Ecological Risk Assessment of Persistent Organic Pollutants in Wetlands of the Remediated Sydney Olympic Park, NSW, Australia

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Certificate of Authorship/Originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements of a degree except as fully acknowledged within the text.

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Signature of Student

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7-ER	7-ethoxyresorufin
ADI	Australian Defense Industries
AhR	Aryl-hydrocarbon receptor
AMD	Acid mine drainage
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ANOSIM	Analysis of similarity
AR	Androgen receptor
ARE	AhR response element
ARNT	Aryl hydrocarbon receptor nuclear translocator
BAF	Bioaccumulation Factor
BCD	Base catalysed decomposition
BCF	Bioconcentration factor
BCI	Below detection limit
CCA	Canonoical correspondance analysis
CPOM	Coarse particulate organic matter
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CYP1A	Cytochrome P450-1A
DCC	Dextran coated charcoal
DCM	Dichloromethane
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichloro-diphenyl-ethane
DDT	Dichloro-diphenyl-trichloroethane
DES	Diethylstilbestrol
DMSO	Dimethylsulphoxide
DO	Dissolved oxygen
DWG	Drinking water guideline
EDCs	Endocrine disrupting compounds
E_1	Estrone

E_2	17β-estradiol
E_2eq	17β-estradiol equivalent concentration
EE_2	17α-ethynylestradiol
EI	Electron ionisation
EPT	Ephemeroptera, Plecoptera, Trichoptera
ER	Estrogen receptor
ERA	Ecological risk assessment
EROD	Ethoxyresorufin-*O*-deethylase
ETR	Ecotoxicological rating
EWQCP	Eastern Water Quality Control Pond
FPOM	Fine particulate organic matter
GC-MS	Gas-chromotography – mass spectrometery
GL	Gonopodial length
GSI	Gonado-somatic index
Gx	Gonopodial extension
HpCDD	Hepta-chloro dibenzo dioxin
ICP-MS	Inductively coupled – mass spectrometry
ITD	Indirect thermal desorption
LR	Gonopodial length ratio
LOE	Line(s) of evidence
LOI	Loss on ignition
NADPH	Nicotinamide adenine dinucleotide phosphate + H^1
nMDS	Non-metric multi-dimensional scaling
NOAA	National Oceanic and Atmospheric Administration
NSW	New South Wales
OCA	Olympic Coordination Authority
OCDD	Octo-chloro dibenzo dioxin
OCP	Organochlorine pesticides
PAH	Pulycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD/F	Poly chlorinated dibenzo dioxins/furans

POPs	Persistent organic pollutants
RBA	Relative binding affinity
RO	Reverse osmosis
SGp	Standardised gonopodial length
SIM	Selected ion monitoring
SIMPER	Similarity percentages
SIMPROF	Similarity profile permutation tests
SL	Standard length
SMA	Standardised major axis
(S)MATR	Standardised major axis tests and routines
SPE	Solid phase extraction
SOP	Sydney Olympic Park
SOPA	Sydney Olympic Park Authority
SWQCP	Southern Water Quality Control Pond
TCDD	Tetra-chlorinated dibenzo-*p*-dioxin
TCDDeq	Tetra-chlorinated dibenzo-*p*-dioxin equivalent concentration
TOC	Total organic carbon
TPH	Total petroleum hydrocarbons
UNEP	United Nations Environment Program
USEPA	United States Environmental Protection Agency
WOE	Weight of evidence
WR	Gonopodial width ratio
WQG	Water quality guideline
WRAMS	Water reclamation and management scheme

Abstract

Disruption to the endocrine systems of wild fauna by anthropogenic compounds (endocrine disruption) has received significant scientific attention over the past 50 years. Compounds with reported reproductive effects (e.g., natural and synthetic estrogens, organochlorine pesticides) have received particular attention due to their potential population level effects. Endocrine disruptors which bind to the aryl hydrocarbon receptor (AhR) (e.g., polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs)) are being increasingly studied. The toxic action of these compounds can be directly (by endogenous metabolisation to mutagenic products) or indirectly (causing carcinogenesis) mediated by the AhR. The presence of these compounds in the environment is cause for ecological concern. The manufacture and/or release of many endocrine disruptors (particularly chlorinated aromatic compounds) have been restricted in many countries but, due to their long half-lives, significant concentrations are still present in the environment.

Sydney Olympic Park (SOP) is constructed on remediated land. Prior to remediation there were significant concentrations of persistent organic pollutants (POPs) (PCBs, PCDDs, organochlorine pesticides) on the site. The remediation effort in the lead-up to the Sydney Olympic Games in 2000 involved the excavation and treatment of large volumes of POP-contaminated soil and sediment, and storage of treated and translocated wastes in containment mounds on the site. A number of wetlands was created during the restoration process and the area is now habitat for native and migratory fauna. It is also an area of high recreational and educational amenity.

This research investigates the presence of endocrine disrupting chemicals (EDCs) in SOP in the post-remediation context. The studies in this research project investigate different lines of evidence from the concentrations of these chemicals, to *in vitro* bioassays, to an *in vivo*

biomarker of exposure, to population and community level assessment of the presence and effects of POPs in the wetlands within the Park. The results of these individual studies form the basis of a weight-of-evidence semi-quantitative ranking of the wetlands within a gradient of contamination at reference sites.

There was no evidence in the water of the wetlands in SOP to support the presence of EDCs with affinity for estrogen receptor (ER) (17β-estradiol equivalency (E_2eq) quantified by an estrogen receptor radioligand binding assay). In the sediments of the wetlands there was quantifiable E_2eq but there was no effect of this potential estrogenicity on the reproductive morphology of the male mosquitofish, *Gambusia holbrooki*. Chemical analysis showed measurable concentrations of ΣPAHs (272 – 14461 ng/g dry weight), ΣDDT (4 – 98 ng/g) and ΣPCBs (5 – 47 ng/g) within the sediments of most of the wetlands in SOP and an *in vitro* bioassay (H4IIE) indicated the presence of compounds able to bind to the AhR in all sediment samples (0.016 – 7.06 ng/g). Both the chemical and *in vitro* bioassay data for sites within SOP were within the range measured for urban impacted sites throughout Sydney. A biomarker of exposure to POPs (CYP1A induction) was measured in fish (mosquitofish) populations inhabiting the wetlands of SOP and was found to be significantly increased above basal level (2308 pmol res/min/mg protein) at one study site (Boundary Ck) (4327 pmol res/min/mg protein). When compared to reference sites around Sydney these were within the range measured at urban reference sites (1211 – 7579 pmol res/min/mg protein).

