2.5, page 50) and evaporated to dryness using a rotary evaporator and the residue was reconstituted in 1 mL methanol and stored at -20° C.

Before analysis, the remaining methanol in the tubes was evaporated off to dryness and the residue was derivatised using dansyl chloride as in the protocol in (2.7.3; page 61) and desalted using the protocol in 2.11.3 and eluted in 1 mL of acetonitrile and 10 µL of the solution was injected into the Shimadzu LC-MS-MS using the method in 2.11.2.

One set of the extractions of the four treatments was removed and analysed by the Agilent HPLC system with FLD detection. These extracts did not need derivatising or desalting.

2.11.8. Conclusion of the method developments for the extraction and analysis of E2 in water and tissues

The tank water from the four treatments were filtered extracted a day after collection using the protocol described in section (2.1; page 44) and were derivatized using dansyl chloride (2.7.3; page 61) There was a delay in analysis as there were problems with the Bruker LC-MS-MS instrument, so the extracts were frozen at -20° C for some months. The analysis of the same extracts was attempted with a Shimadzu LC-MS instrument but there was salt deposition on the cone of the electrospray, so the analysis was discontinued.

As E2 is fluorescent and can be detected using HPLC with an FLD detector, an attempt was made to modify the protocol for underivatized E2 using the Agilent 1100 HPLC system with FLD detection. The dansylated E2 was derivatized using sodium bicarbonate buffer and an alternative ammonium bicarbonate buffer and scanned using a fluorometer to obtain the maximum absorbance for both reactions, However, the derivatised products were not detected using this range of excitation and emission wavelengths, the conclusion being that the derivatised product is

not fluorescent and the observed fluorescence in the reaction mixture was due to the excess Dansyl chloride in the reaction.

Eventually the water extracts were analysed on the Shimadzu LC-MS-MS instrument but there was most likely some degradation. Although the samples were degraded the results were sufficient to determine the presence of E2 in the E2 spiked tank water that was still present at the end of the exposure experiment. There was a little E2 in the other treatments.

The analysis of E2 was explored using GC-MS detection using derivatization by the silylating reagent MSTFA (2.7.2; page 60). Although the method gave a very good calibration curve with a range of E2 standards and using d4E2 as an internal standard, the method was not sufficiently reliable or sensitive when an attempt was made for some of the tissue extracts. Also, the d4E2 internal standard was not chemically stable. Analysis by GC-MS was used to determine the degree of E2 treated by SECs and the amount of residual E2 in the water used in the preparation of the treated SECs.

A protocol was developed using the Agilent 1100 HPLC system with FLD and DAD detection, using Diuron as an internal standard. This protocol worked very well. The Diuron coelutes with the E2 but does not fluoresce, absorbs in UV light and can be detected by DAD detection, whereas the E2 fluoresces and can be detected by FLD but does not absorb in UV light. Both the product and the internal standard are linear at concentrations far higher than required for the present analysis and the results are reproducible with both the E2 and Diuron peaks being symmetrical with full base separation. The LOD is about 17 ng/mL so this protocol is not sufficiently sensitive enough for the tissue analysis of the exposure experiment but is suitable as a tool for future method optimisation for the analysis of other estrogens as well as E2.

The method of QuEChERS extraction that was initially developed for pesticide analysis was investigated as a quicker and cheaper alternative method for extraction of E2 from mussel tissue. This method was compared to a previously published method using solvent extraction and SPE clean up (Cubero-Leon *et al.* 2012). Both methods were comparable for free E2 extraction with very similar results when obtaining calibration curves by standard addition. The QuEChERS method works very well for free E2 analysis using wet gonad tissue without any prior solvent extraction.

Analysis of E2 from E2 gonad tissue is problematic due to the presence of a polar yellow pigment found in gonad tissue. This pigment is strongly retained on C18 reverse phase HPLC columns and eventually compromises the performance of the column. This pigment has not been identified but may be a type of carotenoid pigment. A protocol was developed to remove this pigment from the extraction mixture using a Sep Pak® cartridge.

The chemical hydrolysis protocol fully hydrolyses all types of conjugated steroids Homogenized tissue is initially extracted by a mixture of organic solvents. This extract is then partitioned into parts so that one part of the same extract is analysed for free E2 and the other part of the same extract is analysed, after hydrolysis, for total E2.

The use of enzyme hydrolysis was explored as an alternative to chemical hydrolysis of the tissue extracts. The enzymatic hydrolysis is more selective and the enzyme only cleaves conjugated glucuronates and sulphates of E2. In order to partition the tissue extract, the tissue was digested with the enzyme subtilisin. Both enzyme reactions are pH specific and require several hours of digestion so they are not as straight forward as for chemical hydrolysis.

A protocol to extract E2 from gonad tissue was developed from several protocols that are used to extract E2 from fish tissue and sediments with an extra step using Sep Pak® cartridges to remove the yellow pigment interferent (2.5, page 50) The recovery rate varied depending on the tissue used with an average recovery rate of 75%, the recovery rate of the hydrolysed extracts being slightly less. The amount of E2 extracted from mussel gonad, gill and digestive tract tissue from an exposure experiment in August 2017 was below the LOD for analysis by HPLC with FLD detection.

A method to analyse dansylated E2 using a Shimadzu instrument with MRM mass spectrometry detection. In order to analyse these samples, a method to remove the sodium bicarbonate buffer was developed using C18 filter tips (2.11.3; page 85) This produced a calibration curve that was linear but with more variation than for the previous analytical methods. The sensitivity of this instrument was considerably more sensitive than for the previous instruments used and the extracts needed diluting down, otherwise the signal became too saturated for the detector. The desalting protocol still needs some optimisation, as the resulting calibration curve produced from derivatised E2 standards is not linear, also the LOD and LOQ has not been determined yet. The use of a suitable internal standard still has to be developed and incorporated into the method.

Tissue from an alternative exposure experiment in August 2017 was extracted using the optimised QuEChERs protocol as a trial for the future analysis of the gonad tissue from the January 2018 exposure experiment and analysed using the above Shimadzu LC-MS-MS instrument. The results of these tissue extractions indicate that the QuEChERs protocol is not effective for chemically hydrolysed extracts. This is most likely due to surfactants produced on hydrolysis of the lipids that are altering the partitioning of the E2 into the water layer rather than the acetonitrile layer of the extraction mixture.

Chapter 3. An exposure experiment to investigate the uptake of E2, and SECs loaded with E2, in mussels

3.1. Introduction

The purpose of this experiment was to determine if sporopollenin exine capsules can be used as a means to remove E2 from water supplies by adsorption of E2 onto or within the SECs. There have been several published investigations of Mytilus species exposed to E2, (Janer *et al.* 2005; Puinean *et al.* 2006; Canesi *et al.* in 2007; Ciocan *et al.* 2010; Zabrzańska *et al.* 2015; Schwarz *et al.* 2016) but little work has been done on any detrimental effects of marine or other aqueous species exposed to SECs that have been treated with E2. As SECs are known to adsorb lipophilic molecules (Punt *et al.* 1999; Barrier, 2008; Barrier *et al.* 2011; Diego-Taboada *et al.* 2014). The E2 may be either adsorbed on the surface of the SECs or absorbed within the SECs or a combination of both. It is not known if any absorption or adsorption of E2 is reversible and whether the E2 is held firmly or loosely on the surface of the SECs. If the E2 is absorbed inside the SECs, it is not known if the E2 is permanently contained within the SECs or whether it could be re-released in time. If any E2 is absorbed within the SECs it is not known if the E2 within the E2 is bioavailable if ingested by any marine or other species,

This experiment exposed *M. edulis* to both E2 in the water and SECs that have been treated with water containing the same amount of E2. There are also controls (water only) and SECs (water only) with all four treatments that were positioned randomly in the same controlled environment. In order to determine whether the E2 treated by the SECs is bioavailable, these four treatments were examined for SECs within the tissues to ascertain whether the SECs are ingested by the animals. The tissue was extracted and analysed for the presence of E2 and conjugated E2 and RNA was extracted from the gonad tissue to investigate for differences in gene expression.

3.2. Materials and Methods

3.2.1. Exposure experiment involving three treatments with E2, SECs, E2 plus SECs and a control.

The shared experimental set up consisted of 16 glass aquarium tanks consisting of two 5 litre compartments per tank. These were labelled and colour coded according to the following treatments. These were: -

- 4 control tanks (no treatment) white labelled.
- 4 E2 exposure tanks (a treatment of 200 ng/L of E2 per 5 L compartment) blue labelled.
- 4 sporopollenin tanks (empty SECs) yellow labelled.
- 4 E2 treated sporopollenin (SECs treated with 200 ng of E2 per 5 L compartment) red labelled.
- Plus, an extra three tanks in a separate cold room of: -
- 2 E2 treated sporopollenin (SECs treated with 100 ng of E2 per 5 L compartment) red labelled.
- 1 control tank (no treatment) white labelled.

The tanks were distributed randomly in a controlled cold room with the temperature set at 12° C and a lighting period of 11 hours light and 13 hours dark. The tanks were numbered and their position relative to each other was recorded. Each compartment of the tank was aerated with tubing and an air stone with plastic lines from small diaphragm air pumps. The tanks were covered with lids of aluminium foil to prevent dust or other contaminants falling in the water.

The artificial sea water was made from dissolving 35 g Tropic Marine™ sea salt (Tropical Marine Centre Ltd, Chorleywood, UK.) per litre of purified water to give a salinity percentage of 35% which was checked using a refractometer. The tanks were filled the day before the start of the experiment to allow the water to adjust to the ambient cold room temperature. This temperature was measured on a regular basis, there being a mean temperature of 12.9° C with a gradient of less than 0.2° C across the room. The water was changed every second day and replaced with fresh artificial sea water and the treatments repeated. The used water was collected and filtered with a 2 µm cellulose acetate membrane and stored in 500 mL glass bottles and refrigerated at 5° C for later analysis. 10 mL aliquot of the stored filtered water samples were removed and these were measured for the pH values at two different temperatures, at 10° C and 20° C; the mean pH being 7.88 ± 0.10 at 10° C and 7.78 ± 0.10 at 20° C.

M. edulus were collected from a local, wild population off the coast of Filey, East Yorkshire at 0°16’ latitude and 54°13’ longitude in early January and held for several days in large 20 Litre tanks at about 12° C in a cold room prior to the start of the experiment. Five animals were placed in each compartment of each tank, so a total of 40 animals were used per treatment (Figure 3.1; Figure 3.2; Figure 3.3).

Figure 3.1 Tank set up during the exposure experiment, left side and back

Figure 3.2 Tank set up during the exposure experiment, right side

Figure 3.3 Close up of *M. edulis* in one of the tank compartments

3.2.2. Description of the treatments used in the exposure experiment.

3.2.2.1. E2 treatment

E2 was obtained commercially from Merck,Darmstadt, Germany. A 1 mg/mL stock solution of E2 in methanol was prepared and diluted by 100 in milliQ water by adding 1 mL of the E2 stock solution to 99 mL of water in a 100 mL volumetric flask to give a working solution of 10 ng/µL. A 100 µL aliquot of this solution was added to each 5 L compartment to give a treatment of 200 ng/L concentration of E2 and 50 µL of the E2 solution was added to give a treatment of 100 ng/L. The stock solution in methanol was refrigerated at 5° C between treatments and the diluted treatment solution in water was made directly before the addition to the tanks. The diluted solutions were kept refrigerated for later extraction and chemical analysis to estimate the amount of E2 dissolved in water over time. The water samples from the exposure experiment were extracted by passing through conditioned C18 SPE cartridges (Phenomenex Ltd) and the extracts were kept frozen until LC-MS-MS analysis.

3.2.2.2. SECs treatment

The SECs used are not commercially available so they were previously prepared from acid extracted *Lycopodium olavatum* as described in (Barrier 2008). The modified preparation protocol was as follows:

In a one litre round bottomed flask, equipped with a magnetic stirrer and condenser, acetone washed *Lycopodium olavatum* powder was suspended in 3 M hydrochloric acid and refluxed using a silicone oil bath at 94° C for one hour .After cooling, the resulting hydrolysed particles were filtered through a porosity grade 2 sinter and washed with sufficient water to remove all of the residual acid and the filtrate was neutral. The particles were then rinsed with methanol followed by acetone and air dried.

For a ratio of 1:1 of SECs to the active ingredient to be treated, the amounts of SECs and E2 to be used were calculated as follows. The SECs were estimated to have a mean diameter of 25 µm and therefore a volume of 8184 μm^3 per SEC.

500 ng of E2 would require 500 ng of SECs i.e. 5000 SECs to give a 100 ng/L dose. This approximates to a volume of 5000 x 8184 or 40.9 μm^3. Allowing for air spaces between each of the spheres, an approximate workable volume is about 50 µL of SECs. Therefore, 50 µL of water was pipetted into a pre-weighed 200 µL PCR tube and a line was drawn to the level of the water

surface. All of the water was removed and the tube was filled with SECs to the same level and re-weighed to give a weight of 0.0065g ± 0.002 g. This weight of SECs was used to treat all of the tanks where SECs treatment is required. As the dosing amount of E2 was changed from 100 ng/L to 200 ng/L on the first day of the experiment, there was insufficient time to prepare more aliquots of weighed SECs, so the ratio of E2 to SECs was 2:1(Figure 3.4).The protocol to adsorb E2 onto or encapsulate into the SECs was as follows:

For each treatment dose, 0.0065 ± 0.002 g of SECs was weighed into a 1.5 mL Eppendorf tube and 500 µL of milliQ water was added to wet the SECs. The mixture was vortexed for 20 seconds and the contents were pulse spinned down in a centrifuge. The water was pipetted off and 100 µL of 10 ng/µL working E2 solution was added for the E2 treated SECs or 100 µL of millQ water was added for the SECs controls. The mixture was vortexed for 20 seconds and the contents were pulse spinned down in a centrifuge and the water was removed. The SECs were then briefly washed with 500 µL of milliQ water to remove any free un-adsorbed E2 and mixture was vortexed for 20 seconds and the contents were allowed to settle and the water was removed. The treated SECs and control SECs were kept refrigerated at 5° C until required. Spare aliquots of the treated SECs were refrigerated and all of the water washings during the treatment stages were frozen for later analysis to estimate the amount of E2 treated in the SECs and residual E2 left in the water washings.

For treating each 5 L compartments, the contents of the control or E2 treated SECs in the Eppendorf tubes were then pipetted out into the compartment and the contents rinsed out with the sea water in that compartment, until the Eppendorf tube was fully emptied.

Figure 3.4 E2 treated SECs, ready for adding to a tank.

At the end of the exposure experiment the animals were removed and their length and weight were measured and recorded and dissected and the following tissues were stored for later analysis. The empty shells were also weighed to estimate the ratio of tissue to shell as a percentage body weight to shell weight ratio (IC) (Appendix 1, page I).

Three types of storage tubes were used: -

- Pre-weighed glass specimen tubes for chemical analysis; these were frozen at -20° C.
- Plastic 1.5 mL Eppendorfs containing RNAlater™ for DNA analysis, these were frozen at -80° C
- Plastic Eppendorfs containing 10% formalin solution for histology, these were kept at room temperature.

For all of the treatments, the mantle was removed and separated into three parts for chemical analysis, DNA analysis and histology. For the SECs, the digestive tract and gill tissue was also removed and separated into three parts for chemical analysis, DNA analysis and histology. 60 of the tubes containing the mantle tissue were reweighed to give an approximate weight of wet tissue available for analysis, giving a mean value of 0.192 g of tissue per sample (Appendix 1, page I).

3.2.3. Histological preparation of Mytilus tissue

In order to determine the sex and stages of gametogenesis, formalin fixed gonad tissue from the exposure experiment was prepared as follows:

The tissue was initially washed with PBS buffer and dehydrated with various concentrations of ethanol (Figure 3.5) to enable infiltration with liquified paraffin. The tissue was then embedded in paraffin wax blocks using a Leica EG1160 embedder. The wax blocks were then sectioned into 5 µm thin slices using a Thermoscientific Shandon Finese 325 microtome which were then floated-out on a water bath and picked up with a paint brush and placed on microscope slides. The slides were then allowed to dry before they were stained. The first staining step was to de-wax the sections by melting the wax over a Bunsen flame for a few seconds or in an oven at 65° C for 30 minutes and then immersing the slides in the solvent Histoclear to remove the wax from the slide prior to staining.

The staining method selected was haematoxylin and eosin (H&E) staining. This method provides a very detailed view of the tissue by clearly staining cell structures including the cytoplasm, nucleus, and organelles and extra-cellular components (Anderson, 2018) (Figure 3.6). The prepared slides were then covered with a cover glass and sealed with Depex mountant before viewing using an Olympus S2X10 microscope and photographed with cellsens entry software at 3.2x magnification. The tissue was then classified into the sex of the animal and stages of gametogenesis using Seed's classification (Seed, 1969).

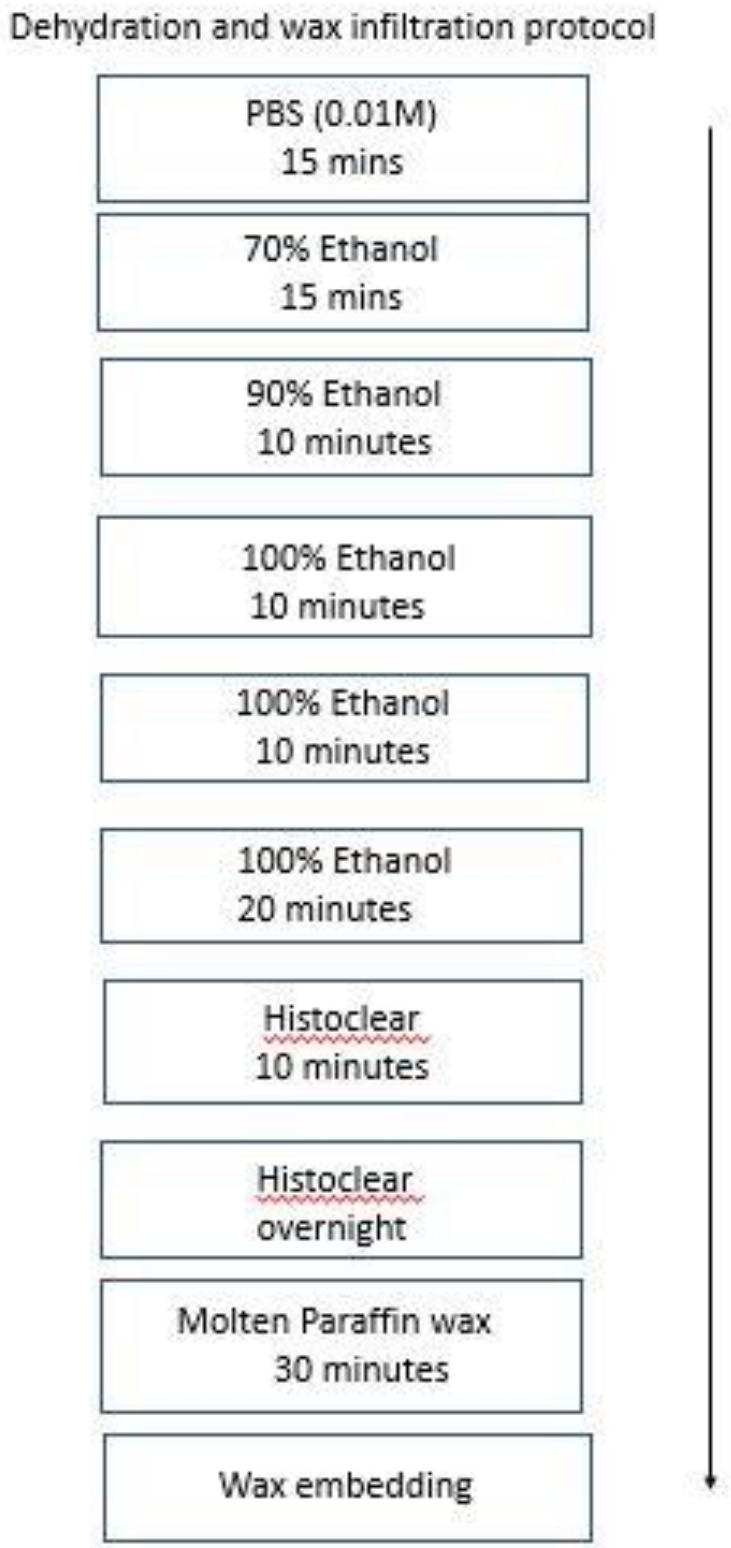


Figure 3.5 Tissue preparation and wax embedding protocol

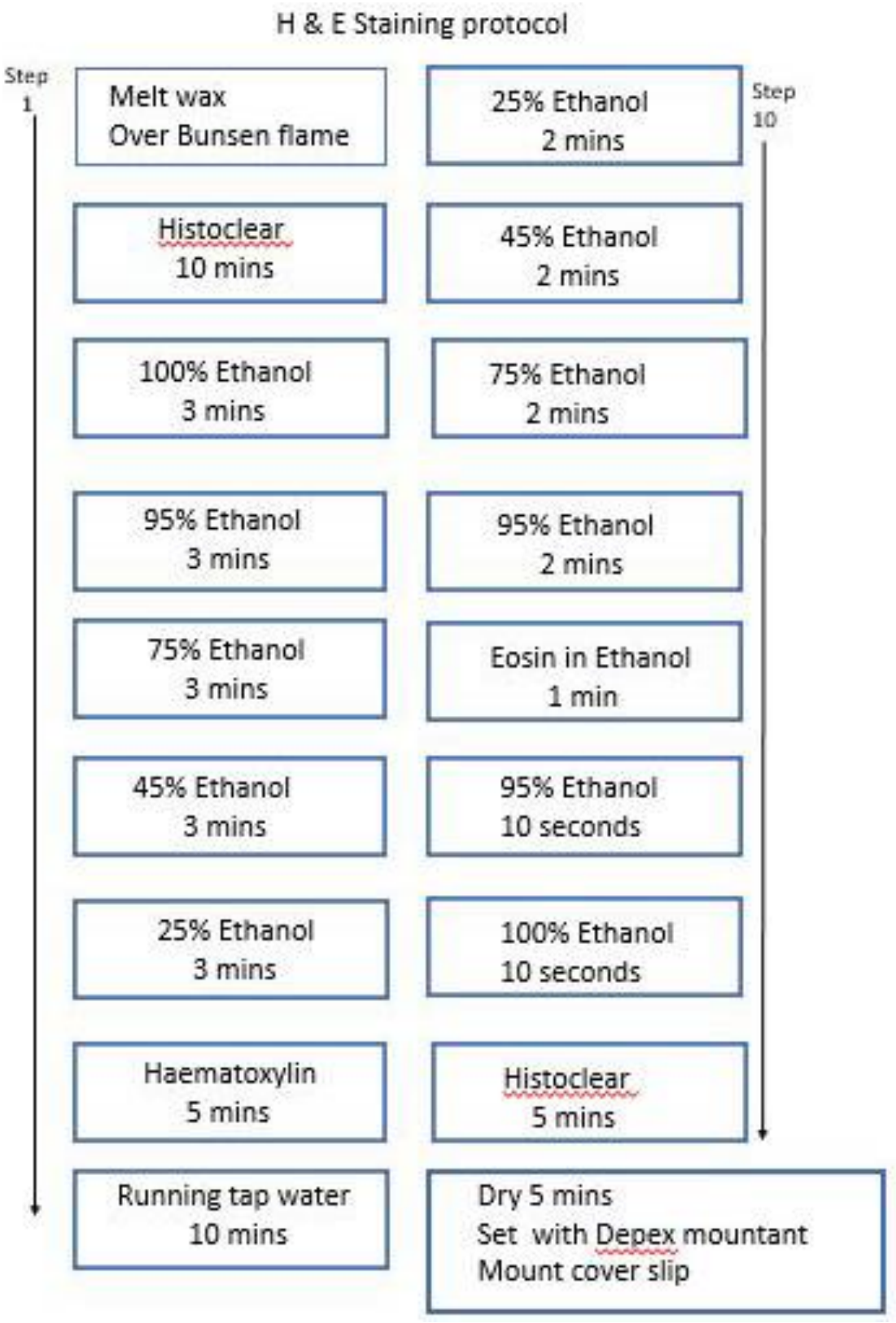


Figure 3.6 Staining protocol

Chapter 4. Determination of the bioavailability of ingested E2 in mussels, via aqueous or attached to SECs exposure, using histology/microscopy, analytical chemistry and molecular biomarker approaches

4.1. Introduction

In order for the E2 to be bioavailable, the SECs must have to be ingested by the mussels. Therefore, the tissue needs to be examined histologically to determine if this is the case and confirm uptake. Although E2 is treated within the SECs, this may or may not be bioavailable to the mussels after ingestion. Bioavailability may be indicated by the several techniques such as the presence of conjugated E2 (Janer *et al.* 2005; Peck *et al.* 2007). In this case, the mussel bio-transforms E2 to the more polar conjugates that are more readily excreted (Ciocan *et al.* 2010; Zabrzańska *et al.* 2015; Schwarz *et al.* 2016). This may be inferred by hydrolysing the tissue extract to convert the conjugated E2 back into E2. An increase in the amount of E2 present in a hydrolysed extract (total E2) compared to the non-hydrolysed extract (free E2) is an indicator that conjugation has taken place. Another indication of bioavailability of E2 attached to SECs would be a change in gene expression by the ER genes in the mussel tissues. This can be evaluated using gene expression and qPCR techniques (Puinean *et al.* 2006; Ciocan *et al.* 2010).

As E2 is hydrophobic (Thomas and Potter, 2013) there is the possibility that the E2 introduced into the tanks may be precipitated onto the glass of the tank, therefore reducing the quantity of E2 in solution. This would have an effect on the potential amount of E2 that the mussels could ingest and therefore the any potential bioavailability of E2. A sample of each of the water from the tanks was analysed half an hour after the initial spiking and at the end of 3 days when the water was changed to ascertain how much of the E2 remained. It is not feasible to extract E2 from the walls of the tanks, air stones and aeration tubing used in the exposure experiment. Instead, the aqueous solutions of E2 stored in the bottles used in the experiments were extracted and the glass walls of the bottles were also rinsed in a solvent to compare the amount of residual E2 on the glass surface and in the water upon storage over a period of days.

