Molluscan biomonitor for quantification and impact assessment of estrogenic and metallic contaminants in Australian marine ecosystems

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A thesis submitted to the Discipline of Biological Sciences, University of Newcastle, in fulfillment of the requirements of the Degree of Doctor of Philosophy

Supervised by: Dr Geoff MacFarlane and Associate Professor Hugh Dunstan

February 2011
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

I hereby certify that some of the work embodied in this thesis has been done in collaboration with other researchers, or carried out in other institutions. I have included as part of the thesis statements clearly outlining the extent of collaboration, with whom and under what auspices.

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Megan Andrew-Priestley
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<td>AGRF</td>
<td>Australian Genomic Research Facility</td>
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<tr>
<td>ALP</td>
<td>Alkali labile phosphates</td>
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<td>ASTM</td>
<td>American Society for Testing of Materials</td>
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<tr>
<td>BCF</td>
<td>Bioconcentration factor</td>
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<td>BPA</td>
<td>Bisphenol A</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BSTFA</td>
<td>Bis-(Trimethylsilyl) trifluoroacetamide</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Diode array detector</td>
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<td>EAL</td>
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<td>ERA</td>
<td>Environmental risk assessment</td>
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<td>ER-CALUX®</td>
<td>Estrogen receptor-mediated, chemical-activated luciferase reporter gene-expression</td>
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<td>ERE</td>
<td>Estrogen related receptor</td>
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<td>GCMS</td>
<td>Gas Chromatography Mass Spectrometry</td>
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<td>GPS</td>
<td>Global positioning system</td>
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<td>High Performance Liquid Chromatography</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
<tr>
<td>TBT</td>
<td>Tributyltin</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>YES®</td>
<td>Yeast estrogen screen</td>
</tr>
</tbody>
</table>
Publications arising from this thesis

Papers


Conference papers


Andrew, M., Dunstan, R. H., O’Connor, W., Van Zwieten, L., Macfarlane, G. R. 2007. 2nd Australasian Symposium on Ecological Risk Assessment and Management of Endocrine Disrupting Chemicals (EDCs), Pharmaceuticals and Personal Care Products (PPCPs) in the Australasian Environment, Canberra, ACT, Australia.

Awards

2010  University of Newcastle, Faculty (Science and IT) RHD Conference Scholarship.

2008  University of Newcastle, Faculty (Science and IT) Award for Outstanding Postgraduate Student Achievement Award.

2008  5th SETAC World Congress, Highly Commended Student Platform Presentation.

2007  University of Newcastle: University of Newcastle Postgraduate Research Scholarship (UNRSC).

2007  Australian Society for Ecotoxicology, Honours prize: “awarded to the best honours thesis submitted at an Australasian University in 2006”.

2006  University of Newcastle, Barry Boettcher Honours Prize: Awarded to the student with the best undergraduate record who is graduating with First Class Honours in the Department of Biological Sciences.
Abstract

Estrogenic compounds have been identified as a potential cause of reproductive effects in aquatic wildlife worldwide. This thesis was focused on the development of a native Australian species, the Sydney rock oyster *Saccostrea glomerata*, as a biomonitor of estrogenic compounds in Australian marine ecosystems. Biomarkers of estrogenic exposure in *S. glomerata* were examined under laboratory and field exposures to estrogenic compound/s.

Laboratory exposure of 17α- ethynylestradiol (EE2) to *S. glomerata* elevated vitellogenin (precursor to the female egg yolk protein) production in both females and males in a dose dependent manner but vitellogenin concentrations declined to basal levels after 49 days exposure. Exposure to EE2 was also capable of inducing accelerated female development, enlarged oocytes and an increase in the proportion of females. Increased proportions of females together with histological observations suggested that a complete sex reversal had occurred from male-intersex-female for a proportion of individuals.

A novel real-time qPCR assay for *S. glomerata* vitellogenin was developed. Sequencing of partial fragments of vitellogenin mRNA and vitellogenin genomic DNA allowed the identification of an intron-exon boundary and the development of real-time qPCR primers. Real-time qPCR revealed that female vitellogenin gene expression responded in a dose response fashion in individuals exposed to a concentration gradient of EE2 for 4 days.

To further test biomarker utility, *S. glomerata* were deployed in the receiving waters of Burwood wastewater treatment plant (WWTP) for 6 weeks, during which effluent was analysed for estrogenic compounds and activity. Sewage effluent from Burwood was found to contain both estrogenic compounds and activity, also *S. glomerata* deployed at Burwood had higher female vitellogenin (both protein and gene expression) and a higher proportion of mature females compared to individuals at reference locations.
As *S. glomerata* is an established biomonitor of metallic contaminants, it was employed for the analysis of heavy metals in individuals deployed at Burwood and reference locations. Concentrations of heavy metals in *S. glomerata* were found to be at low concentrations and within boundaries of spatial variation.

Overall, findings indicate that *S. glomerata* would be suitable as a biomonitor of estrogenic exposure and effects in the marine environment.

**Keywords:** Alkyphenols, biomarker, biomonitor, estrogens, gonadal development, intersex, oyster, sewage effluent treatment, vitellogenin.
Chapter 1: Introduction and Literature review

1.1. Thesis aims

The overall aims of this thesis were to develop the Sydney rock oyster, *Saccostrea glomerata*, as a biomonitor of estrogenic compounds in the Australian marine environment, via validation and development of biomarkers of estrogenic exposure and effect. Biomarkers of estrogenic exposure were selected based on reproductive changes at the cellular level (such as increased vitellogenin [precursor to the female egg yolk protein] and increased oocyte diameter) and at the organism level (increased proportions of female and/or intersex). Chapters were aimed at assessing if biomarkers of estrogenic exposure met the criteria of reliable biomarkers; exhibiting a dose response relationship with exposure to estrogenic compounds, temporal maintenance over constant exposure and early warning utility in laboratory and/or field exposures. As *S. glomerata* is already established as an effective biomonitor of heavy metals, assessment of heavy metals concentrations in the tissue of individuals deployed at a sewage effluent receiving location and at reference locations was also undertaken.

1.2. Estrogenic compounds

It is becoming increasing evident that certain compounds can interfere with the endocrine system of organisms and alter reproductive processes. These compounds are known as “endocrine disruptors”. The United States of America, Environmental Protection Authority (EPA) has defined endocrine disruptors as compounds which “interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior” (EPA, 1997). Estrogenic compounds form a large portion of the list of endocrine disruptors. Estrogenic compounds have structural similarities to the native estrogen; 17β-
estradiol (E2) which may allow binding to estrogen receptors or affect other estrogen related processes.

1.3. Types of estrogenic compounds

Estrogenic compounds can be grouped into those which are naturally occurring estrogens, synthetic estrogens or xenoestrogens. Naturally occurring estrogens include E2, and its metabolites estrone (E1) and estriol (E3). Also naturally occurring are phytoestrogens such as coumestrol, β-sitosterol and genistein (Langston et al. 2005). Others are synthetically created compounds which have been shown to be highly estrogenic, such as pharmaceuticals 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES). Xenoestrogens are man-made compounds created for industrial purposes but have unintended estrogenic action. This includes the industrial group of compounds, alkylphenol polyethoxylates, produced in large quantities worldwide for the production of emulsifiers, detergents, wetting agents and dispersing agents. The main alkylphenol polyethoxylates of concern are 4-nonylphenol (NP) and 4-tert-octylphenol (OP). Another weakly estrogenic xenoestrogen is Bisphenol A (BPA), commonly used in the production of polycarbonate plastics and epoxy resins (Okada et al. 2008). Also with some degree of estrogenic action, albeit very low, are pesticides (for example, atrazine, dieldrin, DDT, endosulfan, heptachlor, lindane, methoxychlor), biocides (pentachlorophenol), plasticisers (phthalates), polychlorinated biphenyls (PCBs which are used in oils, paints and lubricants), food preservatives (butylated hydroxyanisole), metalloestrogens and parabens (used in lotions) (Lintelmann et al. 2003).

Similarities in chemical structure between native estrogens and other estrogenic compounds are an important consideration, as this property allows estrogenic compounds to mimic E2. The chemical structure of E2 consists of an aromatised C18 steroid with hydroxyl groups at the 3β and 17β position (Figure 1.1). Derivatives of E2, E1 and E3, are structurally similar. Estrone (E1) differs from E2 only with a substituted ketone group (instead of a hydroxyl group) at position 17 (Figure 1.1). In comparison to E2, E3 contains an additional hydroxyl group at position 16. Not surprisingly EE2, designed to mimic E2 for its application in the contraceptive pill, is very similar in structure differing only in the addition of an acetylene
group at position 17 (Turan, 1996) (Figure 1.1). Estrogenic compounds OP, NP and BPA all contain a benzene ring with a hydroxyl group and thus possess some structural similarity to E2 and its derivatives (Figure 1.1). It is this structural similarity which allows these estrogenic compounds to potentially bind to estrogen receptors or elicit other non-receptor mediated estrogenic effects.
Figure 1.1: Chemical structure of selected estrogenic compounds of concern
1.4. Sources of estrogenic compounds

Wastewater treatment plants (WWTPs) receive an influx of sewage which is a combination of domestic and industrial wastewater subjected to treatment prior to release into the aquatic environment. While sewage effluent treatment is designed to degrade and remove organic matter and contaminants such as estrogenic compounds, removal is not always 100% efficient. Contaminants such as estrogenic compounds, E1, E2, EE2, E3, NP, OP and BPA have been frequently detected in sewage effluent in trace concentrations. The release of sewage effluent from WWTPs is among the main sources of release of estrogenic compounds into the environment. Intensive agriculture activities such as runoff from cattle feedlots, piggeries or dairies have also been identified as a potential source (Williams et al. 2007). Livestock have been demonstrated to release large quantities of endogenous estrogens, such as E1, E2 and E3 in their waste (Shore et al. 1988; Shemesh and Shore, 1994; Erb et al. 1977). Paper and pulp mills are another potential source of the release of estrogenic compounds into the aquatic environment. Effluent from paper and pulp mills has been shown to contain phytoestrogens, such as β-Sitosterol, β-sitostanol and campesterol, which are released from the wood during the processing procedure (Cook, 1997; Mahmoud-Kahn and Hall, 2003).

1.5. Sewage effluent treatment

Depending on the level of treatment, sewage effluent treatment may be primary, secondary or tertiary. Primary treatment is usually focused on separating the solids, fat and liquid fractions consisting of a sedimentation phase with one or more settling tanks where sludge and heavy particulates sink to the bottom while fats and oils rise to the surface and are mechanically skimmed off (Water Environment Federation, 2008). Primary treatment is far less efficient at removing contaminants, such as estrogenic compounds, compared to secondary and/or tertiary treatment processes (Ying et al. 2008). In comparison, secondary sewage effluent treatment processes are targeted towards biodegradation of contaminants. Thus, estrogenic compounds may be removed, usually via aerobic microbial breakdown which converts estrogenic compounds into non-estrogenically active (or less active) metabolites. Secondary treatment may include the use of filters or rotating membranes, with mixing or the addition of dissolved oxygen to support bacterial and micro-organism growth.
(Water Environment Federation, 2008). Tertiary sewage effluent treatment processes are the most efficient at removal of estrogenic compounds and may involve some or all of the following treatment processes: additional filtration, sedimentation, advanced oxidation (treatment with ozone, oxygen and hydrogen peroxide), ultra violet disinfection, electrolysis or membrane filtration steps to further remove sludge and contaminants from effluent (Water Environment Federation, 2008). Additional filtration may be through sands, lagoons or constructed/natural wetlands. Sewage effluent treatment processes are usually targeted towards reduction of bacterial content, not removal of contaminants such as estrogenic compounds. However, secondary and tertiary processes are usually more efficient at removing estrogenic compounds due to their increased capacity of a) particulate removal (which estrogenic compounds may be bound to) and b) biodegradation of contaminants.

1.6. Heavy metals in sewage effluent

In addition to estrogenic compounds, heavy metals are a group of contaminants which may also not be removed via sewage effluent treatment. Heavy metals have also been shown to associate with particulates and organics (Karvelas et al. 2003) and therefore a proportion of metals are thought to be removed during sewage effluent treatment alongside organics. Similar to estrogenic compounds, removal may not be 100% efficient and therefore heavy metals (especially dissolved metals) may also be released into the aquatic environment via effluent discharge (Karvelas et al. 2003).

Among heavy metals of environmental concern are copper, zinc, cadmium, lead and mercury (Kennish, 1992). Some metals, including iron, magnesium, manganese, cobalt, zinc and copper, have been identified to have essential biochemical roles in organisms (Norris and Carr, 2006). Yet at elevated concentrations all metals can be toxic to aquatic organisms, including those with essential biological roles. Demonstrated effects due to heavy metal exposure include reproductive and growth impairments, behavioural abnormalities and in some cases, mortality (Thomas, 1989; Norris and Carr, 2006). Also of high concern is their potential environmental persistence, capacity to bio-accumulate in
organisms and subsequent biomagnification through the food chain. Thus, monitoring of heavy metals in sewage effluent is of also of high concern and importance.

1.7. Environmental concentrations of estrogenic compounds
Internationally, there has been a vast amount of studies focusing on the detection of estrogenic compounds under the assumption that wastewater treatment plants are a major source. The majority of these studies have focused on the measurement of estrogenic compounds in final treated sewage effluent. International findings have suggested that estrogens, E1, E2 and E3, along with the synthetic estrogen, EE2, are among the main estrogenic compounds of concern detected in sewage. International studies have found that concentrations of natural estrogens (E1, E2, E3 and EE2) in sewage effluent are usually in the low ng/L concentration. Estrone has been detected at concentrations ranging from 3-70 ng/L, with the highest concentration measured in effluent from a German WWTP (Desbrow et al. 1998; Blackburn and Waldock, 1995; Ternes et al. 1999). Others have detected a range of 1-64 ng/L for E2 (Desbrow et al. 1996; Routledge et al. 1998) and 1-8 ng/L for E3 (Baronti et al. 2000). Synthetic EE2 has been detected at concentrations between 0.2-42 ng/L (Ternes et al. 1999; Desbrow et al. 1996). Xeno-estrogens have been detected at much higher concentrations with a range of 0-5370 ng/L for BPA (Sánchez-Avila et al. 2009; Staples et al. 1998a), 20-180 000 ng/L for NP (Blackburn and Waldock, 1995) and 120-17000 ng/L for OP (Lee and Peart, 1995). Fewer studies have reported concentrations of estrogenic compounds in open waters, which is likely to be due to the analytical difficulties associated with detection. However, some examples include reported concentrations of <0.2- 17 ng/L for E2 (Belfroid et al. 1999; Xiao et al. 2001), <0.1- 15 ng/L for EE2 (Desbrow et al. 1998; Ternes et al. 1999) and 0.002- 10 μg/L for NP (Blackburn and Waldock, 1995).

1.8. Estrogenic activity
An important consideration in the management of estrogenic compounds is that the potency of different compounds may vary by orders of magnitude. Estrogenic activity is measured in cell reporter assays via binding to an estrogen receptor and a single value is reported and
expressed as E2 equivalents, EEQ. Furthermore, estrogenic compounds can be compared according to their estrogenic equivalence factor (EEF). Estrogenic equivalence factors are calculated by comparing the response of an estrogenic compound to the response of E2 within a cell reporter assay (Table 1.1).

In terms of EEF factors, E2 and EE2 are highly estrogenic in comparison to all other compounds (Table 1.1) and thus although usually detected in trace concentrations, within low ng/L range, these compounds are of high concern. Several studies have measured both estrogenic activity and individual estrogenic compounds and concluded that E1, E2, E3 and EE2 accounted for over 90% of estrogenic activity (Snyder et al. 2001; Korner et al. 2001; Nakada et al. 2004; Pojana et al. 2004). In terms of their EEF, compounds such as BPA, NP and OP are orders of magnitude less potent. For example 1 ng/L E2 is equivalent to 230 000 ng/L (or 230 µg/L) NP in terms of estrogenic activity. This is an important consideration in terms of the evaluation of the ecological risk of estrogenic compounds. In matrices such as effluent, where there is likely to be a mixture of estrogenic compounds, it may be appropriate to measure the total estrogenic activity or convert concentrations of single compounds into a single value using EEF factors.
Table 1.1: Comparison of estrogenic equivalence factors (EEF) of estrogenic compounds based on response in cell reporter assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of reporter gene/modified cells</th>
<th>EEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Ethynylestradiol</td>
<td>ER-CALUX©</td>
<td>1.2 a</td>
</tr>
<tr>
<td></td>
<td>YES®</td>
<td>1.25 b</td>
</tr>
<tr>
<td></td>
<td>MVLN</td>
<td>0.9 c</td>
</tr>
<tr>
<td></td>
<td>HGLEN</td>
<td>1.6 c</td>
</tr>
<tr>
<td></td>
<td>competitive binding assay (α)</td>
<td>1.25 d</td>
</tr>
<tr>
<td></td>
<td>competitive binding assay (β)</td>
<td>1.25 d</td>
</tr>
<tr>
<td>Estrone</td>
<td>YES®</td>
<td>0.25 b</td>
</tr>
<tr>
<td></td>
<td>MVLN</td>
<td>0.4 c</td>
</tr>
<tr>
<td>Estriol</td>
<td>YES®</td>
<td>0.2 c</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>YES®</td>
<td>5.9 x 10⁻³ b</td>
</tr>
<tr>
<td>Nonylphenol</td>
<td>ER-CALUX©</td>
<td>2.3 x 10⁻⁵ a</td>
</tr>
<tr>
<td></td>
<td>YES®</td>
<td>1.8 x 10⁻⁵ b</td>
</tr>
<tr>
<td></td>
<td>MVLN</td>
<td>0.1 x 10⁻³ c</td>
</tr>
<tr>
<td></td>
<td>HGLEN</td>
<td>0.3 x 10⁻⁴ c</td>
</tr>
<tr>
<td>Octylphenol</td>
<td>ER-CALUX©</td>
<td>8 x 10⁻³ d</td>
</tr>
<tr>
<td></td>
<td>YES®</td>
<td>1.3 x 10⁻⁵ d</td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>1.75 x 10⁻⁴ d</td>
</tr>
<tr>
<td></td>
<td>YES®</td>
<td>2.3 x 10⁻⁵ d</td>
</tr>
</tbody>
</table>

1.9. A National perspective: the issue of estrogenically active compounds in Australian waters

The issue of estrogenic compounds in the environment, and their capacity to cause biological effects, is recognised as a significant issue in Australia (Ying et al. 2008, Williams et al. 2007). However, in comparison to international studies, there is far less known, especially in terms of the effects of estrogenic compounds in native wildlife.

1.9.1. Concentrations of estrogenic compounds

Within an Australian context, the minimum and maximum concentrations of estrogenic compounds are usually lower than reported international concentrations. This could be due to lower Australian population densities compared to European and American cities. Nevertheless, emerging results have indicated that estrogenic compounds are present in Australian sewage effluent. Highly estrogenic compounds have been detected within the range of 0.1-42 ng/L for E1 (Tan et al. 2007), 0.1-14 ng/L for E2 (Ying et al. 2008, Braga et al. 2005), 1-1.38 ng/L for EE2 (Ying et al. 2008) and <0.1 ng/L for E3 (Tan et al. 2007) (Table 1.1). Compared to international findings, within Australia BPA and OP have been detected within the ng/L range only with reported concentrations of 5.48-148 ng/L for BPA (Al-Rafai et al. 2007, Ying et al. 2008) and 5.4-66 ng/L for OP (Tan et al. 2007, Ying et al. 2008). However, NP has been detected at higher concentrations with a range of 0.03-2991 ng/L (Al-Rafai et al. 2007, Ying et al. 2008).

Several Australian studies have measured efficiency of WWTPs at removing estrogenic compounds. Although findings suggest that secondary and tertiary sewage effluent treatment is perhaps more effective at removing estrogenic compounds, there is evidence that removal is usually not 100%. Leusch et al. (2005) and Tan et al. (2007) demonstrated that tertiary WWTPs in Queensland were efficient at removing over 95% of estrogenic activity in sewage effluent. For secondary WWTPs, Ying et al. (2008) found concentrations of selected estrogenic compounds in four WWTPs across South Australia ranged from 64-92% for NP, 0.87-63% for E1, 47-68% for E2 and 0.77-72% for EE2 (Ying et al. 2008). Tan et al. (2007) also studied plants with secondary treatment and found that across five South Australian WWTPs there was 85-99% removal of NP, 38-99% for BPA and >99%
for E1 and E2. Although WWTPs employing primary treatment have not been studied extensively, one study by Braga et al. (2005) demonstrated low efficiency for E1 and E2, between 7 and 0 % respectively. Together, these studies indicate that selected Australian WWTPs are sometimes inefficient at removal of estrogenic compounds.

1.9.2. Effects of estrogenic compounds on native Australian species

At present, there is a lack of information regarding effects of estrogenic compounds on native Australian species, especially invertebrates. However, some recent Australian studies have focused on the development of native fish species as biomonitors of estrogenic exposure. Codi-King et al. (2008) developed an ELISA vitellogenin assay for barramundi, *Lates calcarifer*, and temperate black bream, *Acanthopagrus butcheri*. Laboratory exposure of *L. calcarifer* and *A. butcheri* via injection of E2 (5 mg/kg) resulted in significant vitellogenin induction in treated males compared to control males. Codi-King and Hassell (2008) found that *L. calcarifer* injected with 20 mg/kg E2 displayed significantly higher (p< 0.05) vitellogenin induction (assessed via ELISA and western blot) in comparison to controls. In the same study, Codi-King and Hassell (2008) found that wild *L. calcarifer* collected from contaminated (via agriculture, high population, sugarcane mill effluent and/or commercial ports) rivers had higher, although not significant, vitellogenin levels in comparison to individuals from reference locations. Pollino et al. (2007) suggested rainbow fish, *Melanotaenia fluviatilis* as a suitable model for measuring effects of estrogenic exposure. Pollino et al. (2007) reported that *M. fluviatilis* exposed to 1000 ng/L exhibited increased ALP concentrations (after 3 days) and increased oocyte atersia (after 3 and 14 days) compared to controls. Currently, there are few (if any) studies utilising native biomonitor molluscan species to assess effects in a field situation.

Some studies undertaken in Australia have assessed the effects of estrogenic compounds in an introduced species, the mosquitofish, *Gambusia affinis holbrooki*. One reported effect of estrogenic compounds in *G. holbrooki* is a reduced or modified gonopodium, which is an elongated anal fin used during copulation. Rawson et al. (2006) demonstrated that *G. holbrooki* exposed to 100 and 500 ng/L E2 for 8 weeks experienced a delay in the development of three gonopodium spines crucial for the movement of the gonopodium.
Batty and Lim (1999) found that male *G. holbrooki* collected from receiving waters of an WWTP in New South Wales exhibited a reduced gonopodium compared to individuals collected from reference locations. These findings were also reported by Leusch et al. (2006) who also reported a reduced gonopodium in wild *G. holbrooki* residing within undiluted sewage effluent in Queensland. Following exposure to estrogenic compounds, or mixtures which are suspected to contain estrogenic compounds, other reported effects include reduced reproductive success or alterations to the sex ratio. For example, Doyle and Lim (2005) have reported that males *G. holbrooki* exposed to 20, 100 and 500 ng/L E2 for 84 days had reduced reproductive success (less approaches by control females and lower percentage participating in copulation). Furthermore, Rawson et al. (2008) demonstrated that a population of *G. holbrooki* residing downstream of a WWTP in Sydney, New South Wales, had a decreased proportion of mature males compared to an upstream location which does not receive effluent.

1.9.3. Knowledge gaps
There are many knowledge gaps surrounding the issue of estrogenic compounds in Australia. Findings have demonstrated that estrogenic compounds can be present in sewage effluent from Australian WWTPs and have suggested a need for comprehensive monitoring of WWTPs across Australia to correctly identify and manage this issue. Secondly, as identified in the above review, there is very little known regarding the effects of estrogenic compounds on native biota, especially invertebrates. Combined with the advantages of using invertebrates, particularly molluscs, as biomonitor species (summarised previously in Section 1.14) this has suggested that the development of a native molluscan species would be ideal to monitor estrogenic compounds in the Australian marine environment. Lastly, the majority of Australia’s population is located along the coastal fringe with the bulk of sewage effluent discharged into the ocean. Thus, the marine environment is perhaps most susceptible to estrogenic exposure (via sewage effluent discharge), yet comparatively much less is known of estrogens and their effects in Australian marine waters. A marine species would be ideal for the monitoring of estrogenic compounds.
1.10. Entry and pathways of estrogenic compounds to organisms

Aquatic organisms may be particularly susceptible to estrogenic exposure as the marine environment is the final sink. Following entry into the aquatic environment, there are several routes of potential uptake by organisms. Organisms may be exposed via the ambient water, with uptake via respiratory surfaces such as gills. The lipophilic tendency of estrogenic compounds may contribute to dermal exposure with passive diffusion across epithelial membranes (Walker et al. 2006). Lipophilic compounds also tend to associate with particulates in the water column and filter feeders, such as oysters, may be exposed via food taken in the alimentary tract (Walker et al. 2006). Thus, exposure to estrogenic compounds may occur for aquatic organisms via ambient water or their food source.

Estrogenic compounds may also transfer from the water column into sediments where they may accumulate and concentrate under anoxic conditions (Langston et al. 2005). Sediment dwelling or feeding organisms may also be exposed to sediments via this route. Physical or chemical processes which disturb sediment may cause the re-entry of estrogenic compounds (accumulated in sediment) into the water column. Thus, sediments are also a sink and contribute to the environmental persistence and environmental cycling of estrogenic compounds.

1.11. Effects of estrogenic compounds on fish

The issue of endocrine disruption and the knowledge that certain compounds can mimic endogenous estrogens has been known for a long time, as early as the 1930’s (Cook et al. 1934; Walker and Janney, 1930; Stroud, 1940). However, the identification of reproductive changes in fish residing in receiving waters of WWTPs during the 1990’s prompted significant research effort examining the effects of estrogenic compounds on fish. It was suspected that WWTPs releasing sewage effluent into estuaries were a source of estrogenic compounds. Consequently, researchers have measured vitellogenin (precursor to the oocyte yolk protein) in fish as a sensitive biomarker of estrogenic exposure and effect via effluent exposure in receiving waters of WWTPs. Specifically, vitellogenin induction in males, which do not usually produce the protein, has provided compelling evidence of estrogenic mediated endocrine disruption in fish and has become a widely observed phenomenon worldwide.
Purdom et al. (1994) found that rainbow trout, *Oncorhynchus mykiss*, deployed in cages at 15 marine locations in England which received sewage effluent from WWTPs had increased vitellogenin (precursor to the egg yolk protein) concentrations between 500-1000 fold the concentration measured in individuals from reference locations. Purdom et al. (1994) further demonstrated that male *O. mykiss* may produce vitellogenin following exposure to EE2 at concentrations as low as 0.1 ng/L. Routledge et al. (1998) has also found that male roach, *Rutilus rutilus*, produced significantly higher (p< 0.05) vitellogenin than controls following 21 days exposure to either 100 ng/L E2 or 100 µg/L NP. Thus the occurrence of male vitellogenin induction in fish is a well established biomarker of estrogenic exposure.

The occurrence of intersex, both male and female gametes in a single organism, has also been widely reported as a reproductive effect in fish due to exposure to estrogenic compounds which are released in sewage effluent. In 1998, Jobling et al. reported a high incidence of intersex in wild populations of *R. rutilus* residing within UK and Irish rivers. The occurrence of intersex fish was significantly higher (p< 0.05) in fish populations from sites upstream or downstream of major WWTPs compared to fish populations in reference locations. They were also able to identify a significant linear relationship between the concentration of sewage effluent in river water and percentage of intersex fish (Jobling et al. 1998). Since Jobling et al. (1998), the occurrence of intersex fish due to estrogenic effluent exposure has been reported in fish populations in many countries including the United States of America (Snyder et al. 2004), Canada (Bevens et al. 1996; Folmar et al. 1996; Vajda et al. 2008), Denmark (Bjerregaard et al. 2006), Italy (Viganò et al. 2001) and South Africa (Barnhoorn et al. 2004). Although effects of estrogenic compounds, such as vitellogenin induction and the occurrence of intersex individuals are well established in fish, there is comparatively less known in invertebrates, such as molluscs.
1.12. Effects of estrogenic compounds on molluscs

1.12.1. Bioaccumulation and depuration of estrogenic compounds

Estrogenic compounds are lipophilic in nature suggesting a potential for bioaccumulation and biomagnification. Bioaccumulation is defined as the net product of uptake, metabolism and excretion. Contaminants are bioaccumulated when the rate of intake exceeds the rate of metabolism and/or elimination. A range of factors may affect bioaccumulation including the concentration of exposure, duration of exposure, rate of uptake, mode of uptake, lipid content of organism, metabolism, mode of excretion and the lipophilic nature of the compound of concern.

Accumulation of estrogenic compounds in molluscs may be higher in comparison to vertebrates. In laboratory and field based experiments, invertebrates have been demonstrated to bio-accumulate estrogenic compounds orders of magnitude higher than algae and fish (Staples et al. 1998b). Bioaccumulation in molluscs may be increased due to their lower metabolism. In humans, the metabolic half lives of E2 and EE2 are approximately 20 minutes and 17 hours, respectively (Baumann et al. 1996); however, in molluscs it is thought that this could be much longer as enzyme activities associated with metabolism are up to 10 times lower compared to mammals (Lai et al. 2002).

Molluscan studies provide some evidence that estrogenic compounds may be susceptible to bioaccumulation. Although there have been fewer studies on estrogens (E1, E2, E3 and EE2), these compounds may not be as readily accumulated compared to alkylphenols such as NP. Furthermore, the capacity of molluscs to rapidly metabolise estrogens to an ester (described in Section 1.10.3) may indicate that these compounds are perhaps not likely to be biologically persistent with long term exposure, although their biological effects may be greater due to greater estrogenic potency. Pacific oysters, *Crassostrea gigas* exposed to 0.046 μM E2 had a bio-concentration factor of 31, but this was within a short term exposure of 2 hours (Le Curieux-Belfond et al. 2005). Similarly, Gomes et al. (2004) demonstrated that water fleas, *Daphnia magna*, had a bioconcentration factor (BCF) of 229 after short-term exposure of 400 ng/L E1 over 24 hours. However bioconcentration may be
transient, with Gomes et al. (2004) also reporting that bioconcentration declined with time, reporting a BCF of 165 after 24 hours compared to 278 after 4 hours. In comparison to natural estrogenic compounds, alkyphenol ethoxylates such as NP, may have high bioaccumulation potential and not be as readily metabolised or depurated after days. Higher BCFs have been reported for the xenoestrogen, NP after long term exposures. McLeese et al. (1980) reported BCFs ranging from 1.4-7.9 following 4 days exposure of blue mussels, Mytilus edulis to NP. In addition, very high BCFs of 4120 and 2740 were reported following 16 days exposure of M. edulis to 6.2 µg/L and 5.9 µg/L, respectively (Ekeland et al. 1990). Bioaccumulation was also demonstrated in freshwater snails, Lymnaea stagnalis following exposure to 104 µg/L for 3 days NP and measurement of a BCF of 242 (Lalah et al. 2003). These findings may suggest that estrogens are more readily metabolised/depurated compared to xenoestrogens such as NP.

Compounds with high bioaccumulation potential which are not metabolised or eliminated easily may also biomagnify in a food chain, however, several studies have suggested a low potential for the biomagnification of NP. Ahel et al. 1993 measured the concentration of NP in freshwater organisms from Switzerland and found a high BCF of up to 1000 in algae (up to 38mg/kg in the species Cladophora glomerata) but no bioconcentration in fish species with concentrations ranging from <0.03-1.6 mg/kg. Hu et al. (2005) compared the bioconcentration of NP and dichlorodiphenyltrichloroethane (DDT) metabolites in different trophic levels including plankton, invertebrates, fish and marine birds. They found that there was no trophic magnification of NP in comparison to dichlorodiphenyldichloroethane (DDE). Although further research is required, these findings suggest that alkyphenols may have high potential for bioaccumulation but biomagnification is perhaps not of high concern.

Bioaccumulation of estrogenic compounds does not necessarily translate to estrogenic effects. Potent estrogenic compounds, E2 and EE2, may be rapidly metabolised but are likely to exert greater estrogenic action compared to more persistent compounds such as NP and OP. For example, Andrew et al. (2008) demonstrated that Sydney rock oysters, S. glomerata, exposed to 50 ng/L EE2, accumulated <1% of the total applied estrogenic
activity compared to individuals that were exposed to 100 µg/L NP and accumulated 43% of the applied estrogenic activity. It was suggested that EE2 exerted estrogenic effects prior to a rapid metabolism as estrogenic effects were greater with individuals exposed to 50 ng/L EE2, exhibiting higher levels of vitellogenin (significantly higher than controls by 4-fold in females and 2-fold in males, p< 0.05) compared to individuals exposed to 100 µg/L NP (significantly higher than controls with 2-fold response in females only, p< 0.05). Thus, bioaccumulation may provide insight into the biological persistence of estrogenic compounds but does not necessarily indicate the potential for biological effects.

1.12.2. Vitellogenin

1.12.2.1. Measurement of vitellogenin

Vitellogenin is the precursor to the female oocyte yolk proteins, vitellins, which accumulate in molluscan oocytes during gonadal maturation. Elevated vitellogenin or surrogate estimates of vitellogenin induction are useful as indicators of estrogenic exposure in both sexes but especially in males where the vitellogenin gene is silent. Upon exposure to sufficient concentrations of estrogenic compounds the usually silent male vitellogenin gene can be triggered providing clear evidence of endocrine disruption. The measurement of molluscan vitellogenin may be achieved through a variety of methodologies which may include direct measurement of the gene, protein or surrogate products which are thought to correlate with vitellogenin.

The measurement of vitellogenin gene expression may be achieved via real-time qPCR (qPCR). Although genetic sequence information is not currently available for vitellogenin genes of many molluscan species, real-time qPCR assays are becoming increasingly available for vitellogenin in molluscan species with assays developed in *M. edulis* (Puinean and Rotchell, 2006), *C. gigas* (Matsumoto et al. 2003), in the Calico scallop *Argopecten purpuratus* (Boutet et al. 2008) and *E. complanata* (Gagné et al. 2005).

In terms of the specific measurement of vitellogenin protein, there are very few assays available. Enzyme-linked immunosorbent assay (ELISA) has proved useful for measuring vitellogenin in fish species (Parks et al., 1999; Bon et al, 1997) and has many advantages
including species specificity, accuracy and rapid analysis. However, development of an ELISA assay depends on large amounts of purified vitellogenin for the development of antibody production and for plate coating in ELISA. Due to the difficulty of obtaining adequate amounts of purified vitellogenin in molluscs there are few ELISA assays developed for molluscan species to date.

Measurement of vitellogenin protein in molluscs is usually achieved via an indirect surrogate assay known as alkali labile phosphates (ALP) (Gagné and Blaise, 1999). The ALP assay is widely accepted as a surrogate method of measuring vitellogenin and has been widely employed in studies of effects of estrogenic compounds. This assay is considered indirect because vitellogenin is not measured directly and phosphates are a post-translational characteristic of vitellogenin (Matozzo and Marin, 2008b). Lipid or calcium measurements may also be used as indirect measures of vitellogenin (Gagné et al. 2002). Assays measuring surrogate estimates of vitellogenin, such as the measurement of ALP, calcium or lipids, lack specificity and as acknowledged by Gagné et al. (2005) may be useful as a rapid, inexpensive screening process followed by direct confirmation using specific measurements such as real-time qPCR.

Consequently, this thesis has focused on the development of two novel quantification methodologies for assessment of vitellogenin protein and gene expression in S. glomerata which will be validated via experimental and field exposures of estrogenic compounds.

1.12.2.2. Documented vitellogenin induction due to estrogenic exposure

It is thought that in molluscs, estrogens play a significant role in the synthesis and regulation of vitellogenesis. Matsumoto et al. (1997) demonstrated that E2 increased in ovarian tissue during the early phases of oocyte development in the scallop, Patinopecten yessoensis. Others have provided direct evidence that vitellogenin is under the control of E2 by demonstrating that injections of E2 were capable of stimulating vitellogenesis. Li et al. (1998) injected female C. gigas with E2 (50 µg/day for 10 days) and subsequently measured an increase in vitellins via enzyme-linked immunosorbent assay (ELISA) after 40 days compared to untreated individuals. Similarly, Osada et al. (2003) injected female
mature *P. yessoensis* with E2 (50 µg/day every 10 days, for 90 days) and, after 90 days, measured elevated vitellogenin in treated individuals compared to controls. Vitellogenin gene expression has also been shown to increase in a dose dependent manner following injection of E2 (1-25 nmol) in freshwater mussels, *Elliptio complanata* (Gagné et al. 2005). Together, these lines of evidence suggest that E2 plays a controlling role in the initiation of vitellogenesis.

Alkali-labile phosphates (ALP) are a surrogate measure of vitellogenin. Thus, with evidence that vitellogenin is likely regulated by E2 others have sought to examine induction of ALP in molluscs upon laboratory exposure to xenoestrogens. Various studies have demonstrated that exposure to NP is capable of inducing significant ALP induction. For example, Gagné et al. (2001) exposed male and female *E. complanata*, to 250 nm NP and measured an increase, although not significant from controls, in ALP levels in both male and female exposed mussels. In a similar study, Blaise et al. (1999) found that female soft shell clams, *Mya arenaria*, injected with NP (1 and 500 nM) had significantly higher (*p*< 0.05) levels of hemolymph ALP compared to control clams after 48 hours. Others have demonstrated that exposure to a concentration gradient of NP caused a dose dependent increase in ALP concentrations. For example, Matozzo and Marin (2005), found that the male manila clam, *Tapes philippinarum*, exposed to increasing concentrations of NP (0, 0.25, 0.05, 0.1 and 0.2 mg/L) for 7 days demonstrated a dose dependent increase in ALP levels in the digestive gland and hemolymph. Albeit higher concentrations of exposure, Riccardi et al. (2008) found that male and female adult black mussels, *Mytilus galloprovincialis*, exposed to NP (0, 25, 50 and 100 µg/L) for 7 days also exhibited a dose dependant increase in ALP levels. Induced ALP levels may be maintained over longer term exposures as demonstrated by Quinn et al. (2006) with significant induction of ALP in males and females after 112 days exposure of the zebra mussel, *Dreissena polymorpha*, to 5 and 500 µg/L NP. Other xenoestrogens, such as BPA, have also been demonstrated to induce significant ALP induction. For example, exposure of BPA (50 µg/L) to female *M. edulis* for 3 weeks caused increased induction of ALP levels (Aarab et al. 2006). Collectively, this evidence suggests that xenoestrogens are capable of inducing significant changes in levels of ALP (vitellogenin).
In contrast, some studies have found that exposure to estrogenic compounds does not induce any changes in vitellogenin or ALP. Riffeser and Hock (2002) measured vitellogenin protein by gel electrophoresis and found that male and female *M. edulis* exposed to E2 (2, 20, 200 and 2000 ng/L) and Swan mussels, *Anodonta cygnea* exposed to 2000 ng/L E2 for 3 weeks experienced no changes in vitellogenin. Similarly, no changes were found in vitellogenin protein (measured via gel electrophoresis) in male and female *E. complanata* exposed to E2 (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M) for 7 and 30 days (Won et al. 2005). These results may be attributed to additional factors which influence vitellogenesis, such as stage of gonadal development. It may be possible that individuals at the beginning of a reproductive cycle (when oocytes are pre-vitellogenic) are more likely to induce vitellogenin compared to mature individuals. This theory is supported by the findings of Matozzo and Marin (2008a) who compared ALP induction in female mature and immature *T. philippinarum* exposed to E2 (5, 25, 50, 100 and 1000 ng/L) for 7 days during both a resting and pre-spawning phase. Females close to spawning, demonstrated no changes in hemolymph ALP compared to controls. However, individuals with immature gonadal development had significant increases in the levels of hemolymph ALP in all exposure treatments (Matozzo and Marin, 2008a). Similarly, Puinean et al. (2006a) found that mature (close to spawning) female *M. edulis* exposed to 200 ng/L E2 exhibited no changes in the level of vitellogenin gene expression after 1, 3, 5 or 10 day/s exposure. Together, these findings may indicate that vitellogenin is most responsive to estrogenic exposure during earlier phases of gonadal development.

Under the assumption that sewage effluent is a potential source of estrogenic compounds, others have measured ALP in molluscs after direct laboratory exposures. Quinn et al. (2004) exposed *D. polymorpha* to tertiary treated sewage effluent from an Irish WWTP for 112 days during gametogenesis and found that exposed females displayed elevated ALP levels compared to female control individuals. Similarly, Gagné et al. (2001) found that male and female *E. complanata* induced significant ALP levels upon laboratory exposures to increasing concentrations (0, 10, 25 and 50 %) of sewage effluent collected from a WWTP within the Saguenay Fjord, Canada.
Molluscs deployed or residing within locations receiving sewage effluent have also been demonstrated to induce significant ALP levels. Gagné et al. (2001) found that deployed male and female *E. complanata*, had significant ALP induction following 62 days deployment 1.5 kilometres downstream of an WWTP in the St Lawrence River, Canada. Similarly, Blaise et al. (2003) deployed *E. complanata*, in waters receiving primary treated sewage effluent and found that exposed females individuals had significantly higher (p< 0.05) ALP compared to those from a reference location. In further support, male and female Calico scallops, *Argopecten gibbus*, deployed in-situ for three months at increasing distances (50 metres, 90 metres and 2.7 kilometres) from a municipal dump site at Castle Harbour, Bermuda, had elevated ALP levels compared to individuals deployed at the reference location (Quinn et al. 2005). Wild molluscs residing within receiving waters have also been demonstrated to exhibit significant ALP induction compared to individuals from reference locations. This has been demonstrated for both wild female *M. arenaria* in the Saguenay Fjord (Gagné et al. 2002) and in wild male and female *M. galloprovincialis* from the canals of Venice (Pampanin et al. 2005). Thus, compelling evidence has accumulated suggesting vitellogenin (or surrogate vitellogenin estimates) is responsive to exposure to effluents and is a useful indicator in field and laboratory studies of mixtures with potential estrogenic activity.

1.12.3. Gonadal development and sex change

Estrogenic compounds are capable of promoting female development with reported cases of accelerated female gonadal development in exposed molluscs. Increased embryo production is one reported example of an effect of estrogenic exposure in the live brooding species, the New Zealand mud snail, *Potamopyrgus antipodarum*. Jobling et al. (2004) reported a dose dependent increase in the total number of embryos in *P. antipodarum* following a 6 week exposure to increasing concentrations (1-25 µg/L) of BPA. Similarly, Duft et al. (2003) reported significantly greater (p< 0.05) embryo production in *P. antipodarum* following exposure to NP (10 and 100 µg/kg) and to EE2 (25 and 100 ng/L). Increased oocyte production has also been reported by Oehlmann et al. (2000). They demonstrated that exposure to increasing concentrations of BPA (1-25 µg/L) and OP
caused a dose dependent increase in oocyte production by giant snails *Marisa cornuarietis*. Other cases of accelerated female development include the occurrence of increased oocyte diameters due to estrogenic exposure. Li et al. (1998) demonstrated that injections of E2 (50 μg once every 10 days, for a 40 day period) in adult female *C. gigas* caused a significant increase in oocyte diameter in exposed (46.9 ± 0.9 μm) compared to control individuals (44.1 ± 2.4 μm). Similarly, Furrow Shell (*Scrobicularia plana*) females, had significantly larger (p< 0.05) oocytes following 6 weeks exposure to 100μg/kg E2 (96 ± 19 μm) or 100μg/kg EE2 (85 ± 8.6 μm) compared to control individuals (69 ± 9.2 μm in laboratory control, 52 ± 26.5 in field control) (Langston et al. 2007). Increased oocyte diameters were also found in juvenile sea scallops *Placopesten magellanicus* following injection with 30 μL (of 1000 μg/mL) estradiol solution. Larger oocyte diameters were observed in female scallops (48.5 μm) injected with estradiol compared to control scallops (22.6μm) (Wang and Croll, 2004). Thus, estrogenic compounds may promote embryo production as well as increased oocyte size.

Furthermore, estrogenic compounds may play a role in molluscan sex determination with reports of skewed female sex ratios and occurrence of intersex individuals. An early study by Mori et al. (1969) demonstrated that *C. gigas* injected with E2 (0.04-0.60 mg estradiol-3-benzoate) during the early stages of maturation experienced a full sex reversal from male to female. Similarly, Langston et al. (2007) demonstrated that in-vitro exposure of undifferentiated *S. plana* to spiked sediment (100 μg/kg E2, 100 μg/kg EE2, 1000 μg/kg NP and 1000 μg/kg OP) caused varying degrees of intersex. Forty four % of exposed individuals were intersex compared to only 0-8% of controls. Nice et al. (2000) found that exposure of *C. gigas* embryos (7 days post-fertilisation) to 1 and 100 μg/L NP caused a significantly higher (p< 0.05) proportion of female and intersex individuals at adulthood. Further, others have demonstrated that exposure to estrogenic compounds, via sewage effluent discharge, is capable of inducing intersex. Chesman and Langston (2006) reported that in locations contaminated with sewage effluent discharge within the Avon estuary in southwest England, 17 out of 23 wild populations of *S. plana* (normally gonochoristic; with separate sexes) exhibited intersex with up to 60% of males being affected. The occurrence of intersex was mainly within summer, during gametogenesis, and it was observed that the
proportion of females remained at 50% while the proportion of males decreased to accommodate an increase in intersex individuals (Chesman and Langston, 2006). Blaise et al. (2003) reported a higher proportion of females (64%) in *E. complanata* residing within receiving waters of sewage effluent compared to 41% females in a population from a reference site. Thus, estrogenic exposure may be capable of influencing sex determination with evidence of sex reversal and intersex, though the underlying mechanisms of estrogen-induced sex reversal in molluscs are largely unknown at present.

1.12.4. Trans-generational Effects

Trans-generational effects have not been studied extensively in molluscs, however, Nice et al. (2003) has reported some evidence which suggested that effects of estrogenic exposure could potentially carry over into the subsequent generation. Nice et al. (2003) exposed fertilised *C. gigas* to 1 and 100 µg/L NP for 48 hours and found that when the subsequent generation reached maturity there was a skewed sex ratio towards females and a higher proportion of intersex individuals (up to 30%). Gamete viability of the parent population was also affected which resulted in poor embryonic and larval development of the subsequent generation. This evidence is concerning and could indicate that even in the scenario of short term pulse exposures, there may be long term effects which could occur long after the initial exposure event. The possibility of intersex and/or increased female proportions in subsequent generations could translate decreased fitness and viability of the population and suggests that further research is required to explore the potential mechanisms responsible for such effects and further, whether such trans-generational effects are ubiquitous across molluscs.