Benthic macroinvertebrate communities were relatively depauperate in most wetlands and had low taxon richness. While it was not possible to assign the cause of these effects to the presence of organic pollutants, multivariate analysis of the data suggests a correlation between depauperate communities and increasing sediment concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalency (TCDDeq), ΣDDT and Total Organic Carbon. It was not possible to prove that differences between the life-history traits of mosquitofish inhabiting the study sites were due to POP-contamination. Differences are likely to be due to complex interactions of biotic and abiotic factors.

A weight-of-evidence approach synthesising these lines of evidence was developed to semi-quantitatively assess the ecological risk associated with the presence of POPs in SOP. A ranking of the sites revealed that the study sites within SOP fell between the pristine site (Upper Colo) and the heavily POP impacted reference site (Homebush Bay). It was concluded that there appears to be little or no legacy impact of the pre-remediation contamination levels at SOP. The remediation program has, therefore, been successful in returning SOP wetlands to within the chemical and biological bounds expected in an urban impacted wetland. Measured contamination in the created wetlands in SOP suggests current inputs from the catchment. It is recommended that these findings form the basis of ongoing monitoring, particularly of the most POP-affected wetlands, as identified by the weight-of-evidence assessment (Boundary Ck and Lake Belvedere). Further, it is recommended that current sources of POPs in the urbanised catchments upstream of these sites be investigated and these contamination pathways restricted or closed.

1. General Introduction

Environmental contamination has been a growing issue of concern to the scientific community. The release of anthropogenic chemicals into terrestrial and aquatic ecosystems has been reported to cause a large range of effects at all levels of biological organisation. The synthesis of novel compounds presents challenges to regulatory bodies in ensuring that any release, deliberate or accidental, does not cause ecological impairment in the receiving environment. Many by-products of manufacturing industries are of concern as their toxic effects may not be identified until forensic ecotoxicological examination of a contamination event is conducted.

For many years the acute toxic effects of chemicals such as heavy metals and pesticides have been well-characterised. There is currently significant research into the field of so-called emerging contaminants, compounds and mixtures which may have been released into the environment for some time without full knowledge of their chronic effects that are now being fully realised. Brominated flame retardants have been in use for many decades but their environmental persistence and biomagnification potential are only now coming under scrutiny (Birnbaum and Staskal 2004). The persistence of arsenic in the environment has caused renewed interest in a well-characterised toxic agent (Belluck et al. 2003). Parabens are included in most cosmetics and personal moisturisers but are now suspected to bind to the estrogen receptor (Golden et al. 2005). The environmental distribution of perchlorate is of concern due to its ability to percolate through to groundwater, contaminate drinking water and disrupt thyroid function (Richardson 2003). While it has been known for over half a century that compounds can interfere with the normal function of the endocrine system most research into their chronic effects of the so-called “endocrine disrupting compounds” has been conducted in the last decade.

1.1 Endocrine Disrupting Compounds

1.1.1 Definition and Description

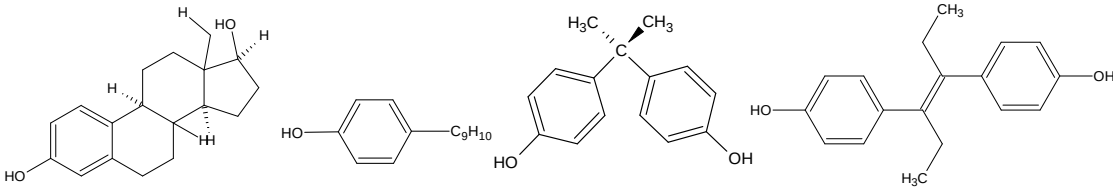
The endocrine system consists of a series of secreting glands, hormones, target cells, cytosolic and membrane associated receptors, and proteins formed as a response to binding to responsive DNA elements, translation and transcription. It is involved in growth, sexual development and the maintenance of homeostasis (Van Der Kraak et al. 1998). Incorrect function of the endocrine system can impair any of these processes and result in sub-optimal individual fitness and even death. The basis of endocrine function is the binding of endogenous hormones to specific receptor sites (which can be located in the cytosol, endoplasmic reticulum or plama membrane of target cells), which initiates protein responses, specific to the target cell. The hydrophobic steroid hormone molecule diffuses directly across the plasma membrane and is bound to a specific estrogen receptor in the cytoplasm. Chaperonin molecules create a homodimer of two ligand-receptor complexes which binds to an estrogen responsive element of DNA. This results in the transcripion of cell-type dependant mRNA and translation for a response protien by ribosmal complexes.

Kavlock et al. (1996) defined an environmental endocrine disrupting compound (EDC) as an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of development processes. Interference can, therefore, occur at any point in the endocrine system from secretion to response protein production. An EDC may elicit a measurable response at any level of biological organisation from sub-cellular to cellular effects, to responses in an individual, or a population (Colborn et al. 1993). Responses to EDCs also include potential intergenerational effects (Damstra et al. 2002). The range of compounds (Table 1-1) and chemical structure classes (Figure 1-1) known to elicit ED responses is large and are from both anthropogenic and natural sources. The presence of EDCs in the environment has attracted wide interest in recent decades with many new sources and the potential environmental and human health impacts described through the investigation of novel pathways of action.

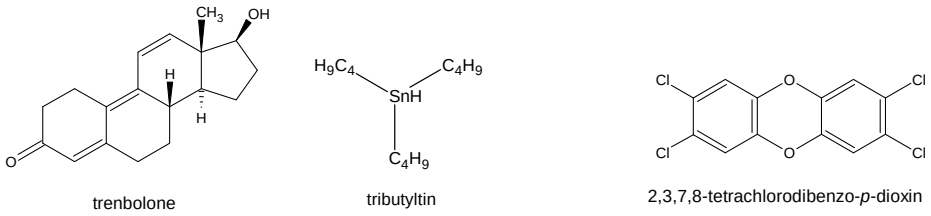
Table 1-1 List of some of the major, known, environmental reproductive and aryl hydrocarbon (AhR) mediated endocrine disrupting compounds (EDCs) their primary receptors, mode of toxic action and source.