The amount of E2 treated within the SECs used in the experiment was estimated by extracting the SECs with solvent to estimate the potential dosage of E2 if the mussels were to ingest them. The water rinsing from the preparation of the SECs were also extracted to estimate the amount of E2 that is not treated or retained during the preparation.

The filter papers used in filtering the water solutions were retained and any trapped particulates were removed in order to determine how many of the SECs were suspended in the water, half an hour after the spiking and three days later when the water was changed.

4.2. Methods

4.2.1. Determination of SECs uptake in gonad tissues and changes in tissue biochemistry using fluorescence microscopy.

Both pollen grains and SECs emit auto-fluorescence with wavelengths that are organelle specific (Pohler *et al*. 2012). SECs emit intense fluorescence in green and red channel (Park *et al.* 2016). Fluorescence microscopy irradiates specimens with specific bands of wavelengths and separates the emitted fluorescence from the excitation light. The resulting emission light is detected, resulting in the fluorescent structure superimposed with high contrast against a dark background. Fluorescence microscopes are equipped with a set of filters to allow the specimen to be illuminated in defined directions. The disadvantage to this type of microscopy is a limited depth of field with thick sections and a resulting poor contrast of the image (Park *et al*. 2005).

In order to ascertain the best filters to visualise the SECs, a micro spatula of SECs was placed on a microscope slide and a drop of Histoclear (National Diagnostics, Nottingham, UK) was added. The SECs and Histoclear were mixed and spread out in a thin layer using a pipette tip. The Histoclear was allowed to dry off in a fume cupboard and the slide was covered in a cover slip and secured with DPX Mountant, 06522, (Merck, Darmstadt) or clear nail varnish. The fluorescence microscope used was an Olympus IX71 inverted microscope with 10x magnification and 40x objective, capturing the image with cellsens entry software the best filter was the YFP filter, which gave a green image (Figure 4.1).

Also, a micro spatula of the SECs was mixed in molten wax to produce a wax section, using the same method as to prepare the sections to visualise the effects of the sectioning and staining processes on the SECs that may be embedded in tissues (Figure 4.2). The resulting images of the sectioned and stained SECs produced images that lacked the defining structure of the untreated SECs. The SECs do not appear to take up the E & H stain. When the SECs are embedded in wax and sectioned the image is blurred due to focusing on images with no fixed depth of field. Also, the SECs structural integrity may be impaired upon desiccation and sectioning and there may be possible collapse in the SECs's morphology (Park *et al*. 2016).

Unfortunately, the eosin and haematoxylin stains also fluoresced producing a high background level when examining the stained tissue sections so extra sections were prepared without any staining using the protocols in Chapter 3. A random selection of ten wax embedded gonad tissue samples were selected from each of the controls, E2 exposed, SECs treated and SECs with E2 treated samples. These were sectioned as 5 µm sections and were treated without staining. The slides were examined under the microscope to see if there were any fluorescent spots that were of a similar diameter to the sectioned SECs slide. Also, slides were prepared from gill and digestive tract tissue from controls and SECs exposed samples.

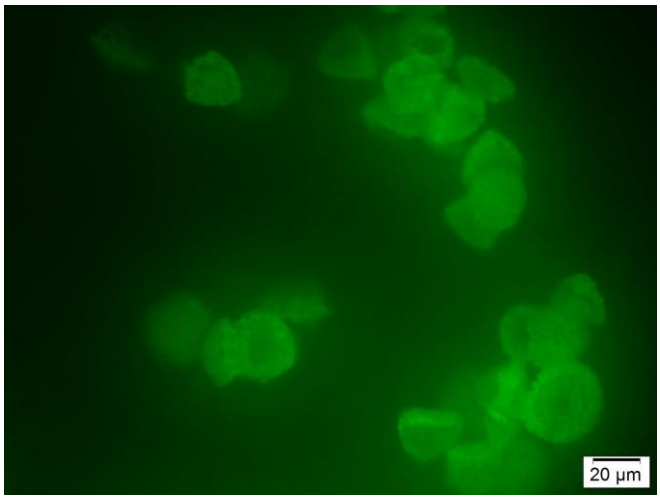


Figure 4.1 Untreated SECs using the YFP filter

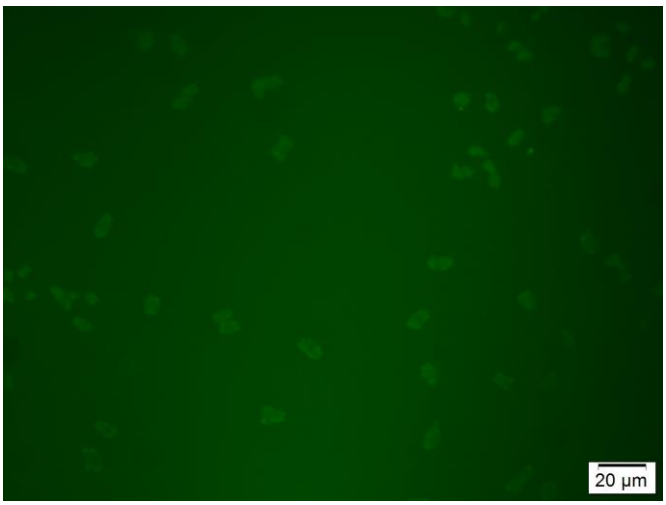


Figure 4.2 SECs that have been embedded in wax and E & H stained using a YFP filter

4.2.2. Chemical analysis methods for determining the presence of conjugated E2 in mussel tissues and encapsulation of E2 by SECs

4.2.3. Introduction

Extraction of E2 from mussel tissue may also extract E2 from the ingested SECs to some degree, or completely, so any level of E2 found in extraction of tissues may not necessary be E2 that is bioavailable. On exposure to E2, mussels it is likely that mussels may reduce levels of E2 in their system by biotransformation to the conjugated forms and the presence of these forms may be an indicator of bioavailability. Therefore, a portion of the tissue must be hydrolysed by either chemical hydrolysis or enzyme hydrolysis, in order to convert any conjugated E2 back into free E2. Therefore, an increase in the amount of E2 in a hydrolysed sample (total E2) compared to the same sample not hydrolysed (free E2) is an indication of biotransformation taking place and therefore bioavailability.

Gonad tissue from the exposure experiments was extracted using the QuEChERS protocol from Chapter 2 and the extracts were further hydrolysed by both chemical hydrolysis protocols and enzyme hydrolysis protocols as detailed in Chapter 2. Also, the 100 ng and 200 ng E2 treated SECs were spiked into commercially purchased gonad tissue and was extracted by the QuEChERS protocol to investigate the efficiency of E2 extraction from SECs ingested by mussels.

4.2.3.1. E2 analysis of E2 adsorption by SECs using GC-MS

Analysis was by GC-MS with derivatization with MSTFA activated with iodine 50992 10 x 1 mL ampoules (Merck, Darmstadt, Germany). 500 µL of anhydrous methanol was added to the dried extract in the test tube and the contents were vortexed for 30 seconds. This solution was transferred to a 2 mL auto-sampler vial and the contents were evaporated to dryness by placing in a condenser for 30 minutes at 30° C. 50 µL of the MSTFA derivatizing reagent was added to the dried extract in the auto-sampler vial and vortexed for 20 seconds. The vial was placed in a heating block at 60° C for 30 minutes. 50 µL of heptane was added to the cooled contents and the solution was vortexed for 20 seconds and removed using a glass Pasteur pipette into a 200 µL glass insert which was replaced back in the same vial. The derivatized extract was run on the GC-MS Agilent 6890N GC with a 5973 Mass Selector in SIM mode (Agilent Technologies Ltd, Cheadle, UK) In order to estimate the amount of recovered extracted E2, an external calibration graph was produced by derivatizing E2 in eight duplicate concentration levels from 0 ng to 1000 ng.

The GC-MS method was as follows, the GC-MS column was a 30 metre HP5-MS capillary column of 0.25 µm film thickness and diameter of 250 µm. The helium carrier gas flow was 1.3

mL/minute. Injection was a pulsed splitless injection with a pulse pressure of 25 psi and pulse time of 1 minute. The inlet was at 250° C and the GC-MS transfer line was at 280° C. There was a solvent delay of 8 minutes. The oven program was an initial temperature of 90° C held for one minute, increasing at a rate of 7.5 degrees per minute until a final temperature of 290° C, this was held for 5 minutes. The GC-MS was run in SIM mode measuring ions at 416 amu with a dwell of 85. The MS source was at 230° C and the MS quad was at 150° C.

Initially the method with MSTFA derivatization produced repeatable results and a good calibration plot (Figure 4.3) but when this method was applied to derivatize spiked extracts, the peak area of the 420 amu ion used to measure the quantity of the internal d4E2 standard diminished and peaks of 416 ions were found in the blank controls.

This is most probably due to the process of hydrogen-deuterium scrambling where the deuterated protons from the d4E2 molecule used as an internal standard are interchanged with that of non-deuterated protons from either position on the molecule or protons from the solvent molecules (Cooper *et al.* 2011). As this process converts the deuterated E2 internal standard into a non-deuterated E2 molecule, the concentration of the internal standard is diminished. The isotope scrambling effect converts the internal standard to E2, and the amount of the internal standard in the sample is reduced. Therefore, the response ratio of the E2 to the internal standard is increased and the amount of E2 in the sample is over estimated. This random effect occurs in solution or in the ion source or at selected transitions in the mass spectrometer source and is not concentration dependent (Cooper *et al.,* 2011). Transition products resulting in any ion scrambling processes were not seen because the GC-MS SIM method was set up to monitor 416 and 420 ions only.

An alternative internal standard was investigated, using DES (Diethylstilbestrol), a synthetic estrogen. This steroid has similar solubility properties to E2 and the derivatized product does not co-elute with that of E2. However, an unsatisfactory calibration graph was produced (Figure 4.4) when derivatizing a range of E2 standards using DES as an internal standard. The plot is not linear and plotting just the E2 peak areas against the concentration of the E2 standards gave results that were not as good as for the external calibration of using E2 without an internal standard.

Eventually an external calibration curve, without the use of an internal standard, was used to estimate the amount of E2 extracted from the SECs and water washings from the preparation of the E2 treated SECs. The range of E2 standard dilutions was in the range of 100 ng to 1000 ng to estimate the extracts (Figure 4.5).

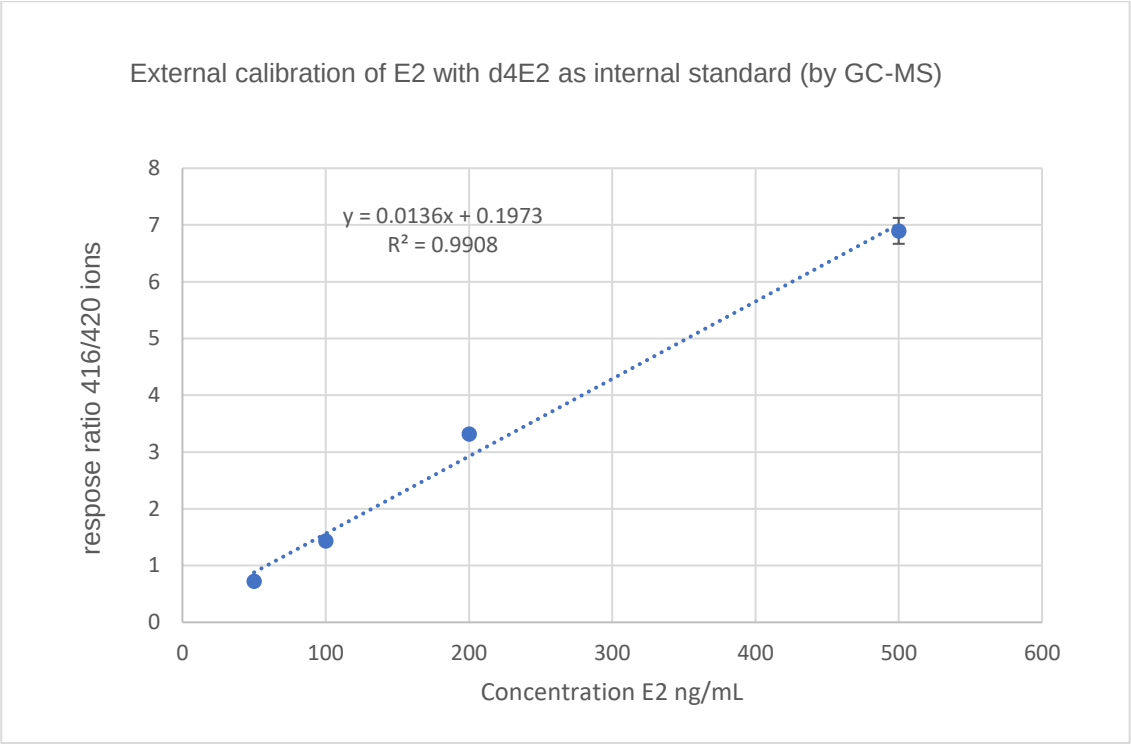


Figure 4.3 External calibration of E2 with d4E2 as an internal standard

(SEM used for error bars, n=3)

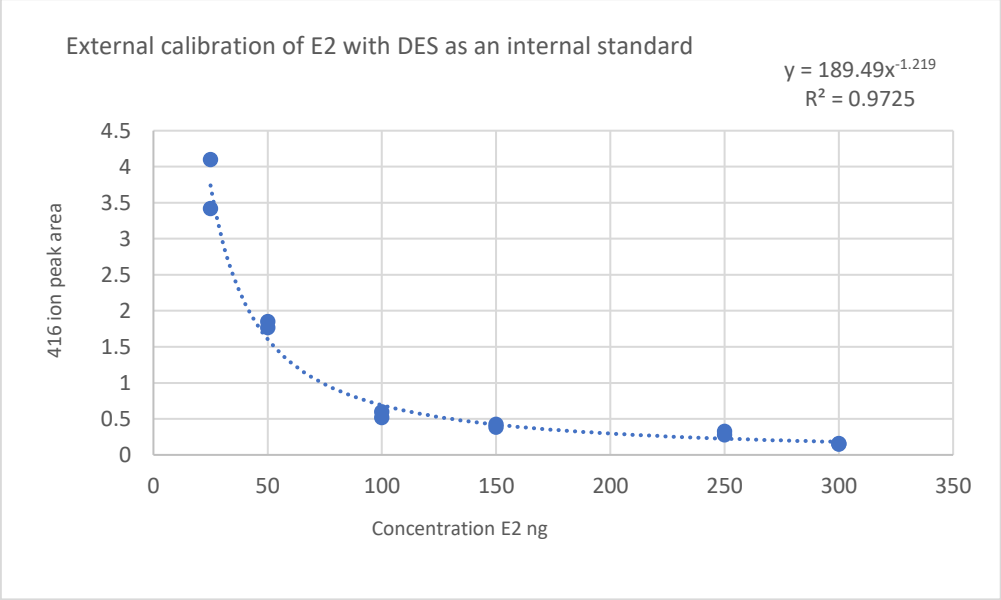


Figure 4.4 External calibration plot of E2 with DES as an internal standard

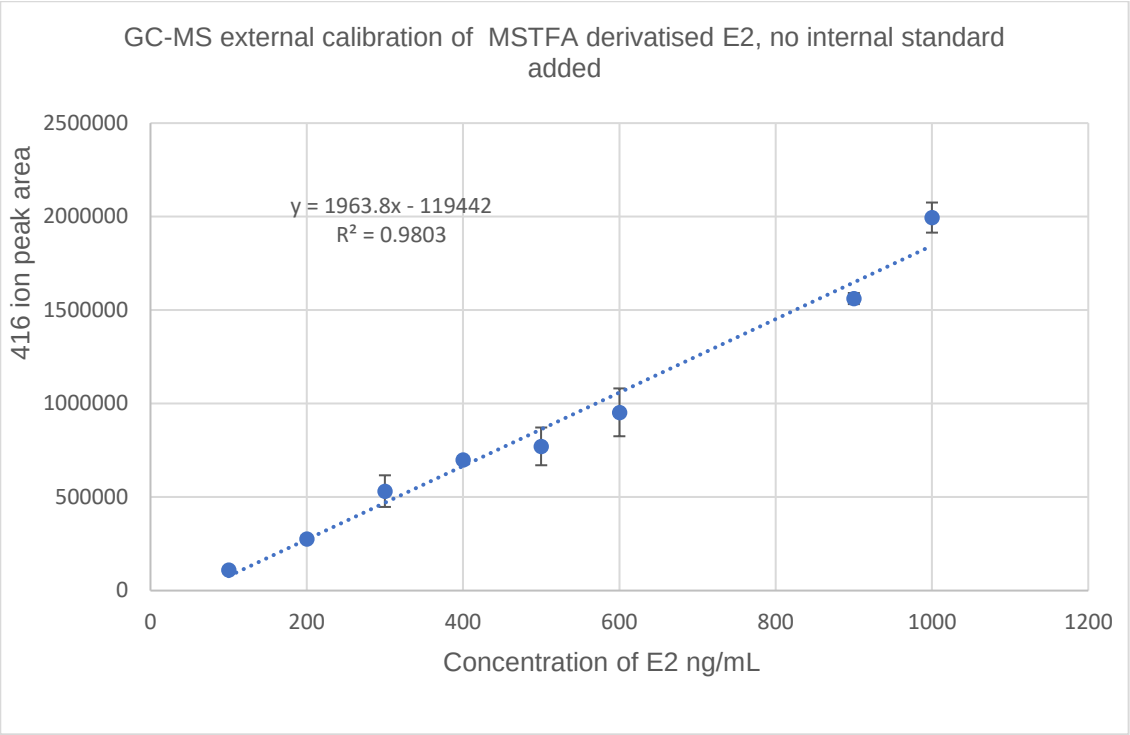


Figure 4.5 External calibration plot of E2 without an internal standard, range 100 ng to 1000 ng (SEM used for error bars, n=3)

4.2.4. Estimation of the amount of E2 in the SECs and water washings and treatment solutions.

4.2.4.1. SECs preparation

The contents of the Eppendorf tubes were removed by adding 500 µL of ethyl acetate to the loaded SECs in 1.5 mL Eppendorf tubes, vortexing the contents for 30 seconds, sonicating for 10 minutes then centrifuging at 13500 rpm for 10 minutes. The ethyl acetate was removed to a glass test tube. The SECs were extracted two more times as above, pooling the ethyl acetate extracts. A spatula of anhydrous sodium sulphate was added to the ethyl acetate extracts to remove any residual water. The dried ethyl acetate solution was evaporated to dryness over nitrogen at 40° C. The dry extract was dissolved in 500 µL of anhydrous methanol, the methanol was removed to a 2 mL sample vial and evaporated to dryness over nitrogen and kept at -20° C until analysis. The contents were derivatised with MSTFA and analysed by GC-MS.

4.2.4.2. Water washings from SECs preparation

The water from loading the SECs and subsequent washings were frozen in 1.5 mL Eppendorfs at -20° C until extraction. The washings were defrosted and 6 of the washings were pooled together, the volume adjusted to 10 mL with milliQ water and acidified to 0.2% with glacial acetic acid and 100 ng of an internal standard (DES) was added. The washings were filtered through a 45 µm cellulose acetate syringe filter and passed through a Phenomenex 3 mL C18U SPE cartridge using the standard method to extract aqueous estrogen samples.

This is as follows: -

- The cartridge was conditioned with 2 x 3 mL of methanol
- Then washed with 2 x 3 mL of 0.2% acidified water (using glacial acetic acid)
- The extract was passed through the cartridge at a slow rate.
- The cartridge was rinsed with 2 x 3 mL of milliQ water
- The cartridge was air dried for 20 minutes
- The estrogen was eluted with 3 x 3 mL of methanol and collected in a test tube
- The solvent was reduced down using a rotary evaporator until a few mL of solvent were left.
- This was returned to the test tube and the contents were evaporated to dryness under a flow of nitrogen at 40° C
- The dried extract was stored at -20° C until analysis.

4.2.4.3. E2 solutions from the exposure experiment and stability on storage

The 100 mL of collected water was extracted using the SPE method above for the water washings and the dried extracts were stored at -20° C until analysis. The water from these bottles was extracted using liquid/liquid extraction with 3 x 15 mL of ethyl acetate in a 250 mL separating funnel and the three ethyl acetate extracts were pooled together in a 50 mL conical flask to which a spatula of anhydrous sodium sulphate drying agent was added.

The empty glass bottles were rinsed with 15 mL of ethyl acetate and this was placed in a 50 mL conical flask to which a spatula of anhydrous sodium sulphate drying agent was added. All of the dried extracts were evaporated to dryness on a rotary evaporator. Then 2 x 500 µL of anhydrous methanol was added to the dried residue in the rotary evaporator flask and the dissolved contents were transferred to a 2 mL auto-sampler vial and the contents were evaporated on a condenser at 30° C for 30 minutes.

4.2.4.4. Water analysis by LC-MS-MS to ascertain the quantity of residual E2 during the exposure experiment

The water samples from the exposure experiment were extracted using SPE with a C18 cartridge following the protocol from 2.1(2.1; page 44) and eluted using methanol and stored at -20° C until analysis by LC-MS-MS.

The methanol extracts were placed in 5 mL Pyrex test tubes with Teflon lined screw tops and the contents evaporated to dryness at 40° C under a flow of nitrogen. The dried extract was derivatised using dansyl chloride using the protocol described in (2.7.3; page 61) and the reaction mixture was placed in a 200 µL glass insert in a 2 mL autosampler vial and stored at -20° C until analysis.

For LC-MS-MS analysis using the Shimadzu LC-MS-MS, 10 µL of the reaction mixture was withdrawn from the autosampler vial and desalted using the protocol from (2.11.3; page 85) and eluted in 1 mL of acetonitrile and 10 µL was injected for analysis. The method for the analysis using the Shimadzu and instrument parameters are detailed in Chapter 2 (2.11.2; page 84).

4.2.4.5. Particulates recovered from the water filtrates to determine the presence and relative amounts of SECs suspended in the water samples

For SPE extraction of E2 from the water samples, it was necessary to filter the water first using 0.45 µm cellulose acetate filter paper under vacuum. These used papers were stored in petri dishes at 5° C the SECs and any other particulates that had been trapped on the papers were recovered by the following method. The papers were folded and pushed down into 15 mL plastic centrifuge tubes so that the surface containing the trapped SECs was facing inwards towards the centre of the tubes and the paper was about 1 cm from the bottom of the tube. Then 10 mL of milliQ water was added, fully immersing the papers and the contents were shaken at 1250 rpm on a Heidoplh Reax shaker for one hour. The tubes were then centrifuged at 5000 rpm for 30 minutes. Most of the water was removed by carefully pipetting it away leaving about 100 µL above any pellet. This pellet was re-suspended in the residual water and all of the contents were removed with a glass pipette and placed on a microscope slide and allowed to air dry and covered with a cover slip. The slides were examined and photographed using the Olympus IX71 inverted fluorescence microscope in brightfield and YFP fluorescence filters at x10 magnification.

The particulates that had adhered to the cellulose acetate filter papers were examined using the Fluorescent microscope in brightfield and YFP fluorescent filters at x10 magnification. The same slides were also photographed in direct ambient light using an Olympus S2X10 microscope at low magnification (x6.4).

4.2.4.6. Tissue extract analysis from the exposure experiment with analysis by LC MS-MS

The tissues for each sex of each of the four treatments were pooled together, omitting any of the samples whose sex could not be determined due to the lack of visible gametes in the histology sections. Some samples did not have any saved gonad tissue due to the small amount of tissue excised during dissection, priority being given to save tissue for RNA extraction and histology. The pooled tissue was collected in pre-weighed 20 mL glass universal bottles, re-weighed and 1 mL of milliQ water was added to aid homogenisation. The homogenised tissue was then freeze dried and weighed again. The weight of the empty universal bottle was subtracted from the weight of the universals containing the wet and freeze-dried tissue to provide information on the percentage water in the tissue and available dry weight for E2 extraction. The freeze-dried tissue was ground to a fine powder in a coffee mill for 30 seconds and stored at -20° C until analysis, the mill being cleaned with ethanol between samples to ensure no cross contamination.

The extraction protocol to extract free and total E2 from the gonad tissue is explained in detail in Chapter 2. In summary, the eventual protocol to extract each of the samples was to initially extract, in triplicate, 25 mg of powdered freeze-dried tissue per sample with methanol and ethyl acetate. The volume of the extractions was made up to 3 mL with ethyl acetate and split into a 1 mL part for total E2 extraction and into a 2 mL part for free E2 extraction. The total E2 extraction portion was hydrolysed chemically according to the protocol in Chapter 2. At this stage the IS was added. Both free and total E2 was extracted by the optimised QuEChERS extraction protocol (Appendix 2; 2.1 page IX), the yellow pigment removed by using a SEP PAK® cartridge and the dry extract derivatised with dansyl chloride and desalted before injection into the Shimadzu LC-MS-MS instrument.

4.2.5. Using *ER* mRNA expression levels as a biomarker of E2 bioavailability in mussel tissues

4.2.5.1. Introduction

Bivalve response to exogenous estrogens at early stages of gametogenesis displays an increase in ER mRNA expression (Ciocan *et al.* 2011). Therefore, if E2 is bioavailable from E2 treated SECs, there should be a higher level of mRNA expression in SECs plus E2 samples compared to control or SECs with no E2 samples. In order to obtain good quality, reliable data the RNA extraction and subsequent qPCR reactions were done following MIQE guidelines (ABI Guide 117GU17-01,2004; Bustin *et al.* 2009). From the histology data, only males in the early stages of gametogenesis were selected for gene expression analysis for each of the four treatments. These samples were randomised before RNA extraction so that the experiments were performed blind.