1.13. Sydney rock oyster

1.13.1. The Sydney rock oyster

The Sydney rock oyster, *S. glomerata* (formerly *S. commercialis*) is endemic to Australia and New Zealand. It has a broad distribution and high commercial value along Australia’s east coast tropical and subtropical waters, from southern Queensland to southern New South Wales. The *S. glomerata* industry also forms the basis of the largest aquaculture industry in New South Wales (White, 2002). Common names for *S. glomerata* include the
Sydney rock oyster, the commercial oyster, the mangrove oyster and the rock oyster. Their habitat is usually within intertidal and sub-tidal estuarine zones. Molluscs are ideal candidates for a biomonitor of contaminants in marine locations due to their sessile nature, high filter feeding capacity and ability to bio-accumulate both organic and inorganic contaminants (Ortiz-Zarragoitia and Cajaraville, 2006).

1.13.2. Oyster anatomy

The anatomy of an oyster has been described in detail by (Galtsoff, 1964) and (Edle and Scro, 1996). An oyster is a soft bodied organism protected by a hard thick shell. The oyster grows to approximately 8-10 cm but can exceed 25 cm in length. The two valves of the shell are attached via an elastic ligament and the abductor muscle. The inner organs of an oyster are fully encased with a fleshy fold of tissue known as the mantle or pallium which consists of two lobes which are connected in the cloacal chamber (Edle and Scro, 1996) (Figure 1.2). The ventral side of the mantle cavity contains the palps and gills and the dorsal side contains the cloacal chamber (Edle and Scro, 1996). The right mantle lobe is separated to form the promyal chamber while the left lobe is connected to the visceral mass. Radial muscles, blood vessels and nerves start in the visceral mass and continue through the mantle forming fan-like enlargement at the base of the sensory tentacles (Edle and Scro, 1996). The border of the mantle is divided into three separate lobes: the outer, middle and inner. The inner lobe has muscles which contract to open or seal the mantle cavity allowing oysters to direct jets of water from the mantle cavity for the purpose of spawning or removing pseudo faeces. The gonads are paired together and cover the outer surface of the digestive gland (Figure 1.2). Each gonad is a system of branching tubules from which the gametdes are attached. The tubules join to form ducts which eventually lead to a short gonoduct (Gosling, 2003).
1.13.3. Endocrine and nervous system of molluscs

In molluscan taxa, major reproductive processes are thought to be under the control of both the nervous system and endocrine system (Defur et al. 1999). The central nervous system consists of a network of neurons which transmit electrochemical signals between different organs within the body. The endocrine system is composed of a system of glands which secrete chemical messages, known as hormones, to regulate processes.

1.13.3.1. Neuropeptides

The molluscan central nervous system consists of the cerebral, pleural, pedal and abdominal ganglia which are responsible for the production of neuropeptides (Geraerts et al. 1988; Nassel, 1996; Defur et al. 1999). Insulin-like neuropeptides are produced by the central nervous system with critical roles in growth, development and metabolism (Pertseva et al. 1996). The neuropeptide known as FMRFamide has been characterised in molluscs...
and thought to be responsible for physiological processes such as the regulation of heart beat (Painter and Greenburg, 1982: Lesser and Greenburg, 1993). Others have identified neuropeptides directly responsible for reproductive processes. For example, Oberdorster and McCellan-Green (2000) located a neuropeptide called APGWamide which is located in the penis and vas deferens in some species of molluscs and is thought to control sexual behaviour. Other research has determined that neuropeptides produced in the cerebral ganglion are responsible for the development of female accessory sex organs, gonad maturation and ovulation (Painter and Greenburg, 1982: Lesser and Greenburg, 1993). For example, the oocyte laying hormone has been identified as responsible for gonadal maturation, oocyte mass production and the oocyte laying behaviour in the sea slug *Aplysia depilans* and *L. stagnalis* (Geraerts et al. 1988).

1.13.3.2. *Endogenous sex hormones*

Endogenous estrogens have been demonstrated to exist in molluscan species and have been shown to exhibit a synchronous profile with gonadal development. Matsumoto et al. (1997) demonstrated that E1 and E2 were present in the ovary of *C. gigas*, and in *P. yessoensis*, concentrations increased with gonadal development and just prior to a spawning event. Gauthier-Clerc et al. (2006) measured concentrations of 150-400 pg/g E2 in *M. arenaria* which also peaked during gametogenesis at the start of gonadal development (during pre-vitellogenic stages) and during spawning.

Evidence suggests that molluscs are capable of the rapid metabolism of estrogens into a lipophilic ester. Molluscs, *C. gigas* and *M. galloprovincalis*, have been shown to be able to esterify E2 (Janer et al. 2004; Janer et al. 2005). The identity of the E2 ester was determined by Labadie et al. (2007) who exposed *M. edulis* to 10 ng/L E2 for 13 days. Following exposure they collected the ester fraction and found three esters, E2-C_{16:0}, E2-C_{16:1} and E2-C_{16:2} were present in both sexes of exposed and unexposed individuals. Janer et al. (2005) and Labadie et al. (2007) suggest that as the majority of E2 is stored as an ester, esterification of E2 may be a mechanism for long term storage and maintenance of free E2 concentrations.
The hypothesised synthesis pathway of endogenous molluscan estrogens via cytochrome P450s and steroid dehydrogenases has been proposed by Porte et al. (2006), based on a review of studies on a variety of molluscan taxa. Cholesterol is converted to pregnenolone by cytochrome P450 side chain cleavage. Pregnenolone may be converted to progesterone by 3β/Δ5-Δ4-hydroxysteroid dehydrogenase and is metabolised by a number of cytochrome P450s to cortisol, but also to 17α-hydroxyprogesterone and androstenedione (an androgen/estrogen precursor). Androstenedione may also be produced from dehydroepiandrosterone (DHEA), and subsequently converted to estrone by cytochrome P450 aromatase. Seventeen β-hydroxysteroid dehydrogenases (17β-HSDs) facilitate production of testosterone from androstenedione and E2 from E1 (Porte et al. 2006) (Figure 1.3). These lines of evidence have suggested that E2 plays an important role in the steroidogenesis of molluscs and suggest that estrogenic compounds (including those which mimic natural estrogens) are likely to affect steroidogenesis, non-genomic estrogen mediated responses and potentially affect estrogen receptor mediated processes, such as vitellogenesis.

1.13.4. Sex Determination

Sex determination in oysters is thought to be partly controlled by environmental factors, such as temperature, food and salinity (Coe, 1936). Oysters, *S. glomerata*, are known to exhibit protandric dioecy (Asif, 1979) whereby sexes are separate. However, a subset of male individuals are thought to be protandric and change to female in later years with a shift in the proportion of females occurring with size and age (Thompson et al. 1996). Sex reversal is thought to occur between reproductive cycles during the resting phase when the gonad is indeterminate. Intersex can be present at low occurrences with reported percentages of 0.4-0.7% (Dinamani, 1974; Cox et al. 1996). The first cycle of gonadal maturation and growth of the individual is highly demanding in terms of energy requirements. Therefore, it is favourable to mature as males due to the lower energy requirements which are required for male gonadal development (Coe 1943).

Guo et al. (1998) studied the sex ratios of *C. gigas* populations and proposed a model for the genetic determination of protandric oysters. They hypothesised that sex determination is strongly influenced by paternal effects and sex determination may involve a single gene locus with a dominant male allele (M) and an allele for protandric females (F). Under this model, oysters with the genotype MF are true males (which will not change sex throughout their life) and oysters with a FF genotype are protandric maturing as males as juveniles and may change sex to fixed females in later years (Guo et al. 1998). Hedrick and Hedgecock (2010) expanded on this research proposed by Guo et al. (1998) by performing hypothetical modelling using the two genotypes, FM for male and FF for protandric females. Their modelling results supported stable polymorphism (i.e. two alleles for sex determination) within a single or multiple age population proposed by Guo et al. (1998). However the two allele model could not explain the heterogeneity of sex ratios which they observed within half-sib families with a single male parent and different female parents. Hedrick and Hedgecock (2010) proposed a three genotype model which supported their sex ratio results with all possible parent crosses in their model. This consisted of two types of female genotypes: FM which are protandric, FF which are fixed females (do not change sex) and one fixed male genotype, MM. It is proposed that further work is needed with manipulation.
of environmental factors which affect sex, combined with genome mapping to identify the allele (and possibly multiple alleles) involved in sex determination.

1.13.5. Reproductive Development

Gonadal development, the maturation of oocytes or sperm, occurs during warmer months between October and March. Oysters may spawn several times during the season which is thought to be triggered by a change in temperature or salinity (Holliday, 1995). Following external fertilisation, embryos develop into free swimming larvae known as the “D” veliger stage (Holliday, 1995). Larvae then develop a clear shell and a retractable foot and swim for up to 3 weeks before developing into the pediveliger stage where they use their foot to attach to a suitable substrate (then known as spat) (Holliday, 1995). The foot is then resorbed, the shell darkens and the spat begins to grow into an adult oyster.

The stages of gonadal development for *S. glomerata* have been described in detail by Dinamani (1974) and are summarised in Table 1.2 below.
Table 1.2: Summary of *Saccostrea glomerata* gonadal developmental stages as described by Dinamani (1974)

<table>
<thead>
<tr>
<th>Gonadal stage</th>
<th>FEMALE</th>
<th>MALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 F1</td>
<td>F1: Follicles contain mainly oogenia and a few primary oocytes.</td>
<td>M1: Follicles contain mainly spermatogonia and a few spermatocytes.</td>
</tr>
<tr>
<td>G2 F2</td>
<td>F2: Oocyte 2 of sizes up to 25 µm with conspicuous nucleolus and granular cytoplasm; follicles small.</td>
<td>M2: Primary and secondary spermatocytes; few spermatids; wide band of early stages in section of follicle.</td>
</tr>
<tr>
<td>G3 F3</td>
<td>F3: Oocyte 2 larger than 25 µm, attached to the follicle with a stalk like connection; a few free in the lumen; apical cytoplasm with dense basophilic granules; follicles increasing in size.</td>
<td>M3: All stages of spermatogenesis up to spermatids; few spermatozoa; follicles do not occupy entire gonad area.</td>
</tr>
<tr>
<td>G4 F4</td>
<td>F4: Large free ova in the lumen, rounded to oval, follicles occupy entire gonadal area with little or no interconnective tissue.</td>
<td>M4: Follicles occupy large area; spermatozoa constituting central area of follicle, with narrow band of spermatocytes peripherally.</td>
</tr>
<tr>
<td>G5 F5</td>
<td>F5: Many follicles are discharged some entirely. Residual oocytes. Few hemocytes present among gonad.</td>
<td>M5: Many follicles are discharged, mass spermatozoa separated from follicular walls.</td>
</tr>
<tr>
<td>GX FX</td>
<td>FX: Residual oocytes sometimes present, follicles collapsed, large numbers of hemocytes</td>
<td>MX: Residual sperms and large hemocytes. Follicles are collapsed and no spermatocytes present.</td>
</tr>
</tbody>
</table>

**Intersex** Presence of both oocytes and spermatozoa which are located within an intact follicle.

**Indeterminate** Absence of oocytes and spermatozoa. There is a high proliferation of hemocytes and follicles are shrunken.

1.13.5.1. Female

The process of female gonad development is described in Table 1.3 with examples of histology photos in Figure 1.4. During F1 (Figure 1.4A) oogonia develop around the perimeter of the follicle wall. The oogonia are quite small in diameter (6-7 µm) and have a very thin layer of cytoplasm (Dinamani, 1974). The stage of F2 development (Figure 1.4 B) is characterised by a higher number of primary oocytes. The primary oocyte has a thicker layer of cytoplasm and the nucleolus is now visible as a small black mass. The main
characteristic of the F3 stage of gonadal development is an abundance of larger irregular shaped oocytes which are attached to the follicle wall by a stalk-like connection (Figure 1.4C). During F4 stage of development (Figure 1.4D) an oyster is referred to as ‘ripe’ when the individual is closest to spawning. The oocytes are more rounded in shape and have been released into the lumen of the follicle. There is very little connective tissue visible as the entire gonad area is filled with follicles of oocytes. Following a spawning event, the oyster moves into a phase of regression classified as F5 (Figure 1.4E). There is a proliferation of hemocytes, the follicles are broken/ shrunken and there are few residual oocytes present in the gonadal area. In gonadal stage F6, the follicles are completely reduced and there are very little, if any, residual oocytes.
Figure 1.4: Gonadal tissue from histological preparation of individual female *Saccostrea glomerata* with the six stages of female gonadal development; A) F1, B) F2, C) F3, D) F4, E) F5 and F) F6. All images are at X 200 magnification. Scale bar= 100 μm.
1.13.5.2. Male

Early male gonadal development can be complicated in terms of identification of gonadal stages, as the sperm cells are much smaller in comparison to oocytes and can look similar to hemocyte cells. However, their nuclei stain much darker compared to hemocytes and they are easy to identify when present in a higher abundance of cells. During M1, the male gonad starts to fill with spermatogonia which are attached to the follicle wall (Figure 1.5A). During gonadal development stage M2 (Figure 1.5B), there is a much higher abundance of spermatocytes which are now detached from the follicle wall. These cells are much darker due to the intense amount of chromatin material in the nucleus. However, there is a higher abundance of connective tissue compared to gonadal cells. During M3 (Figure 1.5C), the spermatids are densely packed within the follicles and the spermatids are aligned with tails in the lumen of follicles (Figure 1.5D and F). At M4 (Figure 1.5D), there is little connective tissue and the entire gonad area is filled. Within follicles, the spermatids are very dark in colour and very densely packed into the lumen of follicle. At M5, the individual has spawned which is evident from the broken follicles (left corner of Figure 1.5E) and high abundance of hemocytes. A male in gonadal development stage M6 (not shown) is difficult to identify as residual spermatozoa are more difficult to locate. It is likely that males in this category will be identified as indeterminate.
Figure 1.5: Gonadal tissue from histological preparation of individual male Sydney rock oysters, *Saccostrea glomerata*. A) M1, B) M2, C) M3, D) M4, E) M5 and F) M4. All images are at X 200 magnification except for F (x 400). Scale bar= 100μm, except for F (200 μm).
1.13.5.3. Indeterminate and intersex

Indeterminate gonadal development is a resting phase where neither oocytes nor sperm are visible (Figure 1.6A). This is the transition phase following F6 and M6 and is also known as a resting phase. Follicles are collapsed or shrunken and there is high hemocyte activity which assists in the clearance of residual oocytes or spermatozoa.

Intersex individuals contain both oocytes and spermatozoa within an intact follicle (Figure 1.6B-F). This is a rare occurrence in *S. glomerata* and is possibly a transition phase in the sex change of male to female for individuals exposed to exogenous estrogens.
Figure 1.6: Gonadal tissue from histological preparation of intersex Sydney rock oysters, *Saccostrea glomerata*. A) Indeterminate gonad, shrunken follicles and hemocyte proliferation X 200, B) Intersex follicle; gonad in regression. Some follicles containing both oogonia (F1) and spermatozoa (M1) X 200, (50 ng/L EE2 exposure for 21 days) C) Intersex follicle containing oogonia and primary oocytes (F1), spermatozoa in centre (M2) X 400(50 ng/L EE2 exposure for 49 days), D) Intersex gonad (lower magnification of C, 50 ng/L EE2 exposure for 49 days), E) Intersex follicle containing oogonia and primary oocytes (F1) with spermatozoa in centre (M3) X 400(12.5 ng/L EE2 exposure for 49 days), F) Intersex gonad (lower magnification of C, 12.5 ng/L EE2 exposure for 49 days), Scale bars, A, B, D and F= 100μm, C and E= 200μm,
1.13.6. Condition index

The condition index provides a valuable insight into the reproduction and health of an individual oyster. Gonadal tissue can account for up to 60% of the body weight of an oyster (Páez-Osuna et al. 1995), and condition index should increase with gonadal development with a dramatic decrease following a spawning event. Furthermore, if condition index does not increase during a phase of gonadal development it may indicate that if the individual is stressed/ diseased or without the energy reserves required for gonadal development (Honkoop, 2003).

There are a range of condition indices that may be employed in bivalve aquaculture. The dry condition index (dry tissue weight: dry shell weight) is generally considered to be the most accurate and preferred index. However a wet condition index may be employed in studies where wet tissue is required for separate analyses such as histology or vitellogenin measurements (Lucas and Beninger, 1985).

1.14. Quantification of estrogenic compounds

1.14.1. Detection of estrogenic compounds GCMS

Quantitative measurement of estrogenic compounds is usually achieved via gas chromatography high spectrometry (GCMS). Analysis via GCMS is (usually) a sensitive and accurate technique that can detect specific compounds within a complex mixture. However, there are several disadvantages for using GCMS to detect estrogenic compounds. Compounds of interest need to be extracted from the sample (e.g. waters, effluent and/or tissues) prior to analysis which can pose difficulties, especially when measuring a suite of compounds, as extraction procedure needs to be specifically tailored towards compound (s) of interest, likely concentrations and the sample matrix (López de Alda and Barceló, 2001). Secondly, there is likely to be a mixture of one or more estrogenic compounds yet it is time consuming and expensive to individually measure each estrogenic compound and its metabolites. The third consideration is that estrogenic compounds may be present in low concentrations, often below the limit of detection. This creates the potential of creating a ‘false negative’ situation where no estrogenic compounds may be detected but the additive
effect of mixture of compounds at a low concentrations may still be sufficient to cause endocrine disruption in organisms (Kinneburg, 2003).

1.14.2. Detection of estrogenic compounds: reporter gene assays

Estrogenic activity may also be measured via cell based assays or reporter-gene assays, such as the Yeast Estrogen Screen (YES® assay) and estrogen receptor-mediated chemical-activated luciferase reporter gene-expression (ER-CALUX®), which measure total estrogenic activity of samples reported in E2 equivalents (EEQ). Reporter gene assays are based on cells modified via the insertion of a reporter gene, such as luciferase, which is up-regulated following binding of an estrogenic compound to the estrogen receptor. Reporter gene assays provide an integrated value for a suite of (likely unknown) estrogenic compounds, taking into account different estrogenic potencies between estrogenic compounds. These assay characteristics are favourable for monitoring of estrogenic compounds reducing sampling and analysis time. Disadvantages to reporter-gene assays may be that estrogenic compounds can exert biological effects through non-genomic mechanisms (other than specific binding to a receptor model) (Thomas, 2003) and that the assay does not identify which compound/s responsible for estrogenic activity causing difficulties in the development of management regimes.

1.15. Biomonitors of estrogenic exposure

A biomonitor is an organism with validated biomarkers which can be used to monitor the health of an ecosystem. There are a number of criteria which should be met for ideal biomonitoring species. For ease of deployment, the species should be sedentary, robust, native, widely distributed and have well described biology (Walker et al. 2006). Filter feeding species are ideal as they have a higher likelihood of exposure and bioaccumulation of contaminants. These criteria are all satisfied by S. glomerata suggesting that this species would be ideal as a biomonitor of estrogenic compounds. In addition, S. glomerata has high commercial value and production in NSW and examination of effects of estrogenic compounds would assist in the protection of this industry.
Biomarkers are quantitative or qualitative biological responses which can be measured and attributed to the contaminant or contaminant class of concern (Huggett et al. 1992). As described by Walker et al. (2006), biomarkers may be biochemical, physiological, histological, morphological and/or behavioural responses. They provide an integrative measure which is highly useful in situations where mixtures of contaminants are likely to be present, such for estrogenic compounds. Furthermore, they provide direct evidence of biological effects in a field situation. Biomarkers should be reliable (i.e. consistent over repeated measurements) and specific to the contaminant/contaminant class of interest. Vitellogenin is a specific biomarker whereby exposure can promote elevated levels (above basal levels or significant induction in comparison to unexposed individuals) in males and females.

Biomarkers may be targeted towards different levels of biological organisation depending on the type of information required for environmental risk assessment (ERA). Biomarkers targeted at the cellular level are likely to be indicative of contaminant exposure only (Walker et al. 2006), for example, measurements of vitellogenin protein/gene expression are indicative of estrogenic exposure but do not provide information about the long term adverse effects of exposure. These types of biomarkers are useful in providing an early warning indication whereby the source of contamination can be removed prior to the onset of later effects. Other biomarkers are targeted at higher levels, such as organism or population, providing evidence of direct adverse effects (Walker et al. 2006). Examples of higher level biomarkers of estrogenic exposure may include measurement of gonadal development or sex, whereby changes may have consequences for reproduction and/or population survival. Changes to biomarkers at higher levels indicate a higher risk in terms of irreversible organism and/or population effects and a requirement to consider management strategies to remove or reduce the source of contamination.

A suite of biomarkers which measures changes at both the cellular and organism level should be included in environmental biomonitoring to assess not only whether estrogenic exposure has occurred, but also whether the exposure has imparted adverse biological effects. Predominantly, this thesis has focused on the development of vitellogenin, both
protein and gene expression, as biomarkers of estrogenic exposure. It is predicted that vitellogenin may increase above basal levels in males and females exposed to estrogens. Higher level effects, such as sex and female gonadal development which are likely to be indicative of adverse effects on reproduction and survival were also utilised as biomarkers of estrogenic exposure. Sufficient estrogenic exposure may promote accelerated female gonadal development, intersex and/or a protandric regression from male to female gametal status.

1.16. Molluscs as biomonitors of metals

Sewage effluent discharge is one of the main sources of estrogenic compounds into the aquatic environment. However, sewage has been long identified as a source of other contaminants, particularly heavy metals. Field experiments, involving the deployment of oysters in receiving waters of sewage effluent, involve a large quantity of resources and time. Therefore, it is rational and opportunistic to monitor for additional contaminants alongside estrogenic compounds, such as heavy metals. Indeed, molluscs are established biomonitors of heavy metals and have proved useful in the assessment of heavy metals in the marine environment. They have been shown to bio-accumulate heavy metals to concentrations orders of magnitude higher than ambient environment concentrations (Phillip and Rainbow, 1993). Their capacity to bio-accumulate has resulted in the development biomonitoring programmes such as the Mussel Watch programme (Goldberg et al. 1983), with other similar biomonitoring initiatives being implemented in Australia (Avery et al. 1996; Lincoln-Smith and Cooper, 2004; Robinson et al. 2005; Scanes, 1996; Scanes and Roach, 1999), the United States (O’Connor, 1998), the United Kingdom, China and Japan.

Metal concentrations in molluscan tissue are the result of uptake, depuration and excretion. Uptake of metals in molluscs occurs via filtering of seawater (containing the soluble form) or via ingestion of food or inorganic particles (Phillips, 1979a). Following ingestion, molluscs are capable of detoxification/regulation of some heavy metals, within a limited range, with the capacity to equilibrate tissue concentrations with the ambient environment (Naimo, 1995). Detoxification of heavy metals may be achieved through the production of
metal binding proteins, metallothioneins, which are capable of intracellular bindings to metals (Roesejadi, 1980; Viarengo, 1985). Once metallothioneins bind to metals they are transferred to the kidney or digestive gland and stored or excreted in membrane bound vesicles or granules (Jenkins and Brown, 1984). The rate of detoxification is highly dependent and variable between metals and molluscan species.

Bioaccumulation of metals occurs when rate of uptake exceeds the rate of detoxification. The capacity of an organism to accumulate or regulate heavy metals varies between different metal types. Although this varies between species, non-essential metals such as cadmium, lead and mercury are perhaps more likely to accumulate in the soft tissue of an organism compared to essential metals such as zinc and copper (Amiard et al. 1987). This is an important consideration in use of molluscan biomonitors in heavy metal analysis, as only metals which are bioaccumulated, not regulated, can provide an indication of relative contaminant loads among locations in waters.

1.17. Development of *Saccostrea glomerata* as a biomonitor of estrogenic compounds: criteria for validating biomarkers

The overall aims of this thesis were to systematically assess whether particular biomarkers in *S. glomerata* were responsive to estrogens and thus whether the species may be exploited as a suitable biomonitor for the assessment of estrogenic compounds in Australian marine waters.

1.17.1. Demonstrated biomarker cause and effect relationship under controlled laboratory conditions

The first criterion of a valid biomarker is an established cause and effect relationship between exposure to the contaminant of interest and biomarker response. Ideally, under controlled laboratory conditions, biomarkers of estrogenic exposure (that are hypothesised to be responsive to estrogenic exposure) should respond in a dose-response fashion (Huggett et al. 1992). As emphasised in a recent review by Ketata et al. (2008), clear evidence of a dose response relationship between estrogenic exposure and reproductive endpoints (for example, vitellogenin and gonadal development) is currently lacking for
invertebrate models. Chapters 2 and 3 explored the dose dependent relationships between EE2 exposure, vitellogenin gene expression and protein induction, gonadal development and sex in *S. glomerata*. In Chapter 2, a HPLC method for direct quantification of the proposed vitellogenin protein biomarker was developed and verified via proteomic sequencing. Secondly in Chapter 3, a new qPCR method for assessing vitellogenin gene expression was developed as potential biomarker for estrogenic exposure. It was hypothesised that vitellogenin gene expression and protein production would increase rapidly with estrogenic exposure, within days of EE2 exposure, in a dose dependent manner, particularly in males, who do not usually produce vitellogenin. Secondly, it was hypothesised that EE2 exposure may cause dose-dependent changes in gonadal development or sex status (such as intersex or complete sex reversal) in *S. glomerata*.

1.17.2. Demonstrated biomarker temporal maintenance and early warning predictive capacity under controlled laboratory conditions

There is evidence to suggest that biomarkers may not exhibit static dose-response relationships temporally, but rather may exhibit variable responses to estrogenic exposure dependent on factors such as exposure regime and duration, adaptive response mechanisms and/or developmental status (Wu et al. 2005). Wu et al. (2005) outlines a number of possible responses of biomarkers temporally depending both on the nature of the biological response to the contaminant and the temporal exposure regime. In the face of constant exposure, the biomarker of interest may remain elevated or alternatively undergo an adaptation to contaminant stress and return to basal levels. Secondly, for those biomarker responses that remain elevated with constant exposure, if a depuration period ensues, a biomarker may return to basal levels quickly, or alternatively remain elevated long after the contaminant exposure has been removed. It is suggested that the ideal temporal response of a biomarker would be a rapid elevation which is maintained temporally in the face of constant exposure to the contaminant class of interest and then exhibit a slow recovery with removal of exposure (Wu et al. 2005). The slow recovery period of this type of temporal biomarker response may provide an extended window for assessing exposure to contaminants, evidence of adverse biological effects and provide advantages over attempting to measure contaminants directly which may fluctuate greatly within the
environment over time. However, such temporal response dynamics may not be useful in the assessment of an organism’s recovery after environmental exposures have ceased. A fast induction of response which is also maintained with constant exposure but instead exhibits a rapid recovery following removal of exposure is likely to closely reflect temporal changes in contaminant exposures and also provide indication of an organism’s capacity for recovery post-exposure. Thus, for application in environmental monitoring it is critical to understand the temporal nature of biomarker responses to exposure to enable: 1. appropriate sampling design which does not under/over estimate exposure and effect; and 2. informs the appropriate choice of biomarker for the question at hand. This will help to determine whether the biomarker could be employed for the detection of contaminant stress which is transient in nature yet leaves a signature of negative biological effects (slow recovery) or alternatively a biomarker which indicates recovery after exposure is removed which may be useful in assessing the effectiveness of remediative interventions to reduce contaminant inputs on biological health.

Regardless of these issues, it is imperative for utility that biomarkers ideally remain elevated with constant exposure temporally and not exhibit an adaptation response. Thus, Chapter 2 also aimed to explore the temporal maintenance of the dose-response relationship between constant EE2 exposure and vitellogenin protein responses over 3 sampling periods during a gonadal development cycle.

Another favourable characteristic of a biomarker is ‘early-warning’ predictive ability (den Besten, 1998; Huggett et al. 1992). It is proposed that vitellogenin may possess this ability and that early vitellogenin responses may correlate with later effects indicative of endocrine disruption at higher organisational levels such as gonadal morphology, i.e. gamete maturation, size, expression of intersex and/or total sex reversal, all of which are likely to have consequences for reproductive success. Chapter 2 aimed to determine if short term (4 days exposure) induction of vitellogenin protein was predictive of later (49 days exposure) higher-level effects. It was hypothesised that early increases in vitellogenin would be predictive of later effects on gonadal development and/or sex status.
1.17.3. Validation in real environmental situations which have demonstrated estrogenic exposure

Once validated under controlled laboratory conditions, biomarkers of estrogenic exposure should then be validated under environmentally relevant exposures (within the field) (Huggett et al. 1992). For a validated biomarker, ideally one should demonstrate that exposure of the biomonitor to estrogenic contaminants under real field situations exhibits significant biomarker induction. Firstly, Chapter 4 aimed to demonstrate, via assessment of estrogenic activity and individual estrogenic compounds in sewage effluent, that the receiving waters of Burwood WWTP were a suitable experimental field scenario where exposure to estrogenic contaminants may occur during the deployment period. It was hypothesised that sewage effluent from Burwood WWTP would contain estrogenic compounds during the experimental window. Secondly, biomarker responses in female and male *S. glomerata*, previously validated via laboratory experiments in Chapters 2 and 3 (vitellogenin [gene expression in both sexes and protein in females], sex, and female gonadal development), were assessed under field exposures. Chapter 5 aimed to determine if estrogenic exposure, via sewage effluent from Burwood WWTP, to *S. glomerata* induced some (or all) of the following reproductive changes: increased female and male vitellogenin induction, enlarged oocytes, intersex, higher proportions of females and mature female gonadal development stages, compared to responses at appropriate reference locations within the region. If these criteria can be validated, then *S. glomerata* may be a suitable biomonitoring species providing reliable information about the presence and biological effects of estrogenic compounds.

1.18. Use of *Saccostrea glomerata* as a biomonitor of metallic contaminants:

As *S. glomerata* has previously been established to be a suitable biomonitoring species of heavy metals in Australian estuarine and marine waters (Avery et al. 1996; Lincoln-Smith and Cooper, 2004; Robinson et al. 2005; Scanes, 1996), then this species may be employed in field situations to detect if there is any evidence which suggests that metals are elevated due to sewage effluent and are bioavailable to marine biota within the vicinity of the effluent outfall. Field deployment of *S. glomerata* in sewage effluent receiving waters in
Chapter 5 provided an invaluable opportunity for multi-contaminant assessment. Chapter 6 aimed to quantify concentrations of heavy metals in *S. glomerata* which were deployed adjacent to Burwood WWTP and at reference locations for 6 weeks. It was hypothesised that if sewage effluent was a potential source of metallic contaminants, then *S. glomerata* deployed in sewage effluent receiving waters may have elevated concentrations of heavy metals in comparison to those deployed at reference locations.

1.19: Chapter aims

The assessment of *S. glomerata* as a suitable biomonitor species for estrogenic compounds via laboratory and field validation was addressed under the following objectives:

Chapter 2: To explore the dose response and temporal relationship between 17α Ethynylestradiol (EE2; a potent estrogenic compound) exposure, vitellogenin induction and gonadal development:

This Chapter aimed to explore the dose dependent and temporal relationships between EE2 exposure, vitellogenin induction, gonadal development and sex in *S. glomerata*. It was hypothesised that vitellogenin would increase rapidly, with days of EE2 exposure, in a dose dependent manner and the relationship would be maintained throughout exposure duration. Secondly, it was hypothesised that EE2 exposure may cause dose dependent changes in gonadal development or sex status (such as intersex or complete sex reversal) in *S. glomerata*. Finally, a successful biomarker should be predictive of later higher-level effects and therefore it was hypothesised that early increases in vitellogenin would be predictive of later effects on gonadal development and/or sex status.

Chapter 3: To develop a sensitive real-time qPCR assay for measuring vitellogenin gene expression in *S. glomerata*:

There is far less known regarding the effects of estrogenic compounds on invertebrate species and one of the large contributing factors is the lack of species specific assays to detect reproductive changes in molluscan taxa. Thus, the main aim of this Chapter was to develop a sensitive qPCR assay for measuring vitellogenin gene expression in *S. glomerata*. It was hypothesised that *S. glomerata* exposed to a concentration gradient of EE2 would
exhibit a dose dependent increase in vitellogenin gene expression, especially at a time soon after exposure, as gene expression is hypothesized to be more sensitive to exposure temporally than protein or indeed organism level responses.

Chapter 4: To assess whether sewage effluent released from Burwood wastewater treatment plant contains estrogenic compounds:
Currently, there is a lack of information on concentrations of estrogenic compounds contained in sewage effluent from Australian WWTPs. The main aim of this Chapter was to assess whether sewage effluent collected from Burwood WWTP over a 6 week period would contain estrogenic activity and estrogenic compounds, and thus, serve as an appropriate impact location for the assessment of biomarker responses in the field examined in Chapter 5.

Chapter 5: To assess the capability of *S. glomerata* as a biomonitor via deployment in waters receiving sewage effluent:
The main aim of Chapter 5 was to validate *S. glomerata* as a biomonitor of estrogenic contaminants via a 6 week deployment of oysters in waters receiving sewage effluent. It was hypothesised that if sewage effluent from Burwood WWTP contained estrogenic compounds, then exposure to *S. glomerata* may induce some (or all) of the following reproductive changes: increased vitellogenin induction, enlarged oocytes, intersex, higher proportions of females and mature female gonadal development stages.

Chapter 6: To assess the presence of a suite of heavy metals in *S. glomerata* deployed in waters receiving sewage effluent:
The main aim of this Chapter was to assess concentrations of heavy metals in *S. glomerata* which were deployed adjacent to Burwood WWTP and at reference locations for 6 weeks. It was hypothesised that *S. glomerata* deployed at Burwood locations would have elevated concentrations of heavy metals in comparison to those deployed at reference locations.
Chapter 2: Exposure to 17α-Ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster

2.1. Summary

Although mounting evidence suggests exposure to estrogenic contaminants increases vitellogenin production in molluscs, demonstration of dose-response relationships and knowledge of the temporal nature of the vitellogenin response with continual exposure is currently lacking for biomarker utility. To address this knowledge gap, adult Sydney rock oysters, *Saccostrea glomerata*, were exposed to a range of environmentally relevant concentrations of 17α-ethynylestradiol (EE2) (0, 6.25, 12.5, 25 or 50 ng/L) in seawater under laboratory conditions. Vitellogenin induction and gonadal development were assessed following 4, 21 and 49 days exposure to EE2. Vitellogenin was found to increase in a dose dependent manner with EE2 exposure for females (4 and 49 days) and males (4 and 21 days). Histological examination of gonads revealed a number of individuals exhibited intersex (ovotestis) in 50 ng/L EE2 (after 21 days) and in 6.25 and 12.5 ng/L EE2 (after 49 days). Furthermore, a significant shift towards females was observed following 49 days exposure at 50 ng/L EE2 suggesting estrogenic exposure is capable of facilitating a progression for protandric males from male-intersex-female gametal status. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages at 21 d and increases in oocyte area following 49 days. Male vitellogenin (4 days) was predictive of decreased male percentages and lower male developmental stages at 49 days. Vitellogenin in *S. glomerata* is a predictive biomarker of estrogenic exposure and effect if sampled soon after exposure and at the commencement of a gonadal development cycle.
A modified version of this Chapter has been published:


Previous experiments, conducted predominantly during an Honours year, also formed the basis of another related publication. Further experimentation was conducted during PhD candidature to enable publication of this data. This predominantly consisted of confirmation of the vitellogenin peak from a HPLC methodology (developed by myself) via SDS-page electrophoresis and proteomic sequencing (Section 2.3.5) and preparation of the manuscript for publication. Although this publication is referred to throughout the thesis it is not presented in detail:


2.2. Introduction

A range of anthropogenic compounds with structural similarity to estrogen, and its functional moieties, has been implicated as primary causal agents responsible for reproductive perturbations in aquatic wildlife (Langston et al. 2005). One such compound is 17α-ethynylestradiol (EE2); a synthetic estrogen widely employed as the active constituent of the female contraceptive pill. Compared to endogenous steroids, such as 17β-estradiol (E2) and its metabolites, EE2 exhibits greater estrogenic potency and is more resistant to metabolism/degradation due to the addition of an ethyl group (Andersen et al. 2003). Consequently when used as the contraceptive pill in humans, EE2 is only partially metabolised, resulting in excretion and entry to sewage effluent. Evidence suggests that during the sewage effluent treatment process EE2, along with a suite of other estrogenic
compounds, may only be partially degraded, and/or removed, resulting in entry of EE2 to streams and surface waters (Andersen et al. 2003). Removal of EE2 from sewage effluent is likely to be largely dependent on the treatment process. Primary and secondary treatment may partially remove EE2 (Braga et al. 2005; Tan et al. 2007; Ying et al. 2008), whereas advanced tertiary treatment is more likely to result in near complete removal (Leusch et al. 2005; Tan et al. 2007). Concentrations of EE2 in sewage effluent have been detected between 0.2- 42 ng/L (Desbrow et al. 1998; Ternes et al. 1999) and in surface waters between 0.1-15 ng/L worldwide (Aherne and Briggs, 1989; Belfroid et al. 1999). Thus, aquatic organisms, including invertebrates, may be exposed to EE2 in aquatic environments receiving sewage effluent.

Aquatic invertebrates, in particular bivalve molluscs, are ideal candidates for assessing effects of anthropogenic contaminants in aquatic systems. Due to their sessile nature, high filter-feeding capacity and bioaccumulation of both organic and inorganic compounds (Ortiz-Zarragoitia and Cajaraville, 2006), molluscs such as oysters and mussels have proved useful in the assessment of contaminant presence, relative contaminant loads among locations and biological responses to contaminant exposure (Scanes, 1996). Molluscs may also prove a valuable tool in the assessment of the effects of estrogenic contaminants. Indeed, available literature on both laboratory and field assessment of the effects of estrogenic compounds in molluscan species suggests that exposure to estrogenic compounds can cause reproductive effects including the induction of vitellogenesis in females and males (evidence of endocrine disruption) (Matozzo et al. 2008b) and gonadal developmental changes (accelerated oocyte development in females and feminisation of male individuals) (Gagné et al. 2006, Gagnon et al. 2006, Ortiz-Zarragoitia and Cajaraville 2006, Langston et al. 2007).

Molluscan vitellogenins are precursors to egg yolk proteins (vitellins) which accumulate in oocytes during gonadal maturation. Evidence suggests that vitellogenesis in molluscs is mediated via endogenous estrogens (reviewed in detail in Chapter 1). In the scallop, *Pinctopecten yessoensis*, E2 is synthesised in the gonad and its levels increase during the early phases of oocyte development (Matsumoto et al. 1997). Li et al. (1998) demonstrated
that E2 treatment *in vivo* stimulated vitellogenesis and increases in vitellins in the gonad of the Pacific Oyster, *Crassostrea gigas*. Similarly, Osada et al. (2003) demonstrated E2 induced synthesis of vitellogenin in the gonad of *P. yessoensis*. The site of synthesis is thought to be within ovarian tissue, with in- situ hybridisation demonstrating localisation of *C. gigas* vitellogenin mRNA within follicle (auxiliary) cells (Matsumoto et al. 2003).

With such evidence suggesting that estrogens play a functional role in vitellogenin induction in molluscs, others have hypothesised that exposure to xenoestrogens may elicit similar effects, and have sought to investigate vitellogenin production as a potential biomarker of estrogenically mediated endocrine disruption. Indeed, Gagné et al. (2002) demonstrated that both male and female freshwater mussels, *Elliptio complanata*, deployed 1.5 and 5 kilometres downstream from a wastewater treatment plant (WWTP) in the St. Lawrence River, Canada, exhibited increases in alkali-labile phosphates (ALP) (a surrogate vitellogenin estimate) compared to reference locations. Male black mussels, *Mytilus galloprovincialis*, deployed in Venice canals (receiving raw sewage) have also been shown to exhibit increased ALP levels in hemolymph compared to reference locations (Pampanin et al. 2005). In terms of controlled exposures to specific xenoestrogens, exposure to 4-nonylphenol (NP) (250 nM) for 72 hours has been shown to increase ALP levels in both male and female mussels, *E. complanata* (Gagné et al. 2001). Similarly, *M. galloprovincialis*, exposed to NP (0, 25, 50 and 100 μg/L) for 7 days exhibited a dose dependent increase in ALP levels (Riccardi et al. 2008). Taken together, these findings provide strong evidence of the role of xenoestrogens in induction of vitellogenin-like proteins in molluscs. However, there has been little research to date exploring the potential effects of xenoestrogens with high estrogenic potency, such as EE2, on molluscan vitellogenesis, excluding the work presented in this and Andrew et al. (2008; 2010).

Estrogenic exposure not only induces vitellogenin production in molluscs, but also influences both the rate of gonadal development and sex determination. In *C. gigas*, injections of E2 (50 μg once every 10 days, for a 40 day period) were shown to accelerate female development by increasing oocyte diameter (46.9 ± 0.9 μm in exposed compared to 44.1 ± 2.4 μm in controls) (Li et al. 1998). Langston et al. (2007) demonstrated that
exposure of clams, *Scrobicularia plana*, to sediment spiked with a mixture of E2, EE2, NP and octylphenol (OP) caused intersex gonadal status in males and enlarged oocytes in both females and in the ovotestis of intersex individuals. Further, injections of E2 (0.04-0.60 mg estradiol-3-benzoate) during the early stages of seasonal gonadal maturation of the oyster *C. gigas*, have been shown to induce full sexual reversal from male to female (Mori et al. 1969). Similarly, exposure to NP (48 hours exposure to 1 and 100 μg/L at 7-9 days postfertilisation) during larval development has resulted in skewed sex ratios towards females during adulthood in *C. gigas* (Nice et al. 2003).

Prior research has focused on the development of, *S. glomerata*, as a potential biomonitoring species for estrogenic contaminants through exploiting vitellogenin induction and gonadal developmental responses following estrogenic exposure. It was previously determined that females exposed to EE2 (50 ng/L) and NP (100 μg/L), during a gonadal development cycle, exhibited significant increases in vitellogenin up to three-fold and double controls respectively. Significant increases in vitellogenin were also found in males exposed to 50 ng/L EE2. Further, exposure to NP (100 μg/L) and EE2 (50 ng/L) induced intersex gametal status for some individuals (Andrew et al. 2008; appendix 2).

As *S. glomerata* exhibit protandric dioecy (Asif, 1979, Guo et al. 1998), it remains unclear whether intersex individuals observed in initial experiments were functionally intersex or the result of an opportunistic sampling of individuals that were in the process of undergoing an estrogenically mediated protandric transition from male to female. If the latter is the case, we may expect to observe shifts in the sex ratio towards females with greater exposure durations to estrogens, at later intervals in the gonadal development cycle and/or with greater sampling frequency during such potential transitions. In the current study, this question was explored by sampling individuals exposed to EE2 at multiple windows temporally during a gonadal development cycle from resting phase to spawning.

Thus, the main aims of the present study were to explore dose-response and temporal relationships between EE2 exposure, vitellogenin induction and gametal status during reproductive conditioning in *S. glomerata*. Specifically, it was expected that vitellogenin
would increase in a concentration dependent manner within days of EE2 exposure (4 days) and that the dose-response relationship would be maintained throughout the exposure duration. Secondly, it was predicted that with time (21-49 days) the presence of intersex individuals in exposure treatments would increase and possibly a shift in the sex ratio towards females. Finally, it was hypothesised that early induction of vitellogenin would be predictive of later effects on gametal maturation, expression of intersex and/or total sex reversal.

2.3. Materials and Methods

2.3.1. Experimental Design

Three hundred and sixty, 18 month old *S. glomerata*, of mixed sex were used for experimentation. Oysters were sourced from an oyster farm in the Port Stephens estuary, NSW, Australia; an area with no known history of sources of estrogenic contamination. Oysters were specifically selected to be in ‘resting’ condition, entering a phase of gonadal development at experimental commencement (Dinamani, 1974). Experiments were conducted during Winter (June-August, 2007) at the Port Stephens Fisheries Centre, Mollusc Hatchery, Taylor’s Beach, NSW according to the protocols of the American Society for Testing and Materials (ASTM) E 729-96 (1996) for static renewal tests. One hundred and eighty individually aerated aquaria (8 L) were maintained at 22 ± 0.5°C in a temperature controlled room. Each aquarium contained 2 oysters. Seawater (3.3% salinity) within each replicate aquarium was completely changed and treatment exposure regimes maintained thrice weekly within a temperature controlled laboratory (maintained at 22°C). Oysters were fed a mixed diet daily containing 660 mL of each algal species culture including: *Pavlova lutheri*, *Chaetoceros muelleri* and Tahitian *Isochysis* aff. *galbana* (1.5×10⁹ cells/oyster/day).

Oysters were exposed to one of six nominal treatments in seawater: 6.25, 12.5, 25 and 50 ng/L EE2 (in 1.2 μg/L ethanol), 1.2 μg/L ethanol control and a seawater control (N= 20 individuals per treatment/time). Water EE2 concentrations were not measured, however, it is acknowledged that dissipation can occur due to adsorption (to the buckets or oyster shells) and uptake by oysters. Thus actual concentrations may have been lower than nominal
concentrations. Oysters were removed (10 aquaria per treatment/time) from the experiment at three separate sampling occasions (4, 21 and 49 days) in order to assess temporal effects. Sampling times were selected to assess whether vitellogenin was induced following short-term exposure (4 days, 96 hour acute exposure) and if vitellogenin responses were likely to be maintained over longer periods of time (chronic effects). The sampling times of 21 and 49 days were considered important time points to measure the shift in sex ratio and sufficient time to allow for gonadal maturation from resting phase to mature gametes prior to spawning (Cox et al. 1996). A group of oysters (from the same population) was maintained in separate aquaria for the purpose of monitoring gonadal development throughout the experiment and to ensure oysters were harvested prior to a spawning event.

2.3.2. Experiment harvest

Upon experiment harvest, oysters were removed from aquaria and whole weight was recorded. Each oyster was opened and the weight of the soft tissue and the shell was recorded. From each oyster a thin slice of tissue (5 mm²) was excised between the labial palps and gills. The gonad was then scraped from the oyster, homogenised with a scapel and stored for vitellogenin analysis. The remainder of the oyster was placed back into the shell and frozen at -20°C for EE2 analysis.

2.3.3. Condition index

Wet condition index was measured to assess the potential confounding effects of estrogenic treatments and/or the solvent carrier on feeding and thus condition. Wet condition index was measured at each harvest. This was calculated using the formula (wet tissue weight X 1000)/ (wet whole weight- wet shell weight) (Lucas and Beninger; 1985).