	Name	Receptor	Mode of Action[a]	Source
Natural Hormones	17β-estradiol	ER[b]	Estrogenic	Sewage effluent
	Testosterone	AR[c]	Androgenic	Sewage effluent
Pharmaceuticals and Personal Care Products	17α-ethynylestradiol	ER	Estrogenic[1,2]	Sewage effluent
	Diethylstilbestrol[d]	ER	Estrogenic[3]	Pharmaceutical
	Tamoxifen[d]	ER	Anti-estrogenic[1]	Pharmaceutical
	Parabens	ER AR	Estrogenic[1] Anti-androgenic[1]	Personal care products
Pesticides	DDT (DDE, DDD)[e]	ER AR	Estrogenic[1] Anti-androgenic[4]	Use and misuse + waste
	Dieldrin	ER AR	Estrogenic[5] Anti-androgenic[6]	Use and misuse + waste
	Chlordane	ER AR	Anti-estrogenic [7] Anti-androgenic[6]	Use and misuse + waste
Manufacturing By-products	Benzo[a]pyrene (PAH)	AhR[f] ER AR	CYP1A inducer[8] Estrogenic[9] Anti-androgenic[10]	Natural and industrial combustion
	2,3,7,8-TCDD	AhR	CYP1A inducer[8]	Natural and industrial combustion
	Bisphenol A	ER AR	Estrogenic[11] Anti-androgenic[11]	Plastic manufacture
Manufactured for industrial uses	Tributyltin (TBT)	AR	Androgenic[12]	Anti-fouling paint
	PCB (some of 209 congeners)	AhR AR	Carcinogenic[13] Anti-androgenic[14]	Industrial manufacture (aerial deposition)
	Nonylphenol	ER[1] AR	Estrogenic[15] Anti-androgenic[16]	Sewage effluent
	Octylphenol	ER[1] AR	Estrogenic[15] Anti-Androgenic[16]	Sewage effluent

[a] Modes of action are reported for both weak and strong action. [b] Estrogen Receptor. [c] Androgen Receptor. [d] Compounds known to exert an endocrine disrupting effects but not reported as environmental contaminants [e] Mode of action of DDT, DDE and DDD is congener dependent. [f] Aryl hydrocarbon receptor. [1]Blair et al. (2000), [2]Kidd et al. (2007), [3] Folmar et al. (2000), [4] Kelce et al. (1996), Soto et al. (1994), Lemaire et al. (2004), [7]Yang and Chen (1999), [8]Whyte et al. (2000), [9]Charles et al. (2000), [10]Vinggaard et al. (2000), [11]Sohoni and Sumpter (1998), [12]Rilov et al. (2000), [13]Silberhorn et al. (1990), [14]Schrader and Cooke (2003), [15]Laws et al. (2000), Xu et al. (2005).

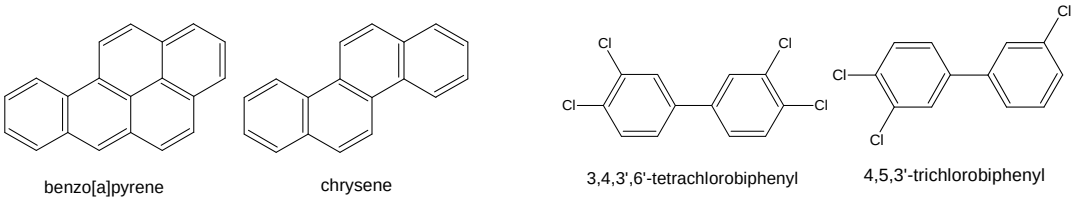


Environmental Estrogens



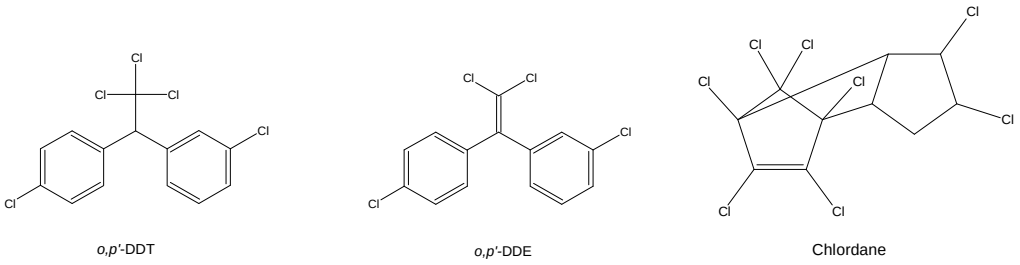
Environmental Androgens

TCDD



Polycyclic aromatic hydrocarbons (PAHs)

Polychlorinated biphenyls



Organochlorine pesticides

Figure 1-1 Chemical structures of representatives of the major groups of known endocrine disrupting compounds (EDCs).

1.1.2 Mode of Action

The normal function of the endocrine system occurs via a wide range of pathwyas and processes. A full review of these is not practical in the current setting but they include such pathwyas as those relating to the amine hormones, peptide/polypeptide hormones and eicosanoids. As described above EDCs may affect any of these pathways. For the purpose of this introduction, the mode of action of endocrine disruption occuring via one particular endocrine sub-system (the steroid hormones) is described as specific chapters of this thesis are based on this pathway. Where an EDC molecule is recognised by a steroid hormone receptor as a native ligand an increase in hormone response will be seen (an agonistic response). Alternatively, the receptor may bind the EDC but the complex is not translocated or bound to the responsive element of the DNA. The action of the native hormone is thereby blocked as receptor binding sites are taken up by the exogenous ligand (an antagonistic response). The important result in any of these cases is an alteration in the end product of the normal steroid hormone receptor binding, transcription and protein translation process. As a regulator of homeostasis, the endocrine system is subject to a number of positive and negative feedback loops. Where receptor antagonism or agonism responses exist there is potential for false positive or negative feedback loops stimulating or stopping the activity of a secretion gland, further enhancing the effect of the EDC.

1.2 Reproductively Active EDCs

1.2.1 Definition, Sources and Fate

Reproductively active EDCs are exogenous ligands for steroid hormone receptors. Their effects fall into four broad categories;

- estrogenic (estrogen receptor agonism),
- anti-estrogenic (estrogen receptor antagonism),
- androgenic (androgen receptor agonism),
- anti-androgenic (androgen receptor antagonism).

The areas of highest current concern are the estrogenic (and anti-androgenic) effects of sewage effluent and certain feminising effects noted in fish downstream of effluent outlets (e.g., Lye et al. 1997, Batty and Lim 1999, Baronti et al. 2000, Jobling et al. 2002, Porter and Janz 2003). Other major groups of reproductively active EDCs include; pesticides (Clark et al. 1998, Baatrup and Junge 2001, Singh and Canario 2004), industrial byproducts such as from manufacture of plastics (Haubruge et al. 2000, Stoker et al. 2003, Watts et al. 2003), industrial detergents (Gray and Metcalfe 1997, Bechmann 1999, Dreze et al. 2000, Kinnberg et al. 2000, Mills et al. 2001, Toft and Baatrup 2001, Kinnberg et al. 2003).