From the RNA extracted from the tissues, cDNA was generated. This was quantified by the technique of qPCR. This is a two-stage process that involves the extraction and quantification of the RNA followed by the generation of the cDNA. The second stage amplifies the cDNA generated which is labelled with a SYBR green fluorescent dye. As the cDNA concentration increases during thermal cycling, the fluorescence increases, the amount of fluorescence being used as a measure of DNA concentration indirectly. A C_T value indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The threshold is chosen at a level that ensures that the PCR is in the exponential phase of amplification. These C_T data values are used to indicate the level of gene expression for the sample. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction, therefore lower the C_T value the greater the amount of amplicon (Schmittgen and Livak, 2008).

4.2.5.2. RNA extraction

The samples selected were randomised and allocated sample numbers to be used for the whole of the experiment. Total RNA was isolated from gonadal tissue using a High Pure RNA tissue kit (High Pure RNA Isolation Kit 11 828 665 001, Roche Applied Science, 68298 Mannheim, Germany), About 10 mg of tissue was homogenised in 400 µL of lysis binding buffer in autoclaved 2 mL Eppendorf tubes and stored on ice during the extraction procedure. The homogenised sample was centrifuged at 13,000 rpm for 2 minutes and the supernatant was transferred to a new autoclaved 1.5 mL Eppendorf tube and 200 µL of ethanol was added and the contents mixed. The mixture was then transferred to the top of a filter cartridge from the kit and briefly centrifuged and the collected liquid was discarded. DNase enzyme (10 µL, 0.182 ku) in 90 µL of DNase buffer was added to remove all of the DNA on the filter. The reaction was left on the cartridge for 15 minutes at room temperature. The contents on the filter were then twice washed with 500 µL of buffer followed by 300 µL of buffer, the liquid passing through the cartridge being discarded each time. After the final wash, the tubes were centrifuged at 13,000 rpm for 2 minutes and the cartridge was transferred across to a new 1.5 mL Eppendorf tube and the residual RNA was eluted with 50 µL of PCR grade water. The eluent was left on the cartridge for 1 minute to soak through then centrifuged for 1 minute. In order to elute as much of the RNA as possible, the eluent was passed through the filter again.

The RNA concentration was measured using a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Basingstoke, UK), 1 µL of the RNA extract was placed in a Qubit assay tube to measure its concentration using the rest of the extract was immediately frozen at -80° C in 1.5 mL Eppendorfs.

The Qubit measurement uses RNA BR buffer (Invitrogen, Thermo Fisher Scientific, Basingstoke, UK) and is calibrated using 2 standards freshly made each time measurements are

taken. The buffer is made up as a master mix of 200 µL of buffer and 1 µL of dye per sample. Each standard is made up of 190 µL master mix and 10 µL of standard and each sample is made up of 199 µL mix and 1 µL of RNA extract. RNA concentration is measured as µg/mL and only the highest quality of RNA can be used for cDNA generation and qPCR amplification. Only RNA of high quality was used with a minimum concentration of 25 µg/mL. The concentration of RNA being diluted so that there was the same amount of RNA per reaction i.e. 225 ng in 9 µL of molecular grade quality water.

4.2.5.3. cDNA preparation

In order to eliminate any contamination, all of the PCR reactions were performed in a UVT-B-AR UV cabinet for PCR operations (Grant-Bio, Grant Instruments, Cambridge, UK), using a range of dedicated pipettors with gamma irradiated filter tips (Greiner, Greiner Bio-One International, Kremsmünster, Austria). The pipettors, aliquots of PCR water, tube racks, plastic tubes and tips were irradiated with UV light for 15 minutes before the addition of the reagents was performed. The cDNA was produced from the RNA extract using the reverse transcription reagents from the Precision nanoscript 2 Reverse transcription kit, (PrimerDesign (Primerdesign Ltd, Camberley, UK).50 reactions RT-nanoscript, 2-RT-Nanoscript2). The same amount of RNA is added to each reaction (225 ng).

The generation of cDNA is in two steps; the first step is to make a mixture of the RNA template, 1 µL of reductase RT primer and sufficient RNase free water to make the total volume 10 µL. This mixture is annealed at 65° C for 5 minutes and then removed from the 65° C heating block and put directly onto ice. The second step is the extension step a master mix is made of 5 µL nanoscript 4x buffer, 3 µL dNTP mix 10mM, 3 µL RNase free water and 1 µL of nanoscript enzyme (160 units) giving a total volume of 10 µL per sample. This is mixed with the RNA and the contents pulse spinned down. This mixture is then incubated at 25° C for 5 minutes, followed by 42° C for 20 minutes. The mixture is then heat in activated at 75° C for 10 minutes. The resulting cDNA mixture is stored at -20° C. A few RNA samples were randomly selected to generate no-reverse transcription negative controls to confirm that there was no residual DNA in the RNA extraction process. This was done by replacing the 1 µL of nanoscript enzyme with 1 µL of water.

4.2.5.4. Primer optimisations

The primers used were two reference genes or housekeeping genes, that are expressed at all time. These reference genes are used to normalise the real time RT-PCR data generated. The primers were obtained from Integrated DNA technologies (IDT) ((Integrated DNA Technologies, Leuven, Belgium) as lyophilised powders. These primers were diluted with molecular grade quality

water (Molecular Biology Reagent water, Merck, Darmstadt, Germany) to a concentration of 100 µM and stored as 10 µL aliquots at -20° C.

18Sr RNA, a ribosomal gene that is basic, conserved gene found in all eukaryotic cells

- Sequence of 1*8S* forward
  5' CAT TAG TCA AGA ACG AAA GTC AGA G-3'
- Sequence of *18S* reverse
  5' GCC TGC CGA GTC ATT GAA G-3'

EF1, an elongation gene, a conserved gene for protein synthesis

- Sequence of the *EF1* forward gene is
- 5'- CAC CAC GAG TCT CTC CCA GA-3'
- Sequence of the *EF1* reverse gene is
- 5' GCT GTC ACC ACA GAC CAT TCC -3'

The gene used to determine E2 induced gene expression is the *ER2* gene

- Sequence of *ER2* forward
- 5'-GGA ACA CAA AGA AAA GAA AGG AAG -3'
- Sequence of *ER2* reverse
  - 5' GCT GGA TTA GGA CTG CCA CTT G-3'

The primers were initially optimised for using 2 µL of a forward and reverse primer mix in a 20 µL qPCR reaction. The optimum concentrations were 100 nm for the *18S* and *EF1* primers and 500 nm for the *ER2* primer. For each time that a qPCR reaction was performed fresh aliquots of the 100 µm primer stocks were diluted in 95 µL or 98 µL of molecular quality water as follows; the reagent volumes for each 20 µL qPCR reaction was (Table 4.1) *18S* and *EF1* primers, 1 µL of forward and 1 µL of the reverse primer in 98 µL of water, *ER2* primer 5 µL of forward and 5 µL of reverse primer.

In order to test for any contamination, negative controls were done, substituting the cDNA template with 1 µL of molecular quality water. The qPCR reactions were amplified using a Bio-Rad CFX96 Real-Time PCR detection system using Hard Shell 96- Well Plates (Bio-Rad HSP9655) with Microseal B Adhesive seals (Bio-Rad MSB1001) and strip tubes (Bio-Rad low profile PCR tubes 8 strip tubes white TLS0851 with optical flat 8 cap strip for 0.2 mL tube strips/plates TCS0803). The qPCR reagents used were10 mL Precision PLUS qPCR Master Mix premixed with SYBRgreen (2-PLUS-SY-5ML) from PrimerDesign. The thermal cycling conditions are shown below (Table 4.2).

Reagent	Volume (µL) per reaction
PrecisionPLUS qPCR Master Mix with SYBR Green	10
Molecular grade PCR water	7
Forward and Reverse Primer mix	2
cDNA template	1

Table 4.1 Reagent volumes used in the qPCR reaction

Number of Cycles	Step	Time	Temperature
1	Enzyme activation hot start	2 minutes	95° C
40	Denaturation	10 seconds	95° C
40	Data collection	60 seconds	60° C
	Melt curve		

Table 4.2 Thermal cycling conditions for the qPCR reaction.

4.2.5.5. mRNA expression of target and housekeeping genes in mussel tissues exposed to E2

The fluorogenic data collected in the data step were collected through the SYBR green channel and the C_T values were obtained using Bio-Rad CFX Manager software. The amplification plots and melt curves were generated for each set of primers using the same random cDNA template for each of the primers plus a negative control. These showed amplification plots with initial low C_T values and signal melt peaks showing no primer dimerization (Figure 4.7; Figure 4.8; Figure 4.9; Figure 4.11; Figure 4.12; Figure 4.13; Figure 4.15; Figure 4.16; Figure 4.17).

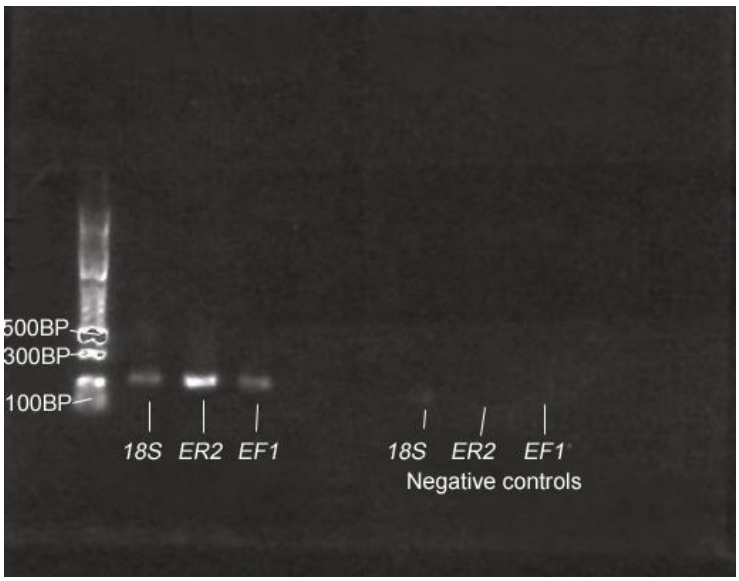


Figure 4.6 Gel of qPCR products for the primers *18S, ER2* and *EF1* with negative controls

The efficiency of the primers was investigated by performing qPCR reactions on the same cDNA template over a series of dilutions from undiluted cDNA template, over a series of serial dilutions from 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. These results were plotted on a graph of log dilution against the C_T value.(Table 4.3; Figure 4.10; Figure 4.14; Figure 4.18). The slopes of each of the regression curves were used to calculate the efficiency of the reaction, using an application from the website http://www.genomics.agilent.com/biocalculators/calcSlopeEfficiency.jsp?_requestid=851955.

The efficiency of all three primers was within the limits of the MIQE guide.

Primer Pair	Slope	Amplification	Correlation coefficient
18S Forward/Reverse	-3.4388	1.954	0.9989
EF1 Forward/Reverse	-3.1000	2.102	0.9926
ER2 Forward/Reverse	-3.055	2.125	0.9521

Table 4.3 Primer efficiency results

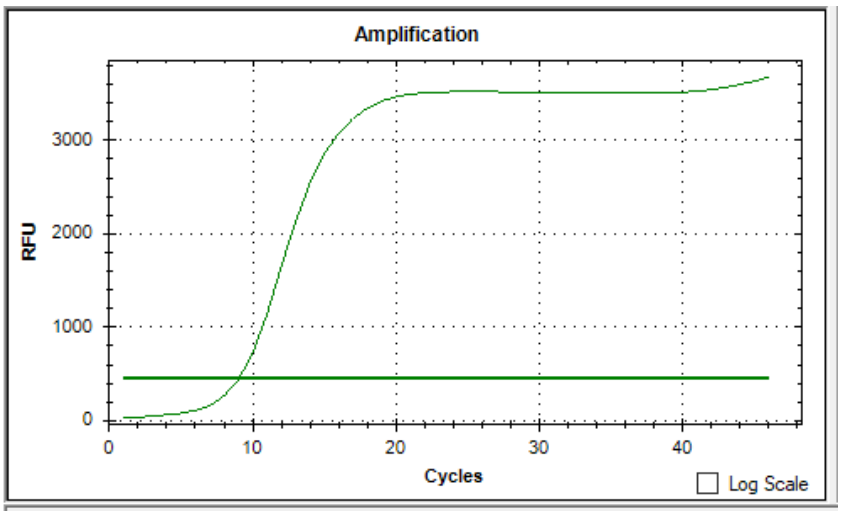


Figure 4.7 *18S* Primer, amplification plot.

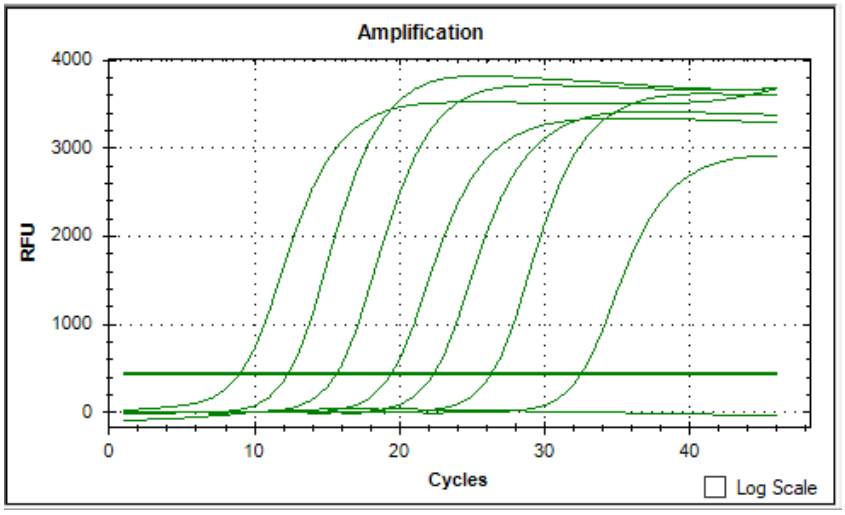


Figure 4.8 *18S* Primer, dilution series overlaid amplification plots.

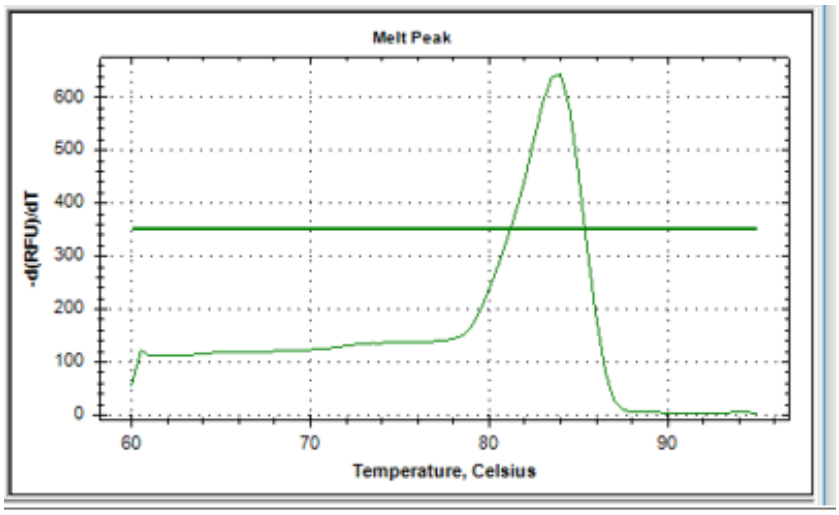


Figure 4.9 *18S* Primer, melt curve

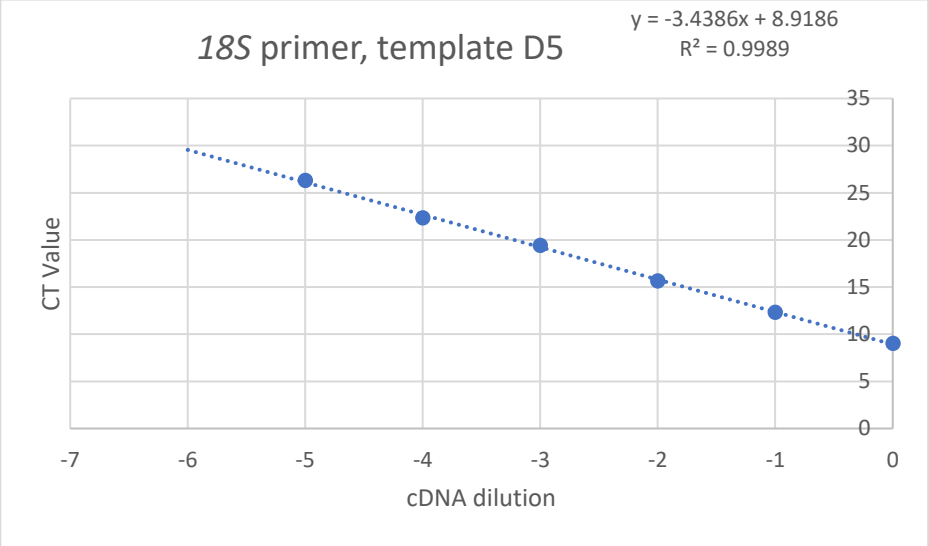


Figure 4.10 *18S* Primer, plot of log dilution against the C_T value.

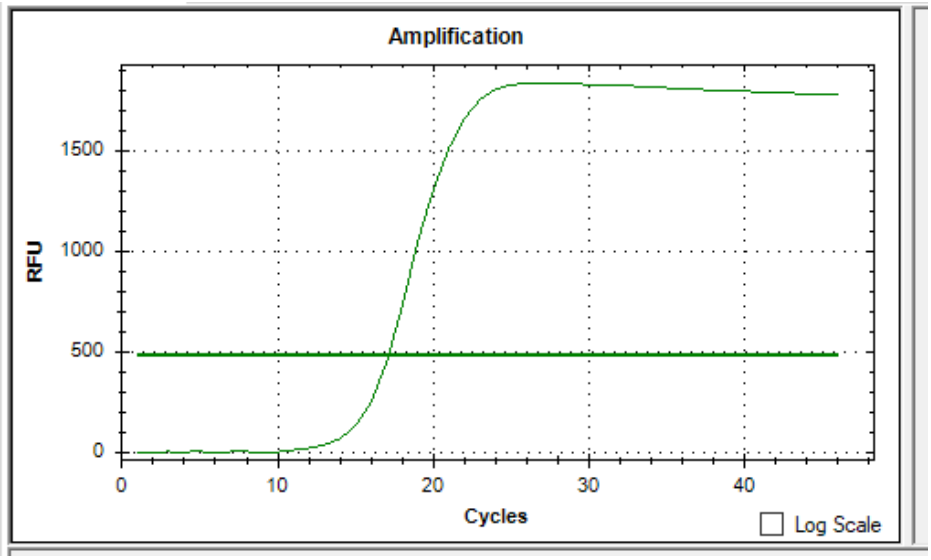


Figure 4.11 *EF1* Primer, amplification plot.

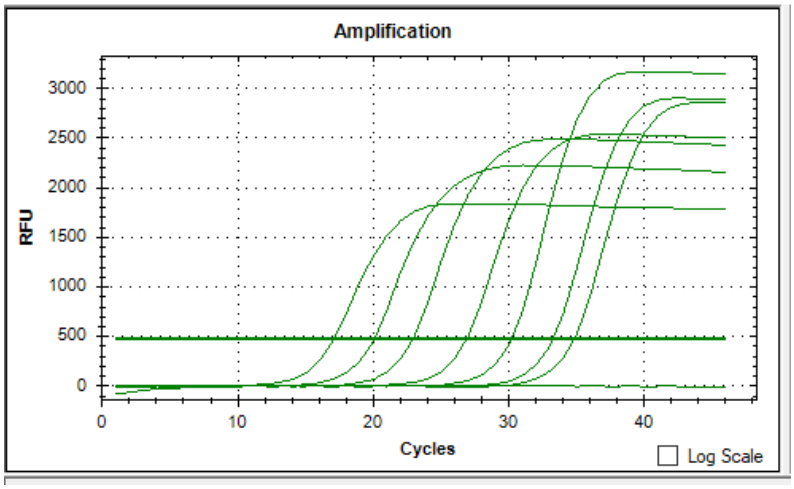


Figure 4.12 *EF1* Primer, dilution series overlaid amplification plots.

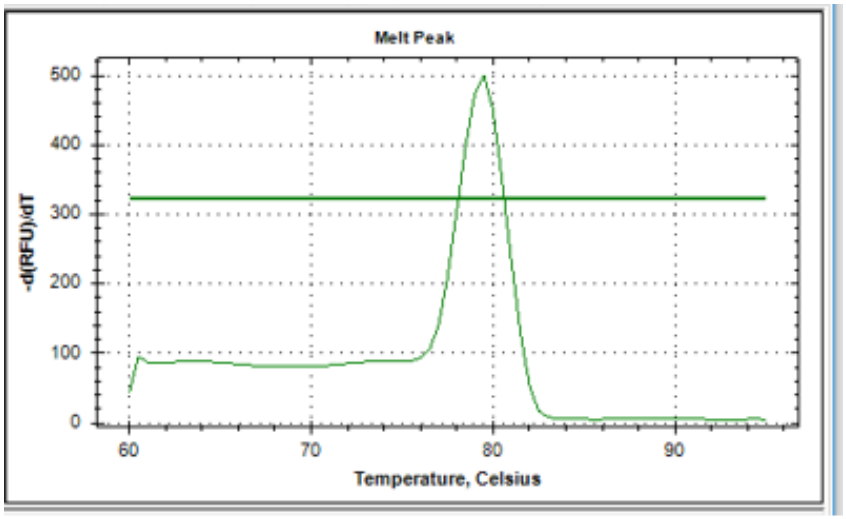


Figure 4.13 *EF1* Primer, melt curve.

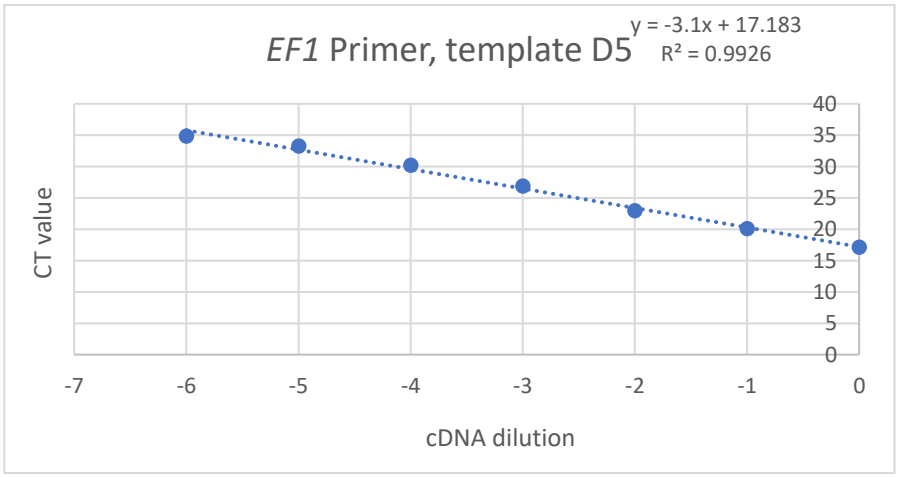


Figure 4.14 *EF1* Primer, plot of log dilution against the C_T value.

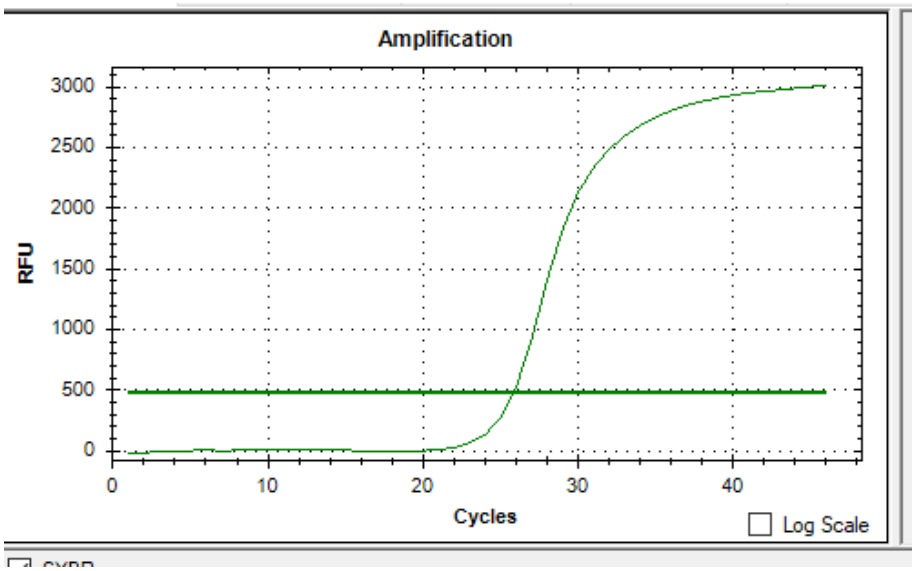


Figure 4.15 *ER2* Primer, amplification plot.

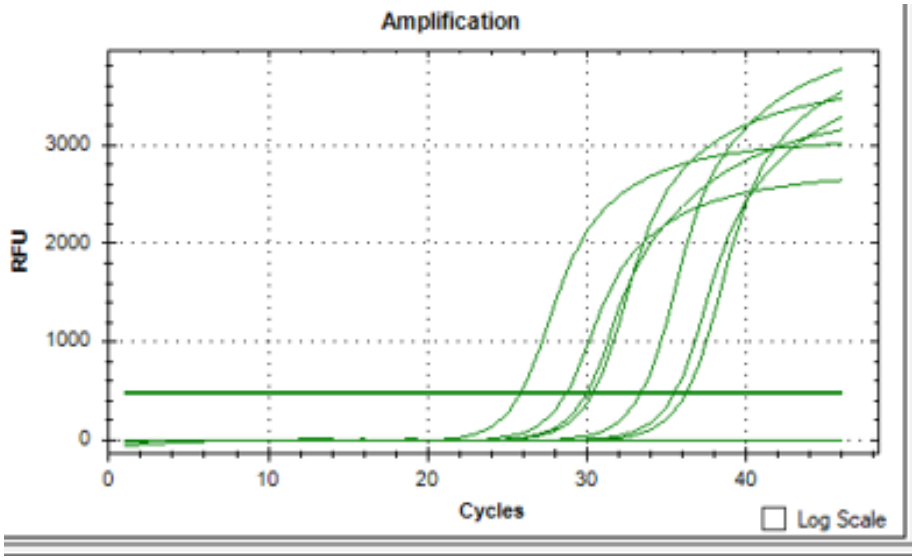


Figure 4.16 *ER2* Primer, dilution series overlaid amplification plots.