2.3.4. Measurement of 17α-ethynylestradiol in *Saccostrea glomerata*

2.3.4.1. Extraction

Whole *S. glomerata* were removed from the freezer (-20 °C) and thawed. Stomach content was excised from the oyster and the remaining tissue was weighed. Whole oyster tissue was excised into smaller cubes (~2 mm²) and then placed into a 50 ml conical flask with 25 ml of 25% acetone in hexane. The mixture was then homogenised via ultra-sonication for 10
minutes. The homogenised mixture was allowed to settle for 10 minutes and the supernatant (25% acetone in hexane) was removed and filtered through 15 grams of androgynous sodium sulphate into a 100 mL volumetric (Sigma Aldrich). The remaining tissue was then rinsed twice by re-homogenising with 15 mL of acetone. Rinse supernatants were also added to volumetric flask. The solvent containing organics (EE2) was then reduced via rotary evaporation and then transferred into a 5 mL glass centrifuge tube and gently evaporated to just dry under a stream of N₂. Sample was then re-suspended into 1% DMSO (Sigma Aldrich) and 10% methanol (Sigma Aldrich).

2.3.4.2. ELISA
ELISA reaction was then carried out using an EE2 EIA Kit according to manufacturer’s guidelines (Biosense laboratories). Each sample/standard was run in duplicate. Each plate consisted of a standard curve including 0.05- 3 µg/L and samples were diluted to fall within this range. One hundred µL of sample or standard was mixed with 100 µL of enzyme conjugate solution (1:1). The mixture was then transferred into the ELISA plate in 100 µL aliquots and incubated for 1 hour at room temperature (~22 ºC). Each well of the plate was washed 3 times using 300 µL of wash solution. One hundred µL of colour development solution was then added to each well followed by incubation at ~22 ºC, reaction was stopped after 30 minutes and absorbance was read at 450 nM. Sample concentration was calculated according to standard curve analysis.

2.3.5. Extraction and HPLC assay for measuring vitellogenin in S. glomerata
A new method for the analysis of vitellogenin in biological samples allowing greater specificity than established ALP surrogate assays was developed (Andrew et al., 2008). The extraction (modified extraction from Gagné and Blaise, 1999) and analysis of oyster gonadal samples were performed using protocols outlined in Andrew et al. (2008). Briefly, 100 mg of oyster gonadal tissue was homogenized in 225 µL citrate buffer (pH 6.5, 10 mM and with 16 mg/mL polyethylene glycol) and 25 µL of the protease inhibitor aprotinin then stored at -80°C. Following storage, samples were thawed and 500µL Tris-HCl buffer was added. One hundred microliters of tissue homogenate was transferred to a glass centrifuge tube and ALPs were extracted using 800 µL of t-butyl methyl ether (Chromsolv grade,
Sigma Aldrich) with a 30 minutes extraction period at 4 °C. The organic fraction was then separated and dried under N₂, then re-suspended in 1000 µL of PBS. Three female and 3 male individuals from separate aquaria were selected for each treatment/time for vitellogenin analysis.

2.3.6. Confirmation of vitellogenin peak in HPLC assay

In order to gain verification of the peak as vitellogenin, aqueous and organic extracts from the t-butyl methyl ether extraction were subjected to gel electrophoresis (Figure 2.1). Extracts were dried in a rotational vacuum concentrator (Model RVC2-25, Martin Christ, Germany), re-suspended in SDS-PAGE sample buffer (2%, v/v mercaptoethanol; 2%, w/v SDS; and 10%, w/v sucrose in 0.1875M Tris, charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 1 s (m/z 100–1600).

![Figure 2.1](image_url)

**Figure 2.1:** Confirmation of Sydney rock oyster, *Saccostrea glomerata*, vitellogenin protein. Aqueous vitellogenin extracts were subjected to gel electrophoresis with subsequent silver staining. Numerous bands were visible ranging from 17 to >170kDa (B). Organic extracts purified and collected via HPLC were subjected to gel electrophoresis with subsequent silver staining. The only prominent protein band in this fraction was >170kDa and was excised for amino acid sequencing.
The LC/MS/MS data was used to search *Other Metazoa* entries using Mascot (Matrix Science, London, UK) in the NCBI protein database and seven peptides provided matches with 43% coverage and an alignment score of 63.9 to an oyster vitellogenin amino acid sequence deduced from a partial cDNA fragment of *S. glomerata* origin (Anderson et al. 2010, GeneBank accession number ABW69671.1 and Chapter 3) (Figure 2.2, Andrew et al., 2008; appendix 2). It should be acknowledged that within this Chapter, the use of the term vitellogenin implies the analyte is a vitellogenin-like protein based on genomic/proteomic sequence with similarity to known vitellogenin from other taxa (molluscs, fish spp. etc) and that it is possible that other isoforms may exist in *S. glomerata*. It is possible that other *S. glomerata* vitellogenin isoforms may exist or that the vitellogenin extraction may include cleaved vitellins.

Figure 2.2: Alignment of the amino acid sequence of peptides (A) (obtained from Sydney rock oyster, *Saccostrea glomerata*, excised protein band via 1D NanoLC ESI MS/MS analysis) with (B) a previously determined vitellogenin amino acid sequence deduced from a partial cDNA fragment of Sydney rock oyster, *Saccostrea glomerata*, vitellogenin (GeneBank accession number ABW69671.1) employing the NCBI protein database BLAST algorithm. Sequenced peptides with matches to the deduced known vitellogenin sequence are underlined.

2.3.7. **Histological analysis of sex and gonadal development**

Sexes in *S. glomerata* are separate with low occurrence of intersex, i.e. less than 0.4- 0.7% (Dinamani, 1974, Cox et al. 1996). Sex assignment was required due to the hypothesised differences in vitellogenin induction between males and females (Blaise et al. 1999, 2003),
that vitellogenin induction in males is strong evidence for estrogen mediated endocrine disruption, and finally due to the established effects of estrogentic exposure on sex ratio (Andrew et al. 2008). Each individual oyster was prepared for histological examination resulting in a total of 360 analyses (20 individuals per treatment per time). A cross-section of approximately 5 mm$^2$ was excised from each oyster between the labial palps and gills. Tissue samples were placed in Davidson’s solution (10% glycerin, 20% formalin, 30% alcohol, 30% sodium chloride solution and 10% glacial acetic acid) for 24 hours (Cox et al. 1996) with successive dilutions of ethanol (70%, 50% and 50%) (Howard and Smith, 1983). Tissue was embedded into paraffin blocks, sectioned transversely at 5 µm intervals and floated on a water bath heated to 80 ºC. Sections were placed onto acid washed glass slides and dried overnight at 60 ºC. Sections were stained with haematoxylin and counterstained with eosin (H&E). Study of the gonadal area was performed under a compound microscope at 200 and 400 X magnification.

The sex of each individual was determined as female, male, intersex or indeterminate under microscopic examination (200 x magnification) using oocytes or spermatozoa as indicators. Intersex individuals were identified via the presence of both oocytes and spermatozoa within an intact gonadal follicle. Other individuals were found to be indeterminate whereby the gonadal cells were undifferentiated and usually accompanied by an abundance of hemocytes.

It may be possible that estrogen exposure promotes female development resulting in acceleration towards higher proportions of mature female gonad stages in individuals exposed to EE2 compared to controls. Thus, each oyster was assigned a gonadal developmental stage for oogenesis, and also spermatogenesis, based on criteria described by Dinamani (1974). Briefly, stages included: 1) follicles contain primary oogonia or spermatogonia, 2) oocytes (<25 µm) or spermatocytes beginning to mature, 3) maturation of oocytes (>25 µm) or all stages of spermatogenesis up to spermatids, few spermatozoa, 4) oocytes or spermatozoa occupying a large proportion of the gonad, 5) following spawning, many follicles are discharged, residual oocytes or spermatozoa, X) characteristic of resting, no oocytes or spermatozoa (indeterminate sex) and high hemocyte activity. Eight female
individuals per treatment from the 49 days sampling period were randomly selected for oocyte area measurement. For each individual, digitalised images of 4 randomly selected areas of the gonad were taken with subsequent measurement of 80-100 oocytes (Imagetool 2.0). Only oocytes with visible nuclei were measured and oogonia were not measured.

2.3.8. Statistical analysis
Linear dose-response relationships between EE2 exposure and vitellogenin concentration in gonadal tissue were assessed to test biomarker utility via linear regression analyses using STATISTICA (Statsoft, 2005). According to Levene’s test for homogeneity of variance, vitellogenin units were not homogenous and thus log transformed, ln (x + 1), prior to statistical analysis.

A Pearson Chi squared analysis (SPSS version 17) was used to determine significant differences in sex ratio (female, male, intersex or indeterminate) among time (4, 21 and 49 days exposure) for each exposure to EE2 (0, 6.25, 12.5, 25 or 50 ng/L). Numbers of males, intersex and indeterminate individuals across treatments and time were insufficient for non-parametric analysis and were pooled in order to gain sufficient frequency for comparison to females. The aim was to determine if the proportion of females in the test population increased throughout the duration of the experiment. It was hypothesised that there would be a difference in the sex ratio, specifically a shift towards a greater proportion of females in exposure treatments over time compared to no difference in the controls.

Pearson’s correlations were performed between treatments and mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) to further determine relationships between exposure and reproductive endpoints. Pearson’s correlations were also performed using early mean vitellogenin levels (4 days) with mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) in order to test the early warning biomarker predictive utility of vitellogenin. As individuals were sacrificed at each sampling period it was not possible to undertake pair wise correlations between vitellogenin replicates from 4 days with reproductive endpoint replicates from separate individuals at 49 days. Thus, correlation analyses were performed
using mean values for each treatment. Due to low N (N=3 per treatment), the power to
detect a significant correlation between variables was greatly reduced.

During the experiment an ethanol control was included due to the addition of ethanol as a
solvent carrier for EE2 in the exposure treatments (1.2 µg/L). Prior to statistical analyses
the seawater and ethanol controls were compared for differences. As no significant
differences were observed, both controls were pooled for the purpose of all analyses.

2.4. Results

2.4.1. Measurement of EE2 in oyster tissue via ELISA
Concentrations of EE2 in oyster tissue at 49 days were measured via ELISA. When
analyzing the sexes separately, it was found that there was no difference in accumulated
EE2 between sexes or treatments (Figure 2.3B). Results were unusual and unexpectedly
EE2 concentrations were detected in oyster from the control treatment. There was also no
significant differences between treatments when sexes were combined to increase
replication, and hence statistical power (Figure 2.3A).
Figure 2.3: Levels of 17α-ethynylestradiol measured via ELISA in oyster tissue from Sydney rock oysters, *Saccostrea glomerata*, exposed to 0, 6.25, 12.5, 25, 50 ng/L 17α-ethynylestradiol in: A) combined sexes, (mean ± standard error, N= 7) and B) female, male and intersex individuals from the 49 day exposure (mean ± standard error, N= 1-6).
2.4.2. Effects of EE2 on wet condition index

Wet condition index was measured to assess the potential confounding effects of estrogenic treatments and/or the solvent carrier on feeding and thus condition. Wet condition index was measured at each harvest. Oyster condition was observed to decline in the 12.5 ng/L, 25 ng/L and 50 ng/L EE2 treatments by 8.5, 7.7 and 12.6% respectively after 4 days (F=2.58, df= 4, p< 0.05), though oysters quickly recovered with no significant differences in condition among treatments at both 21 (F= 2.095, df= 4, p> 0.05) and 49 days (F= 2.269, df= 4, p> 0.05) (Figure 2.4A-C).
Figure 2.4: Comparison of the wet condition index measured in Sydney rock oysters, *Saccostrea glomerata* following exposure to 17α- ethynylestradiol (0, 6.25, 12.5, 25 and 50ng/L) for A) 4 days, N= 18-20, B) 21 days, N= 17-18 and C) 49 days, N= 18-20. Letters indicate statistically similar treatments. (mean ± standard error)
2.4.3. Effects of EE2 on vitellogenin production

2.4.3.1. Females

While there was greater variability in the vitellogenin response at 4 days compared to later time periods, levels were elevated across all EE2 treatments and increased linearly with EE2 exposure ($R^2 = 0.22$, $p < 0.05$) (Figure 2.5A). Yet the vitellogenin response declined over time, with approximately half of the vitellogenin units found at 49 days compared to the vitellogenin units in corresponding treatments at 4 days. At 49 days, albeit with lower vitellogenin concentrations, a linear relationship with EE2 exposure was found ($R^2 = 0.58$, $p < 0.05$) while at 21 days the relationship was not significant ($p > 0.05$) (Figure 2.5A).

2.4.3.2. Males

Males displayed a similar response with vitellogenin exhibiting a positive linear increase with EE2 exposure at 4 days ($R^2 = 0.42$, $p < 0.05$). A positive linear increase with EE2 was maintained at 21 days ($R^2 = 0.31$, $p < 0.05$) (Figure 2.5B) but, similar to the female response, vitellogenin had declined in comparison to 4 days. At 49 days the vitellogenin response had declined to basal levels across treatments ($p > 0.05$) (Figure 2.5B).

2.4.3.3. Sexes combined

Due to the low replication for males at 21 and 49 days (due to a shift in sex ratio, see Figure 2.6 below) analyses were performed with individuals pooled across sex (Figure 2.5C). This included females, males, and where present, indeterminate and intersex individuals. Vitellogenin was found to increase linearly with EE2 at 4 days ($R^2 = 0.28$, $p < 0.05$). Although significant at 21 days ($R^2 = 0.08$, $p < 0.05$) and 49 days ($R^2 = 0.08$, $p < 0.05$) the proportion of explained variance was again very low suggesting the vitellogenin response approached basal levels, largely due to low male vitellogenin contributions at higher exposures (Figure 2.5C).
Figure 2.5: Comparison of the vitellogenin units measured in Sydney rock oysters, *Saccostrea glomerata* following exposure to 17α-ethynylestradiol (0, 6.25, 12.5, 25 and 50ng/L) for A) Females, 4 days ($R^2=0.22$, $p<0.05$), 21 days and 49 days ($R^2=0.58$, $p<0.05$), B) Males, 4 days ($R^2=0.42$, $p<0.05$), 21 days ($R^2=0.31$, $p<0.05$) and 49 days, N= 1-6 per treatment/time (only one male individual was present in 50ng/L at 21 and 49 days) and C) Combined (males, females, intersex and indeterminate), 4 days ($R^2=0.28$, $p<0.05$), 21 days ($R^2=0.08$, $p<0.05$) and 49 days ($R^2=0.08$, $p<0.05$). N= 6-12 per treatment/time. Filled lines indicate a significant relationship $p<0.05$. (mean ± standard error).
2.4.4. Gonadal sex

The proportions of sexes (male ~20%, female ~ 60%, indeterminate ~ 20%) were similar across EE2 exposure treatments at 4 days (Figure 2.6A). This represents what may be expected of sex proportions within a single reproductive season for *S. glomerata*, allowing for sampling variability. A small proportion of individuals were indeterminate at the beginning of the experiment which was expected and reflects individuals that were yet to commence the gonadal maturation process (Figure 2.6A). It is also possible these individuals were male due to the difficulty of identification of small proportions of rudimentary spermatogonia within follicles (M1) or residual gametes in a male gonad post-spawning (M6).

Following 21 days exposure, the proportion of males and indeterminate individuals declined with a concomitant increase in the proportion of intersex individuals in the 50 ng/L EE2 exposure (4/15) (Figure 2.6B). No other treatments showed evidence of intersex at this sampling interval. A higher proportion, although not significant, of females were found in the 25 ng/L EE2 treatment compared to all other treatments. After 49 days exposure, proportions of intersex were also evident at lower exposures of 6.25 and 12.5 ng/L EE2 (Figure 2.6C). At 50 ng/L EE2, the proportion of intersex individuals declined (relative to 21 days), with a significant shift in the proportion of female individuals at 49 days compared to earlier time periods \(\chi^2 = 6.707, df=2, p< 0.05\) (Figure 2.6C). Only one male was observed within this treatment. Other treatments, including controls, did not exhibit significant shifts in the proportion of females over time. Taken together these results suggest a progression from male-intersex-female occurred with time in the 50 ng/L EE2 treatment. No other significant differences in the female proportions across experimental sampling periods were found in exposure or control treatments.
Figure 2.6: Comparison of the sex ratio of Sydney rock oysters, *Saccostrea glomerata* following exposure to 17α- ethynylestradiol (0, 6.25, 12.5, 25 and 50ng/L) in experimental aquaria at A) 4 days exposure, N=17-18, B) 21 days exposure, N=15-16 per treatment and C) 49 days exposure, N=18-20 per treatment. * indicates a significant shift in the proportion of females via chi square analysis.

Oyster gonadal development occurs through the sequential addition of oogonia or spermatogonia from the basal layer of the follicular wall. As gametes mature, they are
released to fill the lumen of the follicle. At 21 days, it was observed that intersex individuals from the 50 ng/L exposure all contained male gametes (stage 2) within the lumen along with the development of a layer of oogonia (stage 1) around the inner follicle wall (Figure 2.7A). The modal stage of development for males at 4 days was also stage 2 (Figure 2.7B), suggesting that the developmental fate of gametes switched soon after exposure to EE2. A similar pattern of development was observed in intersex individuals in 6.25 and 12.5 ng/L at 49 days, whereby male gametes were at stages 2-4 and the new layer of female gametes were consistently at stage 1 (Figure 2.7C). It was also observed that for all intersex individuals (particularly where the proportion of oogonia was higher than male gametes) there was a proliferation of hemocytic activity within interstitial tissue (Figure 2.7D). The combination of maturing male, immature female gametes and enhanced hemocytic activity further suggests that some individuals begin gonadal maturation as male, but with exposure to EE2 the developmental pathway of germ cells is redirected from a male to female trajectory. Male gametes are eventually removed via hemocytic clearance and the individual continues gametogenesis along a female developmental pathway.
Figure 2.7: Gonadal tissue from histological preparation of individual Sydney rock oysters, *Saccostrea glomerata*. A) Intersex follicle with both oogonia and primary oocytes (F1) and spermatozoa (M2) x 200 (21 days, 50 ng/L EE2), B) Male gonad in stage M2 x 200 (4 days, 50 ng/L), C) Intersex follicle with both oogonia and primary oocytes (F1) and spermatozoa (M4) x 200 (49 days, 12.5 ng/L), D) Intersex follicle with proliferation of hemocyte cells x 400 (higher magnification of C, 49 days, 12.5 ng/L). O= Oocyte cell, S= Spermatozoa with the lumen of follicle filled with tails and H=hemocyte. Scale bars, A, B and C = 100 μm, D=50 μm.

### 2.4.5. Female gonadal development stages

**Gonadal Development**

At 4 days, the proportions of female gonadal development stages were similar among all treatments (Figure 2.9A). After 21 days exposure, it was observed that all exposure treatments had higher proportions of female development stages F2, F3 and F4 (Figure 2.8A-C) compared to the controls (where 95% females were within stages F1 or F2) suggesting EE2 exposure accelerated female gametal development (Figure 2.9B). However
at 49 days within higher exposures (e.g. 25 and 50 ng/L EE2), the proportions of female stages (F2-F4) were once again similar to the controls (F1-F5) (Figure 2.8D and Figure 2.9C). The significant increase in female proportions in 50 ng/L at 49 days together with histological observations of increased proportions of intersex individuals within this treatment at earlier intervals (21 days) suggested that the trend towards higher proportions of earlier female gonadal development stages within these treatments was perhaps the outcome of sampling individuals that have transitioned from a male to female developmental mode. This resulted in a ‘mixture’ of female developmental stages, with ‘new’ females in earlier maturation stages such as F2 (Figure 2.8D) (that have recently transitioned from intersex or indeterminate gonadal status) and individuals initially female at higher maturation stages (F3 and F4). It is unlikely that EE2 exposure initiated a spawning event in the 25 and 50 ng/L exposure at 49 days and individuals were re-commencing a secondary gonadal development cycle, as spawning (even at low levels) is evident within aquaria.
Figure 2.8: Gonadal tissue from histological preparation of individual Sydney rock oysters, *Saccostrea glomerata.* A) Female gonad in stage F2 x 200 (21 days, 50ng/L), B) Female gonad in stage F3 x 200 (21 days, 50ng/L), C) Female gonad in stage F4 x 200 (21 days, 25ng/L), D) Female gonad in stage F2 x 200 (49 days, 50ng/L). O= Oocyte cell. Scale bars= 100μm.
Figure 2.9: Comparison of the frequency of development stages in female Sydney rock oysters, *Saccostrea glomerata* following exposure to 17α-ethynylestradiol (0, 6.25, 12.5, 25 and 50ng/L) in experimental aquaria at A) 4 days exposure, B) 21 days exposure, N=8- per treatment and C) 49 days exposure, N=18-20 per treatment.
2.4.6. Does vitellogenin induction and EE2 exposure correlate with later effects on gonadal development?

The effects of EE2 exposure was further explored through examining relationships between EE2 exposure concentration and mean oocyte area, mean reproductive stages and sex percentages from 21 and 49 days. Increasing EE2 exposure negatively influenced male development with a decrease in the percentage of males and decreases in mean male developmental stage following 49 days exposure ($r= -0.91$, $p< 0.05$ and $r= -0.95$, $p< 0.05$ respectively) (Table 2.1).

Secondly, the capability of vitellogenin as a predictive early-warning biomarker of later reproductive effects was examined by performing correlations between mean female vitellogenin at 4 days with mean oocyte area, mean reproductive stages and sex percentages from 21 and 49 days. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages following 21 days ($r= 0.96$, $p< 0.05$) and increases in oocyte area following 49 days ($r= 0.93$, $p< 0.05$) (Table 2.1). Mean male vitellogenin (4 days) may also have useful predictive application of later negative effects on male development with a negative relationship evidenced between early vitellogenin responses and male percentage at 49 days ($r= -0.92$, $p< 0.05$) and between early vitellogenin and mean male development stage at 49 days ($r= -0.97$, $p< 0.05$) (Table 2.1).
Table 2.1: Pearson’s correlation co-efficient between Ethynylestradiol exposure and early mean vitellogenin response from 4 days with sex percentages and mean gonad development stages from 21 and 49 days and mean oocyte area from 49 days.

<table>
<thead>
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<th>Treatment (EE2)</th>
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<th>Male Vitellogenin (4 days)</th>
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</tr>
<tr>
<td>Male development stage</td>
<td>-0.95</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

2.5. Discussion

Evidence from this Chapter suggests that the application of ELISA is unsuitable for detection of accumulated EE2 in oyster tissue. This assay is designed to analyse water samples and has not been validated with tissue samples. It was hypothesised that, at 49 days, EE2 concentrations in *S. glomerata* tissue would increase with exposure concentrations. Furthermore, concentrations of EE2 were statistically similar among treatments and high concentrations of EE2 were also measured in unexposed individuals. Although it is possible that EE2 was regulated in exposed oysters this is unlikely as the concentrations of EE2 were similar in controls (which should not have had any EE2 present at all) and, for each EE2 treatment, accumulated EE2 exceeded the total sum of EE2 which was applied over the course of the experiment.

The EE2 ELISA assay is designed for water samples and has not yet been validated for tissue samples (Pers. Comm. Biosense Laboratories) and it is hypothesised that there was
interference with the ELISA assay which prevented binding of the enzyme labeled EE2 and resulted in an inaccurate estimation of EE2 concentration. The ELISA analysis is based on a competitive reaction for EE2 specific antibodies between enzyme labeled EE2 and free EE2 in the sample. Enzyme labeled EE2 and the sample are mixed and then added to the ELISA with pre-coated EE2 specific antibodies (Biosense Laboratories). After rinsing, a colour development substrate is added which converts the enzyme labeled EE2 into a coloured (yellow) product. The colour intensity of each well is measured via a spectrophotometer for absorbance (at 450 nm) which is inversely proportional to sample EE2 concentration (Biosense Laboratories). It may be possible that oyster extracts, which are yellow-milky coloured, interfere with absorbance measurements. Oyster tissue has a high lipid content and it is possible that lipids coated the bottom of the ELISA well (which is pre-coated with EE2 specific antibodies) and prevented or decreased binding of enzyme labeled EE2 in samples. It is suggested that future experiments employ alternative methods (such as HPLC or Gas Chromatography Mass Spectrometry [GCMS]) for the analysis of EE2 in oyster tissue.

Individuals exposed to EE2 exhibited increased vitellogenin in a linear dose dependent fashion, however this was not maintained throughout experimental sampling periods. At 4 days, both sexes exhibited a significant relationship between EE2 exposure and vitellogenin production however, this was not evident at 21 days for females or at 49 days for males. Thus, the temporal maintenance of the vitellogenin response varies between sexes.

Vitellogenin production, especially for females, is likely to be dependent on stage of gonadal development. Results indicated that immature females beginning a reproductive cycle were more likely to exhibit increased vitellogenin compared to mature individuals ready to spawn and indicated that developmental stage is an important consideration when assessing estrogentially mediated vitellogenin induction. As a precursor to egg yolk protein (vitellins), vitellogenin may be synthesised most actively during the earlier stages of gonadal development, as oocytes are developing (Li et al. 1998). Findings for vitellogenin production in control females supported this assertion, with vitellogenin declining with time. Furthermore, female vitellogenin exhibited a linear increase with EE2 at 4 days and
subsequently at 49 days only. The relationship found at 49 days was driven by the response in high exposures (25 and 50 ng/L EE2) where a transition from male-intersex-female was likely to have occurred while lower exposures (6.25 and 12.5 ng/L EE2) exhibited similar vitellogenin responses to controls. It may be possible that recently transitioned female individuals within these treatments, at earlier stages of gonadal development, were more responsive to EE2 exposure at 49 days. Matozzo and Marin (2008a) have also explored vitellogenin response dynamics at different stages of reproductive development through measurement of ALP in the Manilla clam, *Tapes philippinarum* exposed to E2 (5, 25, 50, 100 and 1000 ng/L) for 7 and 14 days during both a resting and pre-spawning phase. Females exposed to E2 in a pre-spawning phase for 7 days resulted in lower levels of hemolymph ALP in all exposure treatments compared to controls. Yet, following 14 days, only the 50 ng/L exposure exhibited significantly higher ALP (p< 0.05). However, in the resting phase, they found that ALP significantly increased in the hemolymph of females following 7 days exposure to E2 (5, 25, 50, 100 and 1000 ng/L), but ALP levels in exposure treatments had declined following 14 days and were only significant at the highest exposure (1000 ng/L) (Matozzo and Marin, 2008a). Similarly, Puinean et al. (2006) found that blue mussels *Mytilus edulis*, exposed to 200 ng/L E2 displayed no significant differences in vitellogenin gene expression compared to control individuals during the mature stages of gametogenesis. Taken together, these observations imply vitellogenin is most actively synthesised, and most responsive to estrogenic stimulation, during earlier phases of gonadal development, suggesting individuals commencing a gonadal development cycle should be targeted when employing vitellogenin as a biomarker of estrogenic exposure in *S. glomerata*. Individuals in an early stage of development can be identified via visual inspection of the gonad. A thin layer of white gonadal development coats the stomach, although the stomach is usually still visible in areas. These findings also suggest that future studies of estrogenic effects in *S. glomerata*, and other molluscan taxa, should measure gonadal development alongside vitellogenin.

Despite these temporal response limitations, it appears that vitellogenin is induced in a dose-dependent fashion soon after exposure at the commencement of reproductive conditioning for both females and males. The fact that males also exhibit this vitellogenin
induction, suggests estrogens exert a response not observed in unexposed males, indicative of endocrine disruption. Indeed, male molluscs (*E. complanata* and *M. edulis*) have been shown to possess a functional, yet silent, gene for vitellogenin which has been shown to be activated upon exposure to exogenous estrogens in *E. complanata* (Gagné et al. 2005) but not in *M. edulis* (Puinean et al. 2006). Further, others have found that male molluscs can exhibit sensitivity to estrogenic exposure in terms of initiation of vitellogenesis (Matozzo and Marin, 2005, 2008a; Blaise et al. 1999, 2003).

In terms of how estrogens may induce vitellogenesis mechanistically, it is well established in vertebrate models, such as fish, that vitellogenin production is mediated through estrogens binding to intracellular estrogen receptors (predominantly ER-β and to a lesser extent ER-α) which function as ligand-modulated transcription factors, binding to estrogen-responsive elements in the promoter region of the vitellogenin gene(s) (Leaños-Castañeda and Van Der Kraak, 2007). Despite this well characterized pathway in vertebrate models, little is known on how estrogens may affect vitellogenin production mechanistically in invertebrate models such as molluscs. Primarily, the literature to date is inconclusive on the presence of functional estrogen receptors (ER) in Mollusca. cDNA encoding vertebrate-like estrogen receptors (with high homology in the DNA binding domain to vertebrate ER-α and ER-β) have been cloned in a number of molluscan species including the rock shell, *Thais clavigera* (Kajiwara et al. 2006), the common octopus, *Octopus vulgaris* (Keay et al. 2006) and *C. gigas* (Matsumoto et al. 2007). Matsumoto et al. (2007) suggested that such estrogen receptors may act as nuclear receptors regulating the transactivation of reproductive genes, including the vitellogenin gene, with estrogen receptor immuno-reactivity localised in the nuclei of follicle cells, the site of vitellogenin synthesis (Matsumoto et al. 2007). Yet in-vitro observations have found that these estrogen receptors do not bind estrogen both in ligand-binding and cell based gene-reporter assays (Lafont and Mathieu, 2007). Although far from clearly characterised, literature documenting vitellogenin induction upon exposure to estrogens, in a variety of molluscan taxa, including findings presented in this thesis, suggest that estrogens play a functional role in vitellogenesis though the precise mechanism(s) remain to be elucidated.
Along with an acceleration of female gonadal development, EE2 exposure may be capable of initiating a full sex reversal from male to female. The occurrence of intersex individuals present in the 50 ng/L exposure at 21 days together with a significant increase in the proportion of females at 49 days suggested that a number of individuals have undergone a complete sex reversal during this experiment. The histological examinations of intersex individuals suggest that intersex is a transitional event in the switch from male to female gametal status. Within a gonadal follicle of an intersex individual, it appears that the maturing male developmental pathway is interrupted. Subsequent germ cell differentiation is redirected to a female developmental fate. Oogonia are sequentially added and, over time, male gametes may be removed by the proliferation of hemocyte cells (gamete re-absorption). Gamete re-absorption by hemocytes has been described as a process to recycle materials and energy following a spawning event (or in this case, sex transition) or as a survival mechanism in response to stress/diseases (Beninger and Le Pennec, 2003; Pipe, 1987; Steele and Mulcahy, 1999). Without tracking the gametal status of each individual over the entire experimental window it is difficult to unambiguously confirm full sex reversal (as existing sex determination protocols are invasive [smearing] or destructive [histology]). The observation of intersex individuals in lower EE2 exposures (6.25 and 12.5 ng/L) is a significant finding and may indicate that a transition (to higher female proportions) may occur at lower concentrations of EE2 with greater exposure duration. The occurrence of intersex at these lower, environmentally relevant, exposures of EE2 demonstrates that gonad gametal status may be employed as a sensitive biomarker of estrogenic exposure and effect in S. glomerata. Although it is likely that these individuals may follow a transitional switch from male to female with time, in a similar fashion to higher exposures (50 ng/L), it may also be possible that lower exposures do not promote full sex reversal and subsequently result in a static intersex condition. Regardless, there are potentially negative effects associated with full sex reversal and/or intersex at both the individual and population level. This could include decreased individual fitness/reproductive capacity and/or altering the sex ratio and thus the overall fitness of a population. It is well established that oysters spawn in synchronization, during the summer months in response to environmental signals such as the full moon, changes in salinity and warmer water temperatures (Roughley, 1933). An individual that experiences intersex or a
full sex reversal midway during a gonadal cycle is unlikely to develop mature gametes capable of fertilization for a synchronised spawning event compared to individuals that have matured as female only. Thus, estrogentic exposure is perhaps likely to disrupt spawning synchronization among individuals. Further testing is also required to investigate if the gametes from intersex or these (new) female individuals are viable, capable of fertilization and if there is potential for more long term effects in subsequent generations.

Lastly we assessed the utility of vitellogenin as an early warning indicator of later gonadal effects at the individual level. Higher level effects, such as an increase in female development or a full sex reversal may be detrimental in terms of individual fitness, population level related consequences (again via influencing reproductive synchrony). The ability of a biomarker to predict higher level effects is favourable for biological assessment of contaminants under circumstances where a risk of estrogentic exposure can be identified and removed prior to the onset of higher level effects. The results of this Chapter demonstrated that early increases in female vitellogenin (following 4 days) correlated with an increase in female development (21 days) and increased oocyte area (49 days). Male vitellogenin (following 4 days) exhibited a negative correlation with both male percentages (49 days) and mean male development stages (49 days). Together these results suggest that vitellogenin, if measured during the initial stages of gonadal development, may be useful as an early warning indicator for accelerated female development and decreased male development due to estrogentic exposure. These results suggest that monitoring of both sexes is useful and perhaps necessary. Females are likely to be more sensitive to effects of estrogentic contaminants, display more pronounced vitellogenin induction and exhibit accelerated oocyte development. Males, in comparison, provide definitive evidence of endocrine disruption via vitellogenin induction, decreased male development and sex reversal.

Selection of EE2 exposures in this experiment was based on EE2 concentrations in water reported in international literature (0- 42 ng/L) (Ternes et al. 1999; Desbrow et al. 1996) which are perhaps high in comparison to what is currently known for Australian waters/sewage effluent (1-1.38 ng/L in sewage effluent, reported by Ying et al. 2008). While
estrogenic equivalents (EEQ) of some EE2 exposures used in this experiment (13.13- 105 ng/L) are more similar, they are still outside the range of EEQ values measured in Australian sewage effluent (0- 44.5 ng/L, Leusch et al. 2006; Mispagel et al. 2009; Tan et al. 2007; Williams et al. 2007). Although currently not much is known regarding EE2 concentrations in Australian water and/or sewage effluent, future experiments should consider using lower concentrations that are within the range of that which has been measured in Australian waters to validate biomarker utility under environmental concentrations.
Chapter 3 : The Development of a Real-Time qPCR assay for vitellogenin quantification in the Sydney rock oyster

3.1. Summary
There is mounting evidence that molluscan vitellogenin may increase due to estrogenic exposure. Despite these findings, there is a lack of specific assays and vitellogenin increases are usually indirectly measured, such as via the measurement of alkali labile phosphates (ALP). The main aim of this Chapter was the development of a real-time qPCR assay for the measurement of vitellogenin gene expression in the Sydney rock oyster, *Saccostrea glomerata*. To develop the real-time qPCR assay, partial vitellogenin sequences for mRNA and genomic DNA were obtained (via qualitative PCR and nucleotide sequencing). Real-time qPCR primers for *S. glomerata* vitellogenin were then developed to span the intron-exon boundary. Vitellogenin gene expression was assessed via real-time qPCR in *S. glomerata* exposed to 17α-ethynylestradiol (EE2) (0, 6.25, 12.5, 25 or 50 ng/L) for 4 days. Vitellogenin gene expression was found to increase in a dose-dependent manner in both males and females based on vitellogenin gene averages for each exposure. Further, a significant linear relationship was found in females based on regression of replicates with exposure ($R^2= 0.22$, $p< 0.05$). The real-time qPCR assay for the quantification of vitellogenin gene expression in *S. glomerata* would be useful as a predictive biomarker of estrogenic exposure. Multiple isoforms of vitellogenin, which respond differently to estrogenic exposure, have been demonstrated in fish species. Thus this Chapter also explored the possibility of multiple vitellogenin isoforms in *S. glomerata*. Qualitative PCR for vitellogenin gene expression provided some evidence for a second vitellogenin gene isoform in males with the appearance of additional bands. However, sequencing of these bands found that the PCR product was not another vitellogenin isoform but rather a vitellogenin genomic DNA sequence.
Part of this Chapter involved research collaboration with another postgraduate student, Kelli Anderson (University of the Sunshine Coast) which led to participation as a co-author of her publication. My contribution to this publication consisted of providing laboratory reared oyster samples to include as controls (from Chapter 2) and providing comments in the preparation of a manuscript for publication. Anderson et al. (2010) were successful in obtaining a partial mRNA vitellogenin sequence and performed qualitative PCR to assess differences in vitellogenin gene expression. This Chapter was focused on building on this research by obtaining a partial genomic sequence and developing a Real-Time qPCR assay which would enable quantitative and more sensitive measurement of relative vitellogenin gene expression in *S. glomerata*. This publication is referred to throughout this Chapter but is not presented in detail:


3.2. Introduction:
Vitellogenin is a large glyco-lipo-protein which acts as a precursor molecule for smaller egg yolk proteins known as vitellins. Vitellogenin is thought to be present in females of most oviparous species. In vertebrates it is well known that vitellogenin gene transcription is mediated via ligand induced transcriptional activation of estrogen receptors (ER), whereby endogenous estrogens bind to the ligand binding domain of the ER within the cell nucleus which then simultaneously binds to a estrogen responsive element (ERE) upstream from the region encoding vitellogenin and/or other target genes (Bowman et al. 2000, Mueller, 2003). The vitellogenin nucleotide sequence is directly transcribed from genomic DNA into messenger RNA (mRNA). Immediately following translation, the newly formed vitellogenin amino acid chain undergoes extensive folding and modification to become a functional vitellogenin protein. Vitellogenin is then transported to the vertebrate gonad, cleaved into vitellins and incorporated into growing oocyte cells, usually via receptor-
mediated endocytosis (Wallace, 1985). Given that vitellogenin is a crucial component of oocyte development and its induction is under estrogenic control, induction may also be stimulated via exposure to xeno-estrogens with sufficient structural similarity to native estrogen to facilitate ligand binding with ERs. Thus, research has sought to characterise both gene(s) and protein(s) for the development of bioassays of xeno-estrogen exposure and effect.

Vitellogenin gene expression may be a useful indicator of exposure to estrogenic compounds, but the development of such assays firstly requires nucleotide sequence information for the gene. While molluscs are useful as indicator species, they have not been studied as extensively as vertebrates in terms of genomic sequencing. However, several vitellogenin gene transcripts have been cloned and sequenced in a number of molluscs to date. Matsumoto et al. (2003) cloned a full length vitellogenin sequence consisting of 5023 bp in the Pacific oyster, *Crassostrea gigas*. A large (1689 bp) partial fragment has also been cloned for the Yesso scallop, *Patinopecten yessoensis* (Osada et al. 2004). Smaller partial fragments of putative vitellogenin have also been identified, based on homology with known vitellogenin sequences from other molluscan taxa, and are available in the Sydney rock oyster, *Saccostrea glomerata* (Anderson et al. 2010), the Blue mussel, *Mytilus edulis* (Puinean and Rotchell, 2006), the Calico scallop *Argopecten purpuratus* (Boutet et al. 2008) and in Freshwater mussels, *Elliptio complanata* (Gagné et al. 2005).

Vitellogenin sequences from *C. gigas* and *P. yessoensis* were shown to exhibit homology to vitellogenin gene transcripts from other taxa within the N-terminal and central region (Matsumoto et al. 2003, Osada et al. 2004). Sequence homology between *C. gigas* vitellogenin and other taxa included 25 and 22% with rainbow trout vitellogenin (from nucleotides 24-266 and 567-932 respectively) and 28% to kumara prawn *Penaeus japonicus*, vitellogenin (nucleotides 559-940). Osada et al. (2004) found that the vitellogenin nucleotide sequence from *P. yessoensis* exhibited 20-35% similarity within the N-terminal region to the other vitellogenins from oviparous vertebrates and invertebrates.
Comparisons of homology between molluscan vitellogenin gene sequences suggested that portions of the gene exhibiting homology with vitellogenins from other species were confined to the N-terminal or central region of the gene only (i.e. not in the C-terminal region) (Matsumoto et al. 2003, Osada et al. 2004). Similarly, others have reported that vitellogenin was not highly conserved among vertebrate and invertebrate taxa with similarity within short segments only which is mainly confined to the N-terminal region of the gene (Wahli, 1988; Denslow 1999).

In molluscan taxa, vitellogenin genes have exhibited some homology to vitellogenin genes from vertebrate species yet the location of protein synthesis within organisms has been shown to differ among taxa. Within both vertebrates and invertebrates, vitellogenin protein synthesis was shown to be usually external from the gonad such as within the liver of fish, intestines for nematodes, digestive gland of snails and within the fat body for insects (Bride et al. 1992, Wahli, 1988). However in molluscs, evidence suggests that vitellogenin synthesis occurs within the gonadal tissue, in auxiliary cells with close contact to the growing oocytes. Suzuki et al. (1992) suggested that vitellogenin was synthesised within the ovary, as this was the only tissue to react with vitellogenin anti-serum. In-situ hybridization has further demonstrated that vitellogenin mRNA expression was localised within the auxiliary cells close to the growing oocytes in C. gigas (Matsumoto et al. 2003) and P. yessoensis (Osada et al. 2004). Several studies have demonstrated molluscan taxa are capable of synthesising estrogens and both endogenous estrogens and xeno-estrogens may regulate vitellogenin (Gauthier-Clerc et al. 2006; Li et al. 1998; Matsumoto et al. 1997; Osada et al. 2003; Porte et al. 2006; Riccardi et al. 2008), this is reviewed in detail in Chapter 1.

It should be acknowledged that despite comprehensive evidence that vitellogenin gene/protein(s) exist and their production could be regulated by estrogens, literature to date is inconclusive on the presence of a functional ER as a mediator of vitellogenesis in molluscs. In fish, vitellogenin has been shown to be mediated via a classical nuclear ER pathway. Estrogen-related receptors (ERR), with structural similarity to vertebrate ERs have been cloned in a number of species including, sea snails Thais clavigera (Kajiwara et
al. 2006), octopus *Octopus vulgaris* (Keay et al. 2006) and *C. gigas* (Matusmoto et al. 2007). Matsumoto et al. (2007) found that the deduced amino acid sequence of the cDNA sequence of the binding domain of *C. gigas* ERR had respective similarities of 86 and 89% to the human ER-α and ER-β. Further, the deduced amino acid sequence of the ligand binding domain has 45% similarity to human ER-α and ER-β (Matsumoto et al. 2007). Recent in-vitro observations however, have found that the EERs of these species were not responsive to, or did not bind estrogen. Despite the absence of evidence for estrogen binding, Matsumoto et al. (2007) suggested that such ERs may act as nuclear receptors regulating the transactivation of reproductive genes, including the vitellogenin gene, with estrogen receptor immuno-reactivity localised in the nuclei of follicle cells, the site of vitellogenin synthesis (Matsumoto et al. 2007). Collectively, evidence to date is insufficient to allow characterisation of precise mechanism(s) through which vitellogenin is induced upon exposure to estrogens.

It is also unknown whether vitellogenin gene transcription in molluscs is under the control of single or multiple genes. Multiple isoforms of the vitellogenin gene have been shown to be synthesised within single vertebrate species. Gene isoforms identified as vitellogenin A, B or C which code for one or more vitellogenin proteins have been identified in species such as the fathead minnow *Pimephales promelas* (Parks et al. 1999; Miracle et al. 2006), white sturgeon *Acipenser transmontanus* (Bidwell and Carson, 1995), white perch *Morone americana* (Hiramatsu et al. 2002), mummichog *Fundulus heteroclitus* (LaFleur et al. 1995), rainbow trout *Oncorhynchus mykiss* (Mouchel et al. 1996), and in zebrafish *Danio rerio* (Tong et al. 2004). Differences in these vitellogenin gene isoforms may correspond with differences in translated vitellogenin proteins in terms of structure, rate of degradation, buoyancy and ultimately their role in oocyte development (Hiramatsu et al. 2002, Wang et al. 2000). Different isoforms of vitellogenin have been demonstrated to exhibit different transcription responses (Dae-Sik Hwang et al. 2010; Isoe and Hagedorn, 2007) and therefore the possibility of different vitellogenin isoforms (in either gene or translated protein) may be an important consideration for future studies of molluscan vitellogenin gene expression and their responsiveness to estrogenic exposure.
The lack of knowledge of the mechanisms of vitellogenesis has perhaps contributed to the lack of specific assays for vitellogenin quantification in molluscs. The measurement of vitellogenin protein is usually not specific and assessed via an indirect surrogate assay which quantifies levels of ALPs under the assumption that this measure broadly correlates with vitellogenin protein levels (Blaise et al. 1999, Matozzo et al. 2005, Ortiz-Zarragoitia and Cajaraville 2006). The ALP assay is based on the extraction and quantification of all phosphorylated molecules and thus may not accurately reflect true vitellogenin concentrations. Therefore, the ALP assay may be more useful as a screening measure which needs to be followed with specific vitellogenin protein or gene measurement Gagné et al. (2005). An ELISA for quantification of vitellogenin protein has been developed which is specific for the vitellogenin antigen in P. yessoensis yet this has limited use in other species (Osada et al. 2003). Andrew et al. (2008) developed a specific assay for measuring vitellogenin protein via HPLC analysis in S. glomerata, with the identity of the vitellogenin peak confirmed via proteomic sequencing (appendix 2). Unlike the surrogate ALP assay, HPLC analysis directly measures vitellogenin protein.

Studies in fish have suggested that vitellogenin gene expression quantification may be more favourable, in terms of sensitivity and temporal responsiveness, compared to vitellogenin protein measurements (Denslow 1999). Celius et al. (2000) exposed O. mykiss to a concentration gradient (0, 0.01, 0.1, 1 and 10 mg/kg) of E2 for 10 days and suggested that vitellogenin gene expression (measured via real-time qPCR) was more sensitive in comparison to protein induction (measured via ELISA). Vitellogenin gene expression was significantly induced at lower E2 concentrations, 0.1, 1 and 10 mg/kg compared to vitellogenin protein which was significantly induced only at 1 and 10mg/kg E2 (p< 0.05). Vitellogenin gene expression may also be favourable compared to protein measurements in terms of temporal responsiveness to changes in estrogenic exposure. Vitellogenin mRNA transcripts have been demonstrated to rapidly decline following induction (usually within one-two days post exposure) whereas vitellogenin protein levels have been shown to remain elevated for longer periods, with half lives of up to two weeks post-exposure to estrogens (Denslow 1999, Hemmer et al. 2002). Hemmer et al. (2002) observed half lives ranging from 13.3-14.8 days for plasma vitellogenin in male fathead minnows, Cyprinodon
ariegatus, following exposure to E2 (0.89 and 0.71 µg/L) and 4-nonylphenol (NP) (5.6 and 59.6 µg/L) for 16 days followed by regular sampling up to 96 d post exposure. Similarly, Allen et al. (1999) also calculated a half life of 13.5 days for vitellogenin protein in the plasma of flounder, Platichthys flesus, exposed for 21 days to EE2. Thus, vitellogenin gene expression may be favoured over vitellogenin protein measurement in terms of sensitivity (responds at lower concentrations) and temporal responsiveness (more accurately reflects estrogenic exposure at time of sampling in the face of intermittent or pulsed estrogenic exposures).