Many reproductively active EDCs have short environmental half-lives. Natural estrogens break down quickly (half-life = 2-3 days) while the synthetic estrogen 17α-ethynylestradiol persists for slightly longer (half-life = 4-6 days) (Williams et al. 1999). Other reproductively active EDCs are far more environmentally persistent. Some organochlorine pesticides (OCPs) (e.g., DDT, chlorpyrifos) have been reported to cause estrogenic (Faber et al. 1991, Clark et al. 1998, Pesando et al. 2004), anti-androgenic (Kelce et al. 1995) and developmental (see Colborn et al. 1993 for review) effects. DDT has a reported half-life of between 3 and 30 years depending on environmental conditions (Lichtenstein et al. 1971) and the ratio of DDT to its similarly reproductively active metabolites (DDE, DDD) is usually <1 (e.g., Pham et al. 1996) suggesting that these metabolites are even more persistent.

The natural and synthetic estrogens, OCPs and most other estrogenic compounds are hydrophobic (log K_{ow} = 2.81 (estriol), 4.15 (ethynylestradiol), 6.19 (DDT)) with low volatility, so removal from the water column by sorption to sediments is expected (Ying et al. 2002). With their short environmental residence time, natural and synthetic estrogens are not considered to bioconcentrate in biota. On the other hand DDT, DDE and DDD are considered "highly bioaccumulative" under Environment Canada, United States Environmental Protection Authority (USEPA) and United Nationes Environment Program (UNEP) guidelines (bioconcentration factor (BCF) > 3.7) (Arnot and Gobas 2006) and have been reported in lipids of wildlife geographically removed from known sources (e.g., Norstrom et al. 1998).

1.2.2 Current State of Reproductive EDC Research

Endocrine disrupting effects modulated via steroid receptors have been shown in numerous taxa. Male herring gulls were found to have oviducts and otherwise deformed gonads (Fry and Toone 1981). In Lake Apopka, Florida, (a Lake which was subjected to a spill of the OCPs, DDT and dicofol) a population of alligators had reduced penis size, decreased serum testosterone and gonadal abnormalities (Guillette Jr. et al. 1994, Guillette Jr. et al. 1996). Exposure to bisphenol A caused sex reversal in caiman (*Caiman latirostris*) (Stoker et al. 2003) while DDT and DDE caused disruption in the formation of gonadal ducts in tiger salamanders (*Ambystoma tigrinum*) (Clark et al. 1998). Treatment with 17α-ethynylestradiol caused a shift in sex ratio and gonadal abnormalities in the amphipod (*Hyalella azteca*) (van den Berg et al. 2003). Exposure to 17β-estradiol and bisphenol A delayed moulting and caused mouthpart deformity in *Chironomus riparius* (Watts et al. 2003). Takamura (1996) examined the sex ratio of four chironomid species as a biomarker for the presence of endocrine disrupting pesticides in rice fields. The feminising of birds as measured by the presence of ovarian tissue in the testes has been suggested as a quantifiable endpoint for endocrine disruption (Berg et al. 2004).

Fish are considered an important vertebrate taxon in aquatic environments and have, therefore, been used widely as indictors of reproductive endocrine disruption in laboratory and field studies. The sexual behaviour of Japanese medaka (*Oryzias latipes*) exposed to 17β-estradiol is suppressed (Oshima et al. 2003). Studies on the fecundity and the fertility of different fish species have demonstrated that EDCs can affect both these functions (Jobling et al. 2002, Oshima et al. 2003). Gonadal abnormalities, including reduced size, reduced weight and altered shape, in response to exposure to endocrine disrupting chemicals have been widely reported (Jobling et al. 1996, Gimeno et al. 1997, Shioda and Wakabayashi 2000, Kim et al. 2001, Lange et al. 2001, Papoulias et al. 2003). A high incidence of intersex gonads (the presence of ovarian tissue in male gonads) associated with the presence of pollution sources has also been reported in a variety of species (Lye et al. 1997, Jobling et al. 1998, Rodgers-Gray et al. 2001, van Aerle et al. 2001). Treatment

with nonylphenol, methoxychlor, 17β-estradiol and 17α-ethynylestradiol has produced fish populations with heavily biased sex ratios (Nimrod and Benson 1998, Kidd et al. 2007).

At the cellular level, differences in the levels of estrogen receptors have been used as a biomarker of estrogenic effects (Westerlund et al. 2000). Vitellogenin, a female specific hepatic protein involved in egg yolk production, has been induced in male fish exposed to 17α-ethynylestradiol, 17β-estradiol, estrone, diethylstilbestrol, methoxychlor, DDT, *tert*-octylphenol, nonylphenol, and sewage effluent (Anderson et al. 1996, Harries et al. 1996, Panter et al. 1998, Allen et al. 1999, Allner et al. 1999, Pedersen et al. 1999, Folmar et al. 2000, Thorpe et al. 2000, Mills et al. 2001) and has been shown to be a relevant biomarker for EDCs in very early-life-stage fish (Tyler et al. 1999). Gonadotropin secretion from the pituitary was stimulated by treatment with 17β-estradiol and DDT in Atlantic croaker (Khan and Thomas 1998). Common carp exposed to 17β-estradiol and nonylphenol were severely anemic, a general toxic response (Schwaiger et al. 2000).

To date most EDC research has focused on effects modulated via the steroid hormone receptors and the concomitant reproductive effects. Field and laboratory studies currently employ a range of recognised assessment techniques across all levels of biological organisation. Interest has presumably been focused on these compounds as the results are considered likely to infer population level effects. In one of the only field-based manipulative experiment to investigate this important link, Kidd et al. (2007) dosed a whole lake with the synthetic estrogen 17α-ethynylestradiol and recorded the collapse of a population of fathead minnows (*Pimphales promelas*) due to the loss of the annual juvenile cohort. This reproductive failure demonstrated the link between chronic exposure to reproductively active EDCs and population level effects

1.3 Persistent Organic Pollutants (POPs)

1.3.1 Definition and Description

Persistent organic pollutants (POPs) are a broad group of environmental contaminants, most of which are now either banned or highly controlled. They are characterised by high

molecular weight and hydrophobicity (high log K_{ow}) and are present in only low concentrations in the water column with much higher concentrations observed in sediments (Zhou et al. 2001), particularly adsorbed to fine particles with high carbon content (Gustafsson et al. 1997). Their bioavailability to benthic fauna is dependent on physical and chemical characteristics of the sediment (Carlberg et al. 1986, Brannon et al. 1993, Timmermann and Andersen 2003) but they generally have high bioaccumulation and bioconcentration factors (BAFs and BCFs) (Thoman 1989).