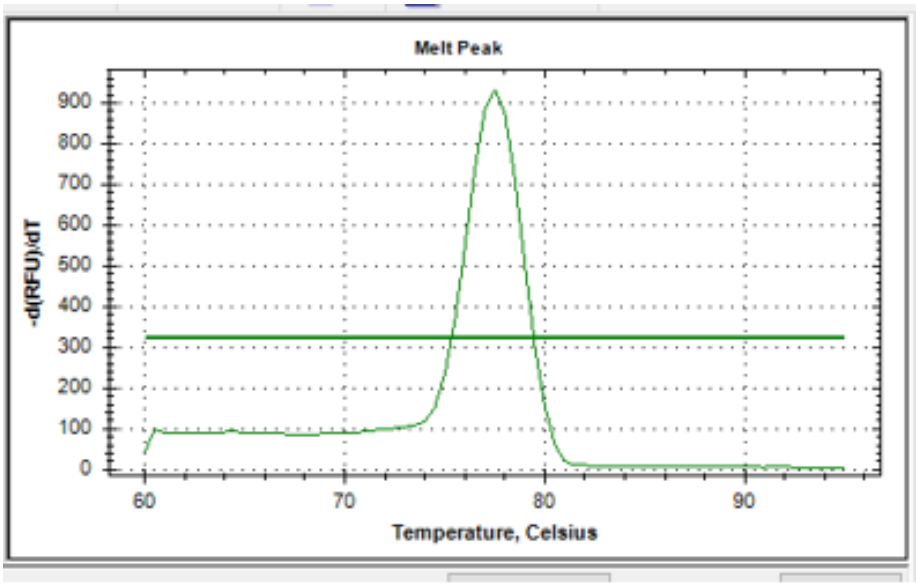


Figure 4.17 *ER2* Primer, melt curve.

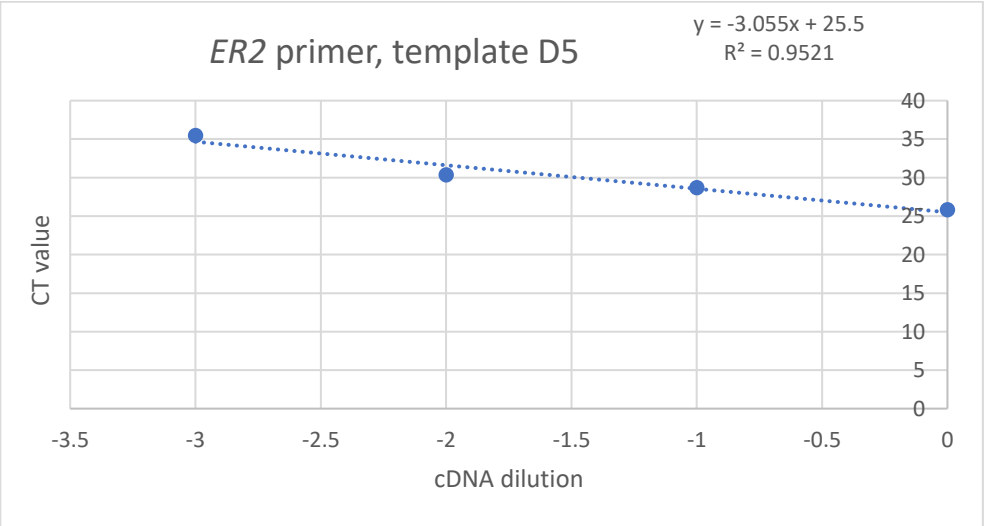


Figure 4.18 *ER2* Primer, plot of log dilution against the C_T value.

4.2.5.6. mRNA expression calculation of target and housekeeping genes in mussel tissues and statistical analyses

The raw C_T values cannot be used directly for analysis as they are not linear terms. A convenient way to analyse relative changes in gene expression from qPCR experiments is to use relative changes in expression of a target gene relative to the same reference groups such as an untreated control, where the untreated control is used as a calibrator. The resulting data is presented as fold change in gene expression normalised to the endogenous housekeeping genes

and relative to the untreated control. For treated samples the evaluation of $2^{(-\Delta\Delta CT)}$ indicates the fold change in gene expression relative to the untreated control. This relative quantification of mRNA is easier to perform than an absolute method as the generation of standard curves are not required, however there are certain criteria to be met with using this method so that the value of $\Delta\Delta C_T$ generated is valid. (Livak and Schmittgen, 2001).

- The amplification efficiency of the target and reference genes must be equal. Therefore, all of the reference genes and the target gene must have similar efficiencies as the method assumes near perfect doubling during the exponential phase of qPCR. This is indicated by the variation of C_T with template dilution.
- The initial amount of RNA per sample must be the same, so that the cDNA derived from the same amount of input RNA.

The $2^{(-\Delta\Delta CT)}$ method or Livak method is as follows (Livak and Schmittgen, 2001).

- The mean C_T values are calculated for the treated and untreated target genes and the equivalent mean C_T values for the housekeeper genes in treated and untreated samples. If more than one housekeeper genes are used, the geometric mean of these two genes are calculated.
- The data by is normalised by subtracting the C_T value of your housekeeper (the smaller C_T value) from the C_T of the target gene in the context of treated or untreated samples
- This yields two sets of normalised integers, (mean C_T Target gene Treated -mean C_T treated housekeeper) and (Mean C_T target gene untreated - mean C_T untreated housekeeper). These normalised values are referred to as ΔC_T values.
- The ΔC_T value derived from the treated sample is subtracted from the untreated sample, i.e. ((Mean C_T target treated - Mean C_T Housekeeper treated) - (Mean C_T Untreated target- Mean C_T untreated housekeeper)) = $\Delta\Delta$ C_T value.
- To compute actual relative expression i.e. fold change of the target gene in the treated sample if $\Delta\Delta C_T$ value= X then relative expression = 2 (to the power of X).

Positive values of fold change are indications that gene regulation has increased or up regulated and negative values of fold change indicate that gene regulation has decreased or down regulated and a value of zero indicates that there is no regulation. As the errors of ΔΔCT values are symmetric, standard statistical procedures can be used for testing and constructing confidence intervals.

Using the information from the histological analysis, only male *Mytilus* at the early developing or mid to late developing stages were selected for qPCR analysis. Also, the RNA extracts of these samples needed to have a concentration greater than 25 µg/mL in order to have a diluted concentration of 225 ng in 9 µL of water for the preparation of the cDNA template.

This gave the following available samples for qPCR analysis,

- Control treatment
- SECs with no other treatment
- SECs plus 200 ng E2 treatment.
- E2 200 ng/L treatment

All of the genes, that is, the reference genes and the *ER* gene were analysed in duplicate on the same plate. For each gene, a no template negative was also included. All of the C_T values greater than 35 were excluded from the analysis.

The control treatments in the exposure experiment were used as an internal reference in the calculation of mRNA expression. The data from the qPCR amplifications were analysed using the comparative C_T method ($\Delta\Delta C_T$ method) (Livak and Schmittgen (2001). The ΔC_T values were calculated by the subtraction of the geometric means of the reference genes (*18S* and *ER1*) from the average C_T value of the target gene (*ER2*). The standard deviation of the C_T values was calculated and the variance of the ΔC_T of each of the treatments was calculated from the standard deviations of the target and reference values using the formula: -

$$S = (S_T^2 + S_R^2)^{½}$$

Where S_T is the standard deviation of the target gene, *ER2*, and S_R is the standard deviation of the reference genes, *18S* and *EF1* and S is the ΔC_T Standard deviation.

The $\Delta\Delta C_T$ values are calculated by subtracting the ΔC_T of the control sample from that of the treatment samples. The standard deviation of the $\Delta\Delta C_T$ values is the same as that of the ΔC_T (Livak and Schmittgen, 2001). The fold differences relative to the control treatments are calculated by the function below

$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

Where the range for the fold change is calculated by the substituting the $\Delta\Delta C_T$ value in the above fold change function with $\Delta\Delta C_T + S$ and $\Delta\Delta C_T - S$.

All of the ΔC_T values were analysed by the Kolmogorov-Smirnov test of Normality and the result of the K-S statistic (D) was 0.28065 with a resulting p value of 0.0192 which indicated that this data is not normally distributed. Therefore, the non-parametric Krusal-Wallis test was used to analyse the *ER2* mRNA expression values for the control and three other treatments

4.3. The results of Microscopy, RT-PCR and chemical analysis to determine uptake and bioavailability of SECs and E2 in the mussel gonad tissue.

4.3.1. Confirming SEC uptake in mussel gonad tissue using microscopy

SECs were detected in the gonads of both the SECs treated without E2 and SECs treated with E2 (Figure 4.19). There were a some of the controls containing spots that were the same as those in the above treatments but these could be pollens from the environment that have fallen into the sea water when prepared and transported. There was a higher background fluorescence for the gill and digestive tissue which made it difficult to detect any SECs, so only gonad tissue was used for analysis.

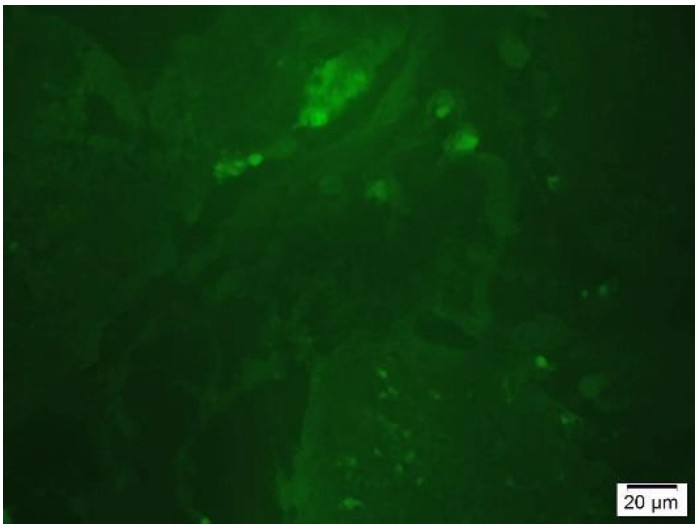


Figure 4.19 Gonad tissue that has been exposed with SECs.

In order to determine the presence of SECs within gonad tissue, unstained slides were also prepared and examined under fluorescence microscopy. The slides were viewed with the same fixed exposure settings of 2.1 seconds using the YFP filter, sensitivity at ISO 100 and resolution of 1600 x 1200. Only the focus was adjusted between the slides. Initially only ten sections of each treatment were prepared and examined and as well as showing fluorescent spots that are likely to indicate the presence of SECs in tissue, fluorescent patches were seen in some of the samples indicating that some biochemical change may be occurring. The control treatments tended to show

a dull background. Therefore, extra sections of each of the treatments were prepared and the sample size was increased to 40 of each treatment. The slides were examined impartially by relabelling the slides with new random sample numbers that could be cross referenced with the samples during analysis.

The slides were graded on the following criteria and a check list was produced for grading the slides as follows (Figure 4.20): -

- Presence or absence of fluorescent spots. In the case of the presence of spots, they should be of a similar size and shape to the SECs embedded in wax.
- Spots of a similar size and shape to the SECs embedded in wax surrounded by fluorescent patches of tissue
- Fluorescent backgrounds higher than normal or patches of fluorescence in the tissue.
  The results were tabulated as contingency tables for analysis using the Fisher Exact test.

Other comments were included on the check list for other unusual properties, for example pearl formation occurring (Figure 4.21) or faint spots or irregular shaped spots of a different size and shape to the SECs embedded in wax. The untreated slide sections were graded for the presence or absence of fluorescent spots. Here, one slide is equivalent to one gonad tissue section from one mussel, representing an individual mussel. Forty slides were analysed per treatment. Where spots were present, the slides were given a count of one and slides were no spots were detected were given a count of zero. These counts were summated for each treatment and were tabulated in a contingency table (Table 4.4).

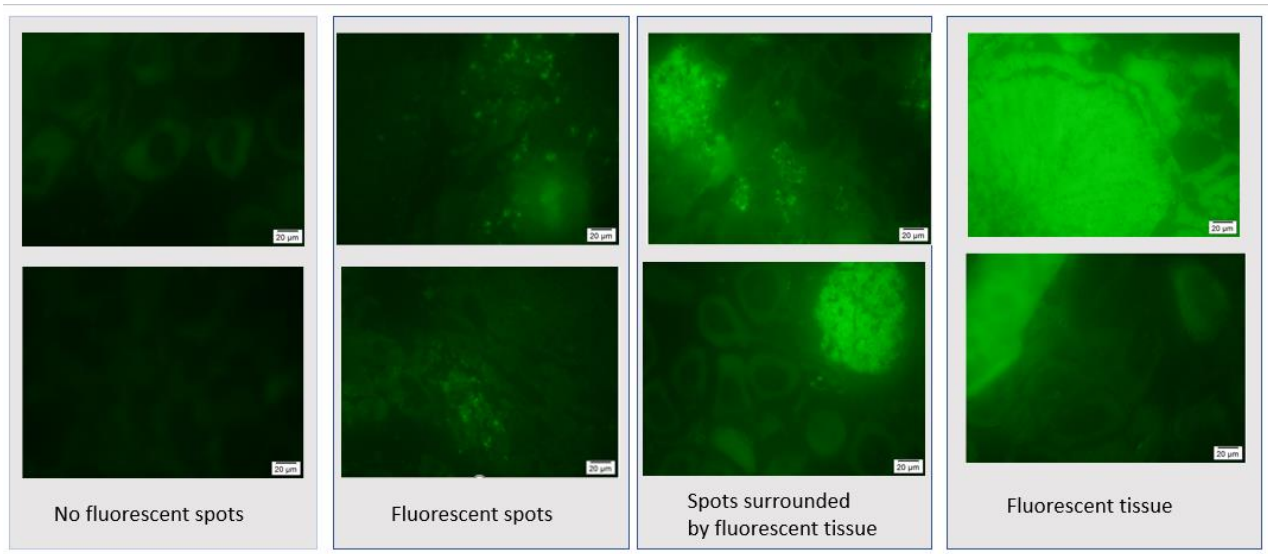


Figure 4.20 Criteria used to grade the histology sections.

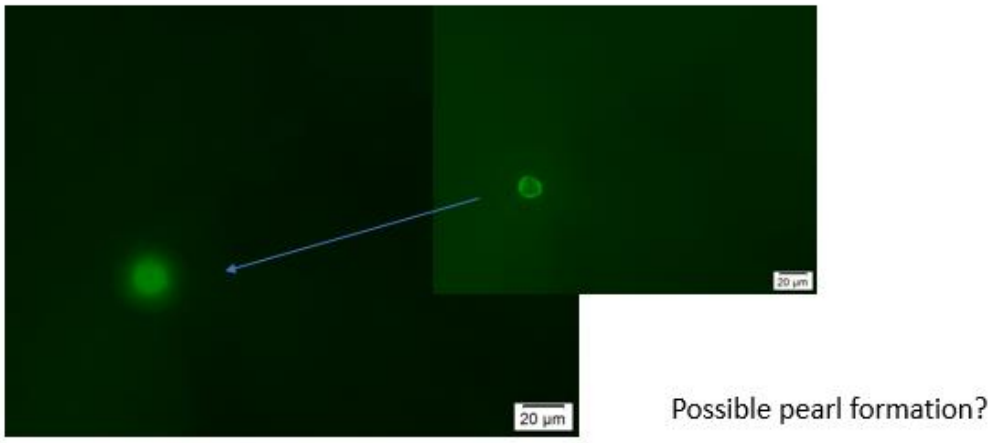


Figure 4.21 Spherical object found in the gonad section

Contingency Table for the Presence or absence of fluorescent spots				
Treatment	No Spots	Spots	Bright Spots	Faint Spots
Control	25	15	5	10
SECs no E2	10	30	22	8
SECS plus E2 (200 ng/L)	4	36	26	10
E2 200 ng/L	18	22	8	14

Table 4.4 Contingency table for presence or absence of fluorescent spots.

4.3.1.1. Analysis of all spots present in the gonad tissue

As some of the counts were less than 5, the Fisher exact test was applied rather than the chi squared test to analyse the counts,

The null hypothesis criteria are

H_o; there is no difference in the number of spots per treatment

H_1; the difference in the number of spots per treatment is significant

Using the Fisher exact test with significance levels of p= 0.05, the p values are tabulated below (Table 4.5).

Results of the Fisher exact Test for the presence or absence of fluorescent spots			
	No Spots	**Spots**	**Fisher test statistic**
Control	25	15	**0.0014**, result is significant at $p<0.05$
SECs no E2	10	30	
Control	25	15	**<0.00001**, result is significant at $p<0.05$
SECs plus E2	4	36	
Control	25	15	**0.1782**, result is not significant at $p<0.05$
E2 200 ng/L	18	22	
E2 200 ng/L	18	22	**0.1**, result is not significant at $p< 0.05$
SECs no E2	10	30	
E2 200 ng/L	18	22	**0.0009**, result is significant at $p<0.05$
SECs plus E2	4	36	
SECs no E2	10	30	**0.1395**, result is not significant at $p<0.05$
SECs plus E2	4	36	

Table 4.5 Results of the Fisher exact test for the presence or absence of fluorescent spots. Easy Fisher Exact Test Calculator (Stangroom).

The count results are also displayed as a pie chart (Figure 4.22).

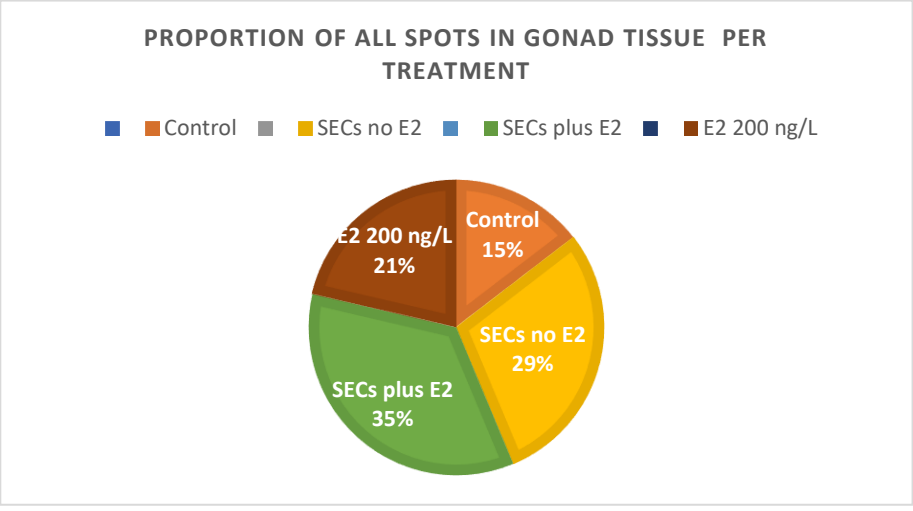


Figure 4.22 Observations of the presence or absence of all spots in the four treatments.

On initial observation it appears that there are more observed spots in the gonad tissue for the treatments where SECs have been added to the tanks compared with the control tanks and tanks spiked with E2. Statistical analysis of differences between the four treatments using the Fisher Exact test show that the differences between the number of spots between the tanks where SECs have been added and that of the tanks where SECs have not been added is statistically significant. However, there is no statistically significant difference between the tanks with E2 added and the tanks with SECs without E2 exposure

4.3.1.2. Analysis of Bright spots present in the gonad tissue

The results are different if only bright fluorescent spots are counted. For the difference in treatments where there is the presence of bright fluorescent spots the Fisher exact tests statistics are tabulated below (Table 4.6) and the count results are displayed in the pie chart (Figure 4.23). The statistical analysis shows that the difference in the number of bright fluorescent spots between treatments are statistically significant between the samples that have had SECs added during the experiment and those that have not had SECs added. The results are the same as for the presence of all spots with the exception that the difference between the tanks with E2 added and the tanks with SECs without E2 encapsulation is now statistically significant as well.

Results of the Fisher exact Test for the presence or absence of Bright fluorescent spots			
	No Spots	**Bright Spots**	**Fisher test statistic**
Control	25	5	**0**, result is significant at $p<0.05$
SECs no E2	10	22	
Control	25	5	**<0.00001**, result is significant at $p<0.05$
SECs plus E2	4	26	
Control	25	5	**0.3416**, result is not significant at $p<0.05$
E2 200 ng/L	18	8	
E2 200 ng/L	18	8	**0.0077**, result is significant at $p< 0.05$
SECs no E2	10	22	

E2 200 ng/L	18	8	**0**, result is significant at $p<0.05$
SECs plus E2	4	26	
SECs no E2	10	22	**0.1306**, result is not significant at $p<0.05$
SECs plus E2	4	26	

Table 4.6 Results of the Fisher exact test for the presence or absence of bright fluorescent spots. Easy Fisher Exact Test Calculator (Stangroom).

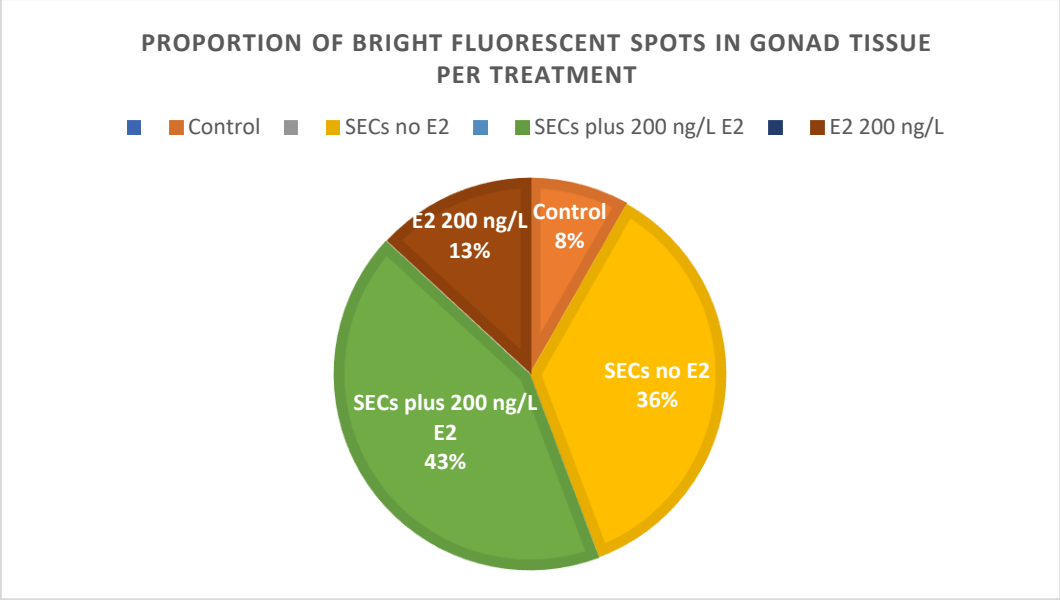


Figure 4.23 Observations of the presence or absence of fluorescent spots in the four treatments.

4.3.1.3. Analysis of faint spots present in the gonad tissue

Statistical analysis of any difference between faint fluorescent spots was also done and the contingency table and results of the Fisher exact test are tabulated (Table 4.7). In this case the presence of faint spots is not significant apart from two treatments, the control and E2 treated tanks and the control and E2 treated SECs. The presence of similar spots in both the control and E2 tanks may be due to natural pollens or particulates that may be present in the atmosphere or partially fluorescent particulates from the seawater. There are slightly less counts of SECs treated with E2 in the tanks compared to those tanks with SECs only but this is statistically insignificant.

Results of the Fisher exact Test for the presence or absence of Faint fluorescent spots			
	No Spots	**Faint Spots**	**Fisher test statistic**
Control	25	10	**0.359**, result is not significant at $p<0.05$
SECs no E2	10	8	
Control	25	10	**0.0096**, result is significant at $p<0.05$
SECs plus E2	4	10	
Control	25	10	**0.2142**, result is significant at $p<0.05$
E2 200 ng/L	18	14	
E2 200 ng/L	18	14	**1.0**, result is not significant at $p< 0.05$
SECs no E2	10	8	
E2 200 ng/L	18	14	**0.1141**, result is not significant at $p<0.05$
SECs plus E2	4	10	
SECs no E2	10	8	**0.1649**, result is not significant at $p<0.05$
SECs plus E2	4	10	

Table 4.7 Results of the Fisher exact test for the presence or absence of faint fluorescent spots.

Easy Fisher Exact Test Calculator (Stangroom).

4.3.2. Presence or absence of fluorescent tissue in the gonad tissue sections and whether this is due to the presence of ingested SECs

The untreated slide sections were graded for the presence or absence of fluorescence in the tissue and whether there was any localised fluorescence around the spots and the observations were tabulated (Table 4.8).

Contingency Table for the presence or absence of Fluorescent tissue			
Fluorescence Present	Fluorescence Not Present	Fluorescence Around Spots	Percentage Fluorescence around the spots compared to total spots
17	23	7	46.67
22	18	10	33.33
25	15	12	33.33
21	19	3	13.64

Table 4.8 Contingency table for presence or absence of fluorescent tissue.

For the presence or absence of fluorescent tissue.

The null hypothesis criteria are

H_o; there is no difference in the number of fluorescence tissue samples for each treatment

H_1; there is a difference in the number of fluorescence tissue samples for each treatment

Using the Fisher exact test with significance levels of p= 0.05, the *p* values are tabulated (Table 4.9). For all the observations, the null hypothesis is accepted and the presence of fluorescent tissue in the slides between treatments has no statistical significance.