Real-time qPCR (quantitative) assays quantifying vitellogenin gene expression are increasingly available in molluscan species with assays developed in M. edulis (Puinean and Rotchell, 2006), C. gigas (Matsumoto et al. 2003), A. purpuratus (Boutet et al. 2008) and E. complanata (Gagné et al. 2005). Several of these assays have been trialed via laboratory experiments of individuals exposed to E2. For example, Puinean and Rotchell (2006) found that exposure to E2 failed to induce significant vitellogenin gene transcription after 72 hours in M. edulis injected with 25 µg E2 and found there were no differences between sexes in either control or exposed treatments. Furthermore they failed to detect differences in vitellogenin gene expression (between sexes or treatments) following exposure to 200 ng/L E2 for 10 days (Puinean et al. 2006). Later experimentation from the same group, exposed adult M. edulis in different stages of gametogenesis to E2 (5, 50 and 200 ng/L) and EE2 (5 and 50 ng/L) were sampled (Ciocan et al. 2010). Vitellogenin gene expression was significantly induced (p< 0.05) during early gametogenesis in all exposures compared to controls (except 200 ng/L E2), while in mature individuals there was no difference between controls and individuals exposed to E2 or EE2. Gagné et al. (2005) trialed their real-time qPCR assay for vitellogenin in E. complanata using individuals exposed to E2; with significant vitellogenin gene transcription observed at a concentration of 2.2 nmole. The development and findings of these assays suggest strong potential for vitellogenin gene induction as a biomarker but also highlights that there are many research gaps in terms of understanding the kinetics of vitellogenin gene transcription in molluscs and confounding factors such as the relationship between gonadal development, exposure (timing and concentration) and the sex of the organism.
Predominantly, this Chapter addressed the need for a molluscan real-time qPCR assay to assess vitellogenin gene expression in *S. glomerata*. Prior to development of a real-time qPCR assay, it is necessary to confirm the presence of a vitellogenin gene in *S. glomerata* and obtain partial mRNA and genomic vitellogenin sequence information for the development of primers for real-time qPCR. Therefore, the initial aim was to identify, clone and sequence a partial mRNA and genomic sequence for the vitellogenin gene in *S. glomerata*. Phylogenetic analysis sought to determine the similarity between the partial vitellogenin sequence of *S. glomerata* and other taxa. Once the presence of vitellogenin in *S. glomerata* was confirmed and partial sequence information was obtained, the aim was to develop a real-time qPCR assay (using partial mRNA and genomic sequence to develop real-time qPCR primers) for use as a biomarker of estrogenic exposure in *S. glomerata*. The final aim of this Chapter was to trial the developed real-time qPCR assay via analysis of individuals exposed to a concentration gradient of 17α-ethynylestradiol (EE2) for 4 days, as gene expression was predicted to be most likely elevated soon after exposure, rather than at later experimental windows.

3.3. Materials and Methods

3.3.1 Experimental Design

Oysters used in the development of the real-time polymerase chain reaction (qPCR) assay were sourced from a previous experiment which examined the dose response relationship between EE2 exposure and reproductive parameters (see Chapter 2 for a full description of experimental methods). Briefly, *S. glomerata* were exposed to a concentration gradient of 6.25, 12.5, 25 and 50 ng/L EE2 (in 1.2 µg/L ethanol); 1.2 µg/L ethanol control; and seawater control (N= 20 per treatment/time). Oysters were removed from the experiment at three separate sampling occasions, 4, 21 and 49 days. Oysters were dissected and gonadal tissue (approx 200 mg) was removed and stored at -80 °C in RNA later (Ambion, catalogue number AM7020, Victoria, Australia). Oyster tissue was employed from the 4 and 49 day sampling periods for the development of a real-time qPCR assay. As vitellogenin protein was most elevated after short term (4 days) compared to long term (49 days) exposure (Chapter 2), oyster tissue from the 4 day sampling period was employed to assess
vitellogenin gene expression with estrogenic exposure once the qPCR method was established.

3.3.2. RNA extraction

Total RNA from oyster gonadal samples (~100 mg) was extracted using RNAqueous®-4PCR and then treated with DNase inactivation agent according to the manufacturer’s guidelines (Ambion® catalogue number AM1914, Victoria, Australia). Quality of the RNA was assessed on a denaturing gel; 15 µL of formaldehyde loading dye was added to 1 µg RNA and heated for 15 minutes to denature RNA. Samples were then loaded on the denaturing gel (consisting of 0.4 M 3-[N-Morpholino]-propanesulfonic acid (MOPS) pH 7, 0.1 M sodium acetate and 0.01 M ethylenediaminetetraacetic acid (EDTA)) and visualised on an Ultraviolet (UV) transilluminator (BioRad) (Figure 3.1).
For each sample, the quality of RNA was assessed via a denaturing gel (Figure 3.1). In a denaturing gel, samples with sharp intense ribosomal bands were used for analysis. The RNA concentration was assessed via a NanoDrop 1000A Spectrometer (Thermo Scientific). Quality of the RNA was also assessed via the NanoDrop 1000A Spectrometer (Thermo Scientific) which was used to calculate the ratio of 260 to 280nm absorbance. The 260/280 nm absorbance ratio is a measure of the purity of the DNA with a low ratio (<1.8) indicative of protein contamination while a high ratio (> 2.1) can be indicative of RNA degradation (Manchester, 1996; Fleige and Pfaffl, 2006). Samples which had an OD 260/280 ratio between the range of 1.8-2.1 were considered to have high purity and low protein contamination and were employed for analysis.

3.3.3. First strand cDNA synthesis
First-strand cDNA synthesis was carried out using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™ catalogue number 18080-051, Vic, Australia) according
to manufacturer’s guidelines. Briefly, 1 μg of RNA was mixed with 1 μL of 50 μM oligio (dT)$_{20}$, 1 μL of 10 mM dNTP mix and 7 μL of DEPC-treated water and then incubated at 65 °C for 5 minutes then on ice for 1 minute. Components were then added in the following order; 2 μL 10x RT buffer, 4 μL 25 mM MgCl$_2$, 2 μL 0.1 M DTT, 1 μL RNaseOUT (40 U/μL) and 1 μL of Superscript™ III RT (200 U/μL) and briefly collected by centrifuge. The mixture was incubated for 50 minutes at 50 °C with termination of reaction for 5 minutes at 85 °C and then chilled on ice. One μl of RNase H (2 U/μL) was added followed by incubation for 20 minutes at 37 °C in order to remove the RNA template to prevent contaminating cRNA: DNA hybrids. Synthesised cDNA was then stored at -20°C prior to PCR analysis.

3.3.4. Qualitative PCR using total RNA

Qualitative PCR was utilised to confirm the presence of the vitellogenin gene in S. glomerata. A partial mRNA sequence of S. glomerata vitellogenin has been previously published (GeneBank accession no. EU179754.1) (Anderson et al. 2010) and this sequence was used to develop primers for qualitative PCR using Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Table 3.1). Primers developed and used by Anderson et al. (2007) were also tested for comparison (Table 3.1). All primers were synthesised by Sigma Genosys.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence</th>
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| Forward: Vitellogenin (F23)  
Reverse: Vitellogenin (R333) | 5’-CAAACACAGGTGCCTGAGAA-3’  
5’-TCACATTTCACCACCAATGCT-3’ |
| Forward: Vitellogenin (F212)  
Reverse: Vitellogenin (R435) | 5’-AACCTGGACAAGAGGAAGCA-3’  
5’-TACATTGGTCGGTCCAGACA-3’ |
| Forward: Vitellogenin (F12)*  
Reverse: Vitellogenin (R08)* | 5’-ATCTGTTCAGGCCCCCTCAC-3’  
5’-GCTTCCTCTTGTCCAGGTTG-3’ |

* Primer pair F12 and R08 developed by Anderson, (2007)
Two female and 2 male oysters were selected from the 49 days sampling period from the control (seawater) and 50 ng/L EE2 exposures. The PCR amplification mix for each tube contained the following components: 2.5 µL 10x PCR buffer (-MgCl₂), 0.5 µL 10 mM dNTP mixture, 0.75 µL MgCl₂ (50 µM), 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 0.5 µL of gonadal template cDNA, 0.1 µL of Platinum® Taq DNA polymerase (5 U/µL) (Invitrogen, catalogue number: 10966-018, Vic, Australia) and 44.65 µl distilled autoclaved water. A negative control was included in all PCR which consisted of a standard reaction mix with nuclease-free water substituted for cDNA template. The PCR cycling conditions consisted of denaturation at 94 °C for 2 minutes, followed by 34 quantification cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 minute. A final incubation at 72 °C for 10 minutes was included and then samples were held at 4 °C. PCR products were then run on a 1 % agarose gel and visualised on a UV transilluminator (Bio-Rad). Gel bands were excised and then DNA/product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, catalogue number TM050).

3.3.5. Cloning
The PCR products were cloned using the pGEM®-T Easy Vector System according to the manufacturer’s guidelines (Promega, NSW, Australia). Three µL of purified PCR product was mixed in a 0.5 mL tube with 5 µL of 2X Rapid Ligation Buffer T4 ligase, 1 µL of pGEM®-T Easy Vector and 1 µL of T4 DNA ligase. A positive control (2 µL of control insert DNA and 1 µL deionized water substituted for PCR product) and a back-ground control (3 µL of deionized water substituted for PCR product) were also prepared. Reactions were carefully mixed by pipetting and incubated for 1 hour at room temperature. The ligation reaction was briefly collected by centrifugation and 2 µL of the ligation was transferred into a sterile 1.5 mL centrifuge tube on ice, 2 µL of control reaction was transferred to a separate tube. Competent cells (JM109) were thawed on ice and 50 µL was added to each reaction and gently mixed by flicking tube. The cells were heat-shocked by incubation in a 42 °C water bath for 50 seconds and then transferred back to ice for 2 minutes. Nine hundred and fifty µL of autoclaved LB broth (10 g Bacto®, 5 g Bacto®-Yeast extract, 5 g NaCl, made up to 1 L, adjusted to pH 7) was added to the reactions followed by
a 1.5 hour incubation with shaking (150 rpm) at 37 °C. For reaction, 100 μL was plated onto 4 pre-prepared antibiotic plates (15 g of agar was dissolved in 1 L LB broth and autoclaved, when cool 100 μg/mL ampicillin was added; 30 mL were poured into 85 mm Petri dishes and then stored at 4 °C) and incubated overnight (16 hours) at 37 °C. Positive control plates were observed to be normal, containing both white colonies (cells with control insert DNA) and blue colonies (undigested pGEM®-T Easy Vector). Background plates were also as expected and contained blue colonies only. From PCR plates, the white colonies (containing the vitellogenin PCR insert) were selected under sterile conditions with a pipette tip and placed into a sterile 50 mL centrifuge tube containing 5 mL of LB broth (1 μg/mL Ampicillin) and incubated at 37 °C overnight with shaking (250 rpm). The tubes were then centrifuged and the supernatant (LB broth) was removed and tube was inverted on paper towel to remove excess LB Broth. Plasmid DNA from the cell culture was then purified using Wizard® Plus SV Minipreps DNA Purification System (Promega). Two-hundred and fifty μL of cell re-suspension solution was added to cell pellet and mixed using pipette, 250 μL of cell lysis solution was added and tube was inverted four times. The mixture was incubated for 5 minutes at room temperature, 10 μL of alkaline protease solution was added and then tube was inverted four times. The mixture was centrifuged at 10,000x g for 10 minutes, 850 μL of the supernatant was passed through a spin column via 1 minute centrifugation. The spin column was washed once with 750 μL of column wash, centrifuged for 1 minute at 10,000x g, washed once 250 μL of column wash and centrifuged for 2 minutes at 10,000x g. The plasmid DNA (vector containing PCR product) was then eluted from spin column using 100 μL of nuclease-free water. Nine μL of the plasmid (200ng/μL) was mixed with either 1 μL of forward primer (F212 (10 μm) or 1 μL of reverse primer (R435) (10 μm) and sent to the Australian Genome Research Facility (AGRF) for sequencing.

3.3.6. Genomic DNA extraction
Genomic DNA was isolated and purified from S. glomerata gonadal tissue from one female (control, 49 days), male (control, 49 days) and intersex oyster (12.5 ng/L EE2, 49 days) using the Wizard® Genomic DNA Purification System (Promega, catalogue number A1120). Tissue was homogenised, centrifuged to form a pellet and then washed twice with
200 µl of PBS. Cells were then re-suspended in 200 µL of PBS and 600 µL of Nuclei Lysis Solution and pipetted to lyse cells. Two hundred µL of Protein Precipitation Solution was added and mixture was vortexed for 20 seconds and then chilled on ice for 5 minutes. Mixture was then centrifuged for 4 minutes at 14,000x g until precipitated protein formed a white pellet. Supernatant was transferred to a new tube and 600 µL of isopropanol was added. Mixture was inverted several times until DNA formed white threads, and then centrifuged at 14,000x g at room temperature for 1 minute. Supernatant was then removed and discarded, DNA pellet was washed with 600 µL of 70% ethanol (analytical grade), centrifuged at 14,000x g at room temperature for 1 minute. Ethanol was removed carefully by a Pasteur pipette and pellet was left to air-dry for 15 minutes. One hundred µL of DNA Rehydration Solution was added and then mixture was incubated at 65 ºC for 1 hour with periodic mixing by gentle tapping. DNA was stored at 4 ºC prior to PCR.

3.3.7. Qualitative PCR using genomic DNA
The PCR conditions were as previously described except with primers F23 and F333 (Table 3.2) and genomic DNA used as the template. PCR products were then visualised on a 1% agarose gel with a product size of ~ 600 bp. Gel bands were excised and DNA purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, catalogue number A9281). PCR products were then cloned using the pGEM®-T Easy Vector System (Promega, New South Wales, Australia) and sequenced by AGRF.

3.3.8. Real-time qPCR development
Partial mRNA and genomic sequences for vitellogenin were aligned to identify the intron-exon boundary. Real-time qPCR primers were then designed with the forward primer to span over an intron-exon boundary to avoid cross-amplification of genomic DNA during real-time qPCR (Primer 3; http://frodo.wi.mit.edu/primer3/). Product size was 134 bp. The 16S ribosomal S. glomerata gene was used as a reference gene with a product size of 173 bp. The CT values for the 16S reference gene were consistent between samples/treatments with an average CT of 12.67 ± 1.29. The optimal melting temperature for primers was initially determined via a temperature gradient analysis (Corbett Life Sciences, Vic, Australia) (Table 3.2).
**Table 3.2**: Primers developed and used in real-time qPCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Efficiency</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: Vitellogenin (GF90)</td>
<td>5'-GGTTATGGAGCCTAGCATTGG-3'</td>
<td>1.00</td>
<td>76.9 °C</td>
</tr>
<tr>
<td>Reverse: Vitellogenin (GR221)</td>
<td>5'-ATTGGTGGTCCAGACAGTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward: 16S (GF16S)</td>
<td>5'-AGTACCTGCCCAGTGCAAAA-3'</td>
<td>1.05</td>
<td>80.7 °C</td>
</tr>
<tr>
<td>Reverse: 16S (GR16S)</td>
<td>5'-GCTGCTCAAGCAAGAGACA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Vitellogenin gene expression was determined by relative real-time qPCR using Syto9 (Invitrogen, Vic, Australia) as the fluorescence dye and the two standard curve method for quantification (Corbett Life Science, NSW, Australia). Serial 1:5 dilutions of cDNA (i.e. 1:5, 1:25, 1:125 and 1:625) were employed to create the standard curve (Figures 3.2 and 3.3). All samples had CT values within the range of the standard curve (11.1- 13.99). A calibrator sample (mixture of cDNA from control individuals) was included within each real-time qPCR run to account for differences in run conditions. The real-time qPCR was carried out in a 96-well Genedisk (Corbett Life Science, NSW, Australia) with 50 µL reaction tubes and a robot was used to mix each reaction tube (Corbett Life Science Robotics, Version 4.9.1, NSW, Australia). Each (15µL) reaction contained 5 µL (2 ng/µL) cDNA template, 1.3 µL Ultrapure water, 0.6 µL of forward primer (10 µM), 0.6 µL of reverse primer (10 µM) and 7.5 µL of 2x master mix (containing 200 mM Tris, pH 8.4, 200 mM KCL, 0.4 mM dNTP each, 100 mM Cyto 9, MgCl₂ and H₂O). Negative controls (with water substituted for cDNA) were included in every run to ensure that no contaminating DNA was present or amplified. Also, mRNA controls (with RNA only) were included in every run to ensure that genomic DNA contamination had not occurred.

All samples, standards, calibrator and negative controls were run in triplicate. The samples were first heated to 95 °C for 2 minutes. Then the PCR reaction was carried out for 40 quantification cycles at 94 °C for 10 seconds, 58 °C for 20 seconds followed by 72 °C for 20 seconds. The cDNA from individuals (i.e. females from 50 ng/L EE2 treatment) which were suspected to have high vitellogenin expression (based on EE2 exposure and earlier qualitative PCR), were pooled to generate a sample which could then be diluted in order to
create a standard curve for both vitellogenin and the reference 16S ribosomal gene (Figure’s 3.2 and 3.3).

**Figure 3.2:** A linear standard curve for vitellogenin gene generated from a serial dilution of Sydney rock oyster, *Saccostrea glomerata*, cDNA in real-time qPCR.

**Figure 3.3:** A linear standard curve for the 16S ribosomal gene generated from a serial dilution of Sydney rock oyster, *Saccostrea glomerata*, cDNA in real-time qPCR

Standard curves were generated by a linear regression between concentration and the CT value of the serial dilutions of the sample. The value of $R^2$ (ideally close to 0.99), M (slope of regression, ideally close to -3.322) and the reaction efficiency (ideally close to 1) were used to assess the quality of the standard curve. The standard curves generated for vitellogenin gene ($R^2= 0.99, M= -3.20$, efficiency= 1.05) and for the 16S ribosomal gene ($R^2= 0.99, M= -3.314$, efficiency= 1), based on this criteria the standard curves were
considered appropriate for calculation of vitellogenin gene expression. Calculation of the relative gene expression was performed using the Rotor-Gene 6000 Series software by using the two standard curve method (Corbett Life Science, NSW, Australia). This method involves first generating a standard curve for the gene of interest (vitellogenin) and the housekeeper gene (16S ribosomal). The vitellogenin gene was assigned arbitrary units “relative mRNA expression” which are calculated by normalising vitellogenin with the 16S reference gene (vitellogenin gene expression = fold change in vitellogenin gene expression/fold change in 16S gene expression). The software normalises samples against the calibrator by assigning this sample a value of 1.

A dissociation (melt) curve was generated for each sample for vitellogenin and the 16S reference gene with a ramp from 60 °C to 95 °C, increasing in increments of 0.5 °C each 5 seconds. Vitellogenin primers generated a melt temperature of ~76.9°C (Figure 3.4) and the 16S ribosomal primers generated a melt temperature of ~80.7 °C (Figure 3.5).

![Figure 3.4](image-url): Melt curve of the Sydney rock oyster, *Saccostrea glomerata*, vitellogenin real-time qPCR product including standards (brown), samples, calibrator and negative controls (light blue).
3.3.9. Statistical Analysis

Linear dose-response relationships between EE2 exposure and vitellogenin mRNA gene expression in gonadal tissue were assessed to test for biomarker utility via linear regression analyses using STATISTICA (Statsoft, 2005).

3.4. Results

3.4.1 Vitellogenin gene expression

Vitellogenin gene expression was detected using qualitative PCR amplification with three different primers sets. In Figure 3.6, vitellogenin gene expression was detected by primer pairs F23 + R333 and F12 + R08 (Anderson et al. 2010) in nearly all individuals.

Figure 3.5: Melt curve of the 16S real-time qPCR product including standards (brown), samples, calibrator and negative controls (light blue).

No other dissociation peaks were found within standards or samples which suggested primers were specific and not binding to other products or forming primer dimers. No response was ever found in any of the negative controls or mRNA controls.
Figure 3.6: Gel electrophoresis image of qualitative PCR using vitellogenin primers sets designed from Sydney rock oyster, *Saccostrea glomerata*, partial mRNA vitellogenin sequence (Anderson et al. 2007). Control females: 1, 2, control males: 3, 4, females treated with 50 ng/L EE2 for 4 days: 5, 6 and males treated with 50 ng/L EE2 for 4 days: 7-8, negative control with water substituted for cDNA: 9.

The primer pairs F23 + F333 and F12 + R08 (Anderson et al. 2010) gave the most satisfactory results with bands appearing in all individuals. The primer pair F212 + R435 gave intense bands in both control and 50 ng/L exposed females, however, in males, bands were either weak (seen in male from control) or not present. Vitellogenin gene expression was highest in females, specifically those treated with 50 ng/L. Figure 3.6 highlights the difficulties in detecting differences in gene expression between treatments using qualitative PCR. A second/third band was sometimes observed in male and intersex individuals following amplification with primer pairs F12 + R08 (Figure 3.6) and F212 + R435 (Figure 3.7).
The appearance of a second band was also reported by Anderson (2007) following amplification of vitellogenin using primer pair F12 + R08 in male *S. glomerata*.

### 3.4.2. Sequencing results

Although a published GeneBank sequence is available for *S. glomerata* (GeneBank accession no. EU179754.1) (Anderson et al. 2010), qualitative PCR and cloning of the vitellogenin product was performed to reconfirm the authenticity of the vitellogenin sequence.

#### 3.4.2.1. 250-bp product: partial vitellogenin cRNA fragment

PCR amplification bands which corresponded to a 250 bp product were excised, cloned and sent to AGRF for sequencing (Figure 3.8). The resulting sequence matched 100% to partial fragment of *S. glomerata* vitellogenin mRNA (Anderson et al. 2010) (GeneBank accession no. EU179754.1). The sequence also matched to other bivalve species with 71 and 70% similarity to partial vitellogenin mRNA sequences from *M. edulis* (GeneBank accession no. AY67911.6.1) and *C. gigas* (GeneBank accession no. AB084783.1), respectively.

![Figure 3.7: Gel electrophoresis image of qualitative PCR using Sydney rock oyster, *Saccostrea glomerata*, vitellogenin primers (F212 and R435). From left: male, female, intersex](image-url)
Figure 3.8: Sydney rock oyster, *Saccostrea glomerata*, partial mRNA vitellogenin sequence obtained from PCR amplification using primer pair F212 and R435. Product size is 223 bp. Primers (F212 and R435) are indicated respectively in purple.

3.4.2.2. 600-bp product (second band in males): vitellogenin genomic fragment

It was suspected that the second band appearing in males may correspond to a second isoform of vitellogenin. PCR amplification bands which corresponded to a 600-bp product were excised, cloned and sent to AGRF for sequencing (Figure 3.9). However, all three clones (Figure 3.9) were found to be vitellogenin genomic DNA fragments with 100% matches to a partial fragment *S. glomerata* vitellogenin genomic DNA (later discovered and sequenced through extraction of genomic DNA and PCR amplification) (Section 3.4.4).

Figure 3.9: Sydney rock oyster, *Saccostrea glomerata*, partial mRNA vitellogenin sequence obtained from PCR amplification using primer pair F212 and R435. Product size is 363 bp.

3.4.3. Analysis of the phylogenetic relationship between *S. glomerata* vitellogenin and vitellogenins from other taxa.

The partial vitellogenin nucleotide sequence obtained (Section 3.4.2.1.) was used to create a phylogram using clustalW software which generated an evolutionary tree based on the most closely related vitellogenin sequences within a range of taxa (Larkin et al. 2007) (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Figure 3.10). The software generated the tree by aligning each pair of sequences and then weighting each sequence based on the similarities in base pairs with subtracted penalties for gaps. This information was then used to generate a tree based on the neighbour joining method (Saitou and Nei, 1987). The
The assumed mode of evolutionary change was Brownian motion and branch lengths are proportional to the amount of inferred evolutionary change.

The *S. glomerata* vitellogenin sequence was most related to other bivalve species for which full-length vitellogenin sequences were available, *C. gigas* and *M. edulis*. The next closely related were vitellogenin sequences from honey bees, *Bombus hypocrite* and *Apis mellifera*. Closer relationships were observed between the *S. glomerata* vitellogenin sequence and those of fish, amphibians and nematodes. The accuracy of phylogenetic analysis could be improved by obtaining and comparing a full length cDNA vitellogenin sequence for *S. glomerata*.

**Figure 3.10:** Phylogenetic tree comparing partial vitellogenin mRNA sequence of Sydney rock oyster, *Saccostrea glomerata*, to known full-length vitellogenin sequences from a range of taxa.

### 3.4.4. Partial genomic sequences for *S. glomerata* vitellogenin

Vitellogenin genomic DNA sequence was required in order to design primers for real-time qPCR. For real-time qPCR, it is important that one primer spans an intron-exon boundary to avoid amplification of potential contaminating genomic DNA. To identify intron-exon boundaries, genomic DNA sequences can be compared to the corresponding mRNA sequence. PCR amplification was performed on genomic DNA template using primer pair F212 + R435 which amplified a product (~600 bp) in a male, female and intersex individual.
Differences in band intensity are likely to be due to differences in template concentration rather than differences among sexes.

Figure 3.11: Gel electrophoresis image of qualitative PCR using vitellogenin primers F212 + R435 on genomic DNA extracted from Sydney rock oysters, *Saccostrea glomerata*.

The partial genomic sequence (Figure 3.12) with introns removed (i.e. blue highlighted sequence only) matched 100% to nucleotides 212-392 from the partial mRNA vitellogenin sequence for *S. glomerata* already published (GeneBank accession no. EU179754.1) (Anderson, 2007). Based on this genomic sequence, primers were designed for real-time qPCR.

```
AACCTGGACACAAGGAAAGGAGCTAGACCAAGATAGTTCCATCCCTCAGGAGTTCTGCCTCCAGCTCCCTCGAAACC
AGGATTTCCATGTATAAGGTTATGGACAGTTGATTGGGTGATGAGCTATTGAAATGAAATGAGTCGTTAACCAGTACAAA
TACACAGTGAATAATAACTGATGAATTGGGCAATAAAATAAAAATGATTTTAAATATATATCCCATTTAAGGTT
ATGAGAAAAATTGATTCTCCTAAAATTGCCGTTTAGATAAAGCAGTTAAACAGATACATTACCAACCACCG
CATTACCCTCTCCTTTTTATATATTATATTATACCCCTAGCATTGGGTGGTGGTGGTGAATCTATACAA
AAGTCAGTCCTCAGCACAATCAAGGTAGTGCTGATCCTAATATATATATAACGAAAGTACGACAACACTGC
TCTGGACCCGACCAATGT
```

Figure 3.12: Partial genomic sequence of Sydney rock oyster, *Saccostrea glomerata*, vitellogenin. Regions of exons are indicated in blue and the introns are indicated in yellow.
3.4.5. Vitellogenin gene expression from *Saccostrea glomerata* individuals exposed to EE2 for 4 days and analysed via real-time qPCR

In order to trial the real-time qPCR methodology developed in this Chapter, individuals exposed to a concentration gradient of EE2 for 4 days were analysed for vitellogenin gene expression (Figure 3.13). For females, vitellogenin increased linearly with increasing EE2 exposure ($R^2=0.22$, $p<0.05$). Although significant, the proportion of explained variance was very low suggesting that there is significant variability between the expressions of different individuals. For males, although vitellogenin gene expression increased with increasing EE2 there was no significant relationship observed. Despite great variability among individuals, average vitellogenin gene expression increased with dose for females ($N=4$, $R^2=0.99$, $p<0.05$) and for males ($N=4$, $R^2=0.93$, $p<0.05$)

![Graph showing vitellogenin gene expression](image)

**Figure 3.13**: Comparison of vitellogenin gene expression measured via real-time qPCR in Sydney rock oysters, *Saccostrea glomerata*, following exposure to ethynylestradiol (0, 6.25, 12.5, 25 and 50 ng/L) for 4 days for females ($R^2=0.22$, $p<0.05$) and males. Filled lines indicate a significant relationship. $N=4$, mean ± standard error.
3.5. Discussion

This Chapter describes the development of a real-time qPCR assay for vitellogenin gene expression in the Sydney rock oyster. The real-time qPCR assay was found to be robust and consistent. The standard curves of both the gene of interest (vitellogenin) and the reference gene (16S ribosomal) were found to be close to the ideal standard curve parameters for the $R^2$, slope and reaction efficiency. The primers developed for real-time qPCR were found to be very specific to vitellogenin cDNA with excellent melt curves and no other non-specific products were ever found in melt curves of any sample for either primer set. Previous results using qualitative primers for vitellogenin indicated some concern regarding the presence of potentially contaminating genomic DNA (due to the presence of multiple bands). As part of the development of a real-time qPCR assay, qualitative PCR was run on the genomic DNA to identify regions of the intron-exon boundary. Primers for real-time qPCR were designed so that one primer spanned the intron-exon boundary to eliminate the possibility of real-time qPCR amplifying potential contaminating genomic DNA. Furthermore products were never observed in negative (water) and mRNA controls (RNA template only) indicating that primers did not bind to a) any unspecific products or b) any possible contaminating genomic DNA.

The partial vitellogenin mRNA sequence obtained was compared to known vitellogenins from other vertebrate and invertebrate taxa to create a phylogram. Phylogenetic analysis of gene sequences has proved a useful tool in molecular biology, both to confirm the authenticity of the gene of interest but also to compare similarities of the gene among taxa. As anticipated, the partial vitellogenin sequence, presented in Section 3.4.2.1., displayed greatest homology to other bivalve species, *C. gigas* and *M. edulis*. The phylogram reflected that vitellogenins currently sequenced in bivalve taxa have a large proportion of evolutionary change/difference with other taxa. Matsumoto et al. (2003) sequenced a full length vitellogenin gene from *C. gigas* and found regions of homology with other taxa, this was confined to particular regions of the sequence. Similarities between their vitellogenin sequence in *C. gigas* and vitellogenins from other taxa were confirmed to the N-terminal and central regions of the sequence with similarities to a vitellogenin in *O. mykiss*, (25 and 22% within nucleotides 24-266 and 567-932 respectively) and also to a vitellogenin in *P.*
*japonicus* (28% within nucleotides 559-940). This suggested that a gene for vitellogenin, in *C. gigas* and possibly other bivalve species, possesses unique qualities which may also translate to differences in protein structure and/or functioning. The partial *S. glomerata* vitellogenin sequence presented in this Chapter also corresponds to the N-terminal region. Further research is needed to obtain a full-length cDNA sequence for vitellogenin in *S. glomerata*. This would allow more accurate homology modeling/phylogenetic analysis, thereby providing more insight into the protein structure and function and allowing further assessment of the possibility of additional isoforms of vitellogenin.

Although not directly related to the development of a real-time qPCR assay, when confirming the presence of vitellogenin using qualitative PCR primers for *S. glomerata*, a second (and sometimes third) band was observed in gel electrophoresis. Band(s) did not consistently appear but were only ever observed in male individuals. It was suspected that the appearance of additional bands could be due to a second isoform for the vitellogenin gene. This was also reported by Anderson (2007) who observed a second band in male individuals yet reported that the sequence results were of insufficient quality. However, sequence results of the second band (which appeared in males) in this study were found to be fragments of genomic vitellogenin DNA (and matched the genomic vitellogenin sequence which was determined via PCR on genomic DNA, followed by excision, cloning and sequencing of the PCR product). While no bands were ever found in the negative (water) or mRNA (RNA template only) controls, which suggested that genomic DNA contamination of samples had not occurred, sequencing results of the second band product in males provided evidence of genomic contamination. Further experimentation is required to determine if additional vitellogenin isoforms exist in *S. glomerata*.

The fact that the additional bands are only observed in males may be attributed to the fact that, in these individuals, the level of expressed vitellogenin gene is likely to be low. Anderson (2007) suggested that the non-target cDNA sequence may be competing with the specific vitellogenin cDNA sequence for primers. In individuals with high vitellogenin expression the vitellogenin-specific primers preferentially bind to the vitellogenin sequence but when there is little or no vitellogenin sequence they may bind to a non-specific
sequence instead. These additional bands were only seen with certain primer sets (F212 and R435, F12 and R08). These findings may indicate that a) primer sets (F212 and R435, F12 and R08) preferentially bind to the vitellogenin sequence, but in individuals with low vitellogenin expression (such as males) they may also bind to potential contaminating DNA b) it is possible that genomic contamination has occurred following excision of the PCR product (second band in males). To truly determine if additional vitellogenin isoforms are present in *S. glomerata*, further research is required by a) repetition of the qualitative PCR performed in this Chapter with more replication of males with high versus low expression to determine if this only occurs in individuals with low expression, again followed by sequencing of the second band which appears in males, or b) exploration of additional vitellogenin isoforms in different invertebrate species and the design of degenerate primers which would potentially match to an unknown *S. glomerata* vitellogenin isoform (although this process is quite difficult unless sequence information is available for closely related species).

It is well known that multiple isoforms of both vitellogenin gene and protein exist in other taxa; recent studies in fish species have described the presence of multiple genes coding for two or more vitellogenin proteins. Distinct functional genes for vitellogenin A and vitellogenin B have been described in vertebrate species, *P. promelas* (Parks et al. 1999; Miracle et al. 2006), *A. transmontanus* (Bidwell and Carson, 1995), *M. americana* (Hiramatsu et al. 2002), *F. heteroclitus* (LaFleur et al. 1995), *O. mykiss* (Mouchel et al. 1996), and in *D. rerio* (Tong et al. 2004). These two vitellogenin isoforms have been shown to be similar in terms of their nucleotide/translated amino acid structure and the analysis of the amino acid sequence has revealed that both isoforms of the protein possess two lipovitellin domains and one phosvitin domain. Matsubara et al. (1999) and Hiramatsu et al. (2002) proposed that while these forms of vitellogenin are structurally similar they may have distinct and slightly different roles in gametogenesis/reproduction. The physiological difference between these vitellogenins has been proposed to correspond to the stage of ovarian/embryonic development. Vitellogenin A is proposed to degrade easier, provide diffusible nutrients and amino acids for the developing embryo and buoyancy for the oocytes while vitellogenin B may be more resistant to degradation and utilized in the later
stages of embryogenesis. A smaller third vitellogenin C protein has also been proposed which is smaller and different in terms of nucleotide homology and structure (lacking or shortened phosvitin domain) to vitellogenin A and B (Hiramatsu et al. 2002, Wang et al. 2000).

Although no conclusive evidence was presented in this thesis, the possibility of additional vitellogenin isoforms in molluscan taxa should be an important consideration for future studies of bivalve vitellogenesis. Physiological differences, such as rate of protein degradation, may highly affect the usage of vitellogenin as a biomarker with potential differences in induction and temporal maintenance between isoforms. In the brackish copepod, Paracyclopina nana, two genes for vitellogenin have been sequenced for the development of real-time qPCR assays (Dae-Sik Hwang et al. 2010). Both vitellogenins had similar induction patterns during a gonadal development cycle and also responded similarly to heavy metal exposure (0.1 mg/L Cd, 0.4 mg/L Cu, and 2 mg/L AsIII). However vitellogenin B displayed much higher transcript response, especially following heavy metal exposure (0.1 mg/L Cd, 0.4 mg/L Cu, and 2 mg/L AsIII). Three gene isoforms of vitellogenin have also been identified in the mosquito, Aedes aegypti (Isoe and Hagedorn, 2007) with vitellogenin A and vitellogenin C shown to have higher levels of gene expression following a blood meal. These results demonstrate that in other invertebrate species there are different isoforms of the vitellogenin gene which may vary in response or level of induction.

Regardless, the main aim of this Chapter was to develop a real-time qPCR assay as a biomarker of estrogenic exposure in S. glomerata. The real-time qPCR assay was trialed using individuals which were exposed to a concentration gradient of EE2. Female individuals exposed to a concentration gradient of EE2 for 4 days exhibited increased vitellogenin gene expression in a linear dose-dependent fashion; however this was not evident in males. It was suspected that the lack of significance in males was due to lack of replication and large variability among individuals as opposed to a lack of response as, although not as pronounced as females, vitellogenin expression did increase with EE2 exposure. Further, when regressions were performed on average values, thereby
minimizing the variability among replicates, significant increases in vitellogenin gene expression with EE2 exposure were observed for both males and females. Regardless, the induction of any level of male vitellogenin gene induction was novel and provides indication of endocrine disruption. Vitellogenin protein induction was concurrently measured in the same individuals via HPLC analysis (Andrew et al. 2010). In females, vitellogenin gene expression appears to be more sensitive in comparison to the HPLC measurement of protein levels. In the highest EE2 treatment (50 ng/L) female vitellogenin gene expression was five times that of the control response, in comparison to the protein levels which were only twice the control. Whereas in males, the level of response was similar in both assays with approximately three times the level of vitellogenin expression/protein found in individuals exposed to 50 ng/L EE2. In males however, a relationship between EE2 and vitellogenin was found for protein analysis ($R^2 = 0.22$, $p < 0.05$, Andrew et al. 2010), but only for gene expression when averages were employed in the analysis suggesting that a higher number of replicates is required. Both assays had high variability in the response, however this was likely to be due to variability between individuals rather than a reflection of the assay. These results suggested that both vitellogenin gene expressions were more sensitive (at least for females) compared to protein analysis and could be a valuable tool for measuring estrogenic compounds.

Based on fish literature and these findings, vitellogenin gene expression may be likely to be more sensitive and responsive to changes in exposure concentrations of estrogenic compounds compared to protein measurement, thus gene expression would serve as a valuable analysis when exposure concentrations are low and further in short term studies which consist of an exposure period of hours to days. In contrast, it would be expected that the levels of protein would take longer to decline and as suggested by Scholz et al. (2004) may have the capacity to integrate changing levels of estrogenic compounds over time. However, as protein analysis is more rapid and cost effective, it may be useful to initially screen individuals by HPLC followed by real-time qPCR to provide more information about the temporal (i.e. past or continuing exposure) nature of the exposure. It should also be considered that this work represents a snapshot comparison of vitellogenin protein and gene expression and further characterisation of vitellogenin kinetics is needed temporally
under acute, pulsed and chronic exposures (see Chapter 2). Additionally, based on the research outlined in Chapter 2, it is clear that gonadal development is a key factor in vitellogenesis and thus the assays should also be compared at different stages of gametogenesis.
Chapter 4: Assessment of the estrogencity in sewage effluent from Burwood wastewater treatment plant

4.1. Summary
The issue of estrogenic compounds in the Australian marine environment is concerning due to potential effects on wildlife. Wastewater treatment plants (WWTP) are identified as one of the main sources of estrogenic compounds into the marine environment, however, concentrations of estrogenic compounds in final treated effluent are still unknown for many Australian WWTPs. This study aimed to determine both total estrogenic activity and concentrations of selected estrogenic compounds for an Australian WWTP, Burwood WWTP. Treated effluent samples were collected over 6 weeks (twice weekly), and extraction was performed on both liquid and solids fractions. Estrogenic activity was detected in the liquid fraction of effluent via the Yeast Estrogen Screen (YES®) with an average of 1.05 ± 0.39 ng/L in the solids fraction and 3.43 ± 0.92 ng/L in the liquid fraction. Although natural estrogens were usually below the limit of detection, mean concentrations were detected including 0.34 ± 0.34 ng/L for E1, 0.69 ± 0.49 ng/L for E2 and 3.26 ± 1.99 ng/L for E3 in the liquid fractions and 1.08 ± 1.08 ng/L for E1, 0.03 ± 0.03 ng/L for E2 and 0.57 ± 0.41 ng/L for E3 in the solids fractions. Mean concentrations of 0.56 ± 0.56 ng/L were detected in the liquid fraction only for 17α-Ethynylestradiol (EE2), which was below the limit of detection in all but one of the effluent samples. Weakly estrogenic compounds, 4-tert-octylphenol (OP) and 4-nonylphenol (NP), were sometimes present with mean respective concentrations 0.98 ± 0.73 ng/L for OP and 1.63 ± 0.92 ng/L for NP in the solids fraction and 4.95 ± 1.37 ng/L for OP and 5.88 ± 2.61 ng/L for NP in the liquid fraction. Bisphenol A (BPA) was consistently detected in sewage effluent with mean concentrations of 61.73 ± 7.66 ng/L in the liquid fraction and 2.47 ± 0.97 ng/L in the solids fraction. Estrogenic equivalence factors (EEF) were used to calculate a theoretical EEQ which accounted for 44% of the estrogenic activity observed via the YES® assay, suggesting that other undetected estrogenic compounds may have been present or that some of the
measured estrogenic compounds were below the limit of detection in particular samples. Together, these findings complement existing studies of estrogenic compounds in effluent of Australian WWTPs.

4.2. Introduction:

Endocrine disrupting compounds are those which interact with the endocrine system of organisms altering reproductive processes. Estrogenic compounds form a portion of the list of endocrine disrupting compounds, due to their potential to mimic endogenous estrogens. This may include naturally occurring estrogens, such as estrone (E1), 17β-estradiol (E2), estriol (E3) and phytoestrogens such as coumestrol, β-sitosterol and genistein (Langston et al. 2005). Other estrogenic compounds are xeno-estrogens, which are synthetically created but due to chemical structural similarities to E2, may bind to the estrogen receptor and exert varying degrees of estrogenic action. Some examples of xeno-estrogens are pharmaceuticals such as 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES) which are specifically designed to target the endocrine system and are highly estrogenic. Other xeno-estrogens are designed for industrial uses but have been found to have unintended estrogen action, albeit lower (by orders of magnitude) in comparison to E2. For example, alkylphenol polyethoxylates, are produced worldwide for the production of emulsifiers, detergents, wetting agents and dispersing agents. Breakdown products of alkylphenol polyethoxylates, such as 4-nonylphenol (NP) and 4-tert-octylphenol (OP), have been found to exhibit weak estrogenic action (Langston et al. 2005). Bisphenol A (BPA) is another weakly estrogenic compound which is commonly used in the production of polycarbonate plastics and epoxy resins (Okada et al. 2008). Also identified to have some degree of estrogenic action, albeit very low, are pesticides (for example, atrazine, dieldrin, DDT, endosulfan, heptachlor, lindane, methoxychlor), biocides (pentachlorophenol), plasticisers (phthalates), polychlorinated biphenyls (PCBs which are used in oils, paints and lubricants), food preservatives (butylated hydroxyanisole), metalloestrogens and parabens (used in lotions) (Lintelmann et al. 2003). However the main compounds of concern appear to be E1, E2, E3 and EE2 (due to their high estrogenic activity), OP, NP and BPA (due to their high concentrations and persistence in the environment).
One major source of release estrogenic contaminants into the environment is via release of effluent from wastewater treatment plants (WWTP). While treatment is designed to degrade and remove estrogenic compounds, removal is not always 100% efficient. Removal of estrogenic compounds during primary treatment is mainly dependent on absorption to particulates and the efficiency of the WWTP at removing particulates via a screening process or biodegradation. Wastewater treatment plants employing only primary treatment appear to be far less efficient at removing estrogenic compounds compared to secondary and/or tertiary treatment processes. In comparison, WWTPs employing secondary treatment may be more efficient and it is proposed that the majority of removal occurs due to aerobic microbial transformation and biodegradation (Pickering and Sumpter, 2003). In addition, removal of estrogenic compounds may be further enhanced via additional advanced tertiary technologies including ozonation, membrane filtration and activated carbon absorption.

In terms of concentrations of estrogenic compounds in treated sewage effluent, available studies indicate that, internationally and within Australia, estrogens (E1, E2, E3 and EE2) are likely to be found within the low ng/L range while xeno-estrogens, such as NP and BPA, can be found at higher concentrations, sometimes within the μg/L range (international concentrations are reviewed in detail in Chapter 1). Although effluent from Australian WWTPs has not extensively studied, available studies indicate that to date, concentrations measured in Australian effluent are usually lower in comparison to what has been detected in international studies. Estrogens have been measured within the range of 0.1-42 ng/L for E1 (Tan et al. 2007), 0.1-14 ng/L for E2 (Ying et al. 2008, Braga et al. 2005), 1-1.38 ng/L for EE2 (Ying et al. 2008) and <0.1 ng/L for E3 (Tan et al. 2007). Xeno-estrogens have been found at 5.48-148 ng/L for BPA (Al-Rafai et al. 2007, Ying et al. 2008), 0.03-2991 ng/L for NP (Al-Rafai et al. 2007, Ying et al. 2008) and 5.4-66 ng/L for OP (Tan et al. 2007, Ying et al. 2008).

In addition to measuring individual estrogenic compounds in sewage effluent, researchers have also sought to measure total estrogenic activity. Measurement of total estrogenic activity may be preferable in comparison to measurement of individual estrogenic
compounds, especially in scenarios where individual compounds may be unknown, as it provides an integrative measurement. Estrogenic activity is measured in cell reporter assays via binding to an estrogen receptor, a single value is reported and expressed as E2 equivalents, EEQ. Leusch et al. (2006) measured EEQ in effluent from WWTPs in Australia and New Zealand and found concentrations ranged from <1-4.2 ng/L. Tan et al. (2007) examined five WWTPs across South QLD and found that EEQ ranged from 1-14.8 ng/L. Comparatively lower EEQ concentrations were found by Williams et al. (2007). They found that the highest EEQ measured in effluent samples from WWTPs across QLD and NSW was 1.9 ng/L. Mispagel et al. (2009) found that EEQ concentrations in effluent from 12 WWTPs across SA ranged from 0-44.5 ng/L. The available literature on both estrogenic activity (EEQ) and individual estrogenic compounds indicates that estrogenic compounds are present in sewage effluent from Australian WWTPs resulting in their release into the aquatic environment.

The removal efficiency of an WWTP can be calculated via measurement of estrogenic compounds and/or activity of influent and effluent. In terms of removal of specific estrogenic compounds, Australian studies have found that efficiency of treatment process is sometimes low and variable between different WWTPs and likely dependent on the type of treatment and amount being processed. Tertiary treatment appears to be much more efficient at removing estrogenic compounds from sewage effluent. For example, advanced tertiary treatments in WWTPs in Queensland, Australia, were able to remove over >95 % of estrogenic activity (measured via reporter gene assays in estrogenic equivalents, EEQ) during the treatment process (Leusch et al. 2005, Tan et al. 2007). Ying et al. (2008) measured concentrations of NP, E1, E2 and EE2 in four WWTPs in South Australia and found efficiency ranged from 64-92 % for NP, 0.87-63 % for E1, 47-68 % for E2 and 0.77-72 % for EE2. They reported lower efficiencies for a treatment plant operating on three bioreactors and a plant with 10 lagoons in series compared to the two other plants (one with activated sludge treatment and the other with two oxidation ditches) (Ying et al. 2008). While Braga et al. (2005) found very low efficiencies of removal at a WWTP operating on enhanced primary treatment for E1 and E2, reporting 7 and 0 % reduction (respectively) between influent and final treated effluent. In another study, of five South Australian
WWTPs (all employing secondary treatment), Tan et al. (2007) found 85-99 % removal of NP, 38-99 % for BPA and >99 % for E1 and E2. Taken together, these findings suggest that efficiencies of removal for estrogenic compounds in Australian WWTP’s are sometimes incomplete indicating potential concern for release into the aquatic environment.