In 2001, the Stockholm Convention defined individual and broad classes of POPs to be regulated by the signatories. These included organochlorine pesticides (OCPs) (e.g., DDT, Aldrin, Chlordane, Dieldrin), polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) polychlorinated dibenzofurans (PCDFs). OCPs were manufactured in large quantities in the first half of the twentieth century and were the most effective insect control measure known at that time. In the 1960s concern grew as to the effect of OCPs (particularly DDT) on wildlife through bioaccumulation and biomagnification (Carson 1962). The Stockholm convention legislated to terminate the use of many OCPs and placed restrictions on the use of DDT. DDT is currently produced for use to control disease vectors in many developing countries, including Ethiopia, India, Myanmar and Mauritius (UNEP 2007).

PCBs were manufactured for a range of industrial uses such as dielectric and fire retardant materials. PCBs occur as 209 congeners and were generally marketed in mixtures such as Aroclor and ClophenA. While the manufacture of PCBs is banned under the Stockholm Convention, termination of their use in equipment will not take effect until 2025 (UNEP 2001). PCBs are ubiquitous in the environment and studies suggest that their atmospheric deposition to aquatic environments and subsequent sequestering to sediments is an important route of entry to such ecosystems (Franz et al. 1998, Tasdemir and Holsen 2005).

Members of the PCDD/Fs are among the most hydrophobic, bioaccumulative and biomagnificative compounds. PCDDs form both naturally from the incomplete combustion of organic matter and through combustive industrial processes. Atmospheric release of

PCDDs can result from natural events such as wildfires (Gullett and Touati 2003) and from numerous industrial processes such as fossil fuel burning and metal production (e.g., aluminium, iron, copper) (Bawden et al. 2004). While their toxicity is highly variable and dependent on chemical structure 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is recognised as one of the most toxic and environmentally persistent chemicals. TCDD was a byproduct of the formation of the herbicides 2,4-D and 2,4,5-T (Barsotti et al. 1979). There was not only significant TCDD contamination of wastes from the manufacture of these herbicides but the products themselves were often highly contaminated. TCDD has a half-life of >100 years in sediment (Sinkkonen and Paasivirta 2000) and despite its release from industrial processes being restricted under the Stockholm Convention (UNEP 2001) it is still found in high concentrations in historically contaminated soils and sediments and can enter waterways through atmospheric deposition (Lohmann and Jones 1998).

While the Stockholm Convention did not address the issue of polycyclic aromatic hydrocarbons (PAHs), they are worthy of mention here as they can elicit similar responses as the traditional POPs. PAHs are generally more volatile than the POPs and therefore less environmentally persistent, but certain PAHs (e.g., benzo[*a*]pyrene) are environmentally persistent in sediments. PAHs, like PCDDs are produced from both anthropogenic and natural activity through the incomplete combustion of organic material such as timber and coal. BAFs for PAHs are not widely published as they are generally metabolised by fish via the cytochrome P450-dependent mixed function oxygenase system (Kennish 1997). Some articles, have however, shown bioaccumulation of some PAHs in some species. Burkhard and Lukasewycz (2000) describe calculated BAFs for five PAHs of between 1.32 (phenanthrene) and 3.98 (benz[a]anthracene) in lake trout in Lake Michigan, USA. Therefore, while some PAHs do not bioaccumulate, others can be categorised as "very bioaccumulative" according to Environment Canada and USEPA thresholds (Arnot and Gobas 2006).

Compounds with high bioaccumulation and biomagnification factors are of significant global concern. Their route of entry to the food chain can be by direct absorption across membranes (usually low), through contaminated sediments (especially in benthic

organisms) and in the dietary fraction (Batterman et al. 1989). The restriction of their manufacture and/or use in one nation/state is generally insufficient to ensure against activity through local food chains, as their long-range transport within the lipid tissue of mobile and migratory animals and in the atmosphere is not bound by political borders.

1.3.2 Mode of Action and Effects of POPs

Traditionally the term "endocrine disruption" has been used to imply interference in the normal function of steroid hormones and their receptors. POPs, however, act via the aryl hydrocarbon receptor (AhR). Limited numbers of endogenous AhR ligands have also been identified (Rannug et al. 1987, Andreola et al. 1997). AhR and its dimerisation partner, ARNT (aryl hydrocarbon nuclear translocator), activate members of the Ah-gene battery. This results in a pleiotropic response including the production of cytochrome P4501A (CYP1A), immunotoxcity, hepatic damage, carcinogenisis, reproductive toxicity, neurotoxcity (see Mandal 2005 for review).

Induction of CYP1A in response to AhR binding of halogenated aromatic hydrocarbons such as 2,3,7,8-TCDD (Hahn 1998) is the best characterised of these responses. CYP1A is a member of the cytochrome P450 family of membrane (endoplasmic reticulum) associated mixed function oxygenases (MFOs). While the direct toxicological implications of small increases in CYP1A concentrations are relatively minor (e.g., CYP1A metabolism of xenobiotics can lead to oxidative stress (Nebert et al. 2000)), the induction of CYP1A above background levels can be used as an early warning biomarker for the exposure of an animal to halogenated aromatic molecules (see Whyte et al. 2000 for review).

The toxic action of POPs can occur either directly or indirectly via the AhR. Direct toxicity results from ligand binding and production of CYP1A which can metabolise the ligand to a toxic intermediate. For example, CYP1A hydroxylates some PAHs but toxicity remains high suggesting a toxic effect associated with the metabolites (Huff et al. 1994, Machala et al. 2001). Other ligands, however, are not catalysed by CYP1A but ligand binding may result in the activation of genes associated with the formation of tumors (oncogenes) or

suppression of immune responses (Puga et al. 2000, Camacho et al. 2001). The mechanism of AhR mediated toxicity is, therefore, complex and research is ongoing.

While the AhR promiscuously binds many compounds (Denison and Heath-Pagliuso 1998), high affinity ligands are generally planar, aromatic and hydrophobic (Denison 1991) and include TCDD, some PCBs, PAHs and some OCPs (Sierra-Santoyo et al. 2000, Fent 2001, Oropeza-Hernandez et al. 2003). Exposure to these persistent organic pollutants (POPs) can cause major physiological damage. POPs are variously carcinogenic, teratogenic, genotoxic and can cause reproductive impairment at low concentrations (reviewed by Delzell et al. 1994a,b,c).

Effects of dioxins and furans include increased mortality in adult (e.g., guppies (Norris and Miller 1974) and rainbow trout (Walker et al. 1992, Giesy et al. 2002b)) and juvenile (e.g., rainbow trout (Helder 1981), pike (Helder 1980)) fish, developmental problems (Walker et al. 1992), gonadal abnormalities (Volz et al. 2005), and multiple hepatic abnormalities (Walter et al. 2000). Developmental toxicity (Wright and Tillitt 1999), early life stage mortality (Walker and Peterson 1991), histopathological changes (Khan 2003) and external lesions have all been attributed to exposure to PCBs (Khan 2003). PAHs have been shown to affect invertebrate molting (Oberdorster et al. 2000), apoptosis (Reynaud et al. 2004), embryo mortality (Chikae et al. 2004), body weight (Chikae et al. 2004), gonado-somatic index (Chikae et al. 2004), cardiovascular system formation (Mizell et al. 1995), carcinogenesis (Hendricks et al. 1985, Metcalfe 1988, Reynaud and Deschaux 2005) and immune function (Tahir and Secombes 1995, Carlson et al. 2002, 2004).