Results of the Fisher exact Test for the presence or absence of fluorescence in tissue			
	No Fluorescence	**Fluorescence**	**Fisher test statistic**
Control	23	17	**0.3771**, result is not significant at $p<0.05$
SECs no E2	18	22	
Control	23	17	**0.1165**, result is not significant at $p<0.05$
SECs plus E2	15	25	
Control	23	17	**0.5021**, result is not significant at $p<0.05$
E2 200 ng/L	19	21	
E2 200 ng/L	19	21	**1.0**, result is not significant at $p< 0.05$
SECs no E2	18	22	
E2 200 ng/L	18	22	**0.4978**, result is not significant at $p<0.05$
SECs plus E2 200 ng/L	15	25	
SECs no E2	18	22	**0.65**, result is not significant at $p<0.05$
SECs plus E2 200 ng/L	15	25	

Table 4.9 Results of the Fisher exact test for the presence or absence of fluorescence in tissue. Easy Fisher Exact Test Calculator (Stangroom).

4.3.3. Particulates recovered from the water filtrates to determine the presence and relative amounts of SECs suspended in the water samples

The SECs are clearly visible at x10 magnification in both brightfield and fluorescent light. There were a considerable number of SECs trapped from all of the water that had been sampled half an hour after addition of both of the SEC treatments. The waste water collected 3 days after treatment also showed a reduced, but still considerable number of SECs.

For the waters samples that had no SECs added, there are no visible SECs in both brightfield light or fluorescent light but in fluorescent light there are other fluorescent particulates of a similar diameter. All of the waste water samples had other particulates including bits of byssal thread and algae as well as some fluorescent particulates that are possibly sediment particulates as they are more angular in shape than that of the SECs (Figure 4.24; Figure 4.25;Figure 4.26).

The same slides were also photographed in direct ambient light using an Olympus S2X10 microscope at low magnification (x6.4) which avoids the fringing effects of the Olympus IX71 inverted fluorescence microscope in brightfield, where the light passes through the slide, rather than onto the specimen on the slide. Natural pollens from the control and E2 treated tank water were observed when using the Olympus S2X10 microscope with direct ambient light that were not as visible using the above inverted microscope as well as crystalline fragments some of which are salt crystals and others may be silicates (Figure 4.27; Figure 4.28).

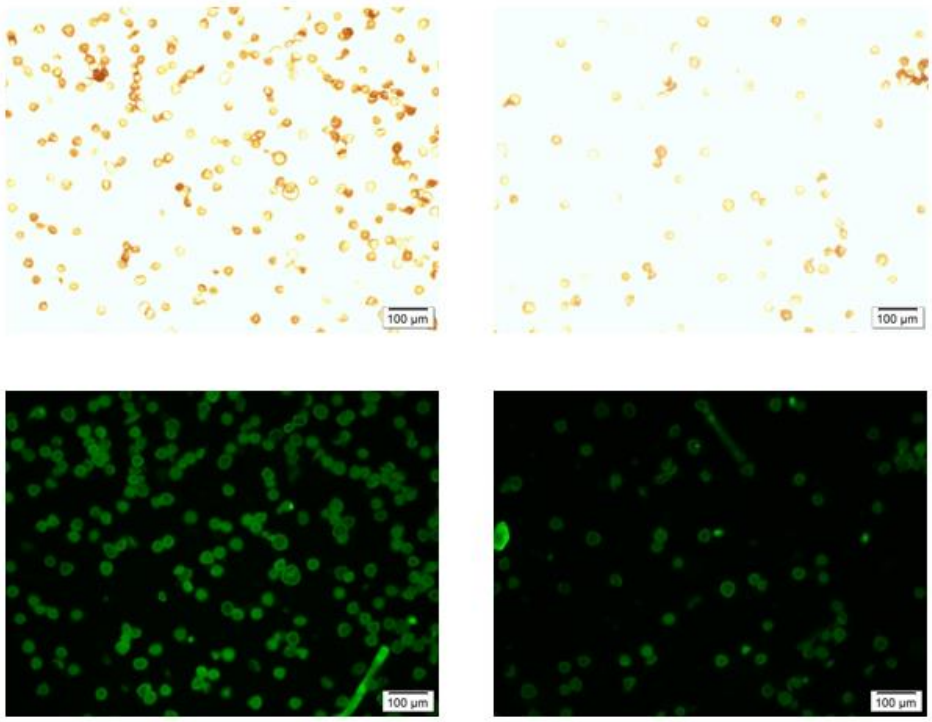


Figure 4.24 Recovered SECs from SEC only treatment on the left and SECs from the same treatment recovered from the waste water 2 days later on the right, Brightfield above and Fluorescent YFP filter light below.

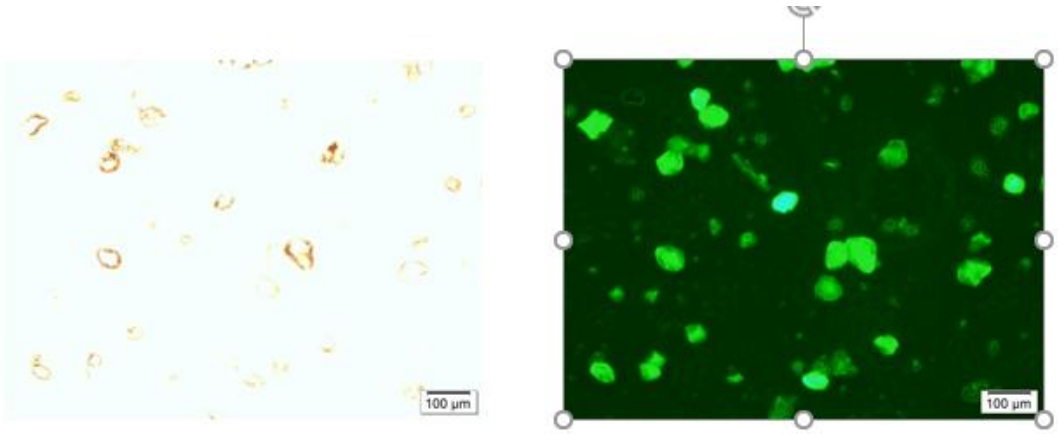


Figure 4.25 Recovered Control water, 30 minutes after treatment, brightfield on the left and fluorescent YFP filter light on the right.

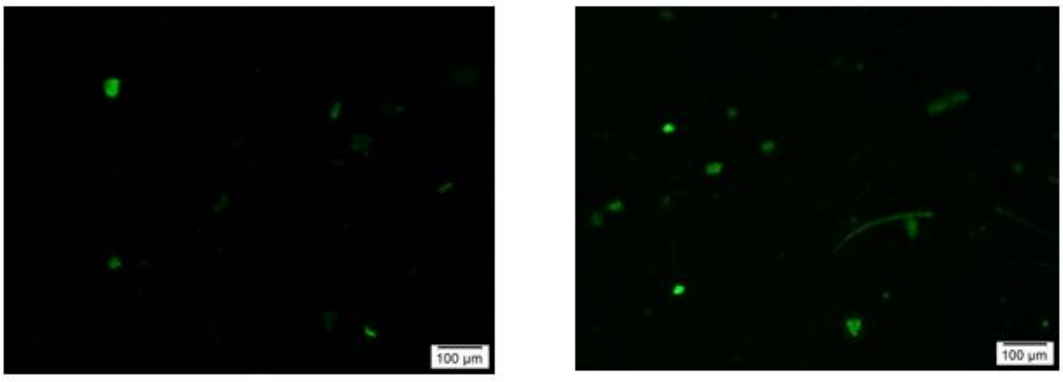


Figure 4.26 Recovered particulates from E2 200 ng treated water on the left and particulates from the same treatment recovered from the waste water 2 days later on the right.

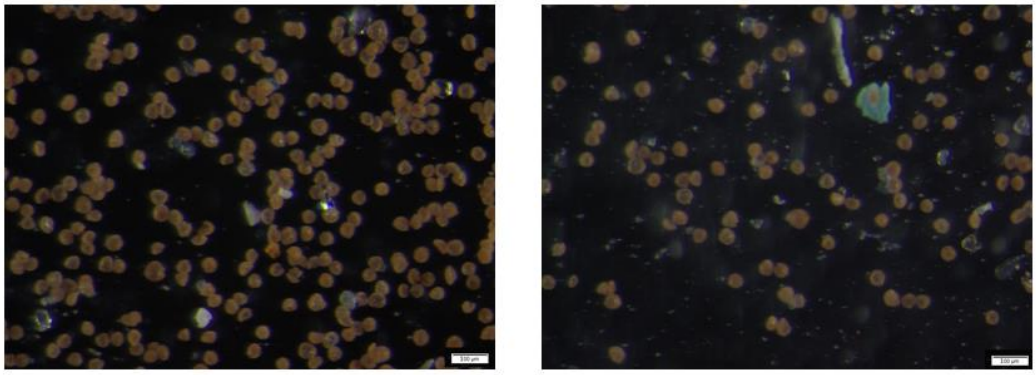


Figure 4.27 SECs recovered from the tank water filtrate just after spiking and SECs recovered from the same tank water 3 days later.

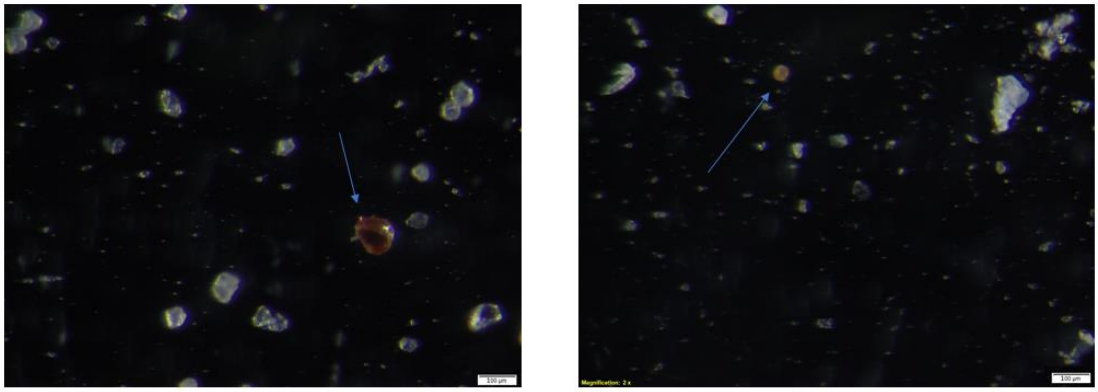


Figure 4.28 Natural pollens recovered from the control water tank (left) and the E2 200 ng water tank (right) both recovered 3 days after the treatments were added.

4.3.4. The Chemical analysis results to determine and quantify the presence of E2 in the SECs, gonad tissue and tank water

4.3.4.1. Estimation of the amount of E2 recovered from the treated SECs and the residual water

The SECs treated with E2 and the water used in the preparation of those SECs were extracted with ethyl acetate, derivatized with MSTFA and analysed by GC-MS as described in (2.7.2; page 60) only a small amount of E2 was recovered from the water washings, with slightly more E2 recovered from the 200 ng/L E2 SEC preparations (Table 4.10). For the E2 treated SECs, there was over 100% recovery for the 100 ng/L treated SECs and about 68% recovery from the 200 ng/L treated SECs (Table 4.11).

The reduction in E2 recovery and the increased amount of E2 in the water washings for the 200 ng/L treated SECs implies that there is an optimum amount of E2 that can be treated. There was a trace of E2 recovered from the control SECs water washes and the SECs but this may be due to “carry over” between GC-MS injections or the calibration graph used to estimate the amount of E2 in the extracts may be over estimating the amount of E2 injected. The results are displayed as a box and whisper plot (Figure 4.29).

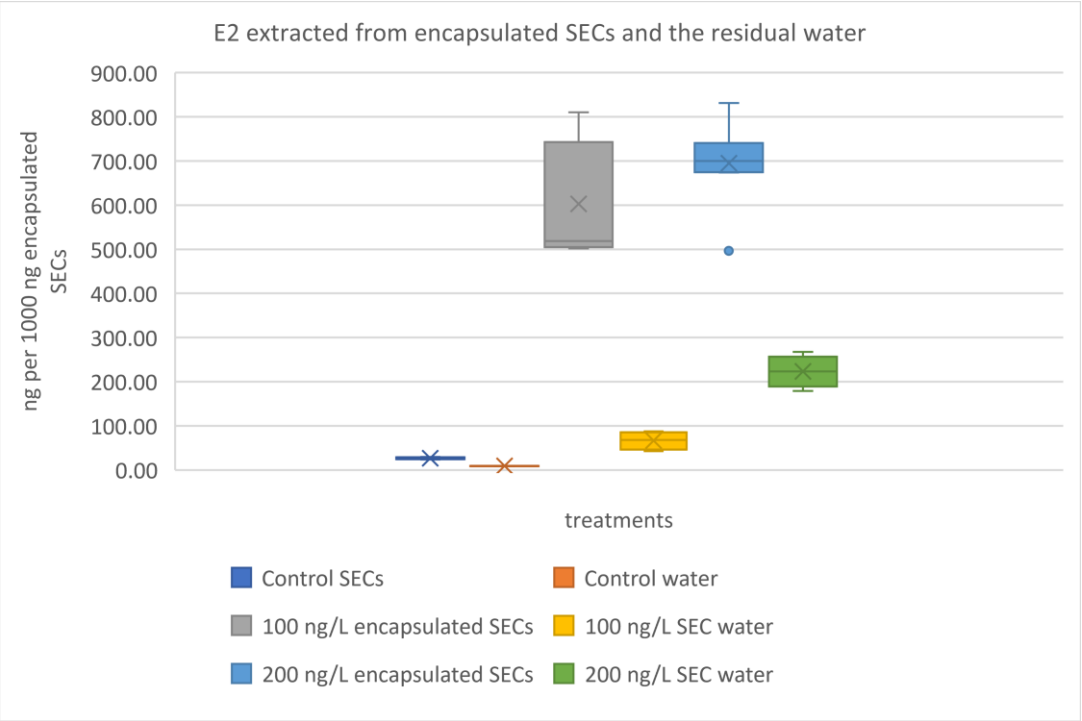


Figure 4.29 Boxplot of the E2 extracted from the E2 treated SECs and the residual water used in their preparation.

E2 extracted from the water washes used in the preparation of the SECs				
Treatment	Amount of E2 recovered from 6 pooled wash samples (ng)	SD	RSD (%)	Recovered E2 per tube (ng)
SECs no E2	11.94	0.25	2.11	1.99
SECs plus E2 100 ng/L	66.77	16.66	24.96	11.13
SECs plus E2 200 ng/L	216.04	29.84	13.81	36.01

Table 4.10 Amounts of E2 recovered from the water used in the preparation of the E2 treated SECs.

E2 recovered from the E2 treated SECs					
Treatment	Amount E2 recovered per tube (ng)	SD	RSD (%)	Mean E2 recovered (ng)	Percentage recovery
SECs no E2	0	3.77	5.31	71.02	
SECs plus E2 100 ng/L	500	116.4	19.9	584.99	117 ± 23.28
SECs plus E2 200 ng/L	100	84.58	12.38	683.08	68.31 ± 8.46

Table 4.11 Amounts of E2 recovered from the E2 treated SECs.

4.3.4.2. Estimation of the residual E2 from the water from the exposure experiment, half an hour after spiking and 3 days later before the water is changed using LC-MS-MS analysis.

On Analysis of the water extracts, all of the samples, including the controls showed a peak at the same retention time as for E2. This may be due to some of the original internal standard, DES, degrading into E2 or some other unexplained reason. The extracts were originally extracted and derivatised in January 2018 and there is likely to be some degradation, even when stored at -20° C. As the calibration curve has more scatter than desired, the quantification of the E2 in the water extracts is therefore prone to error and the estimated quantities can only be used as a guideline as the amount of E2 in the water extracts. Drawing various regression lines through the points on the calibration graph demonstrates that the regression equation varies considerably and the calculated amount of E2 could be under or overestimated depending on the selected regression equation (Figure 4.30).

In order to give an estimate of the amount of E2 extracted from the water samples, some of the lower values of recovered E2 in the data set was removed from the above external calibration set on the assumption that on desalting the DNSC reaction mixture, there has been either incomplete derivatisation or loss of the derivatised product on elution. This calibration plot was used for subsequent analysis to give an estimate of the amount of E2 that was extracted from the samples, until the problem can be resolved by either using an internal standard or troubleshooting

the derivatisation and desalting protocols as to why there is such a variation in the results (Figure 4.31).

The results indicate that there was very little E2 extracted from the water for the control tank, SECs with no treatment tank and SECs exposed to E2 tank. The tanks spiked with E2 at a concentration of 200 ng/L had some residual E2 extracted and after three days the amount of recovered E2 was of a similar value as to that of the initial day of spiking (Table 4.12; Figure 4.32).

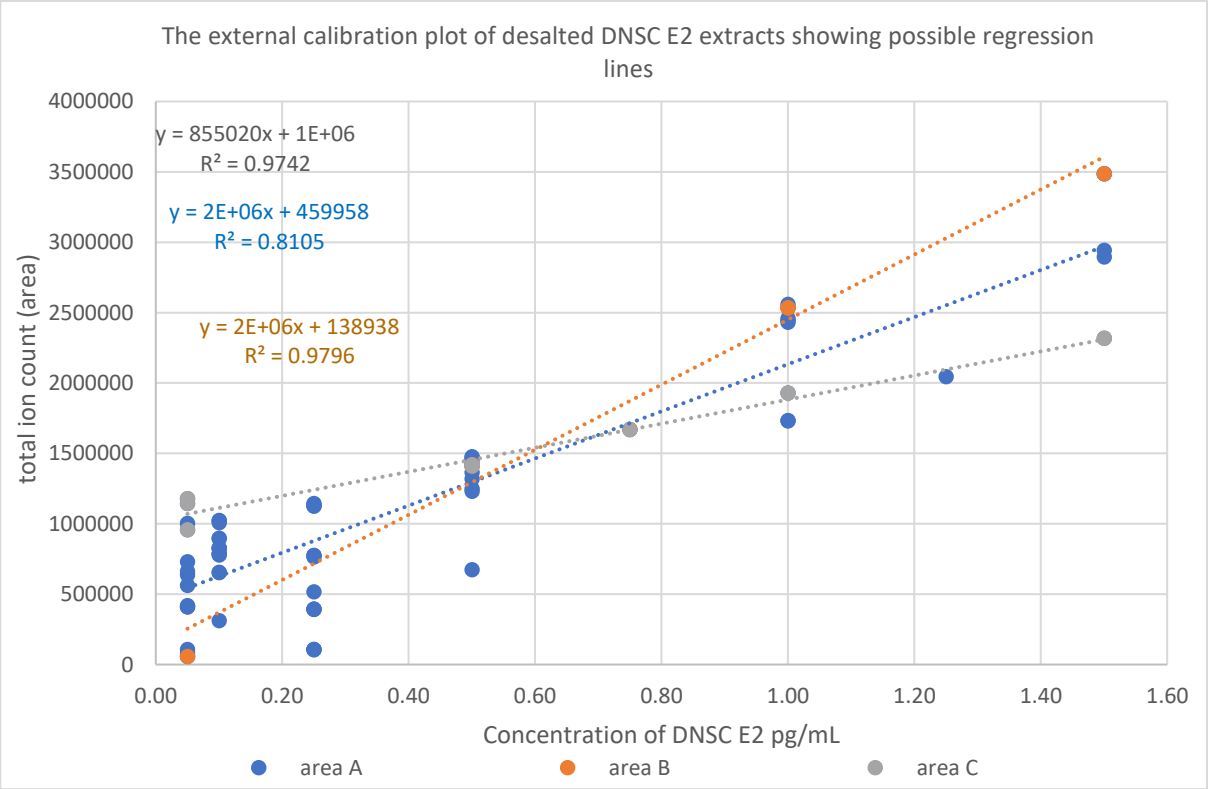


Figure 4.30 Calibration plot of the desalted derivatised E2 standards, demonstrating the possible regression equations, resulting from such scattered data points.

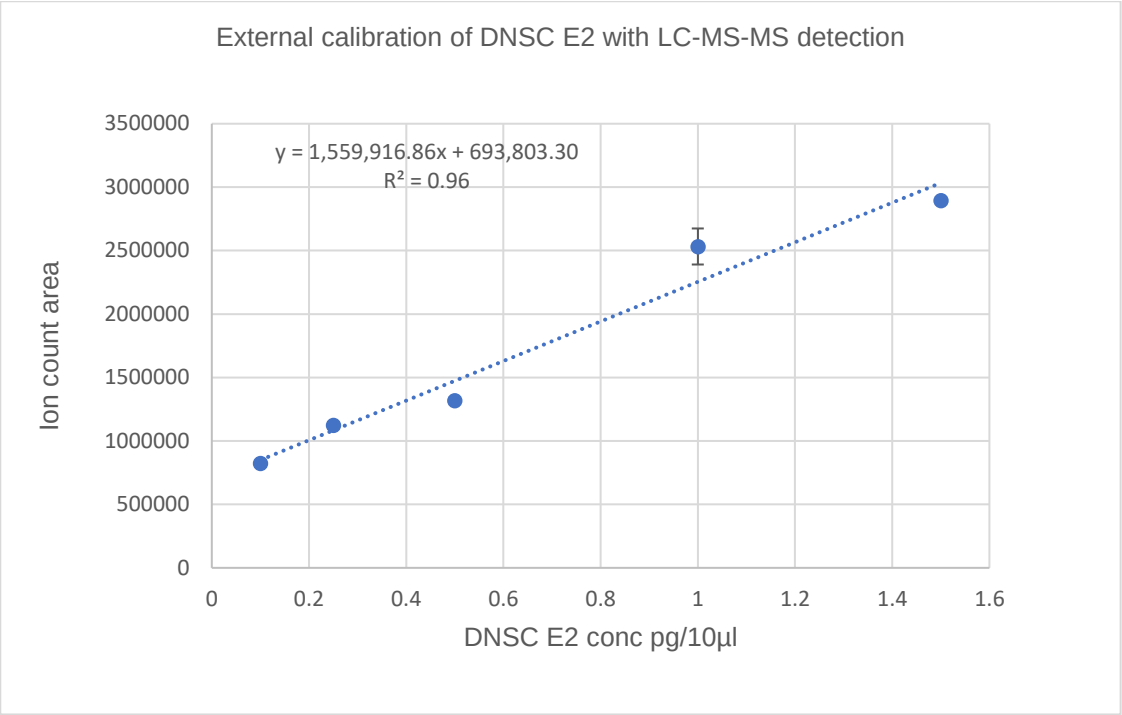


Figure 4.31 Edited calibration plot of the derivatised and desalted E2.

(SEM used for error bars, n=8)

Recovered E2 from the sampled tank water for the four treatments						
	Control, 0 days	SECs, 0 days	SECs plus E2 200 ng/L, 0 days	E2 200 ng/L 0 days	SECs plus E2 200 ng/L, 3 days	E2 200 ng/L, 3 days
Mean ng/L	4.68	2.38	13.39	16.31	16.06	16.31
SD	2.04	0.91	3.15	0.82	0.00	0.82
RSD (%)	43.47	38.34	23.50	5.05	0.00	5.05
SEM	1.02	0.46	1.41	0.41	0.00	0.41

Table 4.12 Estimation of the recovered E2 (ng/L) from the sampled tank water for the four treatments.

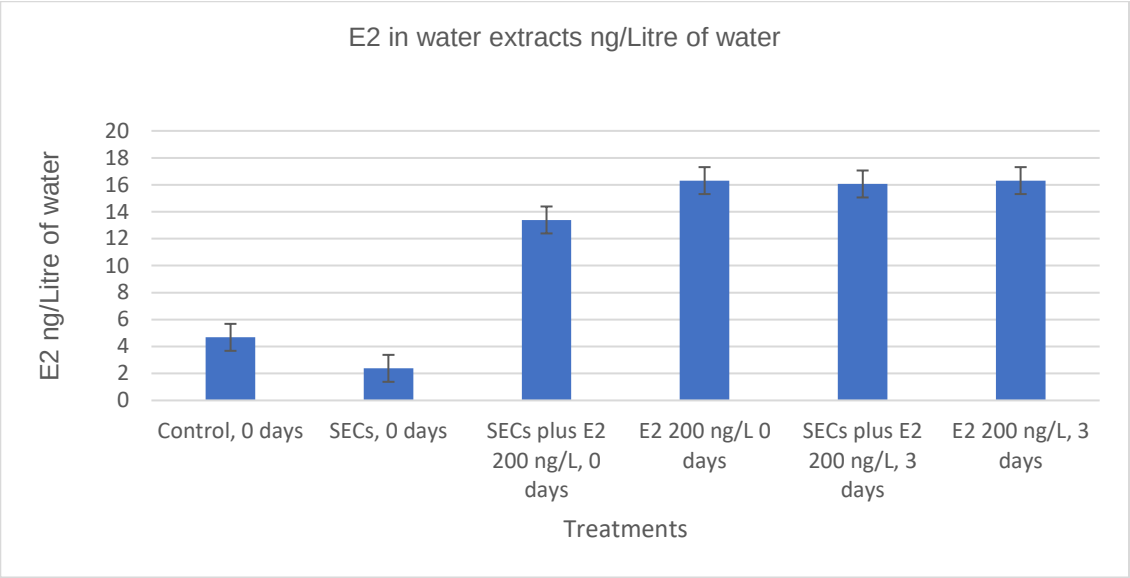


Figure 4.32 Recovered E2 from the water tanks for the four treatments after spiking and for the E2/ SECs E2 treatments after 3 days.

(SEM used for error bars)

4.3.4.3. Stability of the aqueous E2 solution used to spike the treatments after different days storage, using GC-MS analysis.

Analysis by GC-MS of the E2 extracted from aqueous solutions of 1 mg/mL E2 and the glass surface of the storage bottles indicated that the E2 does not stay in solution for very long. After 11 days, almost all of the recovered E2 was extracted from the glass surface of the storage bottle and there was very little residual E2 in aqueous solution (Figure 4.33).