The availability of suitable assays to detect estrogenic compounds coupled with some evidence of estrogenic compounds in Australian effluent has sparked concern initiating further monitoring within Australian WWTPs. Burwood WWTP employs secondary treatment for sewage effluent in the Newcastle region. Little is known regarding the presence of estrogenic compounds in Burwood WWTP effluent, except for Roberts et al. (2007) who found that xeno-estrogens, NP and OP, were not detected in sewage effluent (<LOD, 0.005 mg/kg) from Burwood WWTP. This may be partially attributed to the extraction procedure which was not optimal for the extraction of estrogenic compounds. The lack of knowledge of estrogenic compounds in Burwood WWTP effluent suggests that further monitoring is required which encompasses the measurement of total estrogenic activity and a broader range of estrogenic compounds which includes xeno-estrogens and natural estrogens.

Thus, the main aim of this study was to determine if estrogenic activity was present in sewage effluent discharged from Burwood WWTP in order to establish a suitable impact location for further biomarker studies (Chapter 5). The first aim was to characterize which estrogenic compounds were potentially present in the sewage effluent via GCMS analysis for 7 commonly detected estrogenic compounds. Compounds were selected based on a literature review of their level of estrogenic activity or their persistence and/or likelihood in the environment. The second aim was to assess the total estrogenic activity of the effluent using a validated gene reporter assay, YES®.
4.3. Methods

4.3.1. Burwood WWTP

Burwood WWTP is the major treatment plant for a population of approximately 180,000 people in Newcastle and surrounding suburbs (Figure 4.1). The output of treated effluent is ~44 ML per day. The secondary treatment process consists of physical screening to remove large and fine particulates, biological filtration and activated sludge processing including aeration and settling stages.

![Aerial photograph of Burwood WWTP](https://example.com/burwood_wwtp.jpg)  

**Figure 4.1:** Aerial photograph of Burwood WWTP (© Hunter Water Corporation, 2010)

4.3.2. Effluent collection

One litre of final treated effluent from Burwood Waste Treatment Plant was collected by Hunter Water Staff twice weekly (Monday and Friday) throughout the duration of the experiment (6 weeks). Collection of effluent coincided with the deployment of oysters in waters receiving effluent in order to establish the potential estrogenic exposure during oyster deployment. In total 11 effluent samples were taken. One effluent sample was not collected (5th January, 2009) due to sampling error and thus could not be included in the analysis. Effluent samples were stored in 2.5 litre brown solvent bottles which were triple rinsed and acid wash soaked prior to collection. Following collection Hydrochloric (HCL) acid was added to the effluent until the pH was adjusted to below 2, this prevents bacterial breakdown of estrogenic compounds (Tyler, 2010). Furthermore, effluent samples were extracted within 2 days of collection to avoid degradation of estrogenic compounds.
4.3.3. Effluent Extraction

A two step extraction procedure was performed on effluent samples with the focus of extracting estrogenic compounds from both the liquid and solids fractions of the sample. Others have reported that the majority of estrogenic compounds are dissolved within the liquid fraction of effluent samples (Desbrow et al. 1998, Huang and Sedlak, 2001) and the majority of studies analyse the liquid fraction of effluent samples only. In comparison, Braga et al. (2005) found that 43 % of E2, 24 % of E1 and 100 % of EE2 was associated with the solids fraction of effluent. Thus, both liquid and solids and fractions were considered for analysis.

4.3.3.1. Step one: extraction from solids

One litre of effluent was filtered through a 0.7 μm glass fiber filter (Millipore) under vacuum. Depending on the consistency of the effluent 2-5 filters were used per litre of effluent. Filtrate was collected and temporarily (10 minutes) stored in a brown Schott bottle at 4 ºC. The filters were then divided into smaller pieces, wrapped in aluminum foil and placed into a freeze dryer overnight. The following day, estrogenic compounds were extracted from the glass fibre filters via soxhlet extraction. To clean apparatus, glass wool was placed in the main chamber and apparatus was rinsed using 100 mL of Dichloromethane (DCM) which was heated to 50 ºC for 30 minutes (5 cycles). Dichloromethane was then discarded and replaced with 100 mL of fresh DCM. Glass fiber filters containing the solids component of the effluent sample were removed from the freeze-dryer; the filters from one sample were placed in the main chamber of the Soxhlet apparatus underneath the rinsed glass wool. Dichloromethane was heated to 50 ºC for 1 hour (10 cycles). DCM containing extracted estrogenic compounds was transferred to a 200 mL bulb and reduced to 2 mL by rotary evaporation, then transferred into a 5 mL glass centrifuge tube. Soxhlet apparatus was rinsed by adding 100 mL of fresh DCM to collect estrogenic residues, which was then also reduced by rotary evaporation and combined with original fraction in the 5 mL glass centrifuge tube. Extracted sample in DCM was then temporarily stored in DCM at 4 ºC. Prior to analysis the sample was made to 10 mL in a volumetric flask.
4.3.3.2. Step two: extraction from liquids

Extraction from effluent liquid filtrate was performed using 47 mm Empore C18 Disks (3M), according to the manufacturer’s guidelines. Prior to extraction, Empore C18 disks and acid-washed glassware were washed with 10 mL DCM. The disks were dried using vacuum for 1 minute. Empore disks were conditioned by applying methanol (10 mL, which was allowed to saturate disk for 30 seconds before vacuum), followed by 10 mL milli-Q water. Effluent samples were then filtered through the disk under vacuum. Each disk was dried overnight in a freeze dryer prior to elution of estrogenic compounds. Estrogenic compounds were eluted with two 30 mL DCM aliquots which were collected into a 100 mL glass bulb, reduced to 2 mL under rotary evaporation and temporarily stored at 4 ºC. Prior to analysis the sample was made to 10 mL in a volumetric flask.

4.3.4. Effluent spike series

In order to test the recovery of the extraction procedure a series of spikes were performed in 1L of milli-Q water. The multi-component mixtures contained ether 5 or 50 ng/L of each of the estrogenic compounds, E1, E2, EE2, E3, NP, OP and BPA and were used to spike effluent samples (N= 5). These spiked effluent samples were extracted through identical procedures and also stored in 10 mL of DCM.

4.3.5. GC-MS analysis

The analysis of estrogenic compounds via GCMS was carried out at NSW Industry and Investment, Wollongbar Agricultural Institute with the assistance of Tony Tyler.

4.3.5.1. Derivatisation

One mL (i.e. 10 % of extract) of extracted samples was transferred to a 1.5 mL autosampler vial and reduced to dryness under a stream of N₂. Dried samples were derivatised in 100 μL bis-(Trimethylsilyl) trifluoroacetamide (BSTFA) with 1 % Trimethylchlorosilane (TMCS) and 100 μL Pyridine. Structurally similar estrogen analogs have been found to derivatise to form the same derivative, thus making identification of the parent compound impossible. The addition of pyridine has been found to protect the Ethynyl triple bond in EE2 and the
carboxyl group of E1 to allow derivatisation of two distinct derivatives (Zhang et al. 2003, Tyler, 2010).

4.3.5.2. GCMS conditions

The run conditions for GCMS analysis were developed and optimized by Tyler (2010) in the development of a GCMS assay to analyse E1, E2, E3 and EE2 in sewage effluent. Analysis of samples was accomplished on an automated Agilent 6890 GC/MS in EI (SIM) mode controlled by Chemstation software. The instrument consisted of modules: 5873N Mass Spectrometer with an Agilent SS Source and 30 M J & W DB5-MS column. The carrier gas was helium set at constant flow of 1 mL/minute. The temperature profile consisted of 80 °C, which is then ramped to 180 °C at 20 °C/minute, held for 1 minute, then ramped to 280 °C at 20 °C/minute and held for 18 minutes. The total run time per sample was 30 minutes. Linear calibration curves were generated through injecting increasing concentrations of each of the estrogenic compounds. Analysis of the calibration curve was based on a linear regression analysis. All calibration curves were satisfactory, generating a $R^2$ of > 0.97 (Figure 4.2).
Figure 4.2: Calibration curves for concentration range of 0, 2, 5, 8 and 10 μg/L of 4-tert octylphenol, 4-nonylphenol, bisphenol A, estrone, 17β-estradiol, 17α-ethynylestradiol and estriol. Abundance represents the relative abundance of selected monitored ions.
The retention time and comparison of relative abundances for each estrogenic compound (pure mixed standard, 10 µg/L of each compound) can be seen in Figure 4.3.

![Figure 4.3: Selected Ion Monitoring (SIM) chromatogram of 4-tert octylphenol, 4-nonylphenol, bisphenol A, estrone, 17β-estradiol, 17α-ethynylestradiol and estriol mixed standard (10 µg/L of each compound)](image)

With the exception of NP and OP, the first extraction step (soxhlet extraction of the solids) was inefficient and variable at extracting the estrogenic compounds compared to the second step (empore disk extraction of liquid filtrate) which suggests that, especially for the natural estrogens and EE2, the majority of the estrogenic compounds were dissolved in the liquid fraction (Table 4.1). Poor recoveries have been reported for estrogens, particularly for E3, with C18 solids fraction extraction procedures including SPE cartridges (Ingrand et al. 2003) and Biobeads (Li et al. 2007). Li et al. (2007) suggested that this could be attributed to the high polarity (logarithm of octanol-water co-efficient LogP<sub>ow</sub> of 2.94, compared to 4.13 for E2) which, compared to other estrogenic compounds, is likely to contribute to a poor binding capacity for the C18 disk. Ingrand et al. (2003) also suggested that elution of
E3 may be achieved with a more polar solvent; however, this is likely to also elute other interfering compounds causing difficulties in analysis. Furthermore, in Australian studies, E3 is often excluded from analyses of estrogenic compounds with the exception of Tan et al. (2007).

For the phenolic compounds, NP and OP, the % recovery from the extracted solids samples was closer to 50% of the total recovery. This suggests that these compounds may bind to particulates in the effluent. Parameters of the retention time, target ions, reference ions and recoveries for both liquid and solids extractions are presented below (Table 4.1).

**Table 4.1:** Summary of the GC-MS parameters for selected estrogenic compounds including the retention times, reference ions and recovery for each estrogenic compound

<table>
<thead>
<tr>
<th>Estrogenic Compound</th>
<th>Retention time</th>
<th>Target ion (m/z)</th>
<th>Reference ion (m/z)</th>
<th>Average % recovery (solids)</th>
<th>Empore disk recovery (liquids)</th>
<th>Average % combined recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-tert-octyphenol</td>
<td>8.62</td>
<td>179.2</td>
<td>278.3</td>
<td>53.19</td>
<td>65.91</td>
<td>119.09</td>
</tr>
<tr>
<td>4-n-nonylphenol</td>
<td>9.27</td>
<td>178.98</td>
<td>292.1</td>
<td>58.21</td>
<td>63.78</td>
<td>121.98</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>10.89</td>
<td>357.3</td>
<td>372.3</td>
<td>25.64</td>
<td>45.96</td>
<td>71.59</td>
</tr>
<tr>
<td>Estrone</td>
<td>12.93</td>
<td>342.08</td>
<td>257.01</td>
<td>0</td>
<td>42.46</td>
<td>42.46</td>
</tr>
<tr>
<td>17β- Estradiol</td>
<td>13.16</td>
<td>416.11</td>
<td>285.06</td>
<td>21.06</td>
<td>22.24</td>
<td>43.29</td>
</tr>
<tr>
<td>17α-Ethynylestradiol</td>
<td>13.88</td>
<td>425.1</td>
<td>285.06</td>
<td>0</td>
<td>27.95</td>
<td>27.95</td>
</tr>
<tr>
<td>Estriol</td>
<td>14.58</td>
<td>504.12</td>
<td>311.06</td>
<td>0</td>
<td>43.85</td>
<td>43.85</td>
</tr>
</tbody>
</table>

**4.3.6. YES®**

Effluent and oyster samples were extracted as described above and 1 mL of extract (1:10 dilution) was taken for analysis then gently reduced to dryness under a stream of N2. Analyses of extracted samples via YES® analyses were conducted by Dr Anu Kumar and Dr. Hai Doan at CSIRO Land and Water, Glen Osmond, South Australia. Estrogenic activity of effluent extracts collected from Burwood WWTP was analysed via the YES® assay. The YES® assay was originally developed and described by Routledge and Sumpter.
(1996) and has been widely applied for the measurement of estrogenic activity. Briefly, the assay uses recombinant yeast cells which have been modified via insertion of the human estrogen receptor (ER) to express a reporter gene *laz-Z* (which encodes the enzyme β-galactosidase) upon exposure to estrogenically active compounds.

As described by Routledge and Sumpter (1996), preparation of yeast cells was performed via incubation in growth medium (5 mL glucose solution, 1.25 mL L-aspartic acid solution, 500 μL vitamin solution, 400 μL L-threonine solution and 125 μL copper (II) sulfate), which was added to 45 mL minimal medium (13.61 g KH2PO4, 1.98 g (NH4)2SO4, 4.2 g KOH pellets, 0.2 g MgSO4, 1 ml Fe2 (SO4)3 solution (40 mg/50 ml H2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine- HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 Litre of milli-q water. Then, 125 μL of yeast stock (10X concentrated) was added. Mixture was incubated at 28 ºC for 24 hours on an orbital shaker.

To create a standard curve, E2 was diluted in absolute ethanol (1/20 dilution range within 2724 - 1.3 ng/L) and added to a 96- well microtitre plate in 10 μL aliquots. Aliquots of 10 μL ethanol were added to blank wells and effluent test sample (s) to the sample wells. Ethanol was then allowed to evaporate. Assay medium was prepared by adding 0.5 mL CPRG to 50 mL fresh growth medium. Medium was seeded with 4x 10^7 yeast cells (usually between 0.5 mL and 2 mL) from the 24-hour culture previously prepared. Seeded assay medium was then added (200 μL) to wells using a multichannel pipette. Plates were sealed and shaken for 2 minutes on a plate shaker, then incubated overnight at 32°C. On the second day, plates were again shaken for 2 minutes on a plate shaker, then incubated for a further 48 hours at 32°C. At the end of the 72 hour incubation period, plates were removed and read at an absorbance of 540 nm (optimum absorbance for CPRG ~575 nm) and 620 nm (for turbidity) using a plate reader. Sample EEQs were determined by calculating the response of the effluent sample relative to the E2 standard curve.
4.4. Results

4.4.1. GCMS analyses: concentrations of estrogenic compounds in sewage effluent

It was anticipated that estrogenic compounds would be frequently detected in sewage effluent collected over the experimental period from Burwood WWTP. To address this hypothesis, GCMS analysis was performed on the effluent extract for a selected 7 estrogenic compounds and it was found most estrogenic compounds analysed were present in sewage effluent. It was found that the weakly estrogenic compound, BPA, was detected at high frequency (in every sample) at an average of 61.73 ± 7.66 ng/L in the liquid fraction and 2.47 ± 0.97 ng/L in the solids fraction (Figure 4.4). Alkyphenols, OP and NP, were also detected at high frequency (present in 70% and 63% of samples, respectively) average concentrations in the solids fraction of 0.98 ± 0.73 ng/L for OP and 1.63 ± 0.92 ng/L for NP and average concentrations in the liquid fraction of 4.95 ± 1.37 ng/L for OP and 5.88 ± 2.61 ng/L for NP (Figure 4.4).
Figure 4.4: Concentrations of selected estrogenic compounds, 4-tert-octyphenol, 4- nonylphenol and bisphenol A extracted from sewage effluent, collected from Burwood wastewater treatment plant twice weekly over the 6 week period of Sydney rock oyster, *Saccostrea glomerata*, deployment and analysed via gas chromatography mass spectrometry. N=11. Each data point represents one effluent sample. N/A= sample unavailable on 9/1/2009.

Steroid estrogens were detected at a lower frequency and at lower concentrations. Mean concentrations of 0.34 ± 0.34 ng/L for E1, 0.69 ± 0.49 ng/L for E2 and 3.26 ± 1.99 ng/L for
E3 were detected in the liquid fractions and 1.08 ± 1.08 ng/L for E1, 0.03 ± 0.03 ng/L for E2 and 0.57 ± 0.41 ng/L for E3 in the solids fractions (Figure 4.5). In comparison, EE2 was only detected in one sample in the sampling period with a mean concentration of 0.56 ± 0.56 ng/L in the liquid fraction only (Figure 4.5).

There did not appear to be any direct relationships between the presence and frequency of different estrogenic compounds. For example, it was anticipated that alkyphenols, NP and OP which arise from the same source, would occur at the same time with similar concentrations. However, this was varied across the experimental period. Higher concentrations and frequency of estrogenic compounds were always detected in the liquid fraction of the effluent samples compared with that of the solids fraction.
Figure 4.5: Concentrations of selected estrogenic compounds, estrone, 17β-estradiol, 17α-ethynylestradiol and estriol, extracted from sewage effluent, collected from Burwood wastewater treatment plant twice weekly over the 6 week period of Sydney rock oyster, *Saccostrea glomerata*, deployment and analysed via gas chromatography mass spectrometry. N=11. Each data point represents one effluent sample. N/A= sample unavailable on 9/1/2009.
4.4.2. YES® analysis: effluent

It was hypothesised that effluent from Burwood WWTP would contain estrogenic activity, measured via YES®. Effluent samples were found to contain estrogenic activity/EEQ with an average concentration of $1.05 \pm 0.39$ ng/L in the solids fraction and $3.43 \pm 0.92$ ng/L in the liquid fraction (Figure 4.6). The highest EEQ results (combined liquid and solids total of 8.8, 8.9 and 9.7 ng/L) occurred in the effluent samples during the first and second week of deployment (12th and 15th December 2008, respectively) which co-incided with a period of partial sewage effluent treatment at Burwood WWTP (due to mechanical breakdown). Similar to measurements of individual estrogenic compounds, higher concentrations of estrogenic activity were mostly detected in the liquid fraction of the effluent samples.

![Figure 4.6: Concentrations of EEQ (estradiol equivalent) from extracted sewage effluent samples collected from Burwood wastewater treatment plant twice weekly over the 6 week period of Sydney rock oyster, *Saccostrea glomerata*, deployment and analysed via Yeast Estrogen Screen. Each data point represents one effluent sample. N/A= sample unavailable on 9/1/2009.](/path/to/figure.jpg)
4.4.3. Comparison of YES® to GCMS analyses of effluent

The aim of this Section was to determine if estrogenic compounds, measured via GCMS, accounted for estrogenic activity in effluent samples measured via the YES® assay (liquid and solids fractions combined). It was hypothesised that the 7 estrogenic compounds measured via GCMS would account for a high proportion of estrogenic activity in the sewage effluent. Estrogenic equivalence factors (EEF) have been calculated for individual estrogenic compounds (Legler et al. 2002; Beck et al. 2006) and concentrations of E1, E2, NP, OP and BPA measured via GCMS were multiplied by EEF factors to obtain a theoretical EEQ. Estrogenic equivalence factors used were 1.4 x 10\(^{-6}\) for OP (Legler et al. 2002), 2.3 x 10\(^{-5}\) for NP (Legler et al. 2002), 1.2 x 10\(^{-4}\) for BPA (Beck et al. 2006), 0.25 for E1 (Beck et al. 2006) and 1 for E2. The sum of the calculated EEQ was then compared to the actual EEQ measured via YES® to determine what percentage of EEQ can be accounted using the concentration of estrogenic compounds measured via GCMS analysis (Table 4.2). However it was found that estrogenic compounds measured via GCMS could only account for 44.02 % of the actual EEQ measured via YES® on average, though in particular samples measured estrogenic compounds accounted for up to 99.9% of EEQ via YES®.

**Table 4.2:** Comparison of calculated EEQ (based on EEF factors applied to GCMS result) vs. actual EEQ measured via YES® for each sample analysed. The proportion which is accounted for is indicated on the far right.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calculated EEQ ng/L</th>
<th>Actual EEQ ng/L</th>
<th>% accounted for</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/12/2008</td>
<td>0.005</td>
<td>5.45</td>
<td>0.02</td>
</tr>
<tr>
<td>12/12/2008</td>
<td>8.779</td>
<td>8.79</td>
<td>99.93</td>
</tr>
<tr>
<td>15/12/2008</td>
<td>0.537</td>
<td>8.96</td>
<td>5.99</td>
</tr>
<tr>
<td>19/12/2008</td>
<td>0.748</td>
<td>0.93</td>
<td>80.44</td>
</tr>
<tr>
<td>22/12/2008</td>
<td>8.095</td>
<td>9.71</td>
<td>83.37</td>
</tr>
<tr>
<td>26/12/2008</td>
<td>2.732</td>
<td>4.38</td>
<td>62.38</td>
</tr>
<tr>
<td>29/12/2008</td>
<td>0.001</td>
<td>1.59</td>
<td>0.11</td>
</tr>
<tr>
<td>2/1/2009</td>
<td>0.554</td>
<td>1.78</td>
<td>31.13</td>
</tr>
<tr>
<td>5/1/2009</td>
<td>0.001</td>
<td>1.02</td>
<td>0.13</td>
</tr>
<tr>
<td>12/1/2009</td>
<td>0.002</td>
<td>5.6</td>
<td>0.03</td>
</tr>
<tr>
<td>16/1/2009</td>
<td>0.238</td>
<td>1.06</td>
<td>22.46</td>
</tr>
</tbody>
</table>
4.5. Discussion

The main aim of this Chapter was to determine if sewage effluent from Burwood WWTP contained estrogenic activity. This was firstly assessed via measurements of individual estrogenic compounds in sewage effluent from Burwood WWTP which revealed the presence of several estrogenic compounds in effluent samples. Analysis revealed the frequent presence of Bisphenol A in the sewage effluent with mean concentrations of 61.73 ± 7.66 ng/L in the liquid fraction and 2.47 ± 0.97 ng/L in the solids fraction. Compared to other measurements of BPA in Australian sewage effluent this concentration was high but comparable. Others have detected concentrations of 11.9-86.7 ng/L in treated effluent (Williams et al. 2007; Ying et al. 2009; Tan et al. 2007). High concentrations of BPA in sewage effluent may be attributed to the high domestic and industrial usage of the compounds in production of polycarbonate products, epoxy resins and flame retardants. Leaching from domestic products (for example, plastic bottles, containers, lined aluminum cans, electrical equipment, household appliances and computers), as well as from industrial products (for example, flooring, adhesives, powder coatings, can coatings, circuit boards and automotive primers) may contribute to BPA levels in sewage effluent and subsequent release into the environment (Staples et al. 1998a).

Analysis also revealed the presence of estrogens and EE2 in the sewage effluent with average concentrations over the sampling period of 0.34 ± 0.34 ng/L for E1, 0.69 ± 0.49 ng/L for E2 and 3.26 ± 1.99 ng/L for E3 in the liquid fractions and 1.08 ± 1.08 ng/L for E1, 0.03 ± 0.03 ng/L for E2 and 0.57 ± 0.41 ng/L for E3 in the solids fractions. Xenoestrogens, OP and NP had mean respective concentrations 0.98 ± 0.73 ng/L for OP and 1.63 ± 0.92 ng/L for NP in the solids fraction and 4.95 ± 1.37 ng/L for OP and 5.88 ± 2.61 ng/L for NP in the liquid fraction. Both compounds are considered to be weakly estrogenic (Legler et al. 1999; Legler et al. 2002); however their presence in the aquatic environment is often a concern due to both their persistence and high concentrations (in the μg/L range). Concentrations for E1, E2, EE2, E3, NP and OP were similar to those detected by others in sewage effluent from Australian WWTPs (Williams et al. 2007, Tan et al. 2007, Ying et al. 2009). Together, these findings provide confirmation that estrogenic compounds were
present in sewage effluent from Burwood WWTP during the sampling period. Available Australian studies on concentrations of estrogenic compounds in sewage effluent are compared to the minimum and maximum concentrations measured in international studies in Table 4.3.
Table 4.3: Concentrations of selected estrogenic compounds measured in sewage effluent from Australian and international studies.

<table>
<thead>
<tr>
<th>WWTP location</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>EE2</th>
<th>BPA</th>
<th>NP</th>
<th>OP</th>
<th>EEQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (ng/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solids*</td>
<td>0-11.9 (1.08)</td>
<td>0.027 (0.02)</td>
<td>0-4.12 (0.57)</td>
<td>(0)</td>
<td>0-9.37 (2.46)</td>
<td>0-9 (1.63)</td>
<td>0-7.78 (0.98)</td>
<td>0.11-3.66 (1.05)</td>
</tr>
<tr>
<td>Liquid*</td>
<td>0-3.74 (0.34)</td>
<td>0-5.12 (0.69)</td>
<td>0-21.01 (3.26)</td>
<td>0-6.2 (0.56)</td>
<td>6.16-92.83 (61.73)</td>
<td>0-25.26 (5.88)</td>
<td>0-11.3 (4.95)</td>
<td>0.73-8.7 (3.43)</td>
</tr>
<tr>
<td>Combined*</td>
<td>0-11.9 (1.42)</td>
<td>0-5.12 (0.72)</td>
<td>0-21 (3.84)</td>
<td>0-6.19 (0.56)</td>
<td>6.16-102.19 (64.2)</td>
<td>0-25.26 (7.51)</td>
<td>0-16.38 (5.93)</td>
<td>0.93-9.71 (4.48)</td>
</tr>
<tr>
<td>SA</td>
<td>13.3-39.3 (25.9)</td>
<td>1-4.2 (2.5)</td>
<td>-</td>
<td>0-1.38 (0.48)</td>
<td>12-148 (54.4)</td>
<td>860-2887 (1627.1)</td>
<td>12-66 (36.1)</td>
<td>-</td>
</tr>
<tr>
<td>QLD</td>
<td>&lt;0.1-41.9 (10)</td>
<td>&lt;0.1 (&lt;0.1)</td>
<td>&lt;0.1 (&lt;0.1)</td>
<td>-</td>
<td>11.6-86.7 (31.9)</td>
<td>56.7-335 (139.6)</td>
<td>5.4-23.5 (12.6)</td>
<td>0.2-67.8</td>
</tr>
<tr>
<td>QLD, ACT,</td>
<td>3.1-39.3 (23.9)</td>
<td>0.05-6.3 (3.8)</td>
<td>-</td>
<td>0.01-1.3 (0.45)</td>
<td>(21.5)</td>
<td>514-2991 (1113)</td>
<td>(39.5)</td>
<td>0.05-2.78</td>
</tr>
<tr>
<td>QLD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.75</td>
<td></td>
</tr>
<tr>
<td>NSW</td>
<td>(54)</td>
<td>(14)</td>
<td>-</td>
<td>(&lt; 5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NSW</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(5.48)</td>
<td>(0.03)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NSW</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(23.02)</td>
<td>(1.5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIC</td>
<td>-</td>
<td>1.3-18 (4.3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0-44.5</td>
<td></td>
</tr>
<tr>
<td>VIC</td>
<td>0.1-32</td>
<td>&lt;0.05-18.5</td>
<td>0.05-0.66</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1-18</td>
<td></td>
</tr>
<tr>
<td>International</td>
<td>3-70</td>
<td>1-64</td>
<td>1-8</td>
<td>0.2-42</td>
<td>0-5370</td>
<td>20-180000</td>
<td>120-17000</td>
<td>0.1-151</td>
</tr>
</tbody>
</table>

Mean concentration indicated in brackets and, where available, concentration ranges. Mean concentration and range are sourced from indicated references which measured treated effluent from one or more WWTPs; a= Ying et al. (2008), b= Tan et al. 2007, c= Williams et al. 2007, d= Leusch et al. 2005, e= Braga et al. 2005, f= Al-Rifai et al. 2007, g= Mispagel et al. 2009, h= Allinson et al. 2010, i= Desbrow et al. 1998, j= Ternes et al. 1999, k= Desbrow et al. 1996, l= Routledge et al. 1998, m= Blackburn and Waldock, 1995, n= Lee and Peart 1995, o= Baronti et al. 2000, p= Sánchez-Avila et al. 2009, q= Staples et al. 1998a, r= Matsui et al. 2000, s= Svenson et al. 2003, t= Rutishauser et al. 2004 and u= Tilton et al. 2002. In bold*= this study, values represent extraction of solids, liquid or fractions combined. All other studies represent an extraction from the liquid fraction of effluent only.
Measurement of individual estrogic compounds may not be a comprehensive representation of the estrogic activity of sewage effluent. Therefore total estrogic activity was measured in sewage effluent via YES® to further address the hypothesis that sewage effluent from Burwood WWTP contained estrogic activity over the experimental period. This was confirmed with EEQ ranging from 0.93- 9.71 ng/L (liquid and solids fractions combined. This is similar to EEQ concentrations that have been reported in available Australian studies including <1- 4.2 ng/L (Leusch et al. 2006), 1-14.8 ng/L (Tan et al. 2007) and 0- 1.9 ng/L (Williams et al. 2007) (Table 4.3). High EEQ values were detected during the first two weeks of sampling. It is possible that during this period, partial treatment of effluent (due to a mechanical breakdown in the WWTP) has largely contributed to a decreased efficiency of removal of estrogic compounds. Regardless, these findings have provided further evidence that sewage effluent from Burwood contained estrogic activity during the experimental period.

Concentrations of estrogic compounds (NP, OP, BPA, E2 and E1) were used to calculate EEQ values to determine what proportion of these compounds accounted for the actual concentrations of EEQ measured via YES®. It was hypothesised that estrogic compounds measured via GCMS would account for a high proportion of the estrogic activity measured via YES®. It was found that estrogic compounds measured via GCMS accounted for an average of 44% of the actual EEQ. In general, samples with a higher concentration of EEQ (> 4 ng/L) also had a higher % of estrogic activity accounted for via GCMS analysis. This could be due to the fact that estrogic compounds, not detected frequently in effluent samples via GCMS analysis (such as E1, E2, E3 and EE2), may have been present in the effluent at concentrations below of the limit of quantification but at sufficient concentrations to promote low estrogic activity. It is also possible that EEQ concentrations which are not accounted for with GCMS analysis may be indicative of the presence of other estrogic compounds not measured in this study and this could include compounds such as DES, bisphenol F, phytoestrogens, pesticides or breakdown products of alkylphenol polyethoxylates. A third consideration is that a mixture of estrogic compounds may have a synergistic effect in reporter-gene assays, even if individual compounds are present at concentrations below their limit of quantification (Silva et al.
For example, Silva et al. (2002) has demonstrated that estrogenic effects may be enhanced in mixture. Modelling of a mixture of 8 estrogenic compounds (at 50% concentrations of their No Observed Effect Concentration [NOEC]) demonstrated that the effect of a mixture would produce a significantly greater effect than the sum of their individual effects. Discrepancies between the calculated EEQ and the actual EEQ highlight that it is inappropriate to rely on a single assay for the measurement of estrogenic compounds and demonstrate that valuable insight can be provided from a combined approach.

In conclusion, this Chapter has explored the presence of estrogenic activity and estrogenic compounds in sewage effluent from Burwood WWTP. It was found that sewage effluent from Burwood WWTP contained estrogenic compounds. The xeno-estrogen, BPA, was frequently detected at medium to high concentrations. Steroidal estrogens and EE2 were mostly below the limit of detection, although sometimes present at concentrations of concern. This Chapter involved extraction and analysis of estrogenic compounds from the solids and liquid fraction of final treated sewage effluent samples. Higher proportions of estrogenic compounds were measured in the liquid fraction. However, concentrations of estrogenic compounds detected in the solids fraction suggested that future studies may consider measuring both fractions. Measurement of estrogenic activity via YES® revealed that sewage effluent contained high (but comparable to other Australian studies) EEQ levels, particularly following the period the plant was operating on partial treatment. Comparisons of the EEQ measured via YES® and a theoretical EEQ (based on summation of the E2 equivalents of estrogenic compounds measured via GCMS) may suggest the presence of additional estrogenic compounds or some measured estrogenic compounds were below the limit of detection. Overall, the data has established that effluent entering receiving waters from Burwood WWTP contains estrogenic activity and confirms that the effluent release location will be a suitable estrogenic impact location for further control-impact studies for assessing biological effects of estrogenic contaminants in Chapter 5.
Chapter 5: Biomarkers of estrogenic exposure, vitellogenin and gonadal development, in Sydney rock oysters following 6 weeks deployment in sewage effluent receiving waters

5.1. Summary

Previous findings have suggested that the Sydney rock oyster, *Saccostrea glomerata*, is a useful biomonitor of estrogenic compounds following laboratory exposures. However, assessments of the utility of laboratory validated biomarkers upon field exposures of estrogenic contaminants at environmentally relevant concentrations are lacking. To address this knowledge gap and complete the assessment of Burwood wastewater treatment plant (WWTP) as a source of estrogenic compounds, *S. glomerata* was deployed for 6 weeks in effluent receiving waters (Burwood near, <50 metres and Burwood far, <150 metres from the WWTP outfall) and reference locations (Redhead and Fingal Island) at depths of 4, 8 and 12 metres. Wet condition index was highest in individuals deployed at the Burwood locations and the Fingal Island 1 reference location. Female vitellogenin gene expression was highest in females deployed at 4 metres depth in Burwood locations and significantly greater (p < 0.05) than the majority of reference locations. However, estrogenic exposure was perhaps not sufficient to induce male vitellogenin gene expression which was similar among all locations. Vitellogenin protein was found to increase significantly (p < 0.05) in female individuals deployed at the Burwood far location. There were no significant differences (p > 0.05) in the proportions of sexes and no incidence of intersex individuals, but both Burwood locations had a higher proportion of mature female gonad development stages. Findings suggested that female *S. glomerata* is a suitable biomonitor for field exposures of estrogenic compounds in Australian waters.
5.2. Introduction

The detection of estrogenic compounds in sewage effluent has been considered important due to concern of release into the aquatic environment, with potential effects on aquatic wildlife. In terms of assessment, the development of bio-indicators of estrogenic compounds can be equally important as detection of estrogenic compounds. The presence of estrogenic compounds and/or activity in effluent does not necessarily translate to biological effects in organisms residing or deployed within the receiving waters of sewage effluent. Following release into aquatic environment, and particularly into the open sea, estrogenic compounds may be significantly diluted to concentrations below levels which cause biological effects. On the other hand, biological effects may still be measured even if individual estrogenic compounds are not detectable within sewage effluent. Estrogenic compounds can be environmentally persistent and remain in the environment long after their release with accumulation in sediment (reviewed in detail by Langston et al. 2005, for examples see Blackburn et al. 1999; Lopez de Alda and Barcelo 2001; Lye et al. 1999). Furthermore, it would appear that hydrophobic estrogenic compounds have the potential to accumulate in membranes of organism tissue, thus consistent exposure to low concentrations may not be reflective of contaminants loads in organisms at any one time (Chapter 2; Legler et al. 2002; Lye et al. 1999). Furthermore, in situations where estrogenic compounds are below the limit of detection, there may be synergistic or additive effects when organisms are exposed to a mixture of estrogenic compounds at low concentrations (Silva et al. 2002, example provided in Chapter 4). Therefore, the relationship between concentrations of estrogenic compounds in sewage effluent and effects on biological indicators can be complex. Biological indicators should be used in conjunction with measurement of estrogenic compounds/activity to gain a comprehensive assessment and a true understanding of the biological impacts of estrogenic compounds.

A potential candidate for an Australian biomonitor of estrogenic compounds is *Saccostrea glomerata*. Molluscs have proved useful as monitors due to their sessile nature and filter feeding capacity. Further, it is well established that *S. glomerata* (Avery et al. 1996; Avery et al. 1998) and other molluscan species (O’Connor, 2002; Scanes, 1996) have been useful as biomonitors of other contaminants, such as metals. This thesis and earlier research has
concentrated on laboratory based investigations which demonstrated that *S. glomerata* could be a useful biomonitor of estrogenic compounds. Firstly, estrogenic compounds have been capable of inducing elevated vitellogenin in *S. glomerata*. Females exposed to EE2 (50 ng/L) and NP (100 μg/L) during a gonadal development cycle, exhibited significant increases in vitellogenin up to three-fold and double controls respectively. Significant increases in vitellogenin were also found in males exposed to 50 ng/L EE2 (Andrew et al. 2008). In Chapters 2 and 3, it was determined that *S. glomerata* vitellogenin gene expression and protein production exhibits a dose response relationship with EE2, significantly increasing (p< 0.05) with EE2 exposures (0, 6.25, 12.5, 25 and 50 ng/L). Findings also suggested a role for estrogenic compounds in the sexual differentiation of *S. glomerata*. Exposure to NP (100 μg/L) and EE2 (50ng/L) has induced intersex gametal status for some individuals (Andrew et al. 2008). In a separate experiment, exposure to EE2 for 3- 6 weeks was capable of promoting intersex (at concentrations as low as 6.25 ng/L) but significantly increased (p< 0.05) the proportion of females (at 50 ng/L) (Chapter 2). Thus, it has been demonstrated, via direct laboratory exposure, cause and effect between estrogenic exposure and biological effects in *S. glomerata*. Such findings provide strong evidence that *S. glomerata* may be employed as a successful biomonitor of estrogenic compounds, through harnessing vitellogenin as a biomarker and assessment of gonadal development and gametal sex of exposed individuals. To complete validation of *S. glomerata* as a successful biomonitor, the effects of environmentally realistic concentrations of estrogenic compounds need to be evaluated within a field situation.

A common biomarker of estrogenic exposure is induction of vitellogenin. Increases in alkali labile phosphates (ALP) have been used as a surrogate estimate of vitellogenin to indicate the presence of estrogenic compounds in sewage effluent. Gagné et al. (2001) performed laboratory exposures to sewage effluent and was able to induce significant ALP responses in exposed adult male and female freshwater mussels *Elliptio complanata*, at concentrations 10, 25 and 50 % effluent (v/v). Similarly, Quinn et al. (2004) exposed zebra mussels *Dreissena polymorpha*, to tertiary treated sewage effluent from an Irish WWTP for 112 days during gametogenesis and found that exposed females displayed elevated ALP levels compared to female control individuals. Others have demonstrated increased ALP
levels in molluscan taxa residing or deployed within waters receiving sewage effluent. Wild adult soft shell clams *Mya arenaria*, collected from a location receiving sewage effluent (100 metres from the WWTP) within the Saguenay Fjord, Canada had elevated ALP levels compared to individuals collected from a reference location (Blaise et al. 1999). Similarly, Gagné et al. (2001) deployed adult *E. complanata* 5 km downstream from a WWTP for 62 days and found elevated ALP levels. In Venice canals, wild female and male black mussels *Mytilus galloprovincialis*, were also found to have elevated ALP levels during their season of reproductive development (April) compared to ALP levels in female and male individuals from reference locations (Pampanin et al. 2005). Therefore, the field assessment of vitellogenin (via indirect ALP measurement) in molluscs has been employed as a useful biomarker of potential exposure to estrogenic compounds.

In addition to vitellogenin measurements, changes in male or female development gonadal status have also been indicative of estrogenic exposure in molluscs. This has included increases in proportions of females/intersex, enlarged oocytes, or accelerated female development. Although the majority of evidence has been from direct laboratory exposures to estrogenic compounds there has also been limited evidence from field studies. Langston et al. (2007) suggested that the increased occurrence of intersex (up to 16 % of population) in wild furrow shells *Scrobicularia plana*, within UK locations, was due to the presence of estrogenic compounds released from a nearby WWTP. This study suggested that estrogenic exposure, via sewage effluent, was capable of inducing changes in gonadal status. Further studies examining the effects of sewage effluent on sex determination and gonadal status are clearly required.

Within an Australian context, there has been moderate evidence that suggested estrogenic compounds were present in some wastewater treatment plant (WWTP) effluents (reviewed in Chapter 4, Al-Rifai et al. 2007; Braga et al. 2005; Ying et al. 2008; Tan et al. 2007; Williams et al. 2007). Burwood WWTP has provided secondary treatment for the Newcastle region with the release of treated effluent and bio-solids waste into the ocean. In sewage effluent from Burwood WWTP, estrogenic compounds have not been previously tested, nor have the potential effects on aquatic wildlife. In the first component of this field
study, estrogenic compounds and activity were measured in treated sewage effluent from Burwood WWTP (Chapter 4). Bisphenol A (BPA) was detected at high concentrations (average 65.82 ± 11.53), along with OP and NP (respective averages of 5.15 ± 1.82 and 7.38 ± 3.15) (all combined liquid and solids extraction averages). All detected in effluent were E1 (1.8 ± 1.24 ng/L), E2 (0.82 ± 0.53 ng/L), E3 (3.85 ± 2.13 ng/L) (also combined liquid and solids extraction averages) and EE2 (0.56 ± 0.56 ng/L, only detected in the liquid fraction once). Estrogenic activity, in the form of estradiol equivalents (EEQ), was also detected in sewage effluent ranging from 0.93- 9.71 ng/L (combined liquid and solids extraction). These findings have established that estrogenic compounds are present in sewage effluent and thus provide a suitable impact location to assess *S. glomerata*’s biomonitoring potential for estrogenic contaminants under realistic field-based scenarios.

The aim for this Chapter was to examine whether estrogenic compounds and activity, present in sewage effluent from Burwood STP, were capable of inducing changes in biological indicators of estrogenic exposure and effects in *S. glomerata*. It was hypothesised that *S. glomerata* deployed within receiving waters of Burwood STP would display elevated vitellogenin protein and gene expression compared to reference locations. Furthermore, it was hypothesised that alterations in gonadal development (enlarged oocytes, higher proportions of females, more mature female gonadal development stages and/or intersex individuals) may be evidenced in oysters from Burwood locations compared to reference locations. At Burwood, oysters were deployed at near (within 50 metres from outfall) and far (within 150 metres from outfall) distances from the outfall/diffusers. It was anticipated that there would be a difference in these locations due to different concentrations of effluent exposure. This Chapter will complement previous laboratory findings examining whether *S. glomerata* is a valid biomonitor of estrogenic compounds and provide additional weight of evidence of the presence of estrogenic compounds in Burwood WWTP sewage effluent and potential biological effects.
5.3. Methods  

5.3.1. Oyster deployment  

Sydney rock oysters, *S. glomerata*, approximately 2 years old beginning a phase of gonadal development were deployed in waters receiving sewage effluent adjacent to Burwood WWTP and at reference locations. Sydney rock oysters were deployed within 4 locations which included 2 locations receiving sewage effluent from Burwood WWTP and 2 reference locations. Within each location there were two deployment units. Each deployment unit had 2 bags of 40 oysters attached at 4, 8 and 12 metres depth (Figure 5.1).  

*Figure 5.1.* Photograph of a mesh bag containing 40 *Saccostrea glomerata*. Each deployment unit had a total of 6 bags with 2 bags attached at 4, 8 and 12 metres depth. In total there were 48 bags of *Saccostrea glomerata* which combined contained a total of 1920 *Saccostrea glomerata*.  

For vitellogenin analyses, it was important that *S. glomerata* were deployed during a period favourable for gonadal maturation. Oysters were specifically selected to be entering a phase of gonadal development at experimental commencement (Dinamani, 1974). Oyster gonadal development usually occurs during the warmer months when water temperatures are between 18-26 ºC (Holliday, 1995) and in the Newcastle and Port Stephens region summer months are December-February. It was also important that *S. glomerata* were sampled before a spawning event, which can be dependent on annual weather conditions and typically occurs once or twice in January and/or February in the environment. Oysters were collected after 6 weeks deployment.
Within Burwood WWTP receiving waters, the point of effluent release was divided into two zones based on effluent diffusion and predominant currents (Glamore et al. 2007). The near field zone was defined as a 50 metre radius around the outfall, which extended to the point at which the mixture of seawater and effluent reached its level of neutral buoyancy or the sea surface (Glamore et al. 2007) (Figure 5.2). The far-field began at the edge of the near field boundary and extended to approximately 150 metres from the outfall, in line with the prevailing north-eastern current (Figure 5.2). The edge of the far-field was identified as the region where dilution of sewage effluent was no longer significantly detectable against reference locations or background levels (Glamore et al. 2007). These locations will be referred to as Burwood near or Burwood far. Oysters were deployed within Burwood near and far locations to establish a gradient of effluent exposure with the assumption that oysters deployed at Burwood Near would receive a higher exposure of sewage effluent compared to those at Burwood Far. Oysters were also deployed at reference locations within Redhead and Fingal Island. The period of deployment was for approximately 6 weeks (40 days) in summer from the 8th December 2008 to the 16th January 2009.
Figure 5.2: Map of Newcastle and Port Stephens, NSW, Australia. Deployment units of Sydney rock oysters, *Saccostrea glomerata*, (indicated) were deployed adjacent to Burwood Wastewater treatment Plant in effluent receiving waters. Location of the deployment units at reference locations, Redhead and Fingal Island are also indicated.