1.4 Contaminated Sites

Contaminated sites are aquatic or terrestrial environments in which hazardous substances occur at concentrations above background levels and pose or are likely to pose an immediate or long-term hazard to human or environmental health (NEPC 1999). They are reservoirs of contaminants which may enter local ecosystems or migrate off-site by infiltration to groundwater, volatilisation and aerial dispersal, bioaccumulation and/or

biomagnification to mobile fauna and translocation by abiotic mechanisms (wind or water flows) (Asante-Duah 1996). Where significant ecological impairment, based on the pollutants at a contaminated site is suspected, or where the reuse of a contaminated site is desired, exposure pathways to ecosystems and human populations should be closed. This necessitates a comprehensive assessment of these pathways and the potential biological effects in receptor organisms (Suter 2006).

1.4.1 Contaminated Site Assessment

The process of assessing the risk associated with the contamination of a site is a multilevel procedure involving the characterisation of the presence of contaminants, the likelihood of biota being exposed to the contaminants and the likely effect of this exposure. Ecological risk is the potential for harmful effects to local ecosystems posed by resident or potential pollutants (Riviere 2000). Assessing and quantifying this risk has been the focus of much research over the past few decades and a number of different risk assessment models has been proposed. The basic components of these models are essentially similar although they are presented with different iterative steps and feedback loops (reviewed and compared by Riviere 2000). Firstly they involve a description of the problem which identifies the contaminants (stressors) of concern, and the ecosystem components at risk. This is followed by a characterisation of both the potential exposure (bioavailability and *in vitro* testing) and the ecological risk (individual, population and community level responses) associated with this exposure. Finally, these elements are integrated into a characterisation of the overall ecological risk associated with the site. The endpoints examined at each stage of the risk assessment are highly case dependent. The final data integration step may take qualitative, semi-quantitative or quantitative forms. A variety of different quantification methods and algorithms have been described for this process. Semi-quantitative and quantitative ecological risk assessments can be used in the decision making process to prioritise future monitoring programs and to determine if the risk of ecological effect posed by the contamination is large enough to warrant remediation.

1.4.2 Remediation Methods and Post-Remediation Assessment

Where remediation is decided upon, the appropriate remediation processes and technologies are dependent on the major groups of contaminants present. The basic goal of any remediation process is to reduce or eliminate routes of exposure of contaminants to biota. The construction of physical barriers (barriers, caps) are useful techniques where excavation of contaminated material poses secondary pollution risks (Cairney and Hobson 1998). Washing of soils and sediments is used to remove contaminants (including metals) from particles but results in a product (often of fine particles) of high contaminant concentration which must be further processed (Wilichowski 2001). *Ex situ* aerobic bioremediation technologies have been developed to reduce concentrations of contaminants such as PAHs and PCBs (Kalogerakis 2005). Thermal desorption is used to release bound mercury from soils and sediments into a gaseous phase which can then be further treated (Calmano et al. 2001). For other contaminants thermal desorption techniques can destroy high molecular weight organic contaminants or break them down to non-toxic products (Hamer et al. 2005). A full description of these and other current remediation techniques is beyond the scope of this study but are extensively reviewed elsewhere (e.g., Cairney and Hobson 1998, Stegemann et al. 2001, Lens et al. 2005).

Post-remediation site assessments are (relative to pre-remediation studies) poorly represented in the literature (Anderson et al. 2000). This is presumably because many exist as reports for industry or regulatory bodies. Post-remediation assessment in the form of follow-up risk assessment or ongoing monitoring is essential in determining the success of a remediation effort, yet in their survey of 367 remediated sites in the UK, Rivett et al. (2002) found that post-remediation monitoring was rarely carried out over an extended period (> 3 years) and often only as a one-off validation of effectiveness.

1.5 Homebush Bay and Surrounds

1.5.1 Ecological Degradation and Contamination

Homebush Bay is located 13km west of the central business district of Sydney, Australia. The region of interest for the current study includes the western side of the Bay, the

southern bank of Parramatta River west to Silverwater Bridge, and is bounded on the South and West by Silverwater Rd. and Homebush Bay Drive respectively (refer to Chapter 2 for a detailed site description and map). Prior to European settlement Homebush Bay formed an estuary surrounded by vast intertidal mudflats, fringed with mangrove, saltmarsh and *Eucalyptus* and *Casuarina* woodlands (OCA 1995). There is no direct historical reference to the habitation of the area prior to European settlement of Sydney in 1788 but the oral record suggests that it was used as a meeting place for the aboriginal peoples of the area (Lee and Darwala-Lia 1999).

With the rapid expansion of Sydney during the early twentieth Century, the surrounds of Homebush Bay were cleared of vegetation and developed as an industrial centre. Along with this development, significant reclamation of the Bay and general alteration of the local environment occurred. Streams were diverted and tidal flushing blocked. The Bay gained its present shape in the 1960s but surrounding vegetation continued to be cleared up to the 1980s (Laginestra et al. 2001).

Early industrial occupants of the site included the state brickworks, an abattoir and a naval armaments depot (OCA 1995). These gave way to industries including the manufacture of the herbicides 2,4-D and 2,4,5-T, PCBs and phenols in the 1960s (Rubenstein and Wicklund 1991). Other combustive industries known to produce PAHs as by-products (e.g., manufacture of paints, plastics, industrial alcohols) occurred on the Homebush Bay site until the 1990s (Standing Committee on State Development 2002).

Prior to early 1990s, the Homebush Bay area was also a site for controlled and uncontrolled dumping of industrial and domestic waste (OCA 1997). An estimated total of 9 million m^3 of landfill was dumped over the 760 Ha site in a period spanning over a century (Laginestra et al. 2001).

1.5.2 Sydney Olympic Park Remediation

With the award of the 2000 Olympic Games, to be based at the Homebush Bay site, to Sydney in 1993, a small-scale remediation effort already in progress was provided with further impetus and funding (Laginestra 2003). A key priority of the remediation project was to close contamination pathways to reduce human health and ecological risk (OCA 1997). Alongside this was the goal of returning the existing habitat to an approximation of the natural condition by repairing the degraded habitats, removing introduced species and re-instatement of native vegetation (OCA 2000d). During the bidding process the Sydney team sold the event as the "Green Games" and received the backing of Greenpeace under conditions that the Homebush Bay site (the site for much of the Olympic events) was cleaned up in an environmentally friendly manner.