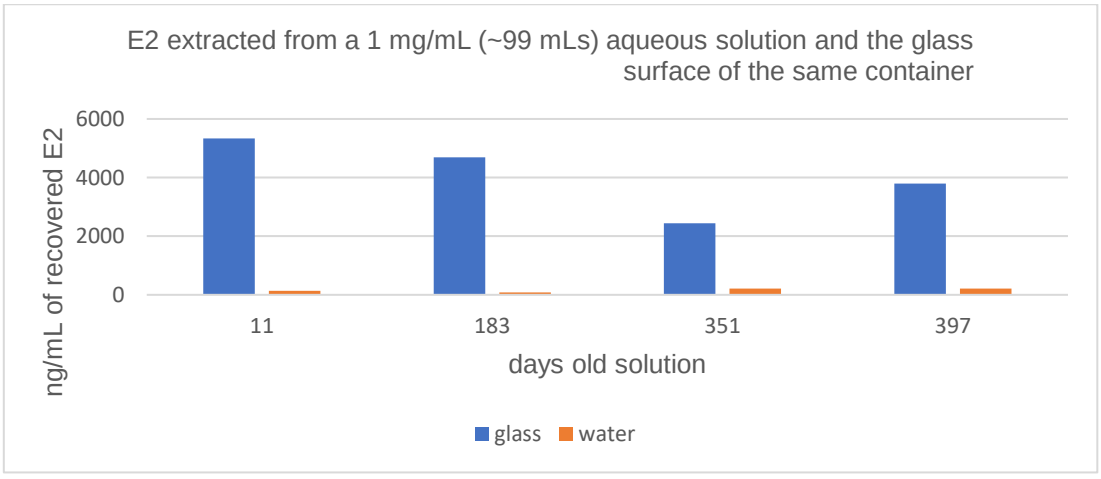


Figure 4.33 The residual E2 extracted from the water and glass surface of the storage container after 11 days and longer-term storage.

4.3.4.4. Estimation of the amount of free and total E2 recovered from the gonad tissue for the four treatments using LC-MS-MS analysis

At the time of writing, there has been insufficient time to extract and analyse any of the tissue from the mussels used in the exposure experiment. Therefore, the only available results are from the trial extraction from mussels exposed to 100 ng/L E2, SECs treated with E2, untreated SECs and Controls in August 2017. This used pooled homogenised tissue of animals whose sex and stage of gametogenesis had not been determined by any histology.

For the set of the extractions of the four treatments analysed by the Agilent HPLC system with FLD detection, there were no visible E2 peaks seen apart from the control extract. There were peaks at the retention time for E2, but were below the LOQ. The peak for the hydrolysed control extract being larger than that for the free extract from the same sample. The other extracts probably had recovered E2 that was less than the LOD. Typical chromatograms obtained show that the peaks of dansyl E2 have full base separation and are symmetrical with a retention time of 4.6 minutes (Figure 4.34; Figure 4.35).

Analysis of the recovered E2 from the tissue samples showed that there was a significant loss of recovered E2 from the hydrolysed extracts and the recovery of free E2 from the extracts that were not hydrolysed was very high, far higher than the range of the external calibration curve. The value of the total recovered E2 should be at least the same amount of free E2 in both extracts, if there was no extra E2 realised from the conjugated E2 on hydrolysis. The reason for the reduced recovery of total E2 in not known but this may be due to either the extraction of E2 from the hydrolysed tissue extract is not as efficient as for the free E2 from the extract that is not hydrolysed, incomplete derivatisation because of the presence of an unknown artefact inhibiting the reaction or the presence of an unknown artefact affecting the adsorption or elution of E2 on the C18 stationary phase on the filter tips in the final desalting protocol.

Because the external calibration plot has such a large amount of scatter the E2 recovered from the tissue extracts cannot be quantified reliably and the amount of conjugated E2 cannot be determined. Also, the amount of recovered E2 from the gonad tissue varies considerably between extracts from tissue of the same treatment. Considering that the tissue was pooled and homogenised for each treatment, the values of recovered tissue should be similar.

The edited calibration plot (Figure 4.31) was used to give an estimation of recovered E2 from the four treatments. From the three extracts analysed for each treatment, the amount of free E2 recovered from the control gonad tissue varied between 1 ng and 13 ng per gram of dry tissue. The treatment with E2 spiked water at 100 ng/L was between 0.73 and 8.8 ng per gram of dry tissue which is not that different from the amount of E2 extracted from the control gonad tissue.

The amount of free E2 recovered from the SECs was between 0.13 and 4.6 ng per gram of dry tissue, which is of a lower amount than both the control tissue and the E2 exposed tissue. The gonad tissue from the mussels exposed to SECs treated with 100 ng/L of E2 showed very high amounts of recovered E2, between 58 ng and 113 ng per gram of dry gonad tissue.

For the hydrolysed tissue samples, the control tissue varied between 13 and 45 ng per gram of dry gonad tissue, the 100 ng/L exposed mussel gonad tissue was between 4 and 26 ng per gram of dry tissue which is in a similar range as to the control tissue. The SEC treated mussel tissue was in a similar range as the free E2, between 0.5 and 22 ng per gram of dry tissue and the SECs treated with E2 had a range of recovered E2 between 5 and 45 ng per gram of dry tissue, a considerably less amount than for the free E2 extracted tissue. The results are summarised (Table 4.13).

Figure 4.34 LC-MS-MS Chromatogram of a tissue extract (100ng E2 exposed), free E2

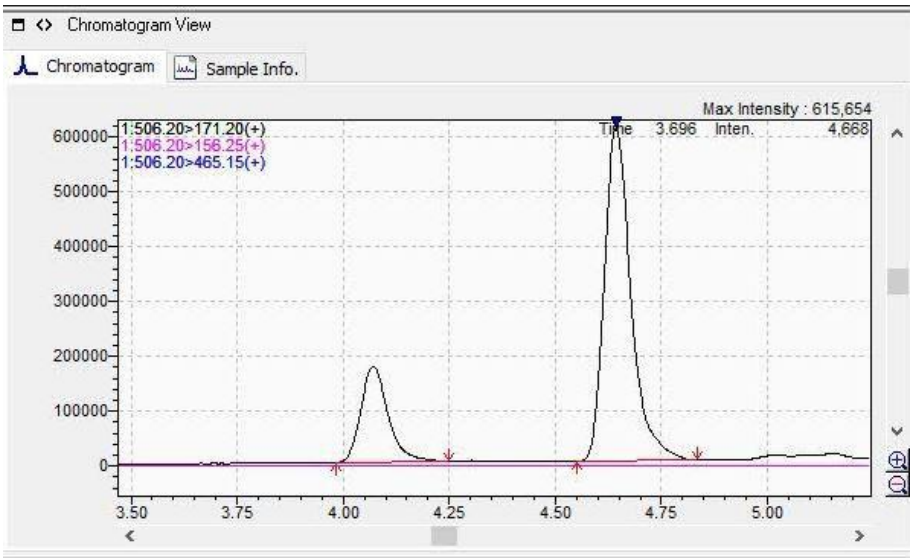


Figure 4.35 LC-MS-MS Chromatogram of a tissue extract (SECs with 100ng), total E2

	Treatments			
	Control	SECs	E2 100 ng/L	SECs plus E2, 100 ng/L
Mean free E2 ng/gm	26.92	7.16	14.63	77.98
SD	14.87	5.30	10.67	22.95
RSD (%)	50.21	1.42	72.93	29.43
SEM	3.97	74.02	2.75	5.74
Mean Total E2 ng/gm	26.39	12.70	13.50	21.71
SD	11.71	5.76	5.70	11.69
RSD (%)	44.39	45.33	42.20	53.83
SEM	3.13	1.66	1.47	2.92

Table 4.13 Summary of the recovered E2 from mussel gonad tissue per treatment.

4.4. Quantitative PCR expression results of the *ER2* mRNA expression of target and housekeeping genes in mussel tissues exposed to E2.

In order to analyse the impact of E2 exposure on the mussel gonadal tissues, only male mussels that were at the stage of gametogenesis of early to late development stage were used for biomarker *ER2* mRNA expression via qPCR analysis. Based on this, there was sufficient data for 7 duplicates for each of the treatments, i.e. n=7.

The biomarker *ER2* mRNA expression for all of the exposure groups are shown in table (Table 4.14) and the significant differences, using the Kruskal-Wallis test, are shown in table (Table 4.15).

The mRNA expression results show that the differences between the *ER2* mRNA expression values for the Control and E2 exposed mussels, and the mussels exposed to the SECs

alone and SECs treated with E2 are not significantly different from each other (Table 4.14; Table 4.15). However, there are significant differences in *ER2* mRNA expression values between the Control mussels and both treatments involving SECs alone and with the mussels exposed to 200 ng/L of E2 and the SECs.

Importantly, the *ER2* mRNA expression-values for the mussels exposed to 200 ng/L of E2 compared with the SEC and SECs with E2 values are different (Figure 4.36) values between the SECs, SECs plus E2 and the E2 exposed mussels (Table 4.14). The box and whisper plot (Figure 4.37) also show that the *ER2* mRNA values of the control and E2 treatments are distinct from the SEC and E2 with E2 treatments.

Sample	**Fold difference in *ER2* mRNA relative to control =$2^{\Delta\Delta C_T}$**	**Range of fold change**
Control	1	0.2-5.2
E2 200 ng/L	1.01	0.38-2.70
SECs only	1010	140.7-7250
SECs + E2	1075	153.5-7329.3

Table 4.14 Mussel gonadal *ER2* mRNA expression, showing fold differences for the four E2 exposure treatments

Kruskal- Wallis Test ($p < 0.05$) ER2 mRNA expression **values**			
Treatment	H statistic	*p* value	Result
Control / E2 200 ng/L	0.03670	0.84801	Not significant
Control / SECs	9.80000	0.00175*	Significant
Control/ SECs E2	9.80000	0.00175*	Significant
E2 200 ng/ SECs E2	9.80000	0.00175*	Significant
SECs E2/ SECs	1.3224	0.25015	Not significant

Table 4.15 Results of the Kruskal-Wallis test for the *ER2* mRNA expression values for the four treatments.

* significant differences between the treatments of the controls and both SEC treatments and the E2 treatments and both SEC treatments, $p < 0.05$

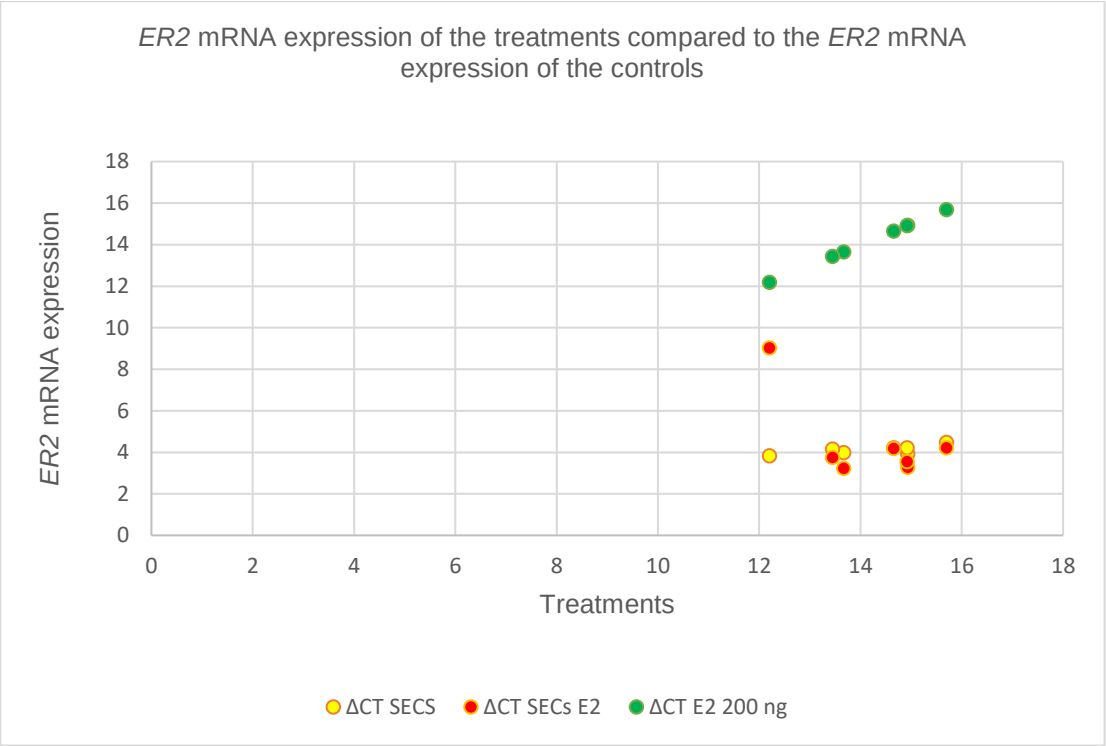


Figure 4.36 Scatterplot of the *ER2* mRNA expression values of the SEC, SEC + E2 and E2 treatments with respect to the equivalent *ER2* mRNA expression values of the control

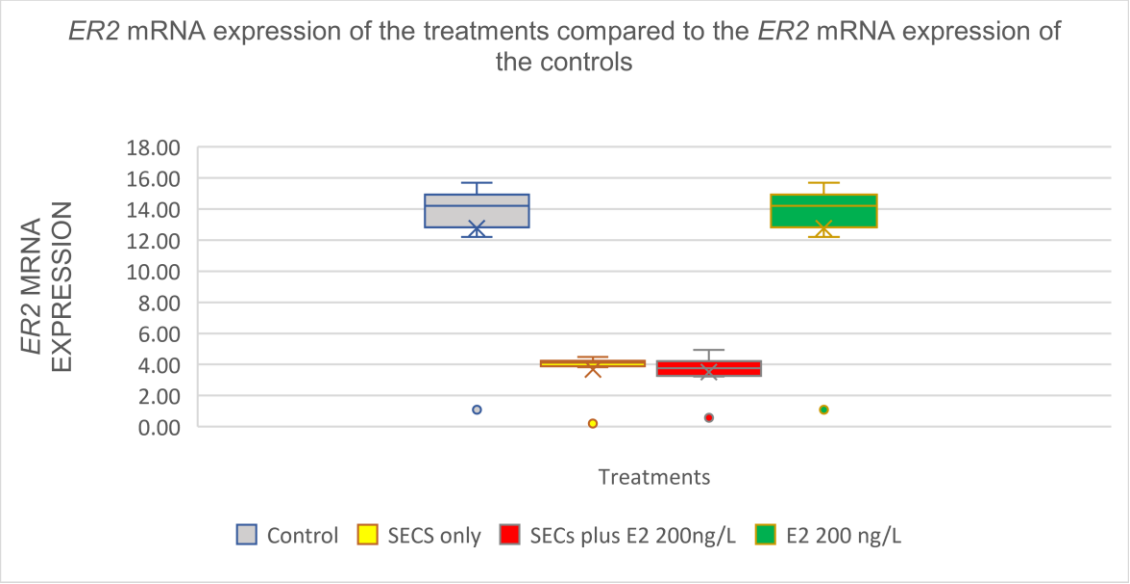


Figure 4.37 Boxplot of *ER2* mRNA expression values of treatments relative to the control

4.5. Discussion of the results from the microscopy, chemical analysis and mRNA gene expression work used in order to determine bioavailability of any E2 uptake by the mussels in the exposure experiment.

4.5.1. Determination of the presence of SECs by ingestion by the mussels and whether the E2 is bioavailable as a consequence of their ingestion.

Auto-fluorescence is an intrinsic property of cells which contain molecules which fluoresce when excited by UV and visible radiation of suitable wavelengths. These endogenous molecules originate from lysomes and mitochondria in the cell cytoplasm and from collagen and elastin in tissues. Such molecules are co enzymes such as NADPH, aromatic amino acids, lipo pigments, pyridinic molecules and flavins (Monici, 2005). Auto fluorescence analysis is a non-destructive process that does not require any treatment or staining and fixing of the specimens and is distinguished from fluorescent signals obtained by adding exogenous markers that are also used in fluorescence microscope (Monici, 2005). Auto fluorescence is generally used as a means to examine biochemical changes occurring in the cells of tissues due to physiological and pathological processes which result in the modification and distribution of endogenous fluorophores. Changes in collagen and elastin are associated in pathological conditions and changes in lipoproteins are associated with lipid oxidation products. Damage in the cell mitochondria results in an efflux of NADPH into the cytoplasm. These fluorescent products accumulate as granules in the cell cytoplasm and are associated with pathological conditions such as tumours (Monici, 2005).

Fluorescence is also found in plant tissues including sporopollenin (Monici, 2005). Both pollen grains and SECs emit auto-fluorescence with wavelengths that are organelle specific (Pohler *et al* 2012). SECs, emit intense fluorescence in green and red channels (Park *et al*. 2016). As autofluorescence analysis yields information about the morphological and physiological state of cells and tissues as well as a method of determination of the presence of any SECs, within the tissue. Fluorescence microscopy irradiates specimens with specific bands of wavelengths and separates the emitted fluorescence from the excitation light. The resulting emission light is detected, resulting in the fluorescent structure superimposed with high contrast against a dark background. Fluorescence microscopes are equipped with a set of filters to allow the specimen to be illuminated in defined directions. The disadvantage to this type of microscopy is a limited depth of field with thick sections and a resulting poor contrast of the image (Park *et al*. 2005).

SECs were detected in the gonads based on their fluorescent properties, confirming uptake by ingestion. The difference in the number of bright fluorescent spots found in the SECs and SECs

treated with E2 was statistically different from that of the control and E2 spiked water tanks. There was also confirmation that the SECs for both treatments were dispersed throughout the tank water for the duration of the experiment as a considerable number of SECs were retrieved from the filter paper used to filtrate the water before analysis, three days after they were introduced into the tanks. The filter papers for the controls and E2 spiked water had some natural pollens and other fluorescent particulates trapped which may account for some fluorescent spots being visible in gonad tissue of the control and 200 ng E2 treatments.

There were fluorescent patches of tissue found in all of the four treatments and fluorescent tissue surrounding both the untreated and E2 treated SECs. The numbers of fluorescent patches of tissue per treatment and the numbers of SECs and E2 treated SECs were statistically evaluated and the results indicated that the observations were statistically insignificant. Although there is fluorescent tissue surrounding both of the SEC treatments this appears that this is not due to the presence or absence of E2 and is due to some unknown reason or reasons.

Therefore, from the above histological and histological evidence above, there is sufficient evidence that the SECs introduced into the treatment water were being ingested by the mussels.

4.5.2. Results of the chemical analyses of the gonad tissue, treatment water and E2 treated SECs

The results of the analysis of the residual tank water for the four treatments at the start of the exposure experiment and three days later at the termination of the exposure experiment showed that there was the presence of E2 in the E2 spiked water throughout the experiment. There was less E2 in the water where SECs treated with E2 were added and very little E2 in the control and SECs with no E2 treatment.

Solvent extraction of the same SECs treated with E2 of both 100 ng and 200 ng E2, recovered most of the E2 that was added with little recovered E2 from the water used in their preparation. The treatment of SECs with E2 was more efficient at a lower concentration of E2, that is 100 ng/L, that for the higher concentration of 200 ng/L. However, there was sufficient E2 recovered from the 200 ng/L treated SECs to be sufficiently potent if the E2 was bioavailable. (Janer *et al.* 2005; Peck *et al.* 2007).

It was found that the concentration of E2 in aqueous solutions of E2 with a concentration of 1 mg/mL were reduced after storage and that most of the E2 was deposited on the glass surface of

the container after 11 days. The E2 spiked into the tank water, is likely to have the same fate and that there will be a reduction of available E2 in the water after some time. As residual E2 was found in the water after three days and the water was spiked with a freshly made solution of E2 after each water change, the amount of E2 that is no longer available for uptake has been minimised.

The chemical analysis of the gonad tissue was not as successful as for the above histology and water analysis. There are some issues with the extraction protocol and the derivatisation and subsequent desalting of the extracted E2 by dansyl chloride before analysis by E2. The extraction of free E2 had considerable variation and the amount of E2 extracted was greater than the maximum concentration of E2 used to generate the calibration regression curve. The hydrolysed extracts used to determine the total E2 in the tissue extracts were recovering E2 that was in the lower concentrations of the regression curve, were there was considerable variation. On retrospection, the extraction of the hydrolysed E2 by using the QuEChERS protocol may be compromised by the chemical hydrolysis of the extract needed to release the conjugated E2. The lipids in the extract are being saponified and producing detergents or surfactants that may be affecting the partitioning of the E2 into the acetonitrile layer during the QuEChERs extraction.

The reason that enzyme hydrolysis was not used was that the enzyme used only cleaves specific sulphates and glucuronates and not all conjugated lipids and the nature of conjugation for the mussels in the experiment is not known.

Therefore, the presence of conjugated E2 in any of the treatments was not determined and the amount of E2 present in the gonad tissue could not be determined accurately. However, comparatively, there was a considerable amount of free E2 extracted from the tissue of the mussel gonads for the SECs treated with E2 and the amount of E2 in the controls and the E2 spiked water was similar to each other. This infers that the mussels collected from the local collection site may have already had some exposure to E2 from their location. There was very little E2 in the tissue exposed to untreated SECs, less than the control gonad tissue.

4.5.3. The results of the *ER* mRNA gene expression as a biomarker for the uptake of E2 by the mussels

The resulting graphical plots of the ΔC_T and $\Delta\Delta C_T$ values for each of the treatments indicate that there is a significant difference in the *ER* mRNA expression between the Control and E2 water spiked treatments and the SECs and E2 treated SECs (Figure 4.36; Figure 4.37). There is no difference in the *ER* mRNA expression between the Control and E2 water spiked treatments and little difference between that of the SECs and SECs treated with E2 (Figure 4.36; Figure 4.37). The statistical analysis of the same ΔC_T and $\Delta\Delta C_T$ values for the four treatments confirm the same results as for the graphical representations (Table 4.14). The fold difference of the E2 spiked water

relative to the control is slightly larger than the value of one, indicating no change or very small up regulation of mRNA gene expression. This result compliments that of the free E2 detected in the gonad tissue by chemical analysis (Table 4.13). Both control tissue and E2 exposed tissue had a similar range of E2 detected (Table 4.13). The fold difference of the SECs and SECs with E2 are both very large, with a similar range, indicating an up regulation of *ER* mRNA expression for both SEC and SECs treated with E2. No explanation can be given as to why both SEC treatments give such a large up regulation in mRNA expression.

4.5.4. General discussion and summary of this chapter

In order for the E2 to be bioavailable, the SECs must have to first be ingested by the mussels. It was observed that the SECs are fluorescent under certain wavelengths of light and that appear as bright fluorescent spots when observed in unstained gonad tissue sections using fluorescence microscopy. There were statistically more bright spots in the tissue sections from the mussels from the tanks where SECs had been added than the tanks where no SECs had been added. The filtrates from the tank water showed that there were large amounts of SECs dispersed in the water throughout the exposure experiment. This infers that these bright spots are indeed SECs that had been taken up into the cavity of the mussels through their syphoning the tank water in which the SECs were suspended.

Although there is evidence that there is uptake of SECs by the mussels, the E2 may not be bioavailable if it remains enclosed within the SECs or remain adhered to the surface of the SECs. Bioavailability can only be determined by either a difference in *ER* mRNA expression for the E2 treated SECs compared to the other treatments or / and by an increase in the amount of conjugated E2.

Solvent extraction of the E2 treated SECs indicated that there was a sufficient concentration of E2 on or inside the SECs to provide a potent dose of E2 should the contents of the SECs be bioavailable after ingestion. The E2 extracted from the E2 spiked tank water indicated that some E2 remained dissolved in the water, even though E2 is hydrophobic. As the tank water was changed every two or three days and the water was spiked with a fresh solution of 200 ng/L E2, there would have sufficient residual E2 dissolved in the water for the mussels to ingest.

The chemical analysis of the gonad tissue was not entirely successful as there was a loss of extracted E2 after hydrolysis, so the presence of any conjugated E2 could not be determined for any of the treatments. However, there was free E2 extracted from the gonad tissue of the SECs treated with E2 and the gonads of the mussels exposed to E2 and a smaller amount of free E2 extracted from the control and SECs not treated with E2.

As the presence of conjugated E2 could not be determined, the only indication of bioavailability of E2 adsorbed on or absorbed in the SECs would be a change in gene expression by the ER genes in the mussel tissues. There was a significant difference in mRNA *ER* gene expression between both of SECs treated mussels than the E2 spiked water with respect to the control mussels (Table 4.14; Figure 4.37). The *ER* mRNA expression change of the SEC exposed mussels was considerably higher than for the E2 spiked water and the difference in change in gene expression between the E2 treated and untreated SECs was statistically insignificant. If SECs plus no E2 has the same ER expression as SECS plus bound E2, then that suggests no change in ER expression and no bioavailability, but the presence of SECs themselves are affecting the ER gene expression relative to the control for some unknown reason.

As there is no difference in the gene expression between that of the SECs and SECs treated with E2, considering a large amount of free E2 was extracted from the gonad tissue and considering a potent amount of E2 is adsorbed or adsorbed on the preparation of these SECs, the E2 bound on or within these SECs is not bioavailable. Hopefully, this can be confirmed by chemical analysis of the gonad tissue from the exposure experiment, when the loss of E2 from the hydrolysed tissue extracts can be addressed.

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Chapter 5. Discussion

5.1. Introduction

The aim is to investigate whether there is any bioaccumulation in mantle tissue of *M. edulis* exposed to a known concentration of E2 in a controlled experiment and whether the introduction of sporopollenin, exposed to the same concentration of E2, has a similar effect with bioaccumulation with regard to the use of SECs as a quenching agent to remove estrogens from water systems.

In this study, the following information was obtained.

- The E2 is either absorbed or adsorbed on or within the SECs for a dose of 100 ng/L of tank water and about 68% treated for a dose of 200 ng/L
- The SECs are taken up in the mantles of the mussels
- The SECs remain suspended in the tank water throughout the experiment
- E2 was recovered from the tank water where a dose of 200 ng/L was added and there is dissolved E2 in the water throughout the exposure experiment
- The *ER2*mRNA gene expression for the SECs and treated SECs is the same and is significantly different from the control water and the water with a dose of 200 ng/L E2. There is a considerable increase in fold change for both the treatments where SECs were added to the tank water and therefore upregulation of the *ER2*mRNA gene in response to the SECs compared to the controls.
- There is a small but insignificant increase in fold change for the mussels in the E2 spiked water.