### 5.3.2. Anchorage and deployment

At the Burwood near location, oyster deployment units were attached directly to the effluent diffuser pipe. For Burwood far, oyster deployment units were deployed in-line with the prevailing North Eastern current. Exposure of oysters to effluent was likely to be variable and highly dependent on the water current. Locations and location descriptions are summarised in Table 5.1.
Table 5.1. Location description including the type of anchorage, depth and GPS co-ordinates of the oyster deployment impact and reference locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Location Description</th>
<th>Anchorage</th>
<th>Depth (m)</th>
<th>GPS co-ordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burwood near 1</td>
<td>Impact location receiving sewage effluent, situated within 50m from effluent release point</td>
<td>120 kg steel beams</td>
<td>23.3</td>
<td>-32.96833 +151.7526</td>
</tr>
<tr>
<td>Burwood near 2</td>
<td>Impact location receiving sewage effluent, situated within 50m from effluent release point</td>
<td>120 kg steel beams</td>
<td>23</td>
<td>-32.96778 +151.7523</td>
</tr>
<tr>
<td>Burwood far 1</td>
<td>Impact location receiving sewage effluent, situated within 150m from effluent release point</td>
<td>Attached to effluent diffuser (no. 8)</td>
<td>20.5</td>
<td>-32.97098 +151.7522</td>
</tr>
<tr>
<td>Burwood far 2</td>
<td>Impact location receiving sewage effluent, situated within 150m from effluent release point</td>
<td>Attached to effluent diffuser (no. 9)</td>
<td>22.1</td>
<td>-32.96932 +151.7525</td>
</tr>
<tr>
<td>Redhead 1</td>
<td>Reference location located ~3.7 km from impact locations</td>
<td>120 kg steel beams</td>
<td>20.5</td>
<td>-33.01483 +151.7274</td>
</tr>
<tr>
<td>Redhead 2</td>
<td>Reference location located ~3.7 km from impact locations</td>
<td>120 kg steel beams</td>
<td>21</td>
<td>-33.01507 +151.7271</td>
</tr>
<tr>
<td>Fingal Island 1</td>
<td>Reference location located ~30 km from impact locations</td>
<td>30 kg concrete</td>
<td>18</td>
<td>-32.73.944 + 152.1963</td>
</tr>
<tr>
<td>Fingal Island 2</td>
<td>Reference location located ~30 km from impact locations</td>
<td>30 kg concrete</td>
<td>18</td>
<td>-32.73834 + 152.2303</td>
</tr>
</tbody>
</table>

Latitude and Longitude in Decimal Degree

Due to region of deployment, the Maritime Authority required use of yellow (coding for ‘special’ activity) 120 cm surface buoys with a flashing solar light at Redhead and Burwood (Figure’s 5.3 and 5.4). The buoyancy provided by the substantial buoys, coupled with the current and potential swell of the region posed a risk to the deployment units moving or breaking during the deployment period. Thus it was important that the structure was able to withstand strong weather conditions of high swell. The anchor system (for Burwood far and Redhead) consisted of 6 steel T bars which weighed 20 kg each (total 120 kg) which were each linked by 1 metre length of 12 mm galvanized chain with a 10 metre (8 mm width) galvanized chain at the end. The 10 metre length of lighter chain (8 mm) was designed to act as a spring during high swell and prevent the system from moving/dragging.
along the ocean floor. This was then attached to 30 metres of 15 mm rope which was attached to the buoy with a galvanized shackle.

Figure 5.3. Photograph of buoys used for deployment units at Burwood near, Burwood far and Redhead. The buoys were 120 cm in diameter and were visible from Merewether, Burwood and Redhead shorelines.

Figure 5.4. Photograph of 2 deployment units being deployed at Burwood near locations.
At each location there were two deployment unit systems and each deployment unit consisted of 2 bags of 40 oysters attached at 4, 8, and 12 metres depth (Figure’s 5.4, 5.7 and 5.8). Deployment depths were selected based on a study of the effluent plume dynamics (Glamore et al. 2007), which suggested that the highest concentration of sewage effluent was likely to be situated within 0-12 metres depth.

**Figure 5.5:** Deployment unit design at Burwood near (within 50 metres from source) and far (within 150 metres from source).
Figure 5.6: Deployment unit design at reference locations: Fingal Island and Redhead.

Figure 5.7. Photograph of the collection after a 6 week deployment period at Burwood.
Surface buoys on deployment units Burwood near 2, Burwood far 1 and Redhead 2 went missing during the period of oyster deployment and thus were not included in analysis. The buoys from two of the missing deployment units were located onshore shortly after their disappearance. Shackles had been removed from the buoys suggesting that there had been interference with the deployment units. Upon collection of the deployment units (Figure 5.5), the remainder of the missing deployment units (anchor with ropes and attached oyster bags) were located on the sea floor. The rope was intact providing further evidence that the deployment units had been tampered with.

5.3.3. Condition index and mortalities

Wet condition index was measured to assess the potential confounding effects of effluent exposure on feeding and thus condition. This was important to determine that potential reproductive effects (such as accelerated reproductive development) were due to estrogenic exposure as opposed to increased essential nutrients from effluent (for example, particulate matter, nitrogen and phosphorus) accelerating development. Wet condition index was measured at each harvest. This was calculated using the formula (wet tissue weight X 1000)/(wet whole weight- wet shell weight) (Lucas and Benninger; 1985). Mortalities were calculated by counting the dead oysters in the bags at experimental conclusion.

5.3.4. Vitellogenin real-time qPCR analysis

Total RNA from oyster gonadal samples (~100 mg) was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s guidelines. Briefly, 100 mg of oyster gonadal tissue in a 2 mL eppendorf tube was homogenised on ice in 500 µl of Trizol Reagent for 1 minute before adding another 500 µL of Trizol Reagent. To pellet cells and remove insoluble material, mixture was centrifuged at 12 000 x g for 10 minutes at 4 ºC. The supernatant was then transferred into a new 1.5 mL eppendorf tube and incubated at 20 ºC for 5 minutes. Two hundred µL of chloroform (pure) was then added to the mixture before shaking for 15 seconds. Mixture was then incubated at 20 ºC for 3 minutes and centrifuged at 12 000 x g for 15 minutes at 4 ºC. Total RNA was then precipitated by mixing with 500 µl isopropyl alcohol (pure) followed by 10 minutes incubation at 20 ºC and centrifugation at 12 000 x g for 10 minutes at 4 ºC. The supernatant was removed from the mixture to
leave RNA, sometimes visible as a clear pellet on inside of tube. The RNA pellet was then washed by adding 1 mL of 75% ethanol (pure), vortexing and centrifuged at 7 500 x g for 5 minutes at 4 ºC. The pellet was then air-dried for 5 minutes and dissolved by mixture with 40 μl of ultra pure water (Invitrogen) and incubation at 58 ºC for 10 minutes.

The quality of the RNA was assessed on a denaturing gel; 15 μl of formaldehyde loading dye was added to 1 μg RNA and heated for 15 minutes to denature RNA. Samples were then loaded on the denaturing gel (consisting of 0.4 M 3-[N-Morpholino]-propanesulfonic acid (MOPS) pH 7, 0.1 M sodium acetate and 0.01 M ethylenediaminetetraacetic acid (EDTA)) and visualised on an Ultraviolet (UV) transilluminator (BioRad). For each sample, quality of RNA was assessed via a denaturing gel, via measurements of the RNA concentration and the 260/280 (the former two assessed using NanoDrop 1000A Spectrometer, Thermo Scientific). The 260/280 nm absorbance ratio is a measure of the purity of the DNA with a low ratio (<1.8) indicative of protein contamination while a high ratio (> 2.1) can be indicative of RNA degradation (Manchester, 1996; Fleige and Pfaffl, 2006). Samples between the range of 1.8-2.1 and were considered to have high purity and low protein contamination. In a denaturing gel, samples with sharp intense ribosomal bands were used for analysis.

First-strand cDNA synthesis was carried out using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™ catalogue number 18080-051) according to manufacturer’s guidelines (see Chapter 3 for a detailed description of methodology). Vitellogenin gene expression was assessed using the real-time qPCR assay developed and described in Chapter 3.

5.3.5. Vitellogenin analysis via HPLC
The extraction and analysis of oyster gonadal samples were performed using protocols outlined in Chapter 2. Briefly, excised gonadal tissue (approximately 100 mg) was stripped from the oyster and homogenized before storage in a 1.5 mL eppendorf tube at -80 ºC in 225 μL citrate buffer (pH 6.5 10 mM and with 16 mg/mL polyethylene glycol) and 25 μL of the protease inhibitor aprotinin (Gagné and Blaise, 1999; Brodeur et al. 2006). Following
storage, samples were thawed and 500 µL Tris-HCl buffer was added prior to homogenization using a micro pestle. One hundred µL of tissue homogenate was transferred to a glass centrifuge tube to which 800 µL of t-butyl methyl ether (Chromsolv grade, Sigma Aldrich) was added. The mixture was vortexed and incubated at 4 ºC for 30 minutes to extract vitellogenin. The organic fraction was separated and dried under N₂ and re-suspended in 1000 µL of PBS.

Analysis of samples was performed using a Hewlett-Packard 1100 Series HPLC (Waldbonn, Germany). Separation of sample analytes was achieved at room temperature (24 ºC) on a Hypersil ODS (C18) (Agilent Technologies, USA) analytical column with dimensions 2.0 mm i.d. × 125 mm, 3µm particle size and 120 Å pore size. The guard column was a Hypersil ODS (C18) cartridge (Agilent Technologies, USA), with dimensions 4.0 mm i.d. × 4 mm, 5 µm particle size. The mobile fraction system was as follows: eluent A was 0.1% trifluoroacetic acid (TFA) in Milli-Q water and eluent B was 0.1% TFA in acetonitrile. The column flow rate was 1.0 mL/minute and the pump gradient program was as follows: 0–50 minutes, 100% A to 100% B, 50–55 minutes, 100% A, 55–60 minutes, 100% B to 100% A. The diode array detector (DAD) parameters focused on signal C, 210 nm (ref. 360 nm).

Unlike previous Chapters, the concentration of vitellogenin in oyster tissue was calculated against a standard curve for pure vitellogenin from rainbow trout, Oncorhynchus mykiss (Biosense Laboratories) to enable quantification in µg/g (Figure 5.8A). To account for variation between sampling runs, an internal standard of purified bovine serum albumin was also included in every run (Figure 5.8B). Standard curves were generated by a simple regression between serial concentrations and their corresponding peak area. Both standard curves have a R² value close to 0.99 suggesting that the response increases with concentrations of standards in a linear fashion. Eight females per location/depth were analysed for vitellogenin protein.
Figure 5.8: Standard curves created through measuring HPLC response following injection of A) purified Rainbow Trout, *Oncorhynchus mykiss*, vitellogenin standard and B) purified bovine serum albumin.
5.3.6. Sex identification and gonadal development stages

Each individual oyster (Figure 5.9) was prepared for histological examination resulting in a total of 540 analyses. A cross-section of approximately 5 mm² was excised from each oyster between the labial palps and gills. Tissue samples were placed in Davidson’s solution (10% glycerin, 20% formalin, 30% alcohol, 30% sodium chloride solution and 10% glacial acetic acid) for 24 hours (Cox et al. 1996) with successive dilutions of ethanol (70%, 50% and 50%) (Howard and Smith, 1983). Tissue was embedded into paraffin blocks, sectioned transversely at 8 µm intervals and floated on a water bath heated to 80 ºC. Sections were placed onto acid washed glass slides and dried overnight at 60 ºC. Sections were stained with haematoxylin and counterstained with eosin (H & E). Study of the gonadal area was performed under a compound microscope at 200 and 400 X magnification.

![Figure 5.9](image)

Figure 5.9. Photograph of a mature Saccostrea glomerata individual following 6 weeks deployment at Burwood.

The sex of each individual was determined as female or male under microscopic examination (200 x magnification) using oocytes or spermatozoa as indicators. Other individuals were found to be indeterminate whereby the gonadal cells were undifferentiated and usually accompanied by an abundance of hemocytes.
If estrogenic compounds are contained within the sewage effluent, it may be possible that estrogen exposure promotes female development resulting in acceleration towards higher proportions of mature female gonad stages in individuals exposed compared to those individuals from reference locations. Thus, each oyster was assigned a gonadal developmental stage for oogenesis, and also spermatogenesis, based on criteria described by Dinamani (1974) (described in detail in Chapter 2).

5.3.7. Oocyte area

Eight female individuals were randomly selected from each location (Burwood near, Burwood far, Redhead, Fingal Island 1 and Fingal Island 2) at 4 metres depth. For each individual, digitalised images of 4 randomly selected areas of the gonad were taken with subsequent measurement of 80-100 oocytes (Imagetool 2.0). Only complete oocytes with a visible nucleus were measured and oogonia were not measured.

5.3.8. Statistical Analysis

Three locations, Burwood near, Burwood far and Redhead, lost one deployment unit and therefore variation within locations could not be assessed. Because the Fingal Island location had both deployment units present at the conclusion of the deployment period, these were treated at separate locations for the purpose of statistical analyses. Each deployment unit had 2 bags of oysters attached at depths 4, 8 and 12 metres. To test for variability between the two bag replicates, student T Test comparisons were performed comparing the results between bag replicates at each depth for each deployment unit for each of the quantitative analyses including wet condition index, vitellogenin gene expression, vitellogenin and mean oocyte area (Statsoft, 2005). The only significant differences found between bags were for wet condition index at 4 metres depth at Burwood near and for vitellogenin levels at 8 metres depth at Burwood near. Importantly, no significant differences were found between bag replicates for the majority of the analyses. Bags were thus pooled in order to simplify analyses and to gain replication and power.

One-way ANOVAs were used to assess differences in mean oocyte area (4 metres depth), mortalities (depths pooled), vitellogenin gene expression for females (4 metres depth) and
males (depths were pooled to gain sufficient replication of males for statistical analysis) among locations. Two-way ANOVAs were not possible due to an unbalanced design, as oysters were not available for sampling from 8 metres depth at the Burwood far location. Thus, each depth (4, 8 and 12 metres) was separately analysed using one-way ANOVAs to assess differences in condition index and vitellogenin levels among locations. According to Levene’s test for homogeneity of variance; vitellogenin units were not homogenous and thus log transformed, ln (x + 1), prior to statistical analysis.

A Pearson Chi squared analysis (SPSS version 17) was used to determine significant differences in sex proportions (female, male, indeterminate) among locations (depths pooled to gain sufficient replication). Proportions of mature gonadal stages were analysed via Kruskal Wallis (one-way analysis of variance by ranks) for each location (depths pooled). Except for the Chi Squared to analyse for significant differences in sex ratio, all analyses were conducted using STATISTICA (Statsoft, 2005).

5.4. Results

5.4.1 Mortalities

Between 20 - 33.3 % mortality was observed across locations following the 6 week deployment. Despite a trend of higher mortalities at both Fingal Island reference locations, no significant differences in mortalities were observed among locations (F= 2.066, df= 4, p> 0.05) (Figure 5.10).
Figure 5.10: Percentage mortality of Sydney rock oysters, *Saccostrea glomerata*, following a 6 week deployment in the receiving waters of Burwood wastewater treatment plant and reference locations. Depths pooled. (mean ± standard error, N= 40).

5.4.2. Wet condition Index

Condition index can provide an indication of the health of individuals. Differences in condition may also indicate different environmental conditions between locations, in terms of nutrient availability, water temperature, stress from pollutants and competition posed by other species for resources (Lucas and Benninger, 1995). It may also be expected that individuals with a higher condition index are more likely to be of greater gonadal maturity compared to individuals with low indices.

At 4 metres depth there was a significant difference in the mean condition index of individuals among locations (F= 5.286, df= 4, p< 0.05) (Figure 5.11A). Individuals deployed at the Burwood near location displayed significantly higher condition indices compared to individuals from Fingal Island 2 and Redhead. Individuals from Burwood far had significantly higher condition indices compared to Redhead only. There were no significant differences among locations in condition indices for individuals deployed at 8
metres (F= 2.17, df= 3, p> 0.05) or 12 metres (F= 1.29, df= 4, p> 0.05) depth (Figures 5.11B and C).
Figure 5.11: Comparison of the wet condition index of Sydney rock oysters, *Saccostrea glomerata* following a 6 week deployment in the receiving waters of Burwood wastewater treatment plant and reference locations at depths 4, 8 and 12 metres (replicates pooled), (mean ± standard error, N= 24).
5.4.3. Vitellogenin gene expression

Vitellogenin gene expression was assessed in female individuals from 4 metres depth under the assumption that this depth would receive the highest effluent, and thus estrogenic, exposure. Female individuals deployed at Burwood near displayed elevated vitellogenin gene expression that was significantly higher than vitellogenin gene expression in individuals from the majority of reference locations with the exception of Redhead (F= 3.77, df= 4, p< 0.05) (Figure 5.12A). Individuals deployed at Burwood far however, had similar vitellogenin gene expression to individuals from reference locations.

In order to gain sufficient replication for the analysis of male vitellogenin gene expression, individuals from all depths (4, 8 and 12 metres) were pooled. There were no significant differences in male vitellogenin gene expression among locations (F= 1.33, df= 4, p > 0.05) and the vitellogenin gene was expressed at very low levels, or not at all, in the majority of male individuals (apart one individual from Redhead) (Figure 5.12B). As this was the case, male vitellogenin protein concentrations were not assessed.
Figure 5.12: Comparison of vitellogenin gene expression analysed via real-time qPCR in Sydney rock oysters, *Saccostrea glomerata*, following 6 weeks deployment in receiving waters of Burwood wastewater treatment plants and reference locations in A) female individuals from 4 metres depth (mean ± standard error, N= 8) and B) males individuals pooled across all depths (mean ± standard error, N= 7). Letters indicate statistically similar locations, p> 0.05.
5.4.4. Female vitellogenin

It was hypothesised that female vitellogenin would be greatest in individuals deployed at the impact locations, particularly at Burwood near. As predicted, individuals deployed at the Burwood near location displayed the greatest vitellogenin response at all depths. Individuals at 4 metres at Burwood near had significantly elevated vitellogenin compared to reference locations Fingal Island 1 and 2 (F= 3.79, df= 4, p< 0.05) (Figure 5.13A). For 8 metres depth, Burwood near was significantly elevated compared to all other locations (F= 6.53, df= 3, p< 0.05) (Figure 5.13B). At 12 metres depth, vitellogenin response in individuals from Burwood near were significantly elevated compared to the individuals from Fingal Island 2 (F= 3.27, df= 4, p< 0.05) (Figure 5.13C).

Although the average vitellogenin response was highest at Burwood near 8 metres depth, there were no significant differences among depths (F= 0.65, df= 2, p>0.05).
Figure 5.13: Comparison of female vitellogenin measured via High Performance Liquid Chromatography in Sydney rock oysters, *Saccostrea glomerata*, following a 6 week deployment in receiving waters of Burwood wastewater treatment plant and reference locations at depths 4, 8 and 12 metres. Letters indicate statistically similar locations, p< 0.05, (mean ± standard error, N= 8 individuals per location/depth).
5.4.5. Sex ratio

It was hypothesised that exposure to estrogenic compounds may alter the sex ratio towards a higher proportion of females. Therefore the proportions of sexes (male, female and interdeterminate) were examined. No intersex individuals were observed at any location. Depths were pooled to gain sufficient replication for a χ² analysis. It was found that there was no significant differences with similar proportions of females (χ² = 1.16, df = 4, p > 0.05), males (χ² = 2.78, df = 4, p > 0.05) and indeterminate (χ² = 4.83, df = 4, p > 0.05) individuals among locations (Figure 5.14).

![Figure 5.14: Comparison of the sex ratio of Sydney rock oysters, Saccostrea glomerata, following a 6 week deployment in receiving waters of Burwood wastewater treatment plant and reference locations at depths 4, 8 and 12 metres. Depths are pooled. (mean ± standard error, N= 120 individuals per location)](image)

5.5.6. Mature gonadal development in females and males

Exposure to estrogenic compounds may also promote female development. Female development may be assessed via the examination of gonadal development stages
(reviewed in Chapter 1; Dinamani, 1974). Female gonadal development stages F1 and F2 are characteristic of early female maturation, where oocytes are in primary and/or secondary stages of development. Stages F3 and F4 are characteristic of a mature female gonad whereby the individual is close to spawning. Following spawning are stages F5 and F6.

Compared to reference locations, both Burwood locations had higher proportions of female individuals in gonad development stage F4 (Figure 5.15). This reflects that a higher proportion of females were in a mature development stage, close to spawning.

**Figure 5.15:** Proportions of gonadal development stages (Dinamani, 1974) of female Sydney rock oysters, *Saccostrea glomerata*, following a 6 week deployment in receiving waters of Burwood wastewater treatment plants and reference locations. Depths are pooled (N= 120 per location).
The finding of higher proportions of mature (F4) individuals at Burwood locations led to further analysis. Proportions of individuals in stages 4-6 are representative of female individuals in mature stages of gonadal stages and includes individual which are close to spawning (stage 4) and also individuals which have spawned (stage 5 and 6). These stages were therefore pooled together, for each location, to gain sufficient replication for statistical analysis for comparisons of mature individuals. It was hypothesised that there would be a higher proportion of mature female individuals at Burwood locations compared to reference locations due to estrogenic exposure via sewage effluent. This was confirmed, with a significantly higher proportion of females in mature gonadal stages (F4-F6) at Burwood locations compared to all reference locations (Kruskal Wallis, H=21.94, p<0.05) (Figure 5.16A).

An acceleration of female development at Burwood locations may be due to an increased availability of nutrients via effluent. If nutrient availability alone was responsible for increased proportions of mature individuals, similar patterns should be evidenced for both males and females. If acceleration of mature females was due to estrogenic exposure (and not due to increased nutrient exposure), then proportions of mature male individuals would be similar across locations or conversely male gonadal development may be impeded at impact locations. As predicted, there were no significant differences observed in proportions of mature males among locations (H=3.51, p>0.05) (Figure 5.16B).
Gonadal development of female and male Sydney rock oysters, *Saccostrea glomerata*, was determined based on histological observations and according to the criteria developed by Dinamani (1974). Gonadal development stages were then grouped into mature stages (stages 4-6; close to spawning and post-spawning gonad). The percentages were compared between locations (mean ± standard error, depths were pooled and N= 89-97 female replicates per location, N= 17-19 male replicates per location).
5.4.7. Oocyte area

Oocyte area increases with female gonadal development and is a reflection of the maturity of a female individual. To further test the hypothesis that exposure to estrogenic compounds promotes female development, mean oocyte area was calculated for female individuals at 4 metres depth. This hypothesis was partially supported with the highest mean oocyte area found in individuals from Burwood near and Burwood far which were significantly higher compared to individuals from the Redhead reference location (F= 6.625, df= 3, p< 0.05) (Figure 5.17).

Figure 5.17: Comparison of mean oocyte area measured in female Sydney rock oysters, Saccostrea glomerata, following a 6 week deployment in the receiving waters of Burwood wastewater treatment plant at 4 metres depth. Letters indicate significantly similar treatments p< 0.05. (mean ± standard error, N=8).
5.5. Discussion

It was hypothesised that estrogenic exposure in waters receiving sewage effluent may have been sufficient to promote an acceleration of female development. A significantly higher proportion (p< 0.05) of mature female gonadal development stages were found at Burwood locations compared to all reference locations. Burwood locations were likely to receive higher nutrients from sewage effluent which could accelerate development, however this would be expected to occur in both sexes and no differences were found among locations in the proportions of mature males. In addition, highest mean oocyte area was found in individuals from Burwood near and far (deployed at 4 metres depth) which was significantly higher (p< 0.05) than mean oocyte area in individuals from Redhead. However, this finding should be interpreted cautiously as oocyte area at impact locations was similar to Fingal Island locations. Others have similarly demonstrated that estrogenic exposure can accelerate female gonadal development. Injections of E2 (50 µg/d for 10 days) accelerated female development inducing significant increases in oocyte diameter (Li et al. 1998). Langston et al. (2007) also found that exposure to sediment spiked with estrogenic compounds (100 µg/kg of E2 and EE2, 1000 µg/kg for OP and NP; for low exposures and ×10 for higher exposures) caused enlarged oocytes in females. In Chapter 2, it was outlined that early female vitellogenin protein (following 4 days exposure to 6.25, 12.5, 25 and 50 ng/L EE2) was predictive of increases in oocyte area following 49 days exposure. These laboratory studies all provide direct evidence that controlled exposures can cause increases in oocyte area, however, this study is among the first to have demonstrated that field exposures can accelerate female gonadal development, at least in terms of the proportion of mature developmental stages.

Exposure to estrogenic compounds has been demonstrated to increase proportions of female (Chapter 2) or intersex (Chapter 2; Langston et al. 2007) individuals. Therefore, following field deployment, proportions of sexes were analysed under the assumption that potential exposure of estrogenic compounds in locations adjacent to the WWTP may cause an increase in female or intersex individuals. Sex proportions were found to be statistically similar among all locations and no intersex individuals were found during this experiment. However, the female: male sex ratio of the oyster population used for the experiment was
perhaps unsuitable for the detection of changes in sex proportions (~80% females across all locations). Power analysis suggested that for a significant detection of a 10% increase from the average 80% proportion of females would have required impractically large sample sizes (200 individuals per depth/location). Sydney rock oysters mature predominantly as males and may change sex to female in later years between reproductive seasons (Asif, 1979; Guo et al. 1998), thus it was likely that the initial population of oysters used for deployment across locations was an older population of oysters. Although not well described, Nell (1993) estimates that under optimal environmental or culture conditions (for example, salinity, temperature, and feeding rates) *S. glomerata* take 3-3.5 years to reach 30-50 grams whole weight. Individuals used in this experiment had an average whole weight of 29.2 grams suggesting that the age of the population may have been close to 2-3 years. Dinamani (1974) found a pre-dominance of females in all populations above 1 year of age. Future field deployments assessing changes in the sex proportions of *S. glomerata* individuals may wish to employ a younger population (<1 year) with a lower proportion of females (i.e. 50% or less). Alternatively, future experiments could assess increases in proportions of females via the deployment of spat in locations receiving sewage effluent, with assessment of sex at oyster maturity. Further, it may be that earlier life stages may be more responsive to low levels of estrogenic exposure than mature individuals.

Another definite indicator of estrogenic exposure is the induction of vitellogenin gene expression. It was hypothesised that *S. glomerata* individuals deployed at Burwood locations would display elevated vitellogenin expression due to exposure of estrogenic compounds via sewage effluent. Female vitellogenin gene expression was found to be significantly elevated (*p* < 0.05) at 4 metres depth at Burwood near compared to the majority of reference locations, suggesting that estrogenic exposure may have occurred. However, lack of vitellogenin gene induction in males (pooled across all depths) deployed at Burwood locations may indicate the level of estrogenic exposure was not sufficient to induce vitellogenin above that of male individuals at reference locations (where vitellogenin gene expression was also not expressed). In fact, male vitellogenin gene expression was low or non-detectable and there were no differences among locations. One explanation may be that estrogenic exposure was not sufficient to induce vitellogenesis in
males. In previous experiments, it was demonstrated that 6 weeks exposure to low estrogenic exposures, 1 µg/L NP or 5 ng/L EE2, was not capable of inducing significant protein induction (Andrew et al. 2008). Alternatively, elevated male vitellogenin gene expression may have occurred early in the experiment but returned to basal concentrations by the sampling period of 49 days. In Chapter 3, it was also demonstrated that no relationship could be found between EE2 exposure (0, 6.25, 12.5, 25 and 50 ng/L) and male vitellogenin gene expression when regressions were performed on individual males. Together, these findings may suggest that much higher exposures of estrogenic compounds are required to elevate vitellogenin gene expression in males.

Similar findings to female vitellogenin gene expression were demonstrated for protein induction of vitellogenin in females. Vitellogenin induction was compared among locations for each separate depth. Female individuals deployed at Burwood near, 8 metres depth displayed significantly elevated (p< 0.05) vitellogenin compared to all reference locations. At 4 and 12 metres, vitellogenin induction in female individuals from Burwood near was significantly elevated (p< 0.05) compared to the majority of reference locations at corresponding depths. Despite impact locations being similar to at least one or two reference locations at 4 and 12m, findings provide strong indication that estrogenic compounds were present in sewage effluent from Burwood WWTP, which was capable of inducing significant vitellogenesis in female *S. glomerata*.

This study is among the first to demonstrate that exposure to estrogenic compounds, via sewage effluent, is capable of inducing significant molluscan vitellogenin induction under field conditions. At present in molluscan taxa, the majority of research has employed a surrogate estimate of vitellogenin, ALP, as an indicator of exposure to estrogenic exposure. Quinn et al. (2004) demonstrated that laboratory exposures to tertiary treated effluent from an Irish WWTP induced significant ALP induction in female individuals exposed to effluent compared to control individuals. Additionally, under the assumption that sewage effluent may contain estrogenic compounds, others have employed ALP as an indicator of estrogenic exposure in molluscs which are deployed or residing within waters receiving sewage effluent. Gagné et al. (2001) found that *E. complanata*, deployed 5 kilometres
downstream of a WWTP for 62 days in the St Lawrence River, Canada, displayed elevated ALP levels compared to individuals 1.5 kilometres upstream. In Venice, where raw sewage has been released into canals, wild male *M. galloprovincialis* were found to have higher ALP levels compared to male individuals collected from reference locations (Pampanin et al. 2005). Surrogate estimates of vitellogenin, such as ALP, may indicative of estrogenic exposure. However, direct measures of vitellogenin provide definite evidence of estrogenic exposure and are favourable over surrogate estimates. This study is therefore among the first to report elevated molluscan vitellogenin due to effluent exposure. Findings of elevated vitellogenin in females deployed at Burwood may indicate exposure to estrogenic compounds and provide further evidence of the presence of estrogenic compounds/activity from sewage effluent released from Burwood WWTP (Chapter 4).

For future studies, slight methodological alterations for improved assessment of vitellogenin and sex are suggested. Future studies may consider measuring vitellogenin gene expression and protein induction following short term exposure (4 days) and during early gonadal development, as earlier findings suggested that this is a crucial period for elevated concentrations of vitellogenin. To determine if sex status is influenced by estrogenic compounds contained in sewage effluent, it is suggested that an oyster population be employed with an even male:female ratio or deploying spat and assessing sex at maturity (after 1 year). Biological effects such as vitellogenin, sex and gonadal development could also be determined in the wild territorial fish population that regularly feed around the sewage release point.

Mortality rates and condition indices of *S. glomerata* are useful indicators of the health of individuals. Highly elevated mortality rates and/or low condition indices may reflect low nutrient availability, disease or high competition. Analysis of mortality numbers and the mean wet condition index was employed to compare environmental conditions between locations and depths. It was hypothesised that there may be differences in mortalities between locations due to different environmental conditions. However for mortalities, there were no significant differences among locations. It was also hypothesised that condition index may be elevated at Burwood locations due to increased availability of
suspended organic particulates via sewage effluent. For 4 metres depth, it was found that condition index was significantly ($p < 0.05$) higher in individuals from Burwood near and far compared to the reference location, Redhead, though was not consistently higher than other reference locations. Individuals deployed at Burwood may have received greater amounts of suspended organic particulates compared to individuals at Redhead. Sewage effluent has been known to contain high amounts of nutrients such as phosphorous and nitrogen (Preston and Chester, 1996). These nutrients are essential and may promote oyster growth (via promoting algae growth and increasing oyster food sources). Redhead is an oceanic location and individuals may not have been able to access organic suspended particulates as efficiently as individuals deployed at Burwood. At 8 and 12 metres depths, there were no significant differences in condition index among locations at corresponding depths. This may indicate that, for at these depths, suspended organics were not sufficiently greater at the Burwood locations to promote elevated condition indices.

In conclusion, this Chapter has provided further complementary evidence that estrogenic compounds were present not only in concentrations of concern (Chapter 4) but had the capacity to significantly alter biological indicators in adjacent waters. Proportions of mature female individuals were significantly higher ($p < 0.05$) at both Burwood locations compared to all reference locations. For all depths, female individuals displayed significantly ($p < 0.05$) elevated vitellogenin which was significantly higher ($p < 0.05$) at Burwood near compared to the majority of reference locations (at corresponding depths). Accelerated gonadal development at Burwood could be attributed to an increased availability of nutrients but as there were no significant differences in condition index among locations for males or females it may be possible that elevated female gonadal development is due to estrogenic exposure via sewage. Female vitellogenin gene expression at 4 metres depth was also significantly elevated ($p < 0.05$) compared to the majority of reference locations at equivalent depths. These findings must be interpreted cautiously however, as vitellogenin biomarkers were not consistently elevated compared to all reference locations, with the exception of vitellogenin protein at 8 metres depth, suggesting effect size was not great and vitellogenin responses at impact locations can fall within the bounds of natural variability in vitellogenin responses. However, estrogenic exposure may not have been sufficient to
promote changes in male vitellogenin gene expression as no differences were observed between locations. Furthermore, it was found that there were no differences in sex ratio between locations. On balance, the findings from this Chapter provide strong evidence that *S. glomerata*, particularly the females, shows potential as a suitable biomonitor for field exposures of estrogenic compounds in Australian marine waters.
Chapter 6: Assessment of heavy metals in oyster tissue of *S. glomerata* deployed in the receiving waters of a wastewater treatment plant

6.1. Summary

Sewage effluent has been identified as a potential source of heavy metal contamination in the aquatic environment. The Sydney rock oyster, *Saccostrea glomerata*, can accumulate most heavy metals and is well established as a successful biomonitor of heavy metals in the marine environment. To determine if Burwood wastewater treatment plant (WWTP) is a source of heavy metals, *S. glomerata* was deployed for 6 weeks in effluent receiving waters (Burwood near, <50 metres and Burwood far, <150 metres) and at reference locations (Redhead and Fingal Island) at depths 4, 8 and 12 metres. In dried oyster tissue, Inductively Coupled Plasma Mass Spectrometer (ICPMS) was employed to assess a suite of heavy metals including arsenic, aluminium, cadmium, chromium, cobalt, copper, lead, manganese, mercury, nickel, selenium, silver and zinc. It was found that concentrations of most heavy metals were not significantly different (p >0.05) in the tissue of *S. glomerata* deployed at Burwood compared to those in the reference locations. Concentrations of heavy metals were similar to those which have been detected in previous studies in NSW. All metals fell below National Food Authority maximum residue levels (MRLs), except for arsenic and this does not appear uncommon for concentrations in biota within NSW. Furthermore, comparisons to historic data suggest that, via measurement in oyster tissue, metal concentrations released into the marine environment via sewage effluent from Burwood WWTP have not increased in the last two decades. Findings suggested that *S. glomerata* was a suitable biomonitor for heavy metals in Australian waters.

6.2. Introduction

Heavy metals enter the sewage treatment process via domestic and industrial sources and incomplete removal during sewage treatment can result in entry into the aquatic
environment via sewage effluent release. Following release into the aquatic environment, metals may dissolve in seawater or bind to particulates such as algae or sediment and be taken up by aquatic species (Naimo, 1995). Uptake of heavy metals by aquatic wildlife is of concern due to potential toxicity effects. Although toxicity varies depending on the exposed species, metal type and concentration, some demonstrated effects include reproductive and growth impairments, behavioural abnormalities and in some cases, mortality (Keller and Zam 1991; Naimo, 1995; Norris and Carr, 2006). Also of high concern, is their potential environmental persistence, capacity to bio-accumulate in organisms and subsequent biomagnification through the food chain. Thus, routine monitoring of heavy metals in the aquatic environment is required.

Monitoring of environmental heavy metal concentrations, such as in seawater or sediment, can pose difficulties as heavy metals can be present in chemical forms which are not bioavailable (Phillips, 1980). Metals in seawater are often at low concentrations, below limits of analytical detection. Furthermore, concentrations may vary greatly temporally, dependent on pulse versus press releases into the aquatic environment. Thus, the measurement of heavy metals in seawater or sediments alone may not provide reliable information on the potential effects in wildlife. Biological monitors, such as molluscs, are capable of accumulating metals to higher concentrations than their surrounding environment and therefore may be used to provide a time-integrated measure of bioavailable heavy metal concentrations. Indeed, molluscs have proved to be useful as biomonitors with the capacity to bioaccumulate heavy metals at concentrations orders of magnitude higher than ambient environmental concentrations. Their capacity to bioaccumulate metals, which reflect environmental concentrations, has resulted in the successful development of the Mussel Watch programme (Goldberg et al. 1983) and, in Australia, the Sydney rock oyster, *Saccostrea glomerata*, is the predominant molluscan species used for monitoring of heavy metals in the marine environment (Avery et al. 1996; Lincoln-Smith and Cooper, 2004; Robinson et al. 2005; Scanes, 1996). For example, Phillips and Yim (1981) found that concentrations of copper and zinc in the tissue of *S. glomerata* collected from contaminated Hong Kong waters were similar to concentrations in sediments. Further, trace metal concentrations in *Crassostrea commercialis* have been
shown to reflect an environmental metal gradient by Mackay et al. (1975). Scanes (1996) also demonstrated that *S. glomerata* was useful as an indicator of metal contamination in waters adjacent to wastewater treatment outfalls in Sydney, New South Wales, demonstrating high contamination of inshore waters was reduced following the commissioning of deepwater offshore discharge. These studies suggest that *S. glomerata* can be successfully employed as a biomonitor of heavy metals in the aquatic environment.

Molluscs can accumulate most metals which are then stored either in a metabolically available form or in a detoxified form. The uptake of heavy metals by aquatic invertebrates is thought to occur via a) the aquatic medium which contains the soluble dissolved form or b) through ingestion of phytoplankton or inorganic particles which are bound to the metal; diet has been suggested to be the major source of exposure (Boisson et al. 1998; Wang, 2002; Rainbow and Wang, 2001). Once taken up, the metal may be used for essential metabolic process, excreted, bound to a biomolecule and/or stored in the body (Rainbow, 2002). All metals are potentially toxic to an organism. Toxicity of the metal depends on concentration, form, whether it is essential or non-essential and the capacity of the organism to excrete or detoxify. As stated by Rainbow (2002, pg. 499) “toxicity occurs when the rate of metal uptake into the body exceeds the combined rate of excretion and detoxification of metabolically available metal”. At the cellular level heavy metals may impart toxic effects via redox recycling, whereby metals accept or donate electrons generating reactive oxygen species which can damage cellular components including lipids (disrupting cellular membranes), proteins and DNA (Kappus and Sies, 1981; Valavanidis et al. 2006). Detoxification of heavy metals may be achieved through the production of metal binding proteins, such as metallothioneins, which are capable of intracellular binding to metals or incorporation into insoluble metaliferous granules with storage in the hepatopancreas or kidney (Phillips and Rainbow, 1989; Roesijadi, 1980; Viarengo, 1985).

An important consideration in the accumulation of metals may be whether the metal has an essential role in the organism. Essential metals are those which have roles in metabolic functioning, certain quantities of these metals are required to meet metabolic needs and these metals cannot be immediately detoxified or excreted. Examples of essential metals
include zinc, iron, magnesium, manganese and copper which are required as components of enzymes and/or respiratory pigments (White and Rainbow, 1985). Other metals are considered non-essential, for example cadmium, lead and mercury (Amiard, 1987; Depledge and Rainbow, 1990), as they have no essential roles in metabolism and therefore need to be excreted or detoxified immediately following uptake (Rainbow, 2002). Both non-essential and essential metals can bioaccumulate in molluscan tissue, however, non-essential metals are perhaps more likely to accumulate within tissue (Bryan et al. 1979). Essential metals have a minimum threshold concentration which is required to meet the organism’s metabolic needs, and to some extent, molluscs may exhibit homeostasis for essential metals such as zinc and copper (Langston et al. 1998; Phillips and Rainbow, 1989). White and Rainbow (1985) calculated the minimum metabolic requirements for zinc and copper in molluscs and crustaceans and this work was later re-evaluated by Depledge (1989) who considered that concentrations may be higher due to the fact that different tissues have different metabolic requirements. Together, their estimates of the metabolic requirements for zinc and copper ranged from 151.3- 177.8 μg/g and 92.8- 275.9 μg/g (dry weight), respectively (White and Rainbow, 1985; Depledge, 1989). If internal concentrations of essential metals exceed maximum internal thresholds (over that which is required for metabolic needs) then essential metals may also bioaccumulate in molluscan tissue. Because non-essential metals have no biological function nor established mechanisms within the organism for their regulation, then they are perhaps more likely to bioaccumulate in comparison to essential metals (Phillips and Rainbow, 1989; Rainbow, 2002). Although oysters are considered to be effective biomonitors of all heavy metals (Phillips and Rainbow, 1989; Scanes, 1996), non-essential metals may have higher potential for bioaccumulation and provide relative concentrations in tissues which more closely reflect environment contaminant loads, in comparison to essential metals. This is an important consideration in the use of heavy metal analysis, as only metals which are bioaccumulated, not regulated, can provide an indication of relative contaminant loads among locations in waters.

Although molluscs may exhibit a degree of homeostasis for some essential metals, there are also examples of bioaccumulation in oysters (Phillips, 1985, 1995; Phillips and Yim, 1981;
Phillips and Rainbow, 1993). This suggests that essential metals can sometimes be bioaccumulated in oysters at high exposure concentrations. George et al. (1978) demonstrated that the European flat oyster, *Ostrea edulis*, can accumulate high concentrations of copper and zinc (up to 13 000 and 25 000 ppm respectively) in cells. Oysters have also been successfully employed to analyse temporal trends and to identify metallic contaminated locations. Brown and McPherson (1992) employed *Saccostrea commercialis* (now known as *S. glomerata*, Iredale and Roughley, 1933) to assess temporal changes in copper and zinc in the Georges River (identified to have a metallic contamination gradient which increased with distance from the mouth of the estuary), demonstrating an increase of 40% for copper and 300% for zinc concentrations since 1975. Further, Spooner et al. (2003) analysed wild *S. commercialis* and found that concentrations of both non-essential and essential metals, including zinc and copper, were significantly elevated (p< 0.05) in oysters which were grown in a contaminated location, Botany Bay, (2600 ± 690 for zinc and 170 ± 45 for copper) compared to oysters grown in reference locations, Jervis Bay and Batemans Bay (which ranged from 980 ± 400 to 1793 ± 392 for zinc and 22 ± 14 to 65 ± 18 for copper). Together, these findings suggest that essential metals, copper and zinc, may also be accumulated by oysters and should be considered alongside non-essential metals in bioaccumulation assessments.

Laboratory exposure studies provide examples of how non-essential metals can bioaccumulate in oysters and remain elevated following depuration periods. This is important as it is likely that metallic inputs into the aquatic environment via sewage discharge are not constant but fluctuate temporally (Luoma et al. 1985; Nielsen and Hrudey, 1983). MacFarlane et al. (2006) exposed the Akoya pearl oyster, *Pinctada imbricata*, to a) constant 180 mg/L lead for 9 weeks and b) two pulse exposures of 180 mg/L for 3 weeks, with a 3 week depuration period. They found that concentrations of accumulated lead were statistically similar in the pulse exposure (539 ± 82 mg/g) compared to the constant exposure (603 ± 173 mg/g) which suggested that the oyster quickly equilibrated internal lead concentrations with exposure and that depuration of accumulated lead was low (MacFarlane et al. 2006). Others have also suggested that, with short term exposures followed by a depuration period, oysters retain much of the accumulated metal load for
extended periods of time. Clams, *Mercenaria mercenaria*, exposed to 220 mg/L lead had similar tissue concentrations after 5 days compared to after a 15 day depuration period (Boisson et al. 1998). Further, Boisson et al. (2003) exposed Pacific oysters, *Crassostrea gigas*, to cadmium (500 ng/L) for 21 days followed by a 41 day depuration period. They found that depuration of cadmium was slow, with 78% remaining after the depuration period (Boisson et al. 2003). Similarly, Riisgard et al. (1985) deployed mussels, *Mytilus edulis*, in waters contaminated with mercury for 3 months followed by a 42 day depuration. They calculated a biological half life of 293 days based on the depuration rate of mercury by *M. edulis*. These studies suggest that oysters are useful as biomonitors of non essential heavy metals due to their capacity to accumulate with slow depuration rates, providing an integration of exposure over time despite possible temporal changes in exposure. Although differences may exist between these species and certainly in other metals, pulse exposures with short term depuration periods may not greatly influence tissue metal concentrations.

The focus of this Chapter was Burwood wastewater treatment plant (WWTP). The main aim was to deploy *S. glomerata* in the receiving waters of Burwood WWTP (Burwood near and Burwood far) and at reference locations (Redhead, Fingal Island 1 and 2) for 6 weeks with subsequent measurement of heavy metals in the tissue. Past studies have identified, via the deployment of *S. glomerata* as a biomonitor, that locations receiving sewage effluent can have higher ambient concentrations of some metals compared to reference locations (Avery et al. 1996; Scanes, 1996). Therefore, it was hypothesised that there would be differences in concentrations of heavy metals measured in *S. glomerata* tissue between locations, with higher concentrations measured in individuals deployed at Burwood location. Heavy metals, particularly non-essential including cadmium, lead and mercury are identified to have high potential for bioaccumulation in molluscan species such as *S. glomerata* (Amiard et al. 1987; Boisson et al. 1998; Boisson et al. 2003; MacFarlane et al. 2006; Riisgard et al. 1985; Robinson et al. 2005). Therefore, it was hypothesised that non-essential metals would be most likely to bioaccumulate following exposure to *S. glomerata*. The final aim of this Chapter was to compare present heavy metal concentrations in *S. glomerata* to historic data of a similar study conducted in 1992-1994 (Ajani and Wansbrough, 1996).
6.3. Methods

6.3.1. Experimental Design

Sydney rock oysters, *S. glomerata*, were deployed at locations receiving sewage effluent from Burwood WWTP along with reference locations (experimental design explained in detail within Chapter 4). Briefly, Sydney rock oysters were deployed at 4 locations which included 2 locations receiving sewage effluent from Burwood WWTP and 2 reference locations. Within each location there were 2 deployment units. Each deployment unit had 2 bags of 40 oysters attached at 4, 8 and 12 metres depth. Unfortunately during the experiment, one deployment unit was lost from each of the locations Burwood near, Burwood far and Redhead and was not included in the analysis. Fingal Island was the only location with 2 deployment units remaining and for the purpose of analyses deployment units were treated as separate locations. The deployment period was 6 weeks. Past studies suggest that 6 weeks is an appropriate length of exposure for the equilibration of metals in molluscan tissue. For example, non-essential metals have been shown to equilibrate within shorter time frames including lead after 5 days in *M. mercenaria* (Boisson et al. 1998) and cadmium after 21 days in *C. gigas* (Boisson et al. 2003).

6.3.2. Preparation of oyster tissue for heavy metal analysis

Eight individuals were selected at random from each bag (two bags/depth). In order to gain sufficient tissue for analyses, tissue from two individuals within the same bag were combined. In total there were 4 replicates (of two pooled females) from each of the two bags at 4, 8 and 12 metres depths at all locations: Burwood near, Burwood far, Redhead, Fingal Island 1 and Fingal Island 2. Whole oyster tissue was excised into small (5 mm² cubes), placed into plastic weigh boats, weighed, wrapped in foil and placed into the freeze dryer for 24- 48 hours until the tissue was completely dry. Dried tissue was then ground to a fine powder within a mortar and pestle and dry weight was recorded.

6.3.2. Heavy metal analysis in dried oyster tissue

The selection of heavy metals was based on a past study performed by (Ajani and Wansbrough, 1996), where *S. glomerata* were also deployed at Burwood and reference locations assess heavy metal bioaccumulation. Metals for analysis included arsenic,
aluminium, cadmium, chromium, cobalt, copper, lead, manganese, mercury, nickel, selenium, silver and zinc.

Extraction of heavy metals was performed using a Hotblock Digestor at 120°C for 1 hour. Each sample consisted of 0.4 grams of dried oyster tissue which was digested with 10 mL concentrated Nitric Acid, which was made to a final volume of 25 mL prior to analysis. Quantification of arsenic, aluminium, cadmium, chromium, cobalt, copper, lead, manganese, mercury, nickel, selenium, silver and zinc was performed using a Perkin Elmer Elan DRC-e Inductively Coupled Plasma Mass Spectrometer. The method was verified using Institute for National Measurement (NIST) Certified Lobster Hepatopancreas Reference Material for trace metals (TORT-2) (Table 6.1). All metals had reasonable recoveries ranging from 107-182%, with the exception of chromium which was 316%. Due to the large recovery, chromium was excluded from results. All concentrations are expressed on a dry weight basis.