An extensive investigation of the area prior to the clean-up, including chemical testing of more than 5000 samples (OCA 2000b), indicated that 160 Ha required some form of remediation (OCA 1997). Under the Environmentally Hazardous Chemical Waste Act NSW (1985) material contaminated with a total concentration of one or more "scheduled compounds" of greater than 2 ppm is defined as "scheduled chemical waste" and requires treatment. Amongst the scheduled chemicals present at the Homebush Bay site were many classed as carcinogens or pro-carcinogens. In particular the presence of OCPs, PAHs, PCBs, dioxins and furans was of concern (Table 1-2). Known sources were industrial processes, including chemical manufacture, and uncontrolled industrial and municipal waste dumping (Waste Services NSW 1994). Many sources, however, remained unidentified. Dumping of TCDD contaminated wastes both in Homebush Bay and the surrounding wetlands has been reported (Standing Committee on State Development 2002).

The remediation of the site was designed to avoid off-site relocation of contamination and four hundred tonnes of material contaminated with "scheduled chemical waste" were excavated from the site and treated in specifically designed modules by indirectly-heated thermal desorption (ITD) and base catalyzed decomposition (BCD) over a 3 year period (OCA and ADI 1999). End of pipe testing of the resulting waste ensured that the levels of scheduled compounds were below that considered of risk. During the initial evaluation

Table 1-2 Organic compounds found in Sydney Olympic Park groundwater, surface waters and sediments samples prior to remediation and leachate from containment mounds post-remediation with Log K_{ow} and Log RBA (Relative Binding Affinity to the Estrogen Receptor (ER)). Unless otherwise specified, data was sourced from the Sydney Olympic Park Ecology Databank. NBC = Non-Binding Competitor, ND = no data available, WB = Weak ER binding only.

Compound	Groundwater (µg/L)	Sediment (µg/Kg)	Surface Waters (µg/L)	Leachate (µg/L)	Log K_{ow}	Log RBA
TPH[a]	1025000	430800	$1x10^{10}$	6159000		
Total PAH	182200	28000	43	5763	3.35[c]	NB[c]
Benzo(a)pyrene	4900	19600	30	33.4	6.35	WB[d]
Benzene	92000	26000	160000	77848	2.03	ND
Toluene	58000	2000	150	3740	2.73	ND
Ethylbenzene	4600	720[b]	4800	1920	3.15	ND
Xylene	5357	30000000[b]	52	1301	3.12	ND
Phenolics	82700	190	50	1171	5.76	-1.43[f]
Total Dioxins/Furans	5.8	316258[b]	<2	$1.3x10^{-10}$		
TCDDeq		14000	0.246[b]	0.000176		
TCDD	0.0092	19500[b]	0.232[b]	0.000029	6.42	NB[g]
Total PCB	<0.03	700	10140[b]	<0.01	4.63[h]	WB[h] 0 – (-1.44)[i]
Total OCP	<600	1500	6800[b]	<0.02		-1.87[j]
DDT	<40	16000	446[b]	ND	6.36	-2.85[d]
DDE	<40	180	744[b]	ND	6.96	NBC
DDD	<40	300	1044[b]	ND	6.02	NBC
Organophosphates	2700	200	ND	ND		ND

[a] Total Petroleum Hydrocarbons. [b] Results from estuarine samples from Homebush Bay and Parramatta River.. [c] Values for naphthalene only. [d] Blair et al. (2000). [e] Hirose et al. (2001). [f] all alkylphenols tested were ER ligands, value for 4-nonylphenol (max RBA). [g] No Vtg induction, anti-estrogenic effects involving the aryl hydrocarbon receptor. [h] Matthews and Zacharewski (2000). [i] Max for 2'5'-Dichloro-4-biphenyl. [j] Kepone (Kepone and Methoxychlor only non- DDT/ DDT derivative ER ligands).

process hotspots of contamination were identified and the amenities on the site were designed around having these non-accessible to the public after remediation.

Landfill with low levels of contamination and treated fill (previously with high concentrations of POPs) were consolidated in clay-capped, geotextile-lined containment mounds on the site (OCA 2000c). Seven thousand tonnes are now contained in seven mounds on site. The mounds were topped with clean soil and landscaped with native grasses and shrubs and now form part of the high visual amenity of the area (OCA 2000a). Each was fitted with an extensive system of drains designed to intercept leachate from the mounds prior to infiltration to groundwater. Leachate is treated in a nearby chemical treatment plant and is not released to the environment (OCA 2000c). Leachate is regularly tested and, when found to contain contaminants in higher than acceptable levels, a secondary level of treatment is triggered.

The sediments of Homebush Bay itself were not included in the remediation program. Examination of these sediments has shown very high concentrations of PCDDs (2,3,7,8-TCDD 1500 pg/g (Birch et al. 2007)), PAHs (ΣPAH $\cong$ 22500 ng/g (McCready et al. 2000)), PCBs (ΣPCB > 200 ng/g (Birch and Taylor 2000)) and OCPs (ΣDDT > 50 ng/g (Birch and Taylor 2000)). The sediments of the Bay have been earmarked for remediation since the late 1990s. The most contaminated parts of the Bay are currently being remediated in conjunction with a residential redevelopment of its eastern shore. The Bay has been closed to commercial and recreations fishing since 1989 and the commercial fishing ban was extended to the remainder of Sydney Harbour in 2006 as a result of elevated TCDD levels in commercial seafood species.

1.5.3 Current State and Uses of Sydney Olympic Park

The region now known as Sydney Olympic Park (SOP) incorporates nearby Bicentennial Park and comes under the jurisdiction of the Sydney Olympic Park Authority (SOPA) (Grant 2002). It has high recreational, educational and ecological values with large

numberof visitors each year. Water-sensitive design has been incorporated into the redevelopment of the area with stormwater and sewage collected and treated in an on-site Water Reclamation and Management Scheme (WRAMS) and reused for irrigation and other non-potable uses such as flushing toilets and garden water in the Games Village (Newington Village) (OCA 2000b).

The Park is home to varied wildlife. Many wetlands were created on the site during the remediation process and aquatic biota including water-birds (some of which are migratory) and amphibians (including the endangered green and golden bell frog (*Litoria aurea*) are of particular importance (OCA 2000d). There are a number of invasive aquatic pests in the wetlands of the Park. Of particular concern is the mosquitofish, *Gambusia holbrooki*, a ubiquitous inhabitant of the urban waterways around Sydney. Mosquitofish are opportunistic feeders (Pen et al. 1993) and may feed on frog eggs (Komak and Crossland 2000) and could therefore pose a threat to populations of the green and golden bell frog (Hamer et al. 2002).