This study failed some of the required objectives

- The gonad tissue has not been chemically analysed, so the amount of free and conjugated E2 for each of the exposure treatments is not known.
- The analysis method for the determination of free and total E2 from the tissue samples using LC-MS-MS has not been fully optimised and validated.

5.2. Uptake and Ingestion of SECS.

There have been no published studies on the absorption or adsorption of E2 by SECs. In this study, solvent extraction of treated SECs recovered sufficient E2 to show that a 100 ng/L dose of E2 was adsorbed or absorbed by the SECs with little residual E2 in the remaining in the water.

SECs were found in the gonad tissue of both treatments where SECs were introduced into the tank water. They could not be differentiated in stained sections of gill and digestive tract with light microscopy. The SECs could be differentiated in unstained sections of gonad tissue using fluorescence microscopy but SECs could not be seen in gill and digestive tract tissue due to a highly fluorescent background.

The presence of SECs in the gonad tissue is evidence that the SECs are taken up into the mussel's body cavity. As the SECs are about 25 µm in diameter (Barrier et al., 2011), they are of a size that mussels would retain them on filtration, consuming particulates of less than 110 microns (Dral 1967; Newall 1979).

Filtration of the tank water at beginning and end of the exposure experiment showed that SECs were present in the water and the number of recovered SECs from the tank water suggests that they remain suspended in the water throughout the exposure experiment.

The rate of ingestion of suspended particulates increases with an increasing concentration of suspended material until the ingestion system is saturated and the excess material is ejected as pseudo faeces. (Atkins 1936, 1937a, 1937b; White 1937; Newall 1979; Jorgensen 1996). Material of a non-nutritive value, such as silt, is removed without ingestion (Davenport *et al* 2000).

Although there is proof of the presence of SECs in the body cavity there is not proof of ingestion. If SECs are considered as not nutritive and are expelled as pseudo faeces, the SECs and the treated E2 within the SECs are not taken up and the E2 is not bioavailable.

If the SECs are digested, what is the fate of the E2 within the SECs? Do the SECs remain undigested and are eventually expelled as pseudo faeces? The SECs consist of a rigid and complex biopolymer that is resistant to chemical degradation (Barrier et al., 2011; Diego-Taboada et al., 2014). The process to prepare the SECs consists of the removal of the exine contents by digestion of Lycopodium spores in a strong acid for several hours, so in effect they are resistant to chemical degradation.

SECs can be degraded biologically by the action of plant and other biological enzymes (Punt *et al.* 1999; Barrier 2008; Barrier *et al*. 2011; Diego-Taboada *et al.* 2014), so there could be digestion of the SECs by enzyme processes within the digestive gland of the mussel.

This study does not provide enough information on fate of E2 adsorbed or absorbed by the SECs that has been taken up by the mussels over a longer period of time than the seven days of the exposure experiment. The length of any digestive process that would degrade the SECs and thus release the E2 from the treated SECs, if such a process does occur is not known. In order to determine if E2 is taken up by ingested E2 treated SECs there needs to be evidence of bioavailability such as biotransformation of E2, mRNA gene expression change or other biomarkers.

5.3. Chemical analysis of the tank water

The water from the tanks was extracted using a published SPE method (Cubero-Leon *et al.* 2012) and derivatised with dansyl chloride for LC-MS-MS analysis. As the d4E2 internal standard was found to be unstable when analysed by GC-MS, DES was added as an alternative internal standard when the water was initially extracted. Unfortunately, the Bruker LC-MS-MS instrument that was intended to be used became unavailable and the samples were stored at -20° C until they could be analysed.

As there was some considerable time before the eventual analysis some degradation of the dansylated E2 was inevitable and the column used with the LC-MS-MS system did not resolve the DES peak from the E2 peak and they co eluted, therefore there is some background interference in all of the E2 peaks which is the residual DES.

The resulting quantification of the residual E2 in the water samples is not absolute and can only be used as a guidance as to the presence or absence of E2 in the samples. There was residual E2 found in the tanks that were spiked with 200 ng/L of E2 and after three days of the water being spiked, there was still residual E2 in the water. There was a small amount of E2 in the water treated with E2 treated SECs and there was the same background peak in the SECs and control water which was presumed to be residual DES.

5.4. Chemical analysis of the gonad tissue

5.4.1. Extraction Methods

The extraction protocols to remove E2 from both sea water and river water tables all use SPE extraction or liquid-liquid extraction protocols (Cubero-Leon *et al.* 2012). The water from the exposure experiment was extracted using a published validated extraction method (Cubero-Leon *et al.* 2012). An extraction protocol to extract E2 from gonad tissue was optimised from several protocols using the QuEChERs method that was originally used to extract pesticides from various animal and vegetable tissues. This optimised method (Appendix 2, page XI) was compared to that of other validated methods to extract E2 from tissue using solvent extraction followed by SPE clean up steps and found to be comparable (Cubero-Leon *et al.* 2012).

Extracting E2 from mussel tissue is challenging because the mussel gonad tissue is rich in both lipids and carotenoids that are interferents in the subsequent analysis by HPLC methods. Any extraction protocol requires their removal otherwise the efficiency of the HPLC column used in the analysis is quickly compromised. A protocol was optimised to remove this yellow pigment, presumed to be a type of carotenoid, from the crude tissue extract, either prior to QuEChERS extraction or after QuEChERs extraction with a 100% recovery rate. This prevented the problems that occurred with

previous work using HPLC chromatography to analyse E2 extracts where the retained carotenoids on the HPLC column resulted in high back pressure and reduced E2 retention over time.

The use of the enzyme, subtilisin, to digest the tissue prior to extraction was investigated. This enzyme was used to produce a resulting liquid that could be partitioned into two parts for the extraction and analysis of free and conjugated E2 from the same tissue extract.

One of these parts was treated with the enzyme β glucuronidase/arylsulfatase to cleave any conjugated E2 into the resulting free E2 for total E2 determination. The disadvantage in using enzymes to digest the tissue and hydrolyse the resulting digest is that the pH of the digest mixture is important and must be adjusted and the digest itself takes several hours and the resulting mixture is colloidal and difficult to pipette. Also, the β glucuronidase/ arylsulfatase enzyme is selective and only cleaves sulphates and glucuronates.

Extraction of E2 from tissue using organic solvents such as DCM and methanol is very efficient and the resulting solution is easy to dispense by pipetting but the solvents used are toxic and unpleasant to use. The chemical hydrolysis using methanolic potassium hydroxide solution results in the release of the E2 from all of the conjugated E2 but was found to interfere with the QuEChERS extraction protocol, resulting in the loss of the extracted E2. Therefore, the chemical hydrolysis protocol needs to be amended to remove these interferents.

5.4.2. Analysis Methods

The chemical analysis for the gonad tissue from the mussels used in the exposure experiment was not completed due to the lack of availability of an instrument of sufficient sensitivity to detect the low levels of E2 required. An attempt was made to develop methods using alternative instrumentation using GC-MS and HPLC with FLD detection.

The analysis of MSTFA derivatised E2 by GC-MS analysis using d4E2 as an internal standard was not entirely successful. Initially, an external calibration curve using the d4E2 as an internal standard gave very good results but the internal standard was not chemically stable, due to an unforeseen problem that has been documented concerning “isotope scrambling”. This problem could have been overcome if an alternative isotope had been used, such as 17β-estradiol-2,3,4-$^{13}C_3$ that does not lose its deuterated proton, however these isotopes are very expensive.

A method using HPLC with FLD detection was developed using the pesticide Diuron as an internal standard with an LOD of about 17 ng/mL for the detection of E2. This method is not sensitive enough for analysing the tissue samples from the exposure experiment, but is useful for the analysis of more concentrated samples and in hindsight, would have been sufficiently sensitive enough to measure the water extracts from the tank water and SEC extracts if they had been concentrated

down enough after SPE or liquid-liquid extraction. This method has the advantage in that the samples require no derivisation steps, the internal standard is detected by DAD, and is thus segregated from the E2 signal and does not require any special HPLC column, only a C18 column, which is a basic column used in most HPLC analysis.

As FLD detection is more sensitive than DAD detection in HPLC analysis, an attempt was made to develop the previous HPLC method using FLD detection for dansylated E2. Several combinations of excitation and emission parameters were used for FLD detection but the results were discouraging and it appears that the dansylated E2 does not fluoresce in any of the FLD parameters that were tried.

Previously, dansylated E2 was analysed using a Bruker LC-MS-MS system but the results were inconsistent. There appeared to be ion suppression and after several samples were run, salt deposits were found on the cone of the electro spray inside the MS detector. These salts were most likely the sodium bicarbonate salt from the derivatisation buffer that are not sufficiently volatile to be dispersed by the electro spray and nitrogen drying gas in the detector. None of the published papers using dansylation of E2 for LC-MS analysis mention this as a problem (Nelson *et al.* 2004; Xu *et al.* 2007; Kohling 2011; Yang *et al.* 2016).

Initially, an alternative derivatisation buffer, ammonium bicarbonate, was used that is known to be volatile but substituting this buffer for that of the sodium bicarbonate buffer was unsuccessful. Eventually, a method was developed to desalt the dansylated E2 for LC-MS-MS analysis. A linear calibration curve was obtained, but there is considerably more variance in the results. This may be overcome by the addition of a suitable internal standard. The internal standard, Diuron, was used but although this internal standard was successful for HPLC analysis with DAD and FLD detection it is not a suitable internal standard for the analysis of dansylated E2 with LC-MS-MS detection. Some of the chemical properties of Diuron differ from that of the dansylated E2, notably its solubility and the retention on the SPE cartridges used in desalting the dansylated E2. Therefore, any internal standard must have the same solubility and SPE retention properties as that of the dansylated E2, which is most likely to be an isotope of E2.

5.4.3. Results from the tissue extracts using LC-MS-MS analysis to determine the amount of free and total E2 in the mussel gonad tissue.

The LC-MS-MS used in the analysis of E2 from tissue extractions became available only towards the end of the study. The tissue samples used for analysis using the LC-MS-MS were from a replicate experiment set up in August of the same year for the purpose of method development

only. The results from the analysis of these samples indicated that the extraction and analysis using the LC-MS-MS required further method development.

Firstly, due to the number of steps in the extraction process, a suitable internal standard need to be added to compensate for losses of sample at each step of the process, especially the final desalting step. The recovery rate for the derivatisation and desalting steps cannot be determined because there is no way to compare the results with that of an unprocessed i.e. underivatised standard.

The results from the tissue analysis showed considerable variation considering that the extracts used were from the same homogenised tissue samples. The hydrolysed samples for all of the extracts were unsuccessful with considerable loss of E2 extracted.

This loss of E2 is most likely due to the hydrolysis step as the hydrolysis method used was by saponification of the tissue extract rather than by enzyme cleavage of the E2 conjugated lipids used in the method validation. The saponified lipids create molecules that are detergents and these are interfering with the partition of the E2 into the acetonitrile layer, resulting in the E2 partitioning into the discarded aqueous and salt layers. This problem can be overcome by removing the resulting saponified salts after chemical hydrolysis before QuEChERS extraction, using hydrolysis by enzyme cleavage as the hydrolysis step in the QuEChERS extraction, or by using an already published method, such as that by Cubero-Leon *et al.* (2012).

Therefore, there is no information regarding the amount of total E2 and therefore the degree of conjugation, from all of the samples extracted and analysed. Although, due to the lack of an internal standard, there is variation in the amount of free E2 extracted from the tissues, it appears that there is a similar amount of free E2 in both the control, E2 spiked samples and the SECs. The E2 exposed SECs had a significantly larger amount of free E2 extracted from the gonad tissue. These results can only be used as a guide to those expected to be obtained from that of the tissue from the exposure experiment. The extracted tissue analysed was homogenised tissue from an undefined proportion of both male and female mussels and the stage of gametogenesis would not have been in the early to mid-development stage as that of the animals from the exposure experiment.

5.5. mRNA ER2 gene expression as a tool to determine bioavailability of E2 bound to SECs.

The number of samples that were available for qPCR analysis was limited due to the required stage of gametogenesis required and the sex of the mussel clearly established as being that of a male mussel (Appendix 1, page I). The response to *ER2* mRNA expression is observed in

mussels exposed to E2 at the early stage of gametogenesis only and the expression is more pronounced in male mussels than that of female mussels (Ciocan *et al.* 2010). Also, the quality of the extracted RNA had to be such that the amount of RNA added to the reaction mixture was 225 ng. Some of the cDNA from the RNA extracts failed to amplify, resulting in a sample size of 7 for each treatment.

The qPCR results from the RNA extracted from the gonad tissue from the four treatments used in the exposure experiment are different to what was expected. The mussels in E2 spiked tank water had fold changes that was slightly larger than that of the controls, but the difference was statistically insignificant. Both the SEC treatments had similar fold changes to each other, the differences between both SEC treatments being statistically insignificant but both of their fold changes were much larger than that of the controls and E2 treated mussels. These differences were statistically significant. The large increase in fold change means that there is up regulation in the cells of both of the SEC treated animals and the quantity of mRNA *ER2* has been increased in response to the SEC exposure. As there is no difference between the untreated SECs and the E2 treated SECs, the change in gene expression is not due to the E2 treated within the SECs but some other unknown reason.

Possible reasons for negative results for gene expression and high expression of *ER2* in controls are the choice of using only one gene, the *ER2* transcript which was found to be non-responsive and prone to contamination during amplification. One possible methodological explanation for the similar fold changes in both control and E2 treated tissue is the presence of SECs in both tissues The SECs may be removing unknown artefacts present in the extraction matrix that have some effect on the qPCR reaction and thus increasing the efficiency of the reaction compared to that of the housekeeping genes. This explanation could be tested by adding a few SECs to a sample of control tissue and extracting m RNA from this tissue alongside the same tissue sample without the addition of SECs and comparing the efficiency of the optimised PCR amplification using both the housekeeping genes and the *ER2* gene. If there is a difference in efficiency between the *ER2* gene and the housekeeping genes on addition of SECs, the results do not satisfy the MIQE guidelines and the resulting observed increase in fold change may be a false positive result.

Although the results of the chemical analysis of the gonad tissue has not been completed, the analysis of the free E2 in the gonad tissue from the experiment in August suggests that there is a similar amount of free E2 in both the controls, the empty SECs and the mussels from the E2 spiked tank water, which would explain the fold change of the mussels exposed to 200 ng/L E2 water being insignificant compared to the controls, but this does not explain the difference in fold change of the untreated SECs compared to the controls. Because there is no information about the

amount of conjugated E2 in any of the samples, the bioavailability of the E2 taken up by the mussels from the treated tank water and the treated SECs cannot be ascertained at this stage.

The mussels collected were at late development stage, stage 3 to 4, according to Seed's classification which was surprising as they were collected in very early January when they should have been at the early development stage, stage 2, according to Seed's classification (Seed, 1969).

Samples of the water from the collection site were not taken for pH measurement and E2 analysis. Janer *et al*. in 2005 suggested that low concentrations of E2 (20 ng/L) behave as an endogenous steroid in mussels and the presence of low levels of E2 in the water from the collection site may be increasing the rate of gonad development. One explanation for estrogens specifically in the high control concentration of E2 may relate to background levels of estrogens in the aquatic environment from which the animals were harvested. Therefore, the potential exposure to mussels to E2 in their natural environment may be having an influence on gonad development and possibly the gene expression results.

5.6. Review of the initial hypothesis from this study

The initial hypothesis before the study started were: -

H1.1 E2 is absorbed by *M. edulis* and this free E2 is conjugated into fatty acid esters and stored mainly in mantle tissue.

H1 0 E2 is absorbed by *M. edulis* and this free E2 is not conjugated into fatty acid esters and stored mainly in mantle tissue.

Results: The work to extract E2 from the mantle tissue for the treatments was not completed so there are no results for the E2 absorbed by the *M. edulis* in any of the mantle tissues. As the information is not yet available, hypothesis H1 cannot be evaluated yet.

H2 1 E2 in the water column exerts an estrogenic effect increases *ER2* gene expression relative to the control group.

H2 0 E2 has no effect upon *ER2* expression in either of the exposure groups relative to the control group.

Results: The *ER2* expression for the E2 in the water column is slightly higher than for the control group, but the difference is not significant. H2 1 is rejected.

However, the *ER2* expression for the SEC and E2 treated SEC groups is significantly higher than for the control group. As there no difference in *ER2* expression between the SEC and E2 treated

SECs, the change in gene expression cannot be explained that it is due to the presence of E2 on or inside the SECs, so H2 is also rejected.

H3 SECs are absorbed by *M. edulis* and the free E2 is bio available and thus conjugated as fatty acid esters.

H3 1 SECs are absorbed by *M. edulis* but the free E2 is not bio-available and thus not conjugated into the fatty acid esters.

H3 0 SECs are not absorbed by *M. edulis* and are passed through their gills and excreted as pseudo faeces.

Results: The presence of SECs was determined in the gonad tissue but could not be determined in the gill or digestive tract tissue. As the gonad tissue has not been extracted and analysed for the presence of free or conjugated E2, hypothesis H3 cannot be evaluated yet.

5.7. Future work

5.7.1. Visualisation of SECs in the gill and digestive tract tissue

The presence of the SECs in the stained sections used to determine the stage of gametogenesis and the sex of the mussels could not be determined because the SECs are also stained by the eosin and haematoxylin stain used. There is no information on staining SECs other than for scanning electron microscopy where SECs are stained with glutaraldehyde followed by osmium tetroxide before being coated with platinum (Barrier 2008).

Future experiments could be performed to investigate the uptake of SECs by mussels if a suitable stain could be found that would be visible in gill and digestive tract tissue that could be visually differentiated from the Eosin and Haematoxylin stain used to stain the tissues. For example, Nile red is that has been used to stain lipid rich materials and microplastics and stained microplastic particulates can be differentiated in eosin and haematoxylin stained sections using polarised light microscopy. The SECs may also take up this stain (Diego-Taboada *et al.* 2014; Tamminga *et al.* 2017). The criteria for the stain selected, must be that it is not toxic to the mussels and it does not impede encapsulation if the SECs are to be treated with an estrogen.

The SECs were visualised in the gonad tissue using fluorescence microscopy on unstained gonad sections but this method was unsuccessful for gill and digestive tract unstained sections because of the high fluorescent background in both of these tissues. Fluorescence microscopy lacks depth of field, so it is difficult to focus on the fluorescent spots seen in the gonad tissue to determine any fine structure to positively identify the bright spot as that of a SEC. The visualisation

of SECs may be improved using confocal light scanning microscopy (CLSM) as the observed items are visualised by lasers at selected excitation and emission values rather than filtered light. This should give an improved depth of field and enable the structure of the SECs within the tissue to be visualised Images from pollens and SECs were captured using CLSM with the following settings: laser excitation wavelengths at 405 nm (10.0%), 488 nm (11.0%), and 561 nm (10.0%) and detected with emission fitters 410–516, 493–556, and 566–685 nm, respectively (Park *et al.* 2016).

5.7.2. Optimising the loading of steroids by SECs

The exposure of SECs by E2 was not optimised so that a known amount of E2 was loaded into a known number of SECs. The number of SECs used to give a treatment of 100 ng/L of E2 was estimated by treating the SECs as a sphere of 25 µm diameter and calculating a volume of 40.9 μm^3 that was equal to 5000 SECs. This volume was rounded up to 50 µL and the weight of SECs equal to this volume was estimated as 0.0065 g ± 0.002 g.

This was a crude estimation that could be improved on. The SECs were exposed to E2 was by the SECs by adding the SECs to water in which sufficient E2 was added to give a load of 100 ng/L or 200 ng/L rather than adding the E2 in an organic solvent such as ethanol where the loading of the E2 would be more efficient (Barrier *et al.* 2011). Extraction of the E2 from the E2 exposed SECs using ethyl acetate showed that there is an optimum loading of E2 in the SECs and this was not fully investigated to find the loading capacity of E2 in SECs.

5.7.3. The use of alternative biomarkers as evidence of bioavailability.

The two biomarkers used in this study were the change in mRNA *ER2* gene expression using qPCR analysis and the presence of conjugated E2 in the gonad tissue.

An alternative biomarker is the presence of vitellogenin, (VTG), a female specific protein involved in the production of egg yolk and in controlling the growth of unfertilised reproductive cells (Palmer and Palmer, 1995) Also, a similar biomarker as used in this study is the qPCR analysis of mRNA *VTG* gene expression (Ciocan *et al.* 2010). Both of these biomarkers are dependent on the mussels being at the early to mid-development stages of gametogenesis (Jobling and Sumpter, 1993; Marin and Matozzo, 2004; Puinean *et al*. 2006; Rotchell and Ostrander, 2013).

Alternative biomarkers that are not dependent on the stage of gametogenesis are changes in levels of antioxidant enzyme activities in gills and digestive tract tissue in response to stress induced xenobiotics. Exposure to E2 may also alter oxidative stress conditions in digestive tissue by transcription of genes that play a role in anti-oxidant defences (Canesi *et al*. 2007). Such enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), glutathione reductase (GR) and malondialdehyde (MDA) concentrations. (Box *et al.* 2007). There are various protocols that are available to measure these enzyme activities including the use of enzyme-linked immunosorbent assay (ELISA) kits (Wang, 2017).

5.7.4. The chemical analysis of the gonad tissue for the presence of free and conjugated E2

It is imperative that the gonad tissue needs to be extracted and analysed to evaluate the amount of free and conjugated E2 in the tissue extracts in order to determine whether the E2 from the E2 treated water and SECs is bioavailable. This is especially important as the results of the qPCR expression show that the addition of both SECs treatments result in up regulation of the mRNA ER2 gene.

The extraction protocol employed is to be the method from Cubero-Leon *et al.* (2012) (Appendix 2, 2.2; page XI) as this is a validated published method. The optimised QuEChERS method (Appendix 2,2.1; page IX) can then be used on a sub set of the same samples to compare the results to eventually validate this method. This could not be done using HPLC with FLD detection because the recovered E2 from actual samples rather than spiked samples is below the LOD The QuEChERS method will use the enzyme glucuronidase to cleave the conjugated E2 and the results can be compared to those of the method from Cubero-Leon *et al*. (2012) where the extract has been chemically hydrolysed in order to determine the nature of conjugation of the E2.

The chemical hydrolysis protocol could be further optimised to remove the saponified salts that are interfering with the QuEChERS extraction. These salts could be precipitated by the addition of a suitable amount of calcium or magnesium hydroxide to convert the saponified lipids into insoluble calcium or magnesium stearates. These stearate salts could then be removed by centrifugation and the pH of the supernatant readjusted by the addition of sufficient acetic acid until the pH was neutral.

The extraction of E2 from mussel tissue could be optimised further by replacing the SEP PAK® extraction after the initial QuEChERS extraction with a dSPE step. The QuEChERs protocol usually uses the addition of a dSPE reagent to remove any residual interferents that were not removed by the addition of the AOAC salts. The dSPE reagents that are used in the published methods (Berge and Vulliet 2015; Syljohn *et al*. 2016) for the extraction of E2 did not remove the

yellow pigment that was assumed to be a type of carotenoid. There are other alternative dSPE mixtures that were not tried out. For example, a dSPE mixture of 25 mg PSA, 150 mg MgSO4, and 2.5 mg graphitized carbon per mL of extract has been used to clean up pesticide extracts with less intensely coloured extracts, or high carotenoid or chlorophyll levels (Restek 2009).

The extraction and analysis by LC-MS-MS and HPLC-FLD was only optimised to separate and detect E2. Further work would be to further optimise these protocols to detect other estrogens, notably, E1, E3 and EE2.

As well as extending the analysis method to incorporate the detection of other estrogens, the derivatisation step, using dansyl chloride could be replaced with alternative derivatisation reagents such as 2-fluoro-1-methylpyridinium p toluene sulfonate (FMPTS), pentafluoro benzoyl bromide (PFBBr) (Domnica Briciu *et al.* 2009) or by using 4-(dimethylamino) benzoyl chloride (DMABC) (Köhling,2011) (Appendix 2, 2.4, page XII) that would not require the removal of buffer salts that are needed to facilitate the derivatisation reaction.

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Appendix 1 Biometric data for the *Mytilus* used in the exposure experiment

1.1 Body weight and shell length information

The length, body weights, empty shell weight and the percentage internal tissue weight to shell weight are displayed below (Appendix Table 1).

The above biometric data were tested for normality using the Kolmogorov-Smirnov test of normality. This test is a measure of divergence from a sample distribution from a normal distribution. A high value of the test statistic value (D) and a low probability value, *p*, is a good indication that the data is not normally distributed (Appendix Table 2).

For the body weight to shell weight ratio data that is normally distributed, a one-way ANOVA test was applied to test if there are any differences in the body to shell weight ratio between treatments (Appendix Table 3; Appendix Table 4).

The one-way ANOVA test gives a *p* value of 0.058134 which is slightly larger than the *p* value of 0.05 for no significance between treatments. This indicates that there is a small difference in body weight to shell weight ratios between the treatments.

Treatment		Length mm	Shell weight grams	Empty shell weight grams	Percentage internal body weight to shell ratio
Control	Mean value	49.26	19.98	12.03	40.02
	SD	4.85	4.88	3.19	6.46
SECs	Mean value	52.68	22.28	14.08	36.62
	SD	5.34	4.61	2.91	4.90
SECs plus E2 (200 ng/L)	Mean value	53.18	24.04	13.30	39.55
	SD	4.69	10.69	3.36	9.07

E2 100 ng/L	Mean value	50.15	22.99	13.31	42.33
	SD	4.04	4.46	3.13	4.20
E2 200 ng/L	Mean value	48.98	21.96	12.86	41.63
	SD	4.39	5.14	3.40	5.52

Appendix Table 1. Biometric data for the Mytilus used in the exposure experiment

Results of the Kolmogorov-Smirnov Test of Normality applied to the Biometric Data					
	Count No.	Skewness	Kurtosis	D Value	*P* Value
Body Length	197	0.286859	-0.55042	0.11439	0.1031 This provides good evidence that the data is not normally distributed
Body weight	197	1.296505	0.641518	0.21976	<0.00001 This provides good evidence that the data is not normally distributed
%tage ratio body weight to shell weight	197	1.542431	11.836568	0.7064	0.26656 The data does not differ significantly from that which is normally distributed.