Table 6.1. Calculated recoveries based on ICPMS analysis of trace metals in certified Lobster Hepatopancreas Reference Material for trace metals (TORT-2).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Measured concentration (mg/kg, dry weight)</th>
<th>Certified reference concentration (TORT-2) (mg/kg, dry weight)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium</td>
<td>30.32 ± 2.27</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Arsenic</td>
<td>23.36 ± 0.10</td>
<td>21.60 ± 1.8</td>
<td>108.15</td>
</tr>
<tr>
<td>Cadmium</td>
<td>30.24 ± 0.16</td>
<td>26.70 ± 0.6</td>
<td>113.25</td>
</tr>
<tr>
<td>Chromium</td>
<td>2.43 ± 0.03</td>
<td>0.77 ± 0.15</td>
<td>316.23</td>
</tr>
<tr>
<td>Copper</td>
<td>114.32 ± 0.21</td>
<td>106.00 ± 10</td>
<td>107.85</td>
</tr>
<tr>
<td>Iron</td>
<td>173.89 ± 10.71</td>
<td>105.00 ± 13</td>
<td>165.61</td>
</tr>
<tr>
<td>Lead</td>
<td>0.64 ± 0.05</td>
<td>0.35 ± 0.13</td>
<td>182.52</td>
</tr>
<tr>
<td>Manganese</td>
<td>16.41 ± 0.15</td>
<td>13.60 ± 1.2</td>
<td>120.66</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.41 ± 0.00</td>
<td>0.27 ± 0.06</td>
<td>151.19</td>
</tr>
<tr>
<td>Nickel</td>
<td>2.76 ± 0.01</td>
<td>2.50 ± 0.19</td>
<td>110.59</td>
</tr>
<tr>
<td>Selenium</td>
<td>8.04 ± 0.02</td>
<td>5.63 ± 0.67</td>
<td>142.76</td>
</tr>
<tr>
<td>Silver</td>
<td>6.75 ± 0.02</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Zinc</td>
<td>234.16 ± 1.15</td>
<td>180.00 ± 6</td>
<td>130.09</td>
</tr>
</tbody>
</table>
6.3.3. Statistical analysis

6.3.3.1. Univariate Analysis

To test for significant differences in metal concentrations measured in *S. glomerata* tissue, two-way ANOVAs assessing effects of location and depth were not possible due to a missing replicate at 8 metres depth, Burwood far. Therefore, differences among locations were assessed in two ways. To determine if there were significant differences among locations (regardless of depth) in heavy metals concentrations in oysters, depths were pooled and one way ANOVAs were performed in STATISTICA (Statsoft, 2005) for each metal. To assess if there were significant differences among locations at each depth (4, 8, 12 metres), one way ANOVAs were performed in STATISTICA (Statsoft, 2005) for each metal at each depth.

6.3.3.2. Multivariate analysis

To identify if differences in accumulated metallic profiles in oysters among location and depth were evident, ordination of metals were performed using non-metric multidimensional scaling (MDS) in PRIMER 6 software (Clarke and Warwick, 1993) for a) all metals at each depth among locations and b) non-essential metals at each depth among locations. Ordinations were constructed based on ranked matrices of dissimilarities between samples, employing the square root transformation Euclidean distance, as a measure of dissimilarity. Goodness of fit (stress) of the resulting two-dimensional plots was measured using Kruskal’s stress formula (Kruskal and Wish, 1978). All generated lots were found to have a stress value lower than the maximum value (0.3) recommended by Sturrock and Rocha (2000) for 2 dimensional nMDS plots.

Differences among locations and depths were assessed via a two-way analysis of similarity in PRIMER 6 software (ANOSIM; Clarke and Warwick, 1993). ANOSIM analyses for differences among locations, contrasted with differences among replicates within locations, based on Euclidean ranked dissimilarities. Significant differences among locations were assessed using multiple pairwise comparisons. ANOSIM were performed to assess for significant differences in metal concentrations among locations for a) all metals at each depth and b) non-essential metals at each depth.
6.4. Results

6.4.1. Summary of ANOVA comparisons of trace metal concentrations in oysters among locations

Under the hypothesis that sewage effluent is a potential source of heavy metals, metal concentrations in *S. glomerata* oyster tissue were compared among deployment locations (with depths pooled) to determine if individuals at outfall locations, Burwood near and Burwood far, displayed significantly greater (*p* < 0.05) concentrations compared to individuals at reference locations.

Significant differences were detected in oyster tissue among locations for nearly all heavy metals analysed (Table 6.2), however, post hoc analysis (via Tukeys) revealed that the majority of these differences were confined to significant differences among reference locations. Arsenic and selenium were the only metals where concentrations were significantly greater (*p* < 0.05) at the Burwood near location compared to some reference locations. Mean arsenic concentrations in oysters deployed at Burwood near were significantly greater (*p* < 0.05) compared to those from both Fingal Island locations. Similarly, the concentration of selenium in oysters from Burwood near was significantly greater (*p* <0.05) compared to concentrations in oysters from Fingal Island 2. Despite this, both arsenic and selenium in oysters at Burwood near were significantly similar (*p* > 0.05) to Burwood far and at least one reference location, implying that Burwood locations are within the bounds of natural variability in terms of accumulation of metals to oysters for the region.
Table 6.2. Summary of one-way ANOVAs of trace metal concentrations (mg/kg) in whole oyster tissue among locations (depths pooled). Abbreviations for locations are as follows; Burwood near (BN), Burwood far (BF), Redhead (RH), Fingal Island 1 (F1) and Fingal Island 2 (F2). Average and standard error are indicated for each location (depths pooled) and letters above indicate the results of the post hoc analysis, Tukeys.

<table>
<thead>
<tr>
<th>Metal</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>BN</th>
<th>BF</th>
<th>RH</th>
<th>F1</th>
<th>F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>4, 103</td>
<td>0.95</td>
<td>0.24</td>
<td>10.24</td>
<td>&lt;0.05</td>
<td>11.13 ± 0.39 &lt;a&gt;</td>
<td>9.73 ± 0.45 ab</td>
<td>11.19 ± 0.26 &lt;a&gt;</td>
<td>9.32 ± 0.3 &lt;b&gt;</td>
<td>8.74 ± 0.36 &lt;b&gt;</td>
</tr>
<tr>
<td>Aluminum</td>
<td>4, 103</td>
<td>14.04</td>
<td>3.51</td>
<td>9.72</td>
<td>&lt;0.05</td>
<td>74.05 ± 23.75 &lt;a&gt;</td>
<td>60.82 ± 13.96 &lt;a&gt;</td>
<td>88.32 ± 13.97 ab</td>
<td>125.77 ± 13.4 ab</td>
<td>130.06 ± 18.78 ab</td>
</tr>
<tr>
<td>Cadmium</td>
<td>4, 103</td>
<td>2.86</td>
<td>0.71</td>
<td>45.14</td>
<td>&lt;0.05</td>
<td>1.03 ± 0.03 &lt;a&gt;</td>
<td>1.2 ± 0.06 &lt;a&gt;</td>
<td>1.9 ± 0.09 b</td>
<td>1.94 ± 0.08 b</td>
<td>1.97 ± 0.09 b</td>
</tr>
<tr>
<td>Copper</td>
<td>4, 103</td>
<td>0.73</td>
<td>0.18</td>
<td>2.56</td>
<td>&lt;0.05</td>
<td>78.9 ± 2.55 &lt;a&gt;</td>
<td>78.35 ± 4.19 &lt;a&gt;</td>
<td>78.45 ± 4.23 &lt;a&gt;</td>
<td>66.12 ± 3.9 &lt;a&gt;</td>
<td>71.01 ± 4.84 &lt;a&gt;</td>
</tr>
<tr>
<td>Lead</td>
<td>4, 103</td>
<td>0.15</td>
<td>0.04</td>
<td>2.61</td>
<td>&lt;0.05</td>
<td>0.52 ± 0.03 ab</td>
<td>0.51 ± 0.03 ab</td>
<td>0.68 ± 0.07 a</td>
<td>0.53 ± 0.03 ab</td>
<td>0.5 ± 0.04 b</td>
</tr>
<tr>
<td>Iron</td>
<td>4, 103</td>
<td>1.02</td>
<td>0.25</td>
<td>2.88</td>
<td>&lt;0.05</td>
<td>140.67 ± 5.66 &lt;a&gt;</td>
<td>165.01 ± 17.89 ab</td>
<td>173.25 ± 21.76 ab</td>
<td>183.46 ± 12.99 ab</td>
<td>190.7 ± 16.99 b</td>
</tr>
<tr>
<td>Manganese</td>
<td>4, 103</td>
<td>0.42</td>
<td>0.10</td>
<td>0.66</td>
<td>0.62</td>
<td>17.46 ± 1.44</td>
<td>15.43 ± 1.19</td>
<td>15.71 ± 1.05</td>
<td>15.44 ± 0.98</td>
<td>14.31 ± 1.25</td>
</tr>
<tr>
<td>Mercury</td>
<td>4, 103</td>
<td>0.01</td>
<td>0.00</td>
<td>3.99</td>
<td>&lt;0.05</td>
<td>0.05 ± 0 a</td>
<td>0.04 ± 0 a</td>
<td>0.06 ± 0 ab</td>
<td>0.06 ± 0 ab</td>
<td>0.06 ± 0.01 b</td>
</tr>
<tr>
<td>Nickel</td>
<td>4, 103</td>
<td>0.77</td>
<td>0.19</td>
<td>3.18</td>
<td>&lt;0.05</td>
<td>0.7 ± 0.04 a</td>
<td>1.43 ± 0.33 b</td>
<td>0.94 ± 0.09 ab</td>
<td>1.09 ± 0.1 ab</td>
<td>1.04 ± 0.1 ab</td>
</tr>
<tr>
<td>Selenium</td>
<td>4, 103</td>
<td>0.45</td>
<td>0.11</td>
<td>15.76</td>
<td>&lt;0.05</td>
<td>4.34 ± 0.09 &lt;a&gt;</td>
<td>4.11 ± 0.12 ab</td>
<td>4.92 ± 0.12 c</td>
<td>4.12 ± 0.08 ab</td>
<td>3.86 ± 0.08 ab</td>
</tr>
<tr>
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<td>0.02</td>
<td>3.32</td>
<td>&lt;0.05</td>
<td>0.39 ± 0.02 a</td>
<td>0.36 ± 0.02 a</td>
<td>0.39 ± 0.03 a</td>
<td>0.31 ± 0.02 a</td>
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</tr>
<tr>
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<td>0.15</td>
<td>2.52</td>
<td>&lt;0.05</td>
<td>1885.14 ± 58.14 a</td>
<td>1899.81 ± 107.41 a</td>
<td>1795.36 ± 92.38 a</td>
<td>1586.02 ± 80.13 a</td>
<td>1713.58 ± 104.1 a</td>
</tr>
</tbody>
</table>
6.4.2. Summary of ANOVA comparisons of trace metal concentrations in oysters among locations and depths

Metal exposure and uptake may vary according to depth of deployment (a previous study of plume dynamics at Burwood WWTP suggested that, following effluent release, there is variability in the concentration of effluent at different depths and distances from the point of release (Glamore et al. 2007)). Thus, concentrations were compared via one way ANOVAs for each depth of deployment to determine if metals were significantly greater at Burwood locations compared to reference locations (Figures 6.1-6.12).

Of particular concern were heavy metals that are non-essential and perhaps more likely to bioaccumulate, such as cadmium, lead and mercury. While it was hypothesised that these non-essential metals may be significantly greater in oyster tissue at Burwood locations compared to reference locations, this was not found for most of these particular metals. In contrast, it was found that cadmium concentrations were significantly greater (p<0.05) in oyster tissue at reference locations compared to Burwood locations, for all depths (Figure 6.3). Mercury concentrations were also found to be significantly greater (p<0.05) at reference locations compared to Burwood locations for 4 and 12 metres (Figure 6.8). The only difference detected for lead was higher concentrations at Redhead compared to Fingal Island locations at 4 metres (Figure 6.5). For the remaining metals, there were also no trends in heavy metal concentrations to indicate that oysters at Burwood had accumulated higher concentrations compared to oysters at all reference locations.

For comparison, available NSW median background concentrations of heavy metals were included on figures, apart from nickel, selenium and lead, all metals analysed were lower in comparison (Scanes and Roach, 1999).
6.4.2.1. Aluminium

Concentrations of aluminium in *S. glomerata* tissue were highest at Fingal Island reference locations, which were significantly higher than Burwood near (4 and 12 metres) and Burwood far (4 metres) (4 metres: \( df = 4, F = 8.79, p < 0.05 \) and 12 metres: \( df = 4, F = 3.93, p < 0.05 \)) (Figure 6.1). There were no significant differences detected at 8 metres (\( df = 3, F = 1.01, p > 0.05 \)).

![Figure 6.1: Comparison of concentrations of aluminium (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.](image)
6.4.2.2. Arsenic

At 4 and 8 metres depth, concentrations of arsenic were highest in *S. glomerata* deployed at Burwood near, however, this was statistically similar to all locations except for Fingal Island 2 (4 metres: \( df = 4, F=3.04, p< 0.05 \) and 8 metres: \( df = 3, F=8.96, p< 0.05 \)) (Figure 6.2). At 12 metres depth, there was variability among reference locations with significantly higher concentrations of arsenic at Redhead compared to Fingal Island 1 and Fingal Island 2 (\( df = 4, F= 3.76, p< 0.05 \)) (Figure 6.2). These differences are likely to be attributed to natural variability. All arsenic concentrations were lower than the NSW median background concentration determined by Scanes and Roach (1999).

![Arsenic concentration chart](chart.png)

**Figure 6.2.** Comparison of concentrations of arsenic (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal...
Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.

6.4.2.3. Cadmium

Concentrations of cadmium were low across all locations in comparison to the median NSW background concentration identified by Scanes and Roach. (1999). Furthermore, concentrations of cadmium were significantly lower in S. glomerata tissue at both Burwood locations in comparison to all reference locations at all depths (4 metres: df = 4, F=22.77, p< 0.05, 8 metres: df = 3, F=52.89, p< 0.05 and 12 metres: df = 4, F=15.99, p< 0.05) (Figure 6.3).

![Cadmium concentrations comparison](image)

**Figure 6.3.** Comparison of concentrations of cadmium (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.4. Copper

Concentrations of copper detected in *S. glomerata* tissue were again much lower in comparison to the median NSW background concentration. For all depths, no significant differences were detected among locations for copper concentrations (4 metres: \( df = 4, F = 2.44, p > 0.05 \), 8 metres: \( df = 3, F = 3.8, p > 0.05 \) and 12 metres: \( df = 4, F = 2.37, p > 0.05 \)) (Figure 6.4).

![Bar chart with copper concentrations](chart.png)

**Figure 6.4.** Comparison of concentrations of copper (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.5. Lead

Concentrations of lead were higher in comparison to the median NSW background concentration at 12 metres (all reference locations) and at 4 metres (Redhead). Furthermore at 4 metres depth, concentrations of lead in *S. glomerata* tissue were significantly higher at Redhead compared to the other Fingal Island reference locations (*df* = 4, *F* = 5.33, *p* < 0.05) (Figure 6.5). No differences among locations were observed at 8 metres and 12 metres depths (8 metres: *df* = 3, *F* = 0.02, *p* > 0.05 and 12 metres: *df* = 4, *F* = 1.53, *p* > 0.05) (Figure 6.5).

**Figure 6.5.** Comparison of concentrations of lead (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.6. Iron

At 8 metres, concentrations of iron in *S. glomerata* tissue were significantly higher at Redhead and Fingal Island 2 in comparison to Burwood near (*df* = 3, *F* = 5.29, *p* < 0.05). Also at 12 metres, concentrations of iron in *S. glomerata* tissue were significantly higher at both Fingal Island reference locations in comparison to both Burwood locations and Redhead (*df* = 4, *F* = 12.54, *p* < 0.05) (Figure 6.6). There were no significant differences detected among locations for iron at 4 metres depth (*df* = 4, *F* = 0.23, *p* > 0.05) (Figure 6.6).

![Iron concentration graph](image)

**Figure 6.6.** Comparison of concentrations of iron (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.7. Manganese

At 8 metres depth, concentrations of manganese were significantly higher at Burwood near compared to Fingal Island 2 but still comparable to the median NSW background concentration ($df = 3$, $F= 7.96$, $p< 0.05$) (Figure 6.7). There were no significant differences detected among locations for manganese at 4 metres ($df = 4$, $F= 0.25$, $p> 0.05$) or 12 metres depth ($df = 4$, $F= 0.85$, $p> 0.05$) (Figure 6.7).

![Manganese concentration graph](https://via.placeholder.com/150)

**Figure 6.7.** Comparison of concentrations of manganese (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.8. Mercury

All concentrations of mercury were lower in comparison to the median NSW background concentration. At 4 metres depth, concentrations of mercury in *S. glomerata* tissue were significantly higher (p< 0.05) at Redhead in comparison to Burwood far (df = 4, F=3.31, p< 0.05) (Figure 6.8). At 12 metres depth, concentrations of mercury in *S. glomerata* tissue at Fingal Island 2 were significantly higher than Redhead and both Burwood locations (df = 4, F= 6.48, p< 0.05) (Figure 6.8). No significant differences were detected among locations for mercury at 8 metres depth (df = 3, F= 1.39, p> 0.05) (Figure 6.8).

![Figure 6.8](image)

**Figure 6.8.** Comparison of concentrations of mercury (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.9. Nickel

Most concentrations of nickel were lower in comparison to the median NSW background concentration, except for nickel in *S. glomerata* from Burwood far (12 metres) which was approximately 2 fold that of the NSW background concentrations. However, this was only significantly higher compared to one reference location only, Redhead (df = 4, F= 4.39, p< 0.05) (Figure 6.9). Interestingly, the opposite was found at 4 metres depth whereby concentrations of nickel in *S. glomerata* tissue were higher at Fingal Island 1 in comparison to Burwood far (df = 4, F= 2.84, p< 0.05) (Figure 6.9). No significant differences were detected among locations for nickel at 8 metres depth (df = 3, F= 2.46, p> 0.05) (Figure 6.9).

![Figure 6.9. Comparison of concentrations of nickel (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth) . At 8m depth, BF replicates were not available for sampling.](image-url)
6.4.2.10. Selenium

For selenium, most concentrations measured exceeded that of the median NSW background concentration. Concentrations were highest at Redhead, significantly higher than all other locations at 4 and 12 metres (4 metres: \( df = 4, F= 12.22, p< 0.05 \) and 12 metres: \( df = 4, F= 6.65, p< 0.05 \) ) (Figure 6.10). At 8 metres, concentrations at Burwood near were significantly higher than Fingal Island 2 (\( df = 3, F= 3.79, p< 0.05 \) ) (Figure 6.10).

![Figure 6.10](image_url)

**Figure 6.10.** Comparison of concentrations of selenium (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.2.11. Silver

For silver, concentrations in *S. glomerata tissue* were highest at Redhead in comparison to both Burwood locations and Fingal Island 2 at 4 metres (*df* = 4, *F* = 5.77, *p* < 0.05) (Figure 6.11). At 8 metres, concentrations of silver in *S. glomerata tissue* at Burwood near were significantly higher in comparison to Redhead and Fingal Island 1 (*df* = 3, *F* = 4.91, *p* < 0.05) (Figure 6.11). There were no significant differences among locations for silver at 12 metres depth (*df* = 4, *F* = 2.12, *p* > 0.05) (Figure 6.11).

![Figure 6.11. Comparison of concentrations of silver (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.](image)

*BN* = Burwood near  
*BF* = Burwood far  
*RH* = Redhead  
*F1* = Fingal Island 1  
*F2* = Fingal Island 2
6.4.2.12. Zinc

All concentrations of zinc were lower in comparison to the median NSW background concentration. For all depths, no significant differences were detected among locations for zinc concentrations (4 metres: \( df = 4, \ F = 1.85, \ p > 0.05 \), 8 metres: \( df = 3, \ F = 2.54, \ p > 0.05 \) and 12 metres: \( df = 4, \ F = 0.74, \ p > 0.05 \)) (Figure 6.12).

![Zinc concentrations graph](image)

**Figure 6.12.** Comparison of concentrations of zinc (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks. Mean ± SE (N= 8 per treatment/depth). At 8m depth, BF replicates were not available for sampling.
6.4.3. Multivariate analysis of metal concentrations at locations and depths for all metals

6.4.3.1. nMDS of heavy metals among locations and depths

Non-metric Multidimensional Scaling (nMDS) is useful to compare similarities among groups and was used to make comparisons of similarities in the suite of heavy metal concentrations in oysters deployed at 5 locations at all depths (Figure 6.13). Two-way analysis of similarities (ANOSIM) indicated that there was a significant difference among locations (Global R= 0.12, p< 0.001), but not between depths (R= 0.04, p> 0.05). Pair wise tests among the 5 locations indicated that heavy metal concentrations at Burwood near were significantly dissimilar to all three reference locations: Redhead (R= 0.08, p< 0.05), Fingal Island 1 (R= 0.28, p< 0.001) and Fingal Island 2 (R= 0.22, p< 0.001). This is likely to be due to the fact that metals at the reference locations were significantly higher than Burwood near for many metals in the previous univariate ANOVA analyses (as opposed to elevated metals due to contamination). Furthermore, Burwood far was significantly different to Fingal Island 1 (R= 0.18, p<0.005) but similar to Burwood near and the other two reference locations: Fingal Island 2 and Redhead.

Figure 6.13. MDS analysis of heavy metals (silver, arsenic, lead, cadmium, chromium, copper, manganese, nickel, selenium, zinc, mercury, iron and aluminium) by location. Metals (mg/kg dry weight) were measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at three depths (4, 8 and 12 metres) in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.3.2. nMDS of heavy metals among locations at 4 metres depth

Separate MDS analyses were performed for each depth to determine if there were differences between locations for each depth (Figure 6.14). Analysis of the nMDS plot of metal concentrations of locations at 4 metres depth reveals that locations are overlapping visually indicating some similarity in metallic composition, and further that there was high variability among replicates within locations. Regardless, one-way ANOSIM analysis indicated that there was a significant difference (Global R= 0.13, p< 0.01). Pairwise comparisons revealed that heavy metal concentrations in oysters at Burwood near were significantly different to Fingal Island 1 (R= 0.22, p< 0.05) and Fingal Island 2 (R= 0.42, p< 0.001). Burwood far was also significantly different to Fingal Island 2 (R= 0.24, p< 0.05). A significant difference was also found between two reference locations, Redhead and Fingal Island 2 (R= 0.18, p< 0.05).

Figure 6.14. MDS analysis of heavy metals (silver, arsenic, lead, cadmium, chromium, copper, manganese, nickel, selenium, zinc, mercury, iron and aluminium) (mg/kg dry weight) in measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at 4 metres depth in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.3.3. nMDS of heavy metals among locations at 8 metres depth

The results of the nMDS of metal concentrations in oysters at 8 metres indicate similarities in the metallic profile among locations (Figure 6.15). One-way ANOSIM analysis confirmed there were no significant differences among locations (Global R= 0.11, p> 0.05).

**Figure 6.15.** MDS analysis of heavy metals (silver, arsenic, lead, cadmium, chromium, copper, manganese, nickel, selenium, zinc, mercury, iron and aluminium) (mg/kg dry weight) in measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at 8 metres depth in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.3.3. nMDS of heavy metals among locations at 12 metres depth

The results of the nMDS at 12 metres indicate a similar distribution of metals in oyster tissue among locations (Figure 6.16). One-way ANOSIM analysis indicated there were no significant differences among locations (Global $R=-0.02$, $p>0.05$).

Figure 6.16. MDS analysis of heavy metals (silver, arsenic, lead, cadmium, chromium, copper, manganese, nickel, selenium, zinc, mercury, iron and aluminium) (mg/kg dry weight) in measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed at 12 metres depth in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.4. Multivariate analysis of metal concentrations at locations and depths for non-essential metals

As non-essential metals tend to bioaccumulate, differential accumulation at impact locations compared to reference locations is more likely to be detected when examining the combination of such metals alone rather than the complete metal dataset where the regulation of essential metals may mask differences among locations despite differential exposure. Multivariate analysis of accumulating non-essential metals, lead, cadmium and mercury (Amiard, 1987; Depledge and Rainbow, 1990) via nMDS plot and ANOSIM revealed that in most cases, both Burwood locations were significantly different (p< 0.05) from all reference locations. Univariate analysis of differences among locations and graphs (Figures 6.1- 6.12) for these metals revealed that concentrations at reference locations were usually higher compared to Burwood locations and this is again demonstrated by the nMDS plots and ANOSIM analysis.

6.4.4.1. nMDS of non-essential heavy metals among locations at 4 metres depth

Analysis of the nMDS plot of concentrations of non-essential metals including lead, cadmium and mercury for locations at 4 metres depth revealed some dissimilarity among locations, with impact locations clustering separately from references locations (Figure 6.17). One-way ANOSIM analysis indicated that there was a significant difference (Global R= 0.49, p< 0.01). Pairwise comparisons revealed that heavy metal concentrations in oysters at Burwood near were significantly different to all reference locations Redhead (R= 0.53, p< 0.01), Fingal Island 1 (R= 0.93, p< 0.01) and Fingal Island 2 (R=0.67, p<0.01), but similar to Burwood far (R= -0.022, p> 0.05). Similarly, Burwood far was different to all reference locations Redhead (R= 0.54, p< 0.01), Fingal Island 1 (R= 0.94, p< 0.01) and Fingal Island 2 (R=0.70, p<0.01). However, differences are likely to be attributed to higher concentrations of non-essential metals detected at reference locations (as opposed to elevated concentrations at Burwood locations). Natural variability was high, some differences also existed among reference locations, Fingal Island 2 was different to Redhead (R=0.26, p<0.01) and Fingal Island 1 (R=0.38, p<0.01).
Figure 6.17. MDS analysis of non-essential heavy metals (lead, cadmium and mercury) at 4 metres depth. Metals (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.4.2. nMDS of non-essential heavy metals among locations at 8 metres depth

Analysis of the nMDS plot of concentrations of non-essential metals locations at 8 metres depth revealed that reference and impact locations were somewhat different for this suite of metals, again clustering separately (Figure 6.18). One-way ANOSIM analysis indicated that there was a significant difference between locations (Global $R= 0.73$, $p< 0.01$). Pairwise comparisons revealed that heavy metal concentrations in oysters at Burwood near were significantly different to all reference locations Redhead ($R= 0.96$, $p< 0.01$), Fingal Island 1 ($R= 0.95$, $p< 0.01$) and Fingal Island 2 ($R=0.97$, $p< 0.01$). Similar to 8 metres results, natural variability was high, some differences also existed among reference locations, Fingal Island 2 was different to Redhead ($R=0.32$, $p< 0.01$) and Fingal Island 1 ($R=0.65$, $p< 0.01$).

![Figure 6.18. MDS analysis of non-essential heavy metals (lead, cadmium and mercury) at 8 metres depth.](image)

Metals (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.4.3. **nMDS of non-essential heavy metals among locations at 12 metres depth**

Analysis of the nMDS plot of concentrations of non-essential metals including lead, cadmium and mercury for locations at 12 metres depth revealed some dissimilarity between Burwood versus reference locations (Figure 6.19). Results were very similar to the other depths except that no differences existed among reference locations. One-way ANOSIM analysis indicated that there was a significant difference (Global R= 0.31, p< 0.01). Pairwise comparisons revealed that heavy metal concentrations in oysters at Burwood near were significantly different all reference locations Redhead (R= 0.57, p< 0.01), Fingal Island 1 (R= 0.79, p< 0.01) and Fingal Island 2 (R=0.82, p< 0.01). Burwood far was different to Fingal Island 1 (R= 0.41, p< 0.01) and Fingal Island 2 (R=0.34, p< 0.01), but similar to Redhead (R=0.15, p> 0.01).

![Figure 6.19](image-url)

**Figure 6.19.** MDS analysis of non-essential heavy metals (lead, cadmium and mercury) at 12 metres depth. Metals (mg/kg dry weight) measured in dried tissue samples of Sydney rock oyster, *Saccostrea glomerata*, deployed in sewage impacted locations (Burwood near and Burwood far) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) for 6 weeks.
6.4.4. Comparisons of heavy metal concentrations in oyster tissue to historical data in sediment and *S. glomerata* individuals at Burwood

In a previous study by Ajani and Wansbrough (1996), heavy metal concentrations were assessed in sediments and in *S. glomerata* deployed in the receiving waters of Burwood WWTP at Burwood near (within 50m of outfall; which corresponds with the Burwood near location employed for the current study). The methodology of this study was similar to the present study with the exception of a longer deployment period, 12 weeks (compared to 6 weeks in present study). It was considered that 6 weeks would be a sufficient period for the equilibration of metals to oyster tissue thus comparisons between studies are possible (Boisson et al. 1998; Boisson et al. 2003).

Concentrations of heavy metals in *S. glomerata* were compared to historic data and it was found that minimum and maximum values were consistently similar and/or lower (Table 6.3). Mean concentrations of heavy metals were also compared to available National Food Authority Maximum Residue Limits (NFA MRLs) (National Food authority, 1992). With the exception of arsenic, all mean concentrations were below NFA MRLs.
Table 6.3. Comparison of present concentrations of heavy metals measured in whole tissue of *S. glomerata* deployed at Burwood near to a) historic data of heavy metal concentrations in *S. glomerata* also deployed within 50m of Burwood outfall, b) heavy metal concentrations in sediment collected within 50 metres of Burwood outfall and c) National Food Authority Maximum Residue Limits (MRLs) for trace metals in oysters. Burwood near is represented by BN.

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<th>a) Concentration in oysters, BN (mg/kg, wet weight) (Ajani and Wansbrough, 1996)</th>
<th>b) Concentration in sediment, BN (mg/kg, wet weight) (Ajani and Wansbrough, 1996)</th>
<th>c) MRL for oysters mg/kg wet weight (NFA, 1992)</th>
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<td></td>
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<tr>
<td>Mercury</td>
<td>0.005</td>
<td>0.003-0.008</td>
<td>0.004- 0.01</td>
<td>0.01- 0.02</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.08</td>
<td>0.05- 0.13</td>
<td>0.13- 0.27</td>
<td>0.69- 5.65</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.48</td>
<td>0.38- 0.57</td>
<td>0.74- 1.33</td>
<td>0- 0.1</td>
</tr>
<tr>
<td>Silver</td>
<td>0.043</td>
<td>0.03-0.06</td>
<td>0.05- 0.95</td>
<td>0.1- 0.26</td>
</tr>
<tr>
<td>Zinc</td>
<td>209.82</td>
<td>162.31- 265.87</td>
<td>285.29- 689</td>
<td>3.86- 23.33</td>
</tr>
</tbody>
</table>

* Data represents a mean value for heavy metal concentration in oyster tissue from the Burwood near location (pooled across depths). Data from present study has been converted to mg/kg wet weight for the purpose of comparisons to historic data.
6.5. Discussion

Comparisons of most heavy metal concentrations in oyster tissue provided contrary evidence to the hypothesis that concentrations of heavy metals would be greatest in oysters deployed in receiving waters of Burwood WWTP due to sewage effluent being a potential source of aquatic heavy metal exposure. In general, concentrations of heavy metals at all locations were low and similar to median background levels detected in oysters in Australian waters. Although in some instances differences were detected between Burwood locations and select reference locations, metals in oysters at Burwood locations were not consistently elevated compared to all reference locations, suggesting metal exposure and accumulation at Burwood was within the boundaries of natural variation observed for the region. For all metals, including those which are non-essential and were expected to accumulate, there was no significant elevation at Burwood locations in comparison to all three reference locations suggesting that during the experimental period, oysters at Burwood locations were unlikely to have been exposed to elevated levels of metallic contaminants. Differences between locations were revealed by multivariate analysis, including nMDS plots and ANOSIM, which were performed for each separate depth using all metals and again using only non-essential metals. The results of these analyses demonstrated that there were significant differences between Burwood locations and all reference locations. However, these differences are likely to be due to the fact that concentrations of many metals, including non-essential metals cadmium, lead and mercury, were higher at reference locations in comparison to Burwood near or Burwood far. Importantly for all metals, including non-essential, there were none that were significantly higher at Burwood locations in comparison to all reference locations suggesting that concentrations of metals at Burwood were within the boundaries of natural and spatial variation.

In previous studies, accumulative biomonitors, such as deployed oysters, have been useful in characterising contaminated locations via comparisons of accumulated heavy metal concentrations (Ajani and Wansbrough, 1996; Scanes 1996). It was hypothesised that non-essential metals would be most likely to bioaccumulate in oyster tissue compared to essential metals. In this study, it was found that there were no significant differences found
among locations for oyster concentrations of essential metals, copper and zinc. This suggests a number of scenarios; that a) concentrations of zinc and copper have been regulated by *S. glomerata* and therefore have not reflected differences in metal loadings, b) there were no differences among locations for these metals and/or c) exposure concentrations did not exceed the minimum required concentration for essential needs. Without testing of environmental concentrations, it is difficult to truly determine whether zinc and copper have been regulated or if they were present at low environmental concentrations.

While oysters have been demonstrated to bioaccumulate essential metals such as, zinc and copper (Brown and McPherson, 1992; George et al. 1978; Phillips, 1995; Phillips and Yim, 1981; Phillips and Rainbow, 1993; Spooner et al. 2003), it has been suggested that molluscs may exhibit a degree of homeostasis for these metals depending on the exposure concentration. Essential metals also have a minimum metabolic requirement and as suggested by Langston et al. 1998 (pg. 233) “copper concentrations may be buffered according to the requirements for the copper containing pigment, haemocyanin”. Concentrations of zinc and copper detected in *S. glomerata* tissue in individuals from Burwood were similar to concentrations from reference locations and were within the range of background concentrations detected in *S. glomerata* within NSW (Scanes and Roach, 1999). This may suggest that for both copper and zinc a) environmental concentrations were similar among locations or b) *S. glomerata* has been exposed to zinc and/or copper but was able to regulate internal concentrations. As significant differences were detected among locations for most other metals, including non-essential metals, it is possible that *S. glomerata* was able to regulate copper and zinc.

Spatial variation in metal concentrations poses difficulties in the measurement of trace metals and the capacity to differentiate between background concentrations and elevated concentrations due to anthropogenic input (Cantillo, 1997; Phillips and Rainbow, 1993; Scanes and Roach, 1999). Therefore, to determine if metal concentrations are significantly elevated at a location, it is useful to compare data to concentrations from reference locations within the same region. Scanes and Roach (1999) calculated background heavy
metal concentrations for *S. glomerata* for 12 locations in NSW which were identified as having a low risk of metal contamination. All mean concentrations of metals in the current study were considerably lower than NSW median background concentrations determined by Scanes and Roach (1999) both at impact and reference locations, with the exception of nickel, selenium and lead. Nickel, selenium and lead exceeded NSW median background concentrations at both Burwood and reference locations but it may be likely that background concentrations of these metals are higher for Newcastle and Port Stephens in comparison to other NSW locations, although there are likely to be some differences as the locations assessed by Scanes and Roach (1999) were estuarine rather than marine locations. For example, Cole (1990) detected similar concentrations of a similar suite of metals in an assessment of heavy metals in fish species, the red morwong, *Cheilddactylus fuscus* and the blue groper, *Achoerodus viridis* in several locations across Newcastle which included Burwood. It was found that concentrations of lead and selenium were detected at similar concentrations in fish in all Newcastle locations, whereas only nickel was elevated at Burwood (Cole, 1990).

With the exception of arsenic, all metals fell below available National Food Authority Authority’s Maximum Residue Limits (MRLs) for concentrations of heavy metals permitted for human consumption of molluscs (National Food Authority, 1992). Arsenic was consistently higher for all locations, but lower than median NSW background concentrations (Scanes and Roach, 1999). However, arsenic has also been detected in similar or higher concentrations for Newcastle locations in fish, *C. fuscus, A. viridis* (Cole et al. 1990) and in *S. glomerata* (Ajani and Wansbrough, 1996). Furthermore, arsenic concentrations in *S. glomerata* from background locations in NSW were also found to exceed the National Food Authority MRL and mean overseas concentrations (Scanes and Roach, 1999). Scanes and Roach (1999) suggested that Australia may be likely to contain higher natural soil concentrations of arsenic.

Concentrations of metals in *S. glomerata* from this study were compared to historic data. An earlier study by Ajani and Wansbrough (1996) similarly deployed *S. glomerata* at Burwood near location (within 50 metres of WWTP) and at reference locations for 3
months which was repeated over 8 sampling periods between 1992-1994, differing from the present study with a longer deployment period (3 months). However, comparisons of present heavy metals in *S. glomerata* deployed at the Burwood near location demonstrate that all metal concentrations are lower than historic data. Several changes in treatment technology to Burwood WWTP has occurred during and since the study of Ajani and Wansbrough (1996) which may have contributed to an improvement in metals removal during the sewage effluent treatment process. In 1992 the plant was upgraded from primary to secondary treatment (CH2M-Hill and Hunter Water Corporation, 2007), which is considered more efficient at removing heavy metals from effluent (Brown et al. 1973; Oliver and Cosgrove, 1974) and in the long term this may have contributed to a decrease in environmental metallic concentrations. Furthermore, in 2004, the point of release of sewage sludge was transferred from the effluent pipe to a separate diffuser pipe (CH2M-Hill and Hunter Water Corporation, 2007) and therefore *S. glomerata* in the current study were less likely to be exposed to sewage sludge compared to *S. glomerata* which were also situated near the effluent pipe in Ajani and Wansbrough (1996). Changes in the usage of metals since 1992 may have also decreased input of metals into the sewage effluent treatment process, for example, the phasing out of lead from petrol since 1985-86 (Spooner et al. 2003). Many other factors can also influence the rate of metal uptake by oysters which potentially contribute to variability among studies, even those which employ the same species as a biomonitor. This could include factors that are biological such as age, sex, size, feeding, gonadal development and/or pre-exposure history to metals (Ayling, 1974; Boening, 1999) or environmental such as organic carbon, temperature, pH, dissolved oxygen and/or hydrologic features such as sewage plume dynamics (Elder and Collins, 1991).

In conclusion, this Chapter has demonstrated that concentrations of most heavy metals were not significantly different (*p >0.05*) in *S. glomerata* deployed at Burwood compared to reference locations. Concentrations of heavy metals were similar to those which have been detected in previous studies in NSW. All metals fell below National Food Authority MRLs, except for arsenic and this does not appear uncommon for concentrations in biota within
NSW. Furthermore, comparisons to historic data suggest that concentrations of metals in sewage effluent have not increased in the last two decades.
Chapter 7: Discussion

7.1. Thesis objectives revisited
This Chapter will attempt to evaluate findings from experimental Chapters and make comment on the utility of *S. glomerata* as a biomonitor of estrogenic contaminants, via assessment of biomarkers of exposure and effect. The assessment of *S. glomerata* as a potential biomonitoring species for estrogenic compounds was achieved via a combination of laboratory investigations and field validation addressed under the following objectives:

1. To explore the dose response and temporal relationship between 17α-Ethynylestradiol (EE2; a potent estrogenic compound) exposure, vitellogenin induction and gonadal development
2. To develop a sensitive real-time qPCR assay for measuring vitellogenin gene expression in *S. glomerata*
3. To assess whether sewage effluent released from Burwood wastewater treatment plant contained estrogenic compounds
4. To assess the capability of *S. glomerata* as a biomonitor of estrogenic contaminants via deployment in waters receiving sewage effluent
5. To assess the presence of a suite of heavy metals in *S. glomerata* deployed in waters receiving sewage effluent

7.2. Evidence to support *S. glomerata* as a biomonitor of estrogenic compounds in the Australian marine environment
This thesis has attempted to collect evidence to determine if the Sydney rock oyster, *Saccostrea glomerata*, is suitable as a biomonitor of estrogenic compounds via validation of biomarkers for the impact assessment and quantification of estrogenic compounds in the marine environment. Aims and hypotheses of experimental Chapters were designed to validate and test proposed biomarkers of estrogenic exposure in *S. glomerata*. Selected biomarkers encompassed biological changes at both the cellular (vitellogenin) and
organism (sex and gonadal development) level and these are discussed below in relation to the criteria of reliable biomarkers.

7.2.1. Demonstrated biomarker cause and effect relationship within controlled laboratory conditions

Initially (Chapter 1), it was proposed that ideally, selected biomarkers would respond to estrogenic exposure in a dose response fashion (Huggett et al. 1992). Chapters 2 and 3 were designed to test this criterion.

Chapter 2 reported on a laboratory exposure of *S. glomerata* to a concentration gradient of 17α-ethynylestradiol (EE2) with subsequent measurement of vitellogenin, gonadal development and sex after 4, 21 and 49 days exposure. Vitellogenin was found to increase in a dose response manner with EE2 exposure for females (after 4 and 49 days exposure) and males (after 4 and 21 days exposure). Exposure of EE2 to *S. glomerata* was also capable of initiating a shift in the proportion of females, which also appeared to be concentration dependent. At the highest EE2 concentration (50 ng/L) a significant shift in the proportion of females was found which, together with histological observations, appeared to be the result of a male-intersex-female transition initiated by EE2 exposure. In lower exposures (6.25 ng/L and 12.5 ng/L EE2 exposure for 49 days) an increase in the proportion of intersex individuals was found. For gonadal development after 21 days EE2 exposure, it was observed that all exposure treatments had higher proportions of ‘mature’ female development stages compared to the controls suggesting EE2 exposure accelerated female gametal development. However this pattern was not observed after 49 days exposure to 50 ng/L and it was suggested that the concurrent transition from male-intersex-female may have resulted in a mixture of female development stages.

Vitellogenin gene expression was also suggested as a biomarker of estrogenic exposure and Chapter 3 largely focused on the development of a real-time qPCR assay for *S. glomerata*. This assay was tested on *S. glomerata* individuals exposed to a concentration gradient of EE2 for 4 days. A dose response relationship between EE2 exposure and vitellogenin gene expression was found for females, but not males. While variable, the average male
vitellogenin gene expression response was still approximately three times the response of control individuals. It is suggested that future studies should employ a higher number of replicates to determine if significant dose-response relationships occur among estrogenic exposure and male vitellogenin gene expression and further to assess temporal maintenance of the dose-response relationship over time for both males and females (7.2.2. below).

Overall, vitellogenin protein (both sexes) and gene expression (for females at least) and selected gonadal and sex endpoints were dose dependent meeting this criterion of a reliable biomarker.

7.2.2. Temporal maintenance of biomarkers within controlled laboratory conditions
It is important to understand the temporal response dynamics of a biomarker to enable appropriate sampling design and correct interpretation of findings. Ideally, biomarkers will have a rapid induction which is maintained in the face of continual long term (weeks) exposure (Huggett et al. 1992; Wu et al. 2005). The temporal kinetics of vitellogenin induction and maintenance with continual exposure was assessed in Chapter 2, via direct laboratory exposure to a concentration gradient of EE2 and measurement over three sampling periods. While vitellogenin increased in a dose dependent manner with EE2 exposure for both females and males, with time, levels declined to basal in females (after 21 days) and males (after 49 days) suggesting that the relationship between estrogenic exposure and vitellogenin induction is not necessarily a direct cause and effect relationship which is temporally maintained. In Chapter 2, it was suspected that temporal declines in vitellogenin over the experiment were due to gonadal development as a concurrent decline was experienced in the male and female controls. Although maintenance of vitellogenin response would be ideal for biomarker utility, the findings of Chapter 2 suggest that the stage of gonadal development should be an important consideration when assessing effects in environmental scenarios. It is likely that vitellogenin production is dependent on the phase of gonadal development and that individuals will respond differently at different phases of gametogenesis. This is not a large issue in controlled laboratory and field experiments where individuals in an immature stage of gonadal development can be selected and additional replicates can also be included to monitor the rate of gonadal
development. It is recommended that all future studies should target specimens commencing a gonadal development cycle (which can be identified via visual inspection), and further, to concurrently assess gonadal development (via histology) alongside all vitellogenin analyses. The temporal kinetics of vitellogenin gene expression has not been explored in this thesis but is highly recommended for future studies to further assess biomarker utility. Direct comparisons were also not performed between vitellogenin gene and protein induction. Therefore, future experiments could be performed to compare the lag between gene expression and protein production, the temporal dynamics with gonadal development and degradation rates of vitellogenin protein and gene expression.

It is also important to identify the type of temporal recovery response following removal of contaminant exposure. Whether biomarkers have a fast or slow recovery following contaminant exposure could affect the interpretation of results and, in terms of experimental design, may dictate when targeted sampling periods should occur, depending on the questions at hand. Biomarkers which are maintained temporally in the face of constant exposure than, with removal of exposure, have a slow recovery over time may be useful in the detection of transient estrogenic inputs in the environment which are unlikely to be detected by water sampling and chemical analysis alone. In contrast, biomarkers which have a rapid recovery following the removal of exposure may be more likely to closely reflect temporal changes in contaminant exposure, and be especially useful in assessing recovery of organisms when remediative action is taken to reduce or remove contaminant stress e.g. post an upgrade to WWTP processes. On the down side, a rapid recovery response may result in missing exposure events if the sampling regime is infrequent (Wu et al. 2005). Thus it is thus important for future work to elucidate recovery responses for vitellogenin in *S. glomerata*. Future controlled experiments should incorporate a depuration period to investigate if vitellogenin induction declines rapidly or slowly after estrogenic exposure/s are removed and whether temporal recoveries differ for gene expression versus protein production. Pulsed short and long term exposures with depuration periods in the laboratory, or reciprocal translocation experiments in the field, could explore the temporal recovery of biomarkers such as vitellogenin to changes in estrogenic exposure for full biomarker utility.
Another consideration, regarding the development of vitellogenin as a biomarker, would be to explore the possibility of additional vitellogenin gene/s (and protein) isoforms which may have different degradation rates which is likely to translate to different temporal responses to estrogenic exposure. Thus, future research could explore the possibility of additional vitellogenin isoforms and if present, determine which isoform is more useful (in terms of sensitivity and responsiveness) for the assessment of estrogenic compounds.