Subsequent to the completion of the remediation program, SOPA was charged with the ongoing monitoring of the area and with maintaining its visual and recreational amenity (Grant 2002). A component of this is the monitoring of the chemical composition of the leachate from the contaminant mounds. Sinclair Knight Merz (SKM) and EVS Environment Consultants (EVS) (2001) found detectable levels of PCDDs (HpCDD, OCDD and ΣTCDD), and some PAHs (naphthalene, fluoranthene, pyrene) in samples collected from leachate drains adjacent to containment mounds. In general the acute toxicity (sea-urchin larvae and Microtox bioassays) of these leachates was due to ammonia and other non-organic contaminants. It is possible that the collection of leachate in mound drainage systems is not complete, resulting in release of POPs to the waterways of the Park. As part of the maintenance of the Park, SOPA undertakes to use the area as an educational facility and as a living laboratory for furthering scientific environmental understanding of such an ecosystem.

1.6 Use of Gambusia *spp. (Poeciliidae) in EDC Ecotoxicology*

Teleosts are widely used in aquatic toxicology as test animals for determining the toxicity of many different pollutants, including EDCs. There are many different species (e.g., Japanese medaka, rainbowfish, trout, perch, bully) whose response to EDCs has been well characterised. Poeciliids (live-bearers) have been used to study many different biological functions and are ideal organisms for the investigation of endocrine disruption (Doyle and Lim 2002). They are generally hardy animals with a high resilience to most environmental variables (e.g., temperature, pH etc) and are relatively insensitive to conventional toxicants (Lee et al. 1992, Jagoe et al. 1996, Chaisuksant et al. 1997). The male of most species is physically smaller than the female and has a readily identifiable elongated anal fin, which acts as an intromittant organ during copulation (Collier 1936). This feature, the gonopodium, is under androgenic control and its development can be delayed or reduced through exposure to estrogenic substances (e.g., Doyle and Lim 2002). Likewise, female poeciliids can be induced to grow a gonopodium-like structure when exposed to androgenic substances (Howell et al. 1980, Bortone et al. 1989, Cody and Bortone 1997, Bortone and Cody 1999).

The mosquitofish, *Gambusia holbrooki* (Poeciliidae), was introduced to Australian freshwaters in an unsuccessful attempt to control mosquito populations. It has since become highly invasive with established populations in most freshwater systems in the country, particulary those located close to urban centres and is thought to pose a threat to native fish and frog populations. *G. holbrooki* has been used in a series of Australian investigations into the effects of EDCs. In the laboratory, exposure of mosquitofish to high levels of 17β-estradiol reduced both the development of the gonopodium and male sex drive (Doyle and Lim 2005) and delayed the development of male-specific skeletal structures (hemal spines) in early-life-stages (Rawson et al. 2006). Field studies investigating the effects of environmental estrogens have shown significant reductions in male secondary sexual characteristics downstream of sewage treatment plants in Sydney (Batty and Lim 1999, Doyle et al. 2003, Rawson et al. 2008).

1.7 Study Aims

The overall aim of this study was to investigate the presence and effects of EDCs in Sydney Olympic Park, Sydney, Australia, after its remediation, using multiple lines of evidence across different levels of biological organisation. This remediated site offers a unique opportunity to evaluate the efficacy of a worlds-best-practice remediation effort in the removal of both reproductive EDCs and POPs. Through the identification of the presence of these pollutants by chemical analysis, examination of their potential biological activity by *in vitro* and *in vivo* assays, and investigation of whole animal, population and community level responses, the study will estimate the ecological risk associated with the freshwater ecosystems present in the Park.

Specifically the hypotheses to be tested are:

1. The fish populations in the remnant and constructed wetlands in Sydney Olympic Park are not affected by estrogenic endocrine disrupting compounds.
2. The remediation has reduced the aqeous and sediment concentrations of POPs to within the bounds expected in an urban impacted wetland in Sydney.
3. The fish populations in the remnant and constructed wetlands in Sydney Olympic Park are not exposed to POPs.
4. The remant and constructed wetlands in Sydney Olympic Park with high concentrations of sediment contamination have depauperate benthic communities with low taxon diversity.
5. Differences in the life-history traits of fish populations wetlands within Sydney Olympic Park can are related to current or past contamination.

By testing these hypotheses and examining the endpoints with reference to urban impacted wetlands in the Sydney metropolitan area, the SOP wetlands will be evaluated within the context of a gradient of impact to appraise the efficacy of the remediation process in closing contamination pathways and returning the site to an approximation of the natural condition. It is anticipated that the results of this study will provide information to managers regarding the current state of the wetlands of the Park, identify wetlands of high

priority for future monitoring and indicate whether future remediation efforts on similarly contaminated lands should follow the same methods used in Sydney Olympic Park.

1.8 Chapter Outlines

The individual studies presented in this thesis follow a tiered approach from chemical analysis through to an *in vitro* assessment of receptor level impacts, an *in vivo* biomarker of exposure, and finally to population and community level responses. The thesis culminates in a weight-of-evidence site assessment incorporating multiple lines of evidence, which ranks the study sites using a semi-quantitative ecological risk assessment. The framework followed is presented in Figure 1-2. The chapters of the thesis are written as manuscripts for publication and some degree of repetition is therefore inevitable.

Chapter 2 introduces the study sites used for the individual studies making up the thesis. Particular focus is placed on the wetlands located within Sydney Olympic Park (SOP) and three reference wetlands – Upper Colo, Macquarie University and Homebush Bay, which represent an expected gradient from pristine to urban impacted to heavily POP contaminated sites.

Chapter 3 follows from a study by Brennan et al. (in prep.) which reports estrogenic effects on the mosquitofish, *Gambusia holbrooki*, inhabiting some of the wetlands within SOP. The current study uses a combination of *in vitro* assessment of the estrogenicity of the waters and sediments of the wetlands using a competitive radioligand binding assay and an *in vivo* assessment of estrogenic impact on resident populations of mosquitofish, a model organism for studying endocrine disruption.

Chapter 4 describes the results of chemical analysis (metals, ΣPCBs, ΣPAHs, ΣDDT) of the sediments of wetlands within SOP and a range of urban impacted reference sites throughout the Sydney metropolitan area. Results of an *in vitro* study (the H4IIE cell line bioassay) to evaluate the 2,3,7,8-TCDD equivalency are presented, as are the results of the characterisation of the sediments of the wetlands (organic and inorganic carbon content, sediment grain size, pH, redox potential etc.). A series of multiple regressions is presented