Appendix Table 2. Results of the Kolmogorov-Smirnov test of normality applied to the biometric data (Stangroom)

Summary of Body weight to Shell weight Biometric Data						
	Treatments					
	1	2	3	4	5	Total
N	49	49	39	40	20	197
∑x	978.83	1076.05	937.53	891.07	459.78	4343.26
Mean	19.98	21.96	24.04	22.28	22.99	22.05
$\sum x^2$	20694.70	24899.98	26879.28	20678.04	10947.17	104099.17
SD	4.88	5.14	10.69	4.61	4.46	6.52

Appendix Table 3. Summary of the body weight to shell weight data, (One-Way Repeated Measures ANOVA Calculator (Stangroom) Treatment 1 Control; Treatment 2 E2 200 ng; Treatment 3 SEC plus E2; Treatment 4 SEC; Treatment 5 E2 100 ng

One-Way ANOVA test					
Source	SS	df	MS	F value	*P* value
Between treatments	385.1563	4	96.2891	2.32309	0.58134 Not significant at *p* <0.05
Within Treatments	7958.1422	192	41.4487		
Total	8343.2985	196			

Appendix Table 4. Results of the one-way ANOVA test as applied to the above data (Stangroom)

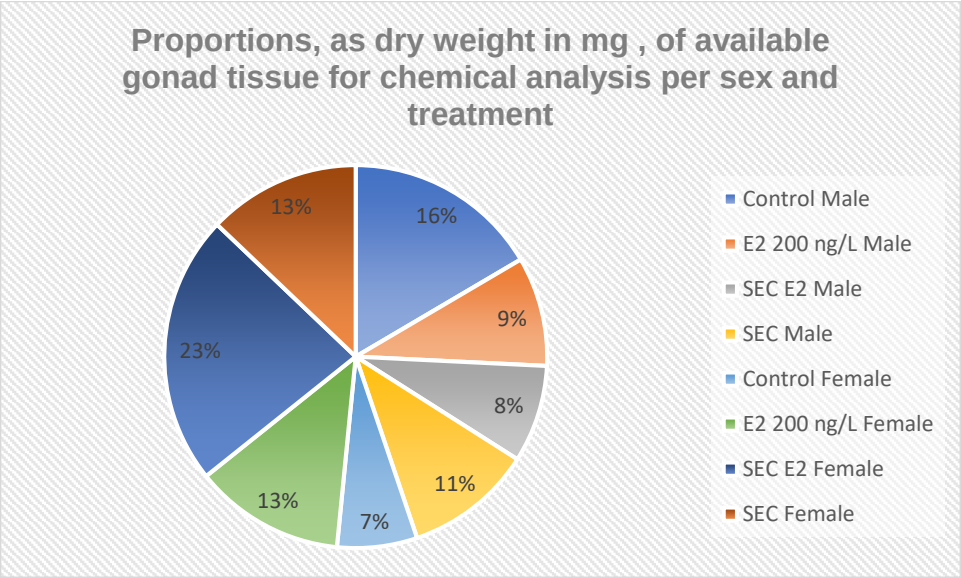
1.2 Results of the amount of pooled of gonad tissue available for analysis

There is slightly more pooled dry tissue available for the male mussels than for the female mussels. The dry weight of tissue for analysis varies from 0.3 mg per animal (for the female controls and 0.48 mg (for the male control). However, there was still ample tissue available for extractions to be done in triplicate for each sex and treatment. The male gonad tissue contained slightly more water than the female gonad tissue, the average pooled male tissue being about 78% water and the average pooled female tissue being about 75% water (Appendix Table 5; Appendix Figure 1) Subtracting the average weight of the wet tissue from that of the average dry tissue gave an approximate estimate that 1 gm of wet gonad tissue is equivalent to 234.42 mg of dry tissue.

		Male pooled tissue			
sample	No animals	weight wet tissue gm	weight dry tissue gm	percentage water	dry weight tissue mg
Control	14	6.78	1.51	77.74	107.84
E2 200 ng /L	21	8.75	1.90	78.30	90.44
SECs + E2	25	11.43	2.40	79.04	95.84
SECs	23	11.60	2.66	77.04	115.76
mean values	19.33	10.01	2.21	77.85	117.19

		Female pooled tissue			
sample	No animals	weight wet tissue gm	weight dry tissue gm	percentage water	dry weightt tissue mg
Control	25	7.44	1.97	73.55	78.74
E2 200 ng /L	16	5.83	1.51	74.03	94.68
SECs + E2	10	4.45	1.06	76.09	106.42
SECs	15	5.34	1.35	74.68	90.07
mean values	17	6.02	1.54	74.50	90.52

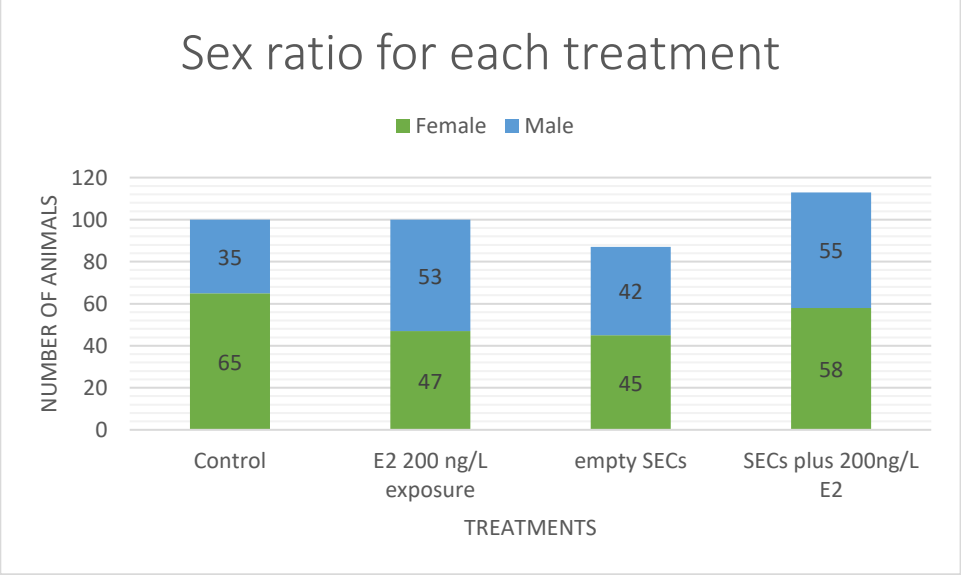
Appendix Table 5 wet and dry weights gonad tissue of female and male mussels per treatment



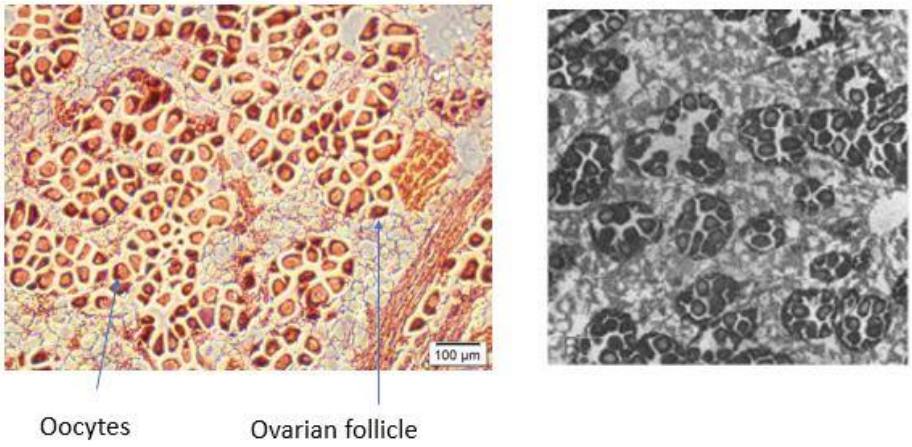
Appendix Figure 1. Proportions of dry tissue per sex and treatment after pooling, homogenising and freeze drying.

1.3 Stages of gametogenesis for the Mytilus used in the exposure experiment.

The majority of the histological sections for both SECs treatments were in the early to mature development stages II to III where there were emerging ripe gametes in the connective tissue of the gonads (Appendix Figure 3; Appendix Figure 4; Appendix Figure 5) The sections for the controls and E2 treatments had more mature than developing stages. The distribution of the sexes was about equal (Appendix Figure 2).

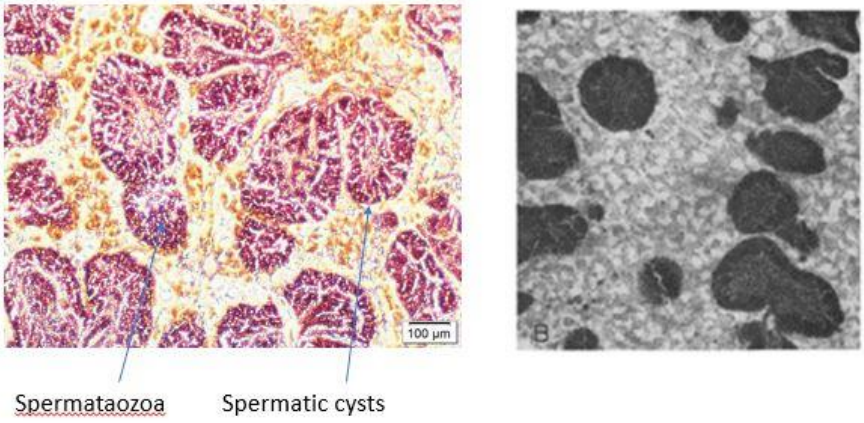


Appendix Figure 2. Sex ratio of mussels in the exposure experiment



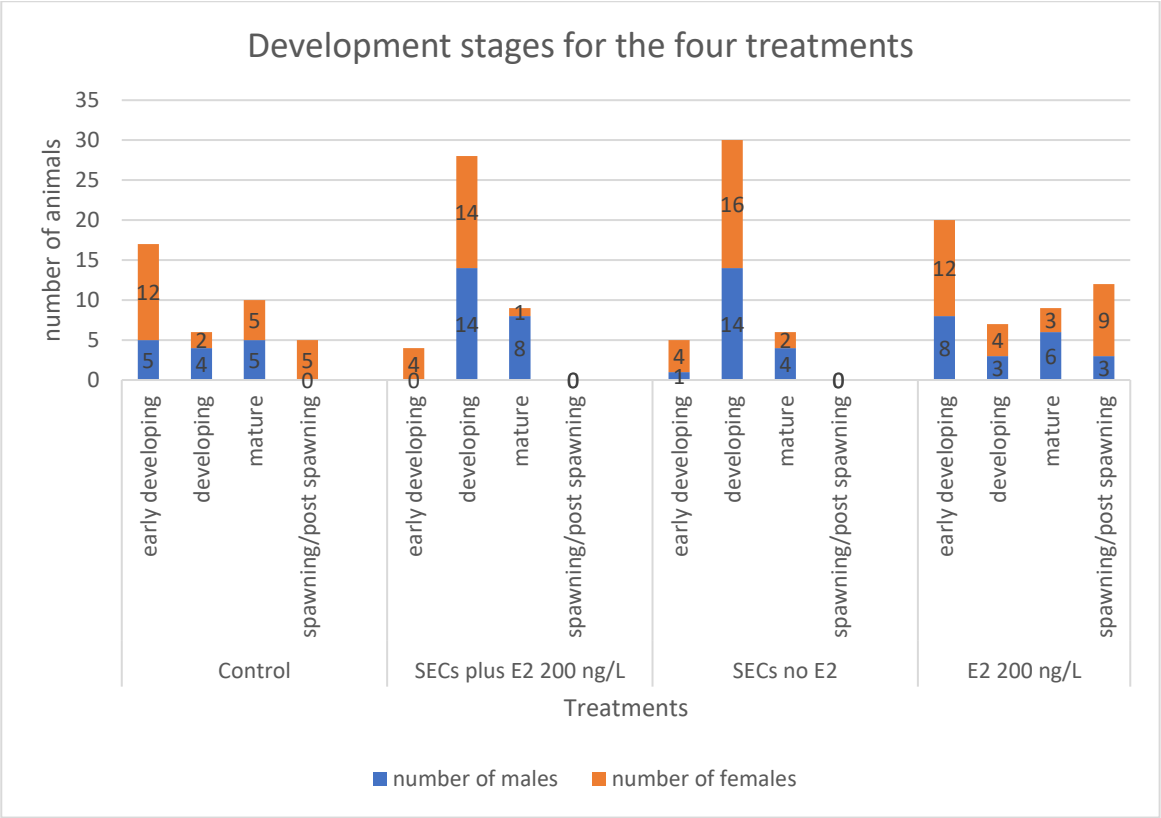
H & E stained preparation of female gonad, Stage III (developing) on the left according to Seed's classification [Seed 1969] on the right

Appendix Figure 3. Typical section of female gonad from the exposure experiment.



H & E stained preparation of male gonad, Stage III (developing) on the left according to Seed's classification [Seed 1969] on the right

Appendix Figure 4. Typical section of male gonad from the exposure experiment.



Appendix Figure 5. Stages of gametogenesis for male and females for each treatment.

Appendix 2 Final proposed extraction protocol used for the analysis of E2 extracted from *Mytilus* gonad tissue and water samples

2.1 Extraction of E2 from mussel gonad tissue using enzyme digestion and analysis by LC-MS-MS.

Mytilus gonad tissue was pre-digested using the enzyme subtilisin (P 5380 Merck, Darmstadt, Germany) using the following procedure:

A working solution was made by dissolving 5 mg of the enzyme in 50 mL of 10 mM sodium acetate plus 5 mM calcium acetate buffer, pH 7.5. to give sufficient enzyme for 50 samples (This solution keeps up to 2 days if refrigerated).

1 mL of the above enzyme solution was added to 25 mg of previously pooled, homogenised freeze dried, tissue in a 1.5 mL Eppendorf tube, and the mixture was incubated in a water bath at 37° C for 2 hours, with periodic vortexing. The digest was transferred to a 10 mL test tube and diluted to a total volume of 3 mL with 100mM sodium acetate buffer pH. 4.5. A 1 mL portion of the digest was retained in the Eppendorf tube for further enzymatic hydrolysis using the enzyme β glucuronidase/arylsulfatase (10127060001 Merck,Darmstadt, Germany) to convert any conjugated E2 in the extract into free E2.

To prepare a working solution of glucuronidase, 5 µL of the above concentrated solution of β glucuronidase/arylsulfatase enzyme was added to 3075 µL of 100 mM sodium acetate buffer pH 4.5, this was sufficient solution for 12 samples. (Aliquots of the diluted enzyme can be frozen at -20° C). Then 250 µL of the above working solution of the β glucuronidase/arylsulfatase enzyme was added to 1 mL subtilisin digest remaining in the test tube from above digest protocol. The mixture was vortexed and incubated in a water bath for 2 hours at 37° C. This digest was then transferred to a 50 mL centrifuge tube for QuEChERS extraction.

The solution was split into two parts (one third of the extract for total E2 extraction and two thirds for free E2 extraction) and both free and total extracts were treated using the same QuEChERS extraction protocol.

The QuEChERS extraction protocol was as follows. The internal standard used was 17 β estradiol 2,3,4, $^{13}C_3$ (719552, Merck, Darmstadt, Germany) A 1 µg/mL stock solution was made in acetonitrile and was serially diluted down to give a working solution of 0.5 pg/µL and 10 µL* of the 0.5 pg/µL working solution was added to each sample.

(* The internal standard peak was small, so for future work, it is recommended to add 20 µL or 25 µL of internal standard instead).

- Water was added to the tissue digest so that the total aqueous volume is 5 mL and the contents were shaken on a Heidoplh Reax shaker for 1 minute at 1250 rpm
- 5mL of acetonitrile acidified with 1% acetic acid was added and the mixture was shaken on a Heidoplh Reax shaker for 1 minute at 1250 rpm.
- 1.5 mL of hexane was added and the mixture was shaken on a Heidoplh Reax shaker for 1 minute at 1250 rpm.
- Half a packet (~ 3.75 g) of the AOAC salt was added (Supel QuE Acetate Extraction tube (55234-U, Supelco, Merck, Darmstadt, Germany) or Phenomenex roQ QuEChERS Extraction kit, KSO-8911, 7500 mg, Phenomenex Ltd., Macclesfield, UK.) and the mixture was hand shaken for 20 seconds, vortexed for 40 seconds and put on a mechanical shaker (Heidoplh Reax, Schwabach, Germany) at 1250 rpm for 2 minutes.
- The mixture was centrifuged at 5000 rpm for 5 minutes and the top hexane layer was removed.
- The tubes were put in the -20° C freezer for two hours. (The bottom aqueous salt layer freezes).
- The top acetonitrile layer was transferred to a new 15 mL centrifuge tube and 2 mL of water was added so that the solvent composition was about 60% aqueous acetonitrile.

The crude extract was cleaned up by passing through Clean-Up C18 Extraction Columns End capped CEC18153 3ml (Chromatography Direct Ltd., Runcorn, Cheshire, UK.). The yellow pigment was retained on the cartridge. The E2 that passed through the cartridge was collected. The SPE protocol used was as follows:

- The cartridge was conditioned by passing 5 mL of methanol through it, this is discarded.
- Then the cartridge was washed with 5 mL of 60% aqueous acetonitrile, this is discarded.
- The extract in about 60% acetonitrile was loaded onto the conditioned cartridge and the eluent was collected.
- The empty centrifuge tube that contained the extract was washed with 2.5 mL of 70% aqueous acetonitrile and these washings were passed through the cartridge and collected with the previous eluent. This step was repeated one more time.
- The collected eluent was placed in a 50 mL rotary evaporator flask and all of the solvent was removed.
- 1 mL of acetonitrile was added to the dry contents in the rotary evaporator flask and the mixture was swirled round to dissolve all of the contents. The solution was removed to a glass sample vial with a glass Pasteur pipette. Another 1 mL of acetonitrile was added to the rotary evaporator flask and the washings were combined with the previous washings.

- The combined 2 mL of extract was stored at -20° C until analysis.

For analysis, 250 µL of the extract was removed to a 2 mL glass vial for derivatisation and the solvent was evaporated under a flow of nitrogen to remove the acetonitrile The vial was then dried in a condenser (Eppendorf concentrator 5301, Scientific Lab Supplies Ltd., Wilford, Nottingham, UK.) at 30° C for 15 minutes to ensure the residue was completely dry before derivatisation.

2.2 Extraction of E2 from mussel gonad tissue using chemical hydrolysis (Cubero-Leon *et al.* (2012).

The gonad tissue was pooled, homogenised and freeze dried. 25 mg of the dry tissue was ultrasonicated in 5 mL of dichloromethane, followed by 3 mL of dichloromethane, 1 mL of methanol and finally 500 µL of methanol. The pooled solvents were combined, evaporated under a flow of nitrogen and re-suspended in 1.5 mL of methanol.

This solution was split into two parts, 500 µL was used to analyse total E2 and 1 mL was used to analyse free E2. The internal standard used was 17 β Estradiol 2,3,4, $^{13}C_3$ (719552, Merck, Darmstadt, Germany) A 1 µg/mL stock solution was made in acetonitrile and was serially diluted down to give a working solution of 0.5 pg/µL and 10 µL* of the 0.5 pg/µL working solution was added to each sample. (* The internal standard peak was small, so for future work, it is recommended to add 20 µL or 25 µL of internal standard instead).

The 500 µL used to analyse total E2 was hydrolysed by heating with 2.2 mL of water, 300 µL of 3 molar potassium hydroxide solution at 80° C for one hour. The cooled solution was then neutralised with 700 µL of 1 molar hydrochloric acid. Water was added to this solution so that the total volume of the extract was 7 mL and the methanol content was less than 10% v/v. Likewise, the other 1 mL of extract (free E2) was evaporated down to almost dryness under nitrogen and diluted down to 7 mL of water, ready for the SPE step.

2.3 SPE clean up steps

This involved two steps as follows:

<u>Step 1</u>

- A 200 mg, 3 mL, Strata X-AW SPE cartridge (Phenonmenex Ltd., Macclesfield, UK.) was conditioned with methanol and washed with 2 x 3 mL of 0.05 M sodium acetate buffer pH 7.0.

- The diluted extract was passed through the cartridge at a rate of about 1 mL/minute, washed with 2 x 3 mLs of the above buffer, followed by 2 x 3 mLs of milliQ water, dried and eluted with 6 mL of ethyl acetate.

The sample was evaporated to dryness under nitrogen and re suspended in 60% aqueous acetonitrile.

Step 2

The crude extract was cleaned up by passing through Clean-Up C18 Extraction Columns End capped CEC18153 3ml (Chromatography Direct Ltd., Runcorn, Cheshire, UK.). The yellow pigment was retained on the cartridge. The E2 that passed through the cartridge was collected.

The SPE protocol used was as follows:

- The cartridge is conditioned by passing 5 mL of methanol through it, this is discarded.
- The cartridge is washed with 5 mL of 60% aqueous acetonitrile, this is discarded.
- The extract in about 60% acetonitrile is loaded onto the conditioned cartridge and the eluent is collected. The empty centrifuge tube that contained the extract is washed with 2.5 mL of 70% aqueous acetonitrile and these washings are passed through the cartridge and collected with the previous eluent. This step is repeated one more time.
- The collected eluent is placed in a 50 mL rotary evaporator flask and all of the solvent is removed. 1 mL of acetonitrile is added to the dry contents in the rotary evaporator flask and the mixture is swirled round to dissolve all of the contents.
- The solution is removed to a glass sample vial with a glass Pasteur pipette. Another 1 mL of acetonitrile is added to the rotary evaporator flask and the washings are combined with the previous washings.

The combined 2 mL of extract was stored at -20° C until analysis. For analysis, 250 µL of the extract was removed to a 2 mL glass vial, evaporated to dryness under nitrogen and dried using a condenser as for the QuEChERS method above.

2.4 Derivatisation of the E2 extract for LC-MS-MS analysis using 4-(dimethylamino)benzoyl chloride

The dry tissue extracts were derivatised using 4-(dimethylamino) benzoyl chloride (DMABC), 526118 Merck, (Sigma Aldrich), Darmstadt, Germany) using a protocol from Köhling (2011). A solution of 0.5 µg/mL DMABC was made in dry acetone (dried over molecular sieves) and a crystal or two of a catalyst dimethylaminopyridine (107700, Merck, (Sigma Aldrich), Darmstadt, Germany) was added to the solution and the mixture was hand shaken to ensure the catalyst was dissolved

completely. 100 µL of this mixture was added to each dry sample, the mixture is vortexed for about 20 seconds and heated at 60° C for one hour and allowed to cool

The cooled sample is diluted with 400 µL of acetonitrile to give a 500 µL solution and 10 µL of this is injected on the LC-MS-MS. The method and parameters for LC-MS-MS analysis are as follows:

The LC-MS-MS instrument used was a Shimadzu model CBM-20Alite, using Shimadzu LabSolutions software

The binary pumps were both model LC-30AD

The mobile phase was LC-MS quality water with 0.1% formic acid at channel A and LC-MS quality acetonitrile with 0.1% formic acid at channel B at a constant flow of 0.5mLs/ minute

The gradient was: -

0.1 minutes 50% B

4.50 minutes 90% B

5.50 minutes 90% B

6.0 minutes 50% B

10 minutes 50% B

The column was a Shimadzu G155-HP 3 µm C18 3 µm x 100 mm

The column oven was model CTO-20AC at constant temperature of 40° C

The autosampler was model SIL-30AC and the injection volume was 10 µL

The acquisition was MRM with positive polarity

The target compound was DMABCl E2, precursor 420.10 m/z and product 148.15 m/z with a dwell time of 90 with a retention time of E2 at about 4.7 minutes (channel 1)

The internal standard was monitored using the precursor 420.10 m/z and product 166.15 m/z with a dwell time of 90 (channel 2)

The Interface was ESI at temperature 300° C

The DL temperature was 250° C

The nebulizer gas flow was 3.00 L/Min

The Heating gas flow was10.00 L/min

The drying gas flow was 10.00 L/min

This derivatisation method has not been validated so far. The recovery rate for the extraction of E2 and the LOD for E2, using the new internal standard still need to be determined.

2.5 Extraction of E2 from water samples (Cubero-Leon *et al.* (2012).

The water sample was filtered through a 0.45 µm cellulose acetate filter and 250 mL of the sample was acidified with 500 µL of glacial acetic acid to give a 0.2% v/v acidified solution. A C18U 3 mL volume cartridge (Phenomenex Ltd., Macclesfield, UK.) was conditioned with 2 x 3 mL of methanol and washed with 2 x 3 mL of acidified water (made by adding 2 mL of glacial acetic acid to one litre of milliQ water). All of the water sample was passed through the cartridge at a rate of about 1 mL a minute. The bottle containing the water sample was rinsed with 6 mL of acidified water and passed through the cartridge. The column was then washed with 2 x 3 mL of milliQ water and air dried for 20 minutes by drawing air through the cartridge under vacuum. About 0.5 g of anhydrous sodium sulphate was added to the top of the dried cartridge. The bottle was then rinsed with 6 mL of methanol and this was added to the cartridge and the methanol eluent was collected. Another 3 mL of methanol was added to the top of the cartridge and collected in a test tube to ensure all of the adsorbed E2 were removed from the cartridge. The methanol was evaporated to about 1 mL in volume using a rotary evaporator and this concentrated extract was returned to the test tube and the contents evaporated to dryness under a gentle flow of nitrogen and stored at -20° C until analysis. These extracts were derivatised using dansyl chloride (2.7.3; page 61).