Biomarkers which assess biological changes at the organism level are more likely to have no recovery (or a slow recovery) following removal of the contaminant exposure in comparison to cellular or biochemical changes. Although not directly assessed in this thesis, it is likely that changes in gonadal development or sex (including those observed in Chapter 2 including accelerated female gonadal development, intersex and/or full sex transitions from male-intersex-female), would be maintained with or without continued estrogenic exposure and may be useful for detection of transient estrogen pulses in environmental situations. It seems likely from experimental evidence that estrogens facilitate a protandric progression of males to intersex status and finally to a female gametal trajectory. Whether this progression is influenced by the temporal nature of exposure is currently unclear. Removal of estrogenic exposure may result in a static intersex condition in males or transient exposure may be sufficient to facilitate a full sex change from male-intersex-female status, but this currently unknown and is perhaps important to ascertain in future investigations.

7.2.3. Early warning capacity of biomarkers

Another favourable characteristic of a biomarker is ‘early-warning’ predictive ability (den Besten, 1998; Huggett et al. 1992). Biomarkers with early warning capacity can be useful in real environmental situations for risk identification and removal of contaminant pressure (via changes to effluent treatment practices) prior to the onset of higher level effects which are potentially irreversible. Higher level effects, such as increased female gonadal development or a full sex reversal, are more likely to be temporally maintained (section 7.2.2.) and can be harmful in terms of individual fitness and population level related effects.
The early warning predictive capacity of female and male vitellogenin was confirmed in Chapter 2. It was found that early female vitellogenin (following 4 days exposure to EE2) correlated with increased female gonadal development (after 21 days) and increased oocyte area (after 49 days). Furthermore, early male vitellogenin (following 4 days exposure to EE2) negatively correlated with the percentage of males (at 49 days) and mean male gonadal development (49 days). This suggested that increases in female and/or male vitellogenin are useful as early warning indicators of later higher level changes of accelerated female development and/or negative male development. It is conceivable that early monitoring of vitellogenin responses could be employed with predictive ability of likely later impacts on invertebrate reproduction and fitness allowing remedial action to be taken prior to onset of higher level effects.

7.2.4. Field validation of biomarkers of estrogenic exposure

The final criterion in validating biomarkers of estrogenic exposure in S. glomerata was assessment under real environmental conditions. In order to assess biomarkers of estrogenic exposure, it was important to first demonstrate that estrogenic compounds were present in the field situation. Wastewater treatment plants (WWTPs) are identified as one of the main sources of estrogenic compounds into the environment and the receiving waters of Burwood WWTP was chosen for Chapters 4 and 5. In Chapter 4, sewage effluent from Burwood WWTP was found to contain estrogenic compounds. Estrogenic compound, BPA, was present at an average of 61.73 ± 7.66 ng/L in the liquid fraction and 2.47 ± 0.97 ng/L in the solids fraction. Alkyphenols, OP and NP, were also detected in the solids fraction with average concentrations of 0.98 ± 0.73 ng/L for OP and 1.63 ± 0.92 ng/L for NP and average concentrations in the liquid fraction of 4.95 ± 1.37 ng/L for OP and 5.88 ± 2.61 ng/L for NP. Whereas, mean concentrations of 0.34 ± 0.34 ng/L for E1, 0.69 ± 0.49 ng/L for E2, 3.26 ± 1.99 ng/L for E3 and 0.56 ± 0.56 ng/L for EE2 were detected in the liquid fractions and 1.08 ± 1.08 ng/L for E1, 0.03 ± 0.03 ng/L for E2 and 0.57 ± 0.41 ng/L for E3 in the solids fractions. Estrogenic activity (EEQ: estradiol equivalents) was also detected in sewage effluent from Burwood WWTP with an average concentration of 1.05 ± 0.39 ng/L in the solids fraction and 3.43 ± 0.92 ng/L in the liquid fraction. These results demonstrated
that the receiving waters of Burwood WWTP were a suitable location for the deployment of *S. glomerata* and subsequent assessment of biomarkers of estrogenic exposure.

Sydney rock oysters, *S. glomerata*, were deployed in the receiving waters of Burwood WWTP and at reference locations for 6 weeks. Several biomarkers were elevated following the deployment period, suggesting that estrogenic exposure, via sewage effluent, had occurred. Firstly, a higher proportion of mature female gonadal development stages were found in individuals deployed at Burwood compared to those deployed at reference locations. Female vitellogenin gene expression was highest in individuals deployed at 4 metres depth at Burwood near (within 50 metres of the outfall) compared to the majority of reference locations. However, male vitellogenin gene expression did not respond with low expression found in individuals deployed at all locations suggesting the estrogenic load within receiving waters was insufficient to elicit significant estrogenic effects.

Further experiments are required to determine if male gene expression responds to estrogenic exposure under field conditions, perhaps via deployment of *S. glomerata* in effluent receiving locations with higher estrogenic contamination. Female vitellogenin protein responded in a similar fashion to gene expression, with significantly higher (p< 0.05) induction at Burwood compared to all reference locations for individuals at 8 metres depth and compared to most reference locations for 4 and 12 metres depth. There were no significant differences among locations in the proportions of males or females. This could be further assessed under field conditions via the deployment of juveniles or embryos (spat) of *S. glomerata*, which are likely to have higher proportions of males and be more sensitive especially in terms of the occurrence of intersex or sex changes. Spat could be deployed at both contaminated and reference locations with proportions of sexes assessed after one year. At one year old, oysters are likely to have a higher ratio of males and significant increases in female proportions would provide definite evidence of the presence of environmental estrogenic compounds.

These findings demonstrate a potential cause and effect relationship between vitellogenin (gene expression and protein), female gonadal development and estrogenic exposure under
field conditions. However, other biomarker criteria were not assessed under field conditions and could perhaps be considered in future studies. Firstly, it is unknown if biomarkers display a dose response relationship with estrogenic exposure under field situations. This could be assessed via deployment of oysters at an increasing sewage effluent concentration gradient (i.e. at a number [5 or greater] of increasing distances from the sewage outfall) which would allow regression analysis between biomarker response and estrogenic exposure. Secondly, the temporal maintenance and early warning capacity was not explored as biomarkers were only assessed following long term (6 weeks) exposure. Future experiments may consider assessing biomarker responses following short term (days) as well as long term exposure (weeks) as laboratory studies suggest vitellogenin is most responsive soon after exposure in the early phases of gonadal development.

7.3. The use of *Saccostrea glomerata* as a biomonitor of metallic contaminants

In the past, sewage effluent has been identified as a source of heavy metals into the marine environment. A large component of this thesis was dedicated to establishing the suitability of biomarkers of estrogenic compounds in *S. glomerata*. However, *S. glomerata* has already been established to be a suitable biomonitoring species for heavy metals in Australian marine and estuarine waters (Avery et al. 1996; Lincoln-Smith and Cooper, 2004; Robinson et al. 2005; Scanes, 1996), and therefore can be directly employed as a biomonitor of metals. The deployment of oysters in a marine location provided an invaluable opportunity for multi-contaminant monitoring and therefore, in addition to estrogenic compounds, heavy metals were assessed in *S. glomerata* that were deployed in the receiving waters of Burwood WWTP and at reference locations for 6 weeks.

At all locations, concentrations of heavy metals in *S. glomerata* were found to be at low concentrations and within the boundaries of spatial variation (i.e. Burwood locations were similar to at least one reference location for all metals). Multivariate analyses found that, for comparisons of metal concentrations among locations, Burwood locations were significantly different ($p< 0.05$) from all reference locations, in contrast to the hypothesis this is likely to be due to the fact that concentrations of heavy metals were higher in *S.
glomerata at reference locations in comparison to the Burwood locations. Comparisons of concentrations of heavy metals in S. glomerata from individuals deployed at the Burwood near location to historic data from a similar experiment (which conducted 8 deployments of 3 months repeated between 1992-1994, Ajani and Wansbrough, 1996) demonstrated that concentrations were slightly lower in the present study and indeed lower than median background levels in oysters in NSW. It was suggested that this could be due to changes in sewage effluent treatment since 1992 or changes in the usage of heavy metals.

Overall, there was little evidence that sewage effluent from Burwood STD was elevated in bioavailable metals, and thus this class of contaminant is unlikely to pose a risk to resident biota.

7.4. The application of S. glomerata as a biomonitoring tool in environmental risk assessment

Environmental risk assessment (ERA) is utilised in situations where the hazard of a contaminant/contaminant class may be unknown and this can assist environmental management authorities in decision making (Walker et al. 2006). Classical ERA consists of analytical measurements of the contaminant of interest which is then compared to known toxic effects from experimental data (Walker et al. 2006). This approach can be limited as toxic effects can vary significantly in the field as exposure concentrations fluctuate often in the presence of mixtures of contaminants. The utilisation of biomonitoring species, with demonstrated reliable biomarkers, in combination with the analytical measurements of contaminant concentrations can be useful in the ERA process allowing detection of contaminants and in situ adverse organism effects within a ‘weight of evidence’ framework.

The findings of this thesis provide environmental management agencies, both nationally and internationally, with a biomonitoring tool for assessing estrogenic exposure and wildlife effects which may now potentially be integrated into existing environmental monitoring programs. Employing S. glomerata as a biomonitor of estrogenic compounds, alongside quantification of estrogenic load of effluents, may increase the capacity of water management agencies (for example, local water authorities such as Hunter Water
Corporation or local government areas (Councils) to identify potential effects of estrogenic effluent discharges and take remedial action to minimise ecological effects. Such remedial action may include secondary or tertiary treatment upgrades (such as activated sludge removal, ozonation, UV treatment or electrolysis [Water Environment Federation, 2008]) to improve the efficiency of removal of estrogenic compounds/activity. The development of *S. glomerata* as a biomonitor may also provide a tool for environmental wildlife protection agencies, such as NSW Industry and Investment, to protect the valuable oyster industry (*S. glomerata* is the largest aquaculture industry in NSW [White, 2002]) from potential contamination and associated human health risks.

In conclusion, although further validation is required, vitellogenin gene expression and protein production, along with assessment of gonadal development and gonadal status are promising biomarkers for estrogenic exposure and effect in *S. glomerata*. It is conceivable, that in the future, the species may be employed as a standard test organism for assessment of exposure and effect of estrogens in marine waters in a similar fashion to its established use a biomonitor for metal contamination in Australian marine and estuarine waters.
Reference List


Andrew, M., O’Connor, W., Dunstan, H. and MacFarlane, G. 2010. Exposure to 17α-Ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster. Ecotoxicology 19: 1440- 1451.


Gagné, F., Blaise, C., Pellerin, J., Pelletien, E and Strand, J. 2006. Health status of *Mya arenaria* bivalves collected from contaminated sites in Canada (Saguenay Fjord) and Denmark (Odense Fjord) during their reproductive period. Ecotoxicology and Environmental Safety 64: 348:361.


Discharges of 12 Wastewater Treatment Plants in Southern Australia. Archives of Environmental Contamination and Toxicology 56: 631-637.


Phillips, D. 1979a. Trace metals in the Common Mussel, *Mytilus edulis* (L.) and in the alga *Fucus vesiculosus* (L.) from the Region of the Sound (Oresund). Environmental Pollution 18: 31-43.


SPSS for Windows, Rel. 17.0.3. 2009. Chicago: SPSS Inc.


Tyler, T. 2010. Horizontal Sub-surface Flow Constructed Wetlands (Reed Beds) effective in reducing Estrogen concentrations from primary treated effluent. MSc Thesis in submission.


Appendix 1: Andrew et al. 2010

Exposure to 17β-ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster

M. N. Andrew · W. A. O’Connor · R. H. Dunstan · G. R. MacFarlane

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Abstract Although mounting evidence suggests exposure to estrogenic contaminants increases vitellogenin production in molluscs, demonstration of dose–response relationships and knowledge of the temporal nature of the vitellogenin response with continual exposure is currently lacking for biomarker utility. To address this knowledge gap, adult Sydney rock oysters, *Saccostrea glomerata*, were exposed to a range of environmentally relevant concentrations of 17β-ethynylestradiol (EE2) (0, 6.25, 12.5, 25 or 50 ng/l) in seawater under laboratory conditions. Vitellogenin induction and gonadal development was assessed following 4, 21 and 49 days exposure to EE2. Vitellogenin was found to increase in a dose dependent manner with EE2 exposure for females (4 and 49 days) and males (4 and 21 days). Histological examination of gonads revealed a number of individuals exhibited intersex (ovotestis) in 50 ng/l EE2 (after 21 days) and in 6.25 and 12.5 ng/l EE2 (after 49 days). Furthermore, a significant shift towards females was observed following 49 days exposure at 50 ng/l EE2 suggesting estrogenic exposure is capable of facilitating a progression for protandric males from male-intersex-female gametral status. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages at 21 days and increases in oocyte area following 49 days. Male vitellogenin (4 days) was predictive of decreased male percentages and lower male developmental stages at 49 days.

Vitellogenin in *S. glomerata* is a predictive biomarker of estrogenic exposure and effect if sampled soon after exposure and at the commencement of a gonadal development cycle.

Keywords Biomarker · Estrogens · 17β-ethynylestradiol · Intersex · Oyster · Vitellogenin

Introduction

A range of anthropogenic compounds with structural similarity to estrogen, and its functional moieties, have been implicated as primary causal agents responsible for reproductive perturbations in aquatic wildlife (Langston et al. 2008). One such compound is 17β-ethynylestradiol (EE2); a synthetic estrogen which is widely employed as the active constituent of the female contraceptive pill. Compared to endogenous steroids, such as 17β-estradiol (E2) and its metabolites, EE2 exhibits greater estrogenic potency and is more resistant to metabolism/degradation due to the addition of an ethyl group (Andersen et al. 2003). Consequently, when used as the contraceptive pill in humans, EE2 is only partially metabolised, resulting in excretion and entry to wastewaters. Evidence suggests that during the sewage treatment process EE2, along with a suite of other estrogenic compounds, may only be partially degraded, and/or removed, resulting in entry of EE2 to streams and surface waters (Andersen et al. 2003). Concentrations of EE2 in sewage effluent have been detected between 0.2 and 42 ng/l (Desbrow et al. 1998; Ternes et al. 1999) and in surface waters between 0.1 and 15 ng/l worldwide (Aherne and Briggs 1989; Bellfroid et al. 1999). Thus, aquatic organisms, including invertebrates, may be exposed to EE2 in aquatic environments receiving sewage effluent.
Aquatic invertebrates, in particular bivalve molluscs, are ideal candidates for assessing effects of anthropogenic contaminants in aquatic systems. Due to their sessile nature, high filter-feeding capacity and bioaccumulation of both organic and inorganic compounds (Ortiz-Zarragoitia and Cajaraville 2006), molluscs such as oysters and mussels have proved useful in the assessment of contaminant presence, relative contaminant loads among locations and biological responses to contaminant exposure (Scanes 1997). Molluscs may also prove a valuable tool in the assessment of the effects of estrogenic compounds. Indeed, available literature on both laboratory and field assessment of the effects of estrogenic compounds in molluscan species suggests that exposure to estrogenic compounds can cause reproductive effects including the induction of vitellogenesis in females and males (evidence of endocrine disruption) (Matozzo et al. 2008) and gonadal developmental changes (accelerated oocyte development in females and feminisation of male individuals) (Gagné et al. 2006; Gagnon et al. 2006; Ortiz-Zarragoitia and Cajaraville 2006; Langston et al. 2007).

Molluscan vitellogenins are precursors to egg yolk proteins (vitellins) which accumulate in oocytes during gonadal maturation. Numerous lines of evidence suggest vitellogenesis in molluscs is mediated via endogenous estrogens. In the scallop, Pecten maximus, E2 is synthesised in the gonad and its levels increase during the early phases of oocyte development (Matsumoto et al. 1997). Li et al. (1998) demonstrated that E2 treatment in vivo stimulated vitellogenesis and increases in vitellins in the gonad of the Pacific Oyster, Crassostrea gigas. Similarly, Osada et al. (2003) demonstrated E2 induced synthesis of vitellogenin in the gonad of P. yessoensis. The site of synthesis is thought to be within ovarian tissue, with in-situ hybridisation demonstrating localisation of C. gigas vitellogenin mRNA within follicle (auxiliary) cells (Matsumoto et al. 2003).

With such evidence suggesting that estrogens play a functional role in vitellogenin induction in molluscs, others have hypothesised that exposure to xenoestrogens may elicit similar effects, and have sought to investigate vitellogenin production as a potential biomarker of endogenously mediated endocrine disruption. Indeed, Gagné et al. (2002) demonstrated that both male and female freshwater mussels, Elliptio complanata, deployed 1.5 and 5 km downstream from a sewage treatment plant in the St. Lawrence River, Canada, exhibited increases in alkali-labile phosphates (ALP) (a surrogate vitellogenin estimate) compared to reference locations. Male blue mussels, Mytilus galloprovincialis, deployed in Venice canals (receiving raw sewage) have also been shown to exhibit increased ALP levels in hemolymph compared to reference locations (Pamparin et al. 2005). In terms of controlled exposures to specific xenoestrogens, exposure to 4-nonylphenol (NP) (250 nM) for 72 h has been shown to increase ALP levels in both male and female mussels, E. complanata (Gagné et al. 2001). Similarly, M. galloprovincialis exposed to NP (0, 25, 50 and 100 µg/l) for 7 days exhibited a dose dependent increase in ALP levels (Riccioti et al. 2006). Taken together, these findings provide strong evidence of the role of xenoestrogens in induction of vitellogenin-like proteins in molluscs. However, there has been little research to date exploring the potential effects of xenoestrogens with high estrogenic potency, such as EE2, on molluscan vitellogenesis, excluding our own work (see below).

Estrogenic exposure not only induces vitellogenin production in molluscs, but also influences both the rate of gonadal development and sex determination. In C. gigas, injections of E2 (50 µg once every 10 days, for a 40 days period) were shown to accelerate female development by increasing oocyte diameter (46.9 ± 0.9 µm in exposed compared to 44.1 ± 2.4 µm in controls) (Li et al. 1998). Langston et al. (2007) demonstrated that exposure of clams, Scrobicularia plana, to sediment spiked with a mixture of E2, EE2, NP and octylphenol (OP) caused intersex gonadal status in males and enlarged oocytes in both females and in the ovotestis of intersex individuals. Further, injections of E2 (0.04–0.60 mg estradiol-3-benzoate) during the early stages of seasonal gonadal maturation of the oyster C. gigas, have been shown to induce full sex reversal from male to female (Mori et al. 1969). Similarly, exposure to NP (48 h exposure to 1 and 100 µg/l at 7–9 days post-fertilisation) during larval development has resulted in skewed sex ratios towards females during adulthood in C. gigas (Niesz et al. 2003).

Our own prior research has focused on the development of, S. glomerata, as a potential bio-monitoring species for estrogenic contaminants through exploiting vitellogenin induction and gonadal developmental responses following estrogenic exposure. We have previously found that females exposed to EE2 (50 ng/l) and NP (100 µg/l), during a gonadal development cycle, exhibited significant increases in vitellogenin up to three-fold and double controls, respectively. Significant increases in vitellogenin were also found in males exposed to 50 ng/l EE2. Further, exposure to NP (100 µg/l) and EE2 (50 ng/l) induced intersex gametial status for some individuals (Andrew et al. 2008).

As S. glomerata exhibits protandry, the sex of individuals observed in our initial experiments were functionally intersex or the result of an opportunistic sampling of individuals that were in the process of undergoing an endogenically mediated protandric transition from male to female. If the latter is the case, we may expect to observe
shifts in the sex ratio towards females with greater exposure durations to estrogens, at later intervals in the gonadal development cycle and/or with greater sampling frequency during such potential transitions. In the current study, we sought to explore this question by sampling individuals exposed to EE2 at multiple windows temporally during a gonadal development cycle from resting phase to spawning.

Among the criteria required for a successful biomarker is evidence of a dose–response relationship to contaminant exposure (Huggett et al. 1989). Yet a recent review by Ketata et al. (2008) emphasised that clear evidence of dose–response relationships between estrogenic exposure and vitellogenin induction is currently lacking for invertebrate models. In the current study, we sought to redress this knowledge gap by assessing the vitellogenin response upon exposure to an environmentally relevant range of EE2 concentrations. It was anticipated that vitellogenin concentrations would increase with estrogenic dose, particularly in males, who do not usually produce vitellogenin.

Further, for a reliable biomarker, it is desirable that observed dose–response relationships are maintained temporally. Yet there is evidence to suggest that biomarkers may not exhibit static dose–response relationships temporally, but rather may exhibit variable responses to estrogenic exposure dependent on factors such as exposure regime and duration, adaptive response mechanisms and/or developmental status (Wu et al. 2005). For application in environmental monitoring it is critical to understand the temporal nature of biomarker responses to exposure to enable an appropriate sampling design which does not underestimate or overestimate exposure and effect. Thus, we chose to assess the temporal maintenance of the dose–response relationship between EE2 exposure and vitellogenin responses over three sampling periods during a gonadal development cycle.

Finally, as a predictive biomarker, vitellogenin should also possess ‘early-warming’ predictive ability. It is desirable that early vitellogenin responses correlate with later effects indicative of endocrine disruption at higher organisational levels such as gonadal morphology i.e. gamete maturation, size, expression of intersex and/or total sex reversal.

Thus, the main aims of the present study were to explore dose–response and temporal relationships between EE2 exposure, vitellogenin induction and gametogenesis during reproductive conditioning in S. glomerata. Specifically, we expected that vitellogenin would increase in a concentration dependent manner within days of EE2 exposure (4 days) and that the dose–response relationship would be maintained throughout the exposure duration. Secondly, we predicted that with time (21–49 days) we would observe the presence of intersex individuals in exposure treatments and possibly a shift in the sex ratio towards females.

Finally, we hypothesised that early induction of vitellogenin would be predictive of later effects on gametogenesis. Expression of intersex and/or total sex reversal.

Materials and methods

Experimental design

Three hundred and sixty, 18 month old S. glomerata were used for experimentation. Oysters were sourced from an oyster farm in the Port Stephens estuary, NSW, Australia; an area with no known history of sources of estrogenic contamination. Oysters were specifically selected to be in ‘resting’ condition, entering a phase of gonadal development at experimental commencement (Dinamani 1974). Experiments were conducted during Winter (June–August 2007) at the Port Stephens Fisheries Centre, Molluscan Hatchery, Taylor’s Beach, NSW according to the protocols of the American Society for Testing and Materials (A.S.T.M.) E 729-96 (1996) for static renewal tests. One hundred and eighty individually aerated aquaria (81) were maintained at 22 ± 0.5°C in a temperature controlled room. Each aquarium contained two oysters. Seawater (33 ± 0.5 g kg/l salinity) within each replicate aquarium was completely changed and treatment exposure regimes maintained thrice weekly within a temperature controlled laboratory (maintained at 22°C). Oysters were fed a mixed diet daily containing 660 ml of each algal species culture including: Pavlova lutheri, Chaetoceros muelleri and Tahitian Isochysis aff. gilben (1.5 x 10⁷ cells/oyster/day).

Oysters were exposed to one of six nominal treatments in seawater: 6.25, 12.5, 25 and 50 ng/l EE2 (in 1.2 μg/l ethanol), 1.2 μg/l ethanol control and a seawater control (N = 20 individuals per treatment/time). Oysters were removed (10 aquaria per treatment/time) from the experiment at three separate sampling occasions (4, 21 and 49 days) in order to assess temporal effects. Sampling times were selected to assess whether vitellogenin was induced following short-term exposure (4 days, 96 h acute exposure) and if vitellogenin responses were likely to be maintained over longer periods of time (chronic effects). The sampling times of 21 and 49 days were considered important time points to measure the shift in sex ratio and sufficient time to allow for gonadal maturation from resting phase to mature gametes prior to spawning (Cox et al. 1996). A group of oysters (from the same population) were maintained in separate aquaria for the purpose of monitoring gonadal development throughout the experiment and to ensure oysters were harvested prior to a spawning event.
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Wet condition index was measured to assess the potential confounding effects of estrogenic treatments and/or the solvent carrier on feeding and thus condition. Wet condition index was measured at each harvest. This was calculated using the formula (wet tissue weight × 1000)/(wet whole weight − wet shell weight) (Lucas and Beninger 1985). Oyster condition was observed to decline in the 12.5 ng/l, 25 ng/l and 50 ng/l EE2 treatments by 8.5, 7.7 and 12.6%, respectively after 4 days (F = 2.58, df = 4, p < 0.05), though oysters quickly recovered with no significant differences in condition among treatments at both 21 (F = 2.095, df = 4, p > 0.05) and 49 days (F = 2.269, df = 4, p > 0.05).

Extraction and HPLC assay for measuring vitellogenin in S. glomerata

The extraction (modified extraction from Gagné and Blaise 2000) and analysis of oyster gonadal samples were performed using protocols outlined in Andrew et al. (2008). Briefly, 100 mg of oyster gonadal tissue was homogenized in 225 µl citrate buffer (pH 6.5 10 mM and with 16 mg/ml polyethylene glycol) and 25 µl of the protease inhibitor aprotonin. The mixture was stored at −80°C. Following storage, samples were thawed and 500 µL Tris–HCl buffer was added. One hundred microliters of tissue homogenate was transferred to a glass centrifuge tube and ALPs were extracted using 800 µl of tert-butyl methyl ether (Chromsolv grade, Sigma-Aldrich) with a 30 min extraction at 4°C. The organic phase was then separated and dried under N2, then re-suspended in 1,000 µl of PBS. Analysis of vitellogenin was performed by HPLC analysis using conditions outlined in Andrew et al. (2008), with the vitellogenin peak initially identified via proteomic sequencing (1DNanoLC ESI MS/MS). It should be acknowledged our use of the term vitellogenin implies the analyte is a vitellogenin-like protein based on genomic/proteomic sequence similarity to known vitellogenin from other taxa (molluscs, fish spp.). Three female and three male oysters were selected from each treatment/time for vitellogenin analysis.

Histological analysis

Sexes in S. glomerata are separate with low occurrence of intersex, i.e. <0.4–0.7% (Dinamani 1974; Cox et al. 1996). Sex assignment was required due to the hypothesised differences in vitellogenin induction between males and females (Blaise et al. 1999, 2003), that vitellogenin induction in males is strong evidence for estrogen mediated endocrine disruption, and finally due to the established effects of estrogenic exposure on sex ratio (Andrew et al. 2008). Each individual oyster was prepared for histological examination resulting in a total of 400 analyses. A cross-section of approximately 5 mm² was excised from each oyster between the labial palps and gills. Tissue samples were placed in Davidson’s solution (10% glycerine, 20% formalin, 30% alcohol, 30% sodium chloride solution and 10% glacial acetic acid) for 24 h (Cox et al. 1996) with successive dilutions of ethanol (70, 50, 50%) (Howard and Smith 1983). Tissue was embedded into paraffin blocks, sectioned transversely at 5 µm intervals and floated on a water bath heated to 80°C. Sections were placed onto acid washed glass slides and dried overnight at 60°C. Sections were stained with haematoxylin and counterstained with eosin (H&E). Study of the gonadal area was performed under a compound microscope at 200 and 400 × magnification.

The sex of each individual was determined as female, male, intersex or indeterminate under microscopic examination (200 × magnification) using oocytes or spermatocytes as indicators. Intersex individuals were identified via the presence of both oocytes and spermatocytes within an intact gonadal follicle. Other individuals were found to be indeterminate whereby the gonadal cells were undifferentiated and usually accompanied by an abundance of hemocytes.

It may be possible that estrogen exposure promotes female development resulting in acceleration towards higher proportions of mature female gonadal stages in individuals exposed to EE2 compared to controls. Thus each oyster was assigned a gonadal developmental stage for oogenesis, and also spermatogenesis, based on criteria described by Dinamani (1974). Briefly, stages included: (1) follicles contain primary oogonia or spermatogonia, (2) oocytes (<25 μm) or spermatocytes beginning to mature, (3) maturation of oocytes (>25 μm) or all stages of spermatogenesis up to spermatids, few spermatocytes, (4) oocytes or spermatocytes occupying a large proportion of the gonad, (5) following spawning, many follicles are discharged, residual oocytes or spermatocytes, (6) characteristic of resting, no oocytes or spermatocytes (indeterminate sex) and high hemocyte activity. Eight female individuals per treatment from the 49 days sampling period were randomly selected for oocyte area measurement. For each individual, digitalised images of four randomly selected areas of the gonad were taken with subsequent measurement of 80–100 oocytes (ImageJ 1.48v). Only oocytes with a visible nucleus were measured and oogonia were not measured.

Statistical analysis

Linear dose–response relationships between EE2 exposure and vitellogenin concentration in gonadal tissue were assessed to test for biomarker utility via linear regression analyses using STATISTICA (Statsoft 2005). According to Levene’s test for homogeneity of variance, vitellogenin units were not homogeneous and thus log transformed, ln (x + 1), prior to statistical analysis. Other curve functions
(i.e. sigmoidal, logistic and exponential) were assessed via relative coefficients of variation, though linear fits were found to best represent the data.

A Pearson χ² analysis (SPSS version 17, 2009) was used to determine significant differences in sex ratio (female, male, intersex or indeterminate) among time (4, 21 and 49 days exposure) for each exposure to EE2 (0, 6.25, 12.5, 25 or 50 ng/l). Numbers of males, intersex and indeterminate individuals across treatments and time were insufficient for non-parametric analysis and were pooled in order to gain sufficient frequency for comparison to females. The aim was to determine if the proportion of females in the test population increased throughout the duration of the experiment. It was hypothesised that there would be a difference in the sex ratio, specifically a shift towards a greater proportion of females in exposure treatments over time compared to no difference in the controls.

Pearson’s correlations were performed between treatments and mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) to further determine relationships between exposure and reproductive endpoints. Pearson’s correlations were also performed using early mean vitellogenin levels (4 days) with mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) in order to test the early warning biomarker predictive utility of vitellogenin. As individuals were sacrificed at each sampling period it was not possible to undertake pair wise correlations between vitellogenin replicates from 4 days with reproductive endpoint replicates from separate individuals at 49 days. Thus, correlation analyses were performed using mean values for each treatment. Due to low N (N = 3 per treatment), the power to detect a significant correlation between variables was greatly reduced.

During the experiment an ethanol control was included due to the addition of ethanol as a solvent carrier for EE2 in the exposure treatments (1.2 µg/l). Prior to statistical analyses the seawater and ethanol controls were compared for differences. As no significant differences were observed, both controls were pooled for the purpose of all analyses.

Results
Effects of EE2 on vitellogenin production

Females

While there was greater variability in the vitellogenin response at 4 days compared to later time periods, levels were elevated across all EE2 treatments and increased linearly with EE2 exposure (R² = 0.22, p < 0.05) (Fig. 1a). Yet the vitellogenin response declined over time, with approximately half of the vitellogenin units found at 49 days compared to the vitellogenin units in corresponding treatments at 4 days. At 49 days, albeit with lower vitellogenin concentrations, a linear relationship with EE2 exposure was found (R² = 0.58, p < 0.05) while at 21 days the relationship was not significant (p > 0.05) (Fig. 1a).

Males

Males displayed a similar response with vitellogenin exhibiting a positive linear increase with EE2 exposure at 4 days (R² = 0.42, p < 0.05). A positive linear increase with EE2 was maintained at 21 days (R² = 0.31, p < 0.05) (Fig. 1b) but, similar to the female response, vitellogenin had declined in comparison to 4 days. At 49 days the
vitellogenin response had declined to basal levels across treatments ($p > 0.05$) (Fig. 1b). 

Gonadal sex development

The proportions of sexes (male ~ 20%, female ~ 60%, indeterminate ~ 20%) were similar across EE2 exposure treatments at 4 days (Fig. 2a). This represents what may be expected of sex proportions within a single reproductive season for *S. glomerata*, allowing for sampling variability. A small proportion of individuals were indeterminate at the beginning of the experiment which was expected and reflects individuals that were yet to commence the gonadal maturation process (Fig. 2a). It is also possible these individuals were male due to the difficulty of identification of small proportions of rudimentary spermatogonia within follicles (stage M1) or residual gametes in a male gonad post-spawning (stage M6).

Following 21 days exposure, the proportion of males and indeterminate individuals declined with a concomitant increase in the proportion of intersex individuals in the 50 ng/l EE2 exposure (4/15) (Fig. 2b). No other treatments showed evidence of intersex at this sampling interval. A higher proportion, although not significant, of females were found in the 25 ng/l EE2 treatment compared to all other treatments. After 49 days exposure, proportions of intersex were also evident at lower exposures of 6.25 and 12.5 ng/l EE2 (Fig. 2c). At 50 ng/l EE2, the proportion of intersex individuals declined (relative to 21 days), with a significant shift in the proportion of female individuals at 49 days compared to earlier time periods ($\chi^2 = 6.707$, df = 2, $p < 0.05$) (Fig. 2c). Only one male was observed within this treatment. Taken together, these results suggest a progression from male-intersex-female occurring with time and EE2 exposure. No other significant differences in the female proportions across experimental sampling periods were found in exposure or control treatments.

Oyster gonadal development occurs through the sequential addition of oogonia or spermatogonia from the basal layer of the follicular wall. As gametes mature, they are released to fill the lumen of the follicle. At 21 days, it was observed that intersex individuals from the 50 ng/l exposure all contained male gametes (stage M2) within the lumen along with the development of a layer of oogonia (stage F1) around the inner follicle wall (Fig. 3a). The modal stage of development for males at 4 days was also stage M2, suggesting that the developmental fate of gametes switched soon after exposure to EE2 (Fig. 3b). A similar pattern of development was observed in intersex individuals in 6.25 and 12.5 ng/l at 49 days, whereby male gametes were at stages M2-4 and the new layer of female gametes were consistently at stage F1 (Fig. 3c). It was also observed that for all intersex individuals (particularly where the proportion of oogonia

![Fig. 2 Comparison of the sex ratio of Sydney rock oysters, *S. glomerata* following exposure to EE2 (0, 6.25, 12.5, 25 and 50 ng/l) in experimental aquaria at a 4 days exposure, n = 17-18, b 21 days exposure, n = 15-16 per treatment and c 49 days exposure, n = 18-20 per treatment. * indicates a significant shift in the proportion of females via $\chi^2$ analysis.](image)
was higher than male gametes) there was a proliferation of hemocytic activity within interstitial tissue (Fig. 3d). The combination of maturing male, immature female gametes and enhanced hemocytic activity further suggests that individuals begin gonadal maturation as male, but with exposure to EE2 the developmental pathway of germ cells is redirected from a male to female trajectory. Male gametes are eventually removed via hemocytic clearance and the individual continues gametogenesis along a female developmental pathway.

**Female gonadal development stages**

At 4 days, the proportions of female gonadal development stages were similar among all treatments. After 21 days exposure, it was observed that all exposure treatments had higher proportions of female development stages F2–F4 (Fig. 4a–c) compared to the controls (where 95% females were in stages F1 or F2) suggesting EE2 exposure accelerated female gametogenesis. However at 49 days within higher exposures (e.g. 25 and 50 ng/l EE2), the proportions of female stages (F2–F4) were once again similar to the controls (F1–F5) (Fig. 4d). The significant increase in female proportions in 50 ng/l at 49 days together with histological observations of increased proportions of intersex individuals within this treatment at earlier intervals (21 days) suggests that the trend towards higher proportions of earlier female gonadal development stages within these treatments was perhaps the outcome of sampling individuals that have transitioned from a male to female developmental mode. This resulted in a ‘mixture’ of female developmental stages, with ‘new’ females in earlier maturation stages such as F2 (Fig. 4d) (that have recently transitioned from intersex or indeterminate gonadal status) and individuals initially female at higher maturation stages (F3 and F4). It is unlikely that EE2 exposure initiated a spawning event in the 25 and 50 ng/l exposure at 49 days and individuals were re-commencing a secondary gonadal development cycle, as spawning (even at low levels) is evident within aquaria.

**Does vitellogenin induction and EE2 exposure correlate with later effects on gonadal development?**

The effects of EE2 exposure was further explored through examining relationships between EE2 exposure concentrations and mean oocyte area, mean reproductive stages and sex percentages from 21 and 49 days. Increasing EE2 exposure
Fig. 4 Gonadal tissue from histological preparation of individual Sydney rock oyster, S. glomerata. a Female gonad in stage F3 ×200 (21 days, 50 ng/l), b female gonad in stage F3 ×200 (21 days, 50 ng/l), c female gonad in stage F4 ×200 (21 days, 25 ng/l), d female gonad in stage F2 ×200 (49 days, 50 ng/l). O = oocyte cell. Scale bars = 100 μm.

<table>
<thead>
<tr>
<th>Treatment (EE2)</th>
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<th>Male vitellogenin (4 days)</th>
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Numbers in bold indicate a significant relationship.

negatively influenced male development with a decrease in the percentage of males and decreases in mean male developmental stage following 49 days exposure (r = -0.91, p < 0.05 and r = -0.95, p < 0.05, respectively) (Table 1).

Secondly, the capability of vitellogenin as a predictive early-warning biomarker of later reproductive effects was examined by performing correlations between mean female vitellogenin at 4 days with mean oocyte area, mean reproductive stages and sex percentages from 21 and
49 days. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages following 21 days \( (r = 0.96, p < 0.05) \) and increases in oocyte area following 49 days \( (r = 0.93, p < 0.05) \) (Table 1). Mean male vitellogenin (4 days) may also have useful predictive application of later negative effects on male development with a negative relationship evidenced between early vitellogenin responses and male percentage at 49 days \( (r = -0.92, p < 0.05) \) and between early vitellogenin and mean male development stage at 49 days \( (r = -0.97, p < 0.05) \) (Table 1).

Discussion

Individuals exposed to EE2 exhibited increased vitellogenin in a linear dose dependent fashion, however this was not maintained throughout experimental sampling periods. At 4 days, both sexes exhibited a significant relationship between EE2 exposure and vitellogenin production however, this was not evident at 21 days for females nor at 49 days for males. Thus, the temporal maintenance of the vitellogenin response varies between sexes.

Vitellogenin production, especially for females, is likely to be dependent on stage of gonadal development. Our results indicate that immature females beginning a reproductive cycle were more likely to exhibit increased vitellogenin compared to mature individuals ready to spawn and indicated that developmental stage is an important consideration when assessing estrogenically mediated vitellogenin induction. As a precursor to egg yolk protein (vitellins), vitellogenin may be synthesised most actively during the earlier stages of gonadal development, as oocytes are developing (Li et al. 1998). Our findings for vitellogenin production in control females support this assertion, with vitellogenin declining with time. Furthermore, female vitellogenin exhibited a linear increase with EE2 at 4 days and subsequently at 49 days only. The relationship found at 49 days was driven by the response in high exposures (25 and 50 ng/l EE2) where a transition from male-intersex-female was likely to have occurred while lower exposures (6.25 and 12.5 ng/l EE2) exhibited similar vitellogenin responses to controls. It may be possible that recently transitioned female individuals within these treatments, at earlier stages of gonadal development, were more responsive to EE2 exposure at 49 days. Matozzo and Marin (2008) have also explored vitellogenin response dynamics at different stages of reproductive development through measurement of ALP in the Manila clam, Tapes philippinensis exposed to E2 (5, 25, 50, 100 and 1,000 ng/l) for 7 and 14 days during both a resting and pre-spawning phase. Females exposed to E2 in a pre-spawning phase for 7 days resulted in lower levels of hemolymph ALP in all exposure treatments compared to controls. Yet, following 14 days, only the 50 ng/l exposure exhibited significantly higher ALP. However, in the resting phase, they found that ALP significantly increased in the hemolymph of females following 7 days exposure to E2 (5, 25, 50, 100 and 1,000 ng/l), but ALP levels in exposure treatments had declined following 14 days and were only significant at the highest exposure (1,000 ng/l) (Matozzo and Marin (2008)). Similarly, Puinean et al. (2006) found that Blue mussels, Mytilus edulis exposed to 200 ng/l E2 displayed no significant differences in vitellogenin gene expression compared to control individuals during the mature stages of gametogenesis. Taken together, these observations imply vitellogenin is most actively synthesised, and most responsive to estrogenic stimulation, during earlier phases of gonadal development, suggesting individuals commencing a gonadal development cycle should be targeted when employing vitellogenin as a biomarker of estrogenic exposure in S. glomerata. These findings also suggest that future studies of estrogenic effects in S. glomerata, and other molluscan taxa, should measure gonadal development alongside vitellogenin.

Despite these temporal response limitations, it appears that vitellogenin is induced in a dose dependent fashion soon after exposure at the commencement of reproductive conditioning for both females and males. The fact that males also exhibit this vitellogenin induction, suggests estrogens exert a response not observed in unexposed males, indicative of endocrine disruption. Indeed, male molluscs (E. complanata and M. edulis) have been shown to possess a functional, yet silent, gene for vitellogenin which has been shown to be activated upon exposure to exogenous estrogens in E. complanata (Bagné et al. 2005) but not in M. edulis (Puinean et al. 2006). Further, others have found that male molluscs can exhibit sensitivity to estrogenic exposure in terms of induction of vitellogenesis (Matozzo and Marin 2005; Blaise et al. 1999, 2003).

In terms of how estrogens may induce vitellogenesis mechanistically, it is well established in vertebrate models, such as fish, that vitellogenin production is mediated through estrogens binding to intracellular estrogen receptors (predominantly ER-β and to a lesser extent ER-α) which function as ligand-modulated transcription factors, binding to estrogen-responsive elements in the promoter region of the vitellogenin gene(s) (Leahos-Castaheda and Van Der Kraak 2007). Despite this well characterized pathway in vertebrate models, little is known on how estrogens may affect vitellogenin production mechanistically in invertebrate models such as molluscs. Primarily, the literature to date is inconclusive on the presence of functional estrogen receptors (ER) in Mollusca, cDNA encoding vertebrate-like estrogen receptors (with high homology in the DNA binding domain to vertebrate ER-α
and ER-$\beta$) have been cloned in a number of molluscan species including the rock shell, *Thais clavigera* (Kajiwara et al. 2006), the common octopus *Octopus vulgaris* (Keay et al. 2006) and *C. gigas* (Matsumoto et al. 2007). Matsumoto et al. (2007) suggested that such estrogen receptors may act as nuclear receptors regulating the transcriptional activity of reproductive genes, including the vitellogenin gene, with estrogen receptor immuno-reactivity localised in the nuclei of follicle cells, the site of vitellogenin synthesis (Matsumoto et al. 2007). Yet in vitro observations have found that these estrogen receptors do not bind estrogen both in ligand-binding and cell based gene-reporter assays (Lafont and Mathieu 2007). Although far from clearly characterised, literature documenting vitellogenin induction upon exposure to estrogens, in a variety of molluscan taxa, including our own findings, suggest that estrogens play a functional role in vitellogenesis though the precise mechanism(s) remain to be elucidated.

Along with an acceleration of female gonadal development, EE2 exposure may be capable of initiating a full sex reversal from male to female. The occurrence of intersex individuals present in the 50 ng/L exposure at 21 days together with a significant increase in the proportion of females at 49 days suggested that a number of individuals have undergone a complete sex reversal during this experiment. The histological examinations of intersex individuals suggest that intersex is a transitional event in the switch from male to female gametial status. Within a gonadal follicle of an intersex individual, it appears that the maturing male developmental pathway is interrupted. Subsequent germ cell differentiation is redirected to a female developmental fate. Oogonia are sequentially added and, over time, male gametes may be removed by the proliferation of hemocyte cells (gamete re-absorption). Gamete re-absorption by hemocytes has been described as a process to recycle materials and energy following a spawning event (or in this case, sex transition) or as a survival mechanism in response to stress/diseases (Beninger and Le Pennec 2003; Pipel 1987; Steele and Mulcahy 1999). Without tracking the gametial status of each individual over the entire experimental window it is difficult to unambiguously confirm full sex reversal (as existing sex determination protocols are invasive [smearing] or destructive [histology]). The observation of intersex individuals in lower EE2 exposures (6.25 and 12.5 ng/L) is a significant finding and may indicate that a transition (to higher female proportions) may occur at lower concentrations of EE2 with greater exposure duration. The occurrence of intersex at these lower, environmentally relevant, exposures of EE2 demonstrates that gonadal gametial status may be employed as a sensitive biomarker of estrogenic exposure and effect in *S. glomerata*. Although it is likely that these individuals may follow a transitional switch from male to female in a similar fashion to higher exposures (50 ng/L), it may also be possible that lower exposures do not promote full sex reversal and subsequently result in a static intersex condition. Regardless, there are potentially negative effects associated with full sex reversal and/or intersex at both the individual and population level. This could include decreased individual fitness/reproductive capacity and/or altering the sex ratio and thus the overall fitness of a population. It is well established that oysters spawn in synchronisation, during the summer months in response to environmental signals such as the full moon, changes in salinity and warmer water temperatures (Roughley 1933). An individual that experiences intersex or a full sex reversal midway during a gonadal cycle is unlikely to develop mature gametes capable of fertilization for a synchronised spawning event compared to individuals that have matured as female only. Thus, estrogenic exposure is perhaps likely to disrupt spawning synchronisation among individuals. Further testing is also required to investigate if the gametes from intersex or these (new) female individuals are viable, capable of fertilization and if there is potential for more long-term effects in subsequent generations.

Lastly we assessed the utility of vitellogenin as an early warning indicator of later gonadal effects at the individual level. Higher level effects, such as an increase in female development or a full sex reversal may be detrimental in terms of individual fitness or population level related consequences (again via influencing reproductive synchrony). The ability of a biomarker to predict higher level effects is favourable for biological assessment of contaminants under circumstances where a risk of estrogenic exposure can be identified and removed prior to the onset of higher level effects. Our results demonstrated that early increases in female vitellogenin (following 4 days) correlated with an increase in female development (21 days) and increased oocyte area (49 days). Male vitellogenin (following 4 days) exhibited a negative correlation with both male percentages (49 days) and mean male development stages (49 days). Together these results suggest that vitellogenin, if measured during the initial stages of gonadal development, may be useful as an early warning indicator for accelerated female development and decreased male development due to estrogenic exposure. Our results suggest that monitoring of both sexes is useful and perhaps necessary. Females are likely to be more sensitive to effects of estrogenic contaminants, display more pronounced vitellogenin induction and exhibit accelerated oocyte development. Males, in comparison, provide definitive evidence of endocrine disruption via vitellogenin induction, decreased male development and sex reversal.

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References


Axl M (1979) Hemorrhagia and sex reversal in the four common oysters species of oysters from the coast of Karachi. Hydrobiol 66:149-155


Gagné F, Blais C, Pellerin J, Pellerin E, Strand J (2006) Health status of Mya arenaria bivalves collected from contaminated sites in Canada (Saguenay Fjord) and Denmark (Odense Fjord) during their reproductive period. Ecotoxicol Environ Saf 64:348-361


SPSS for Windows (2009) Rel. 17.0.3. 2009. SPSS Inc, Chicago

StataCorp (2005) StataCorp for Windows, StataSoft Inc, Tulsa, OK


Appendix 2: Andrew et al. 2008

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Appendix 3: Anderson et al. 2010


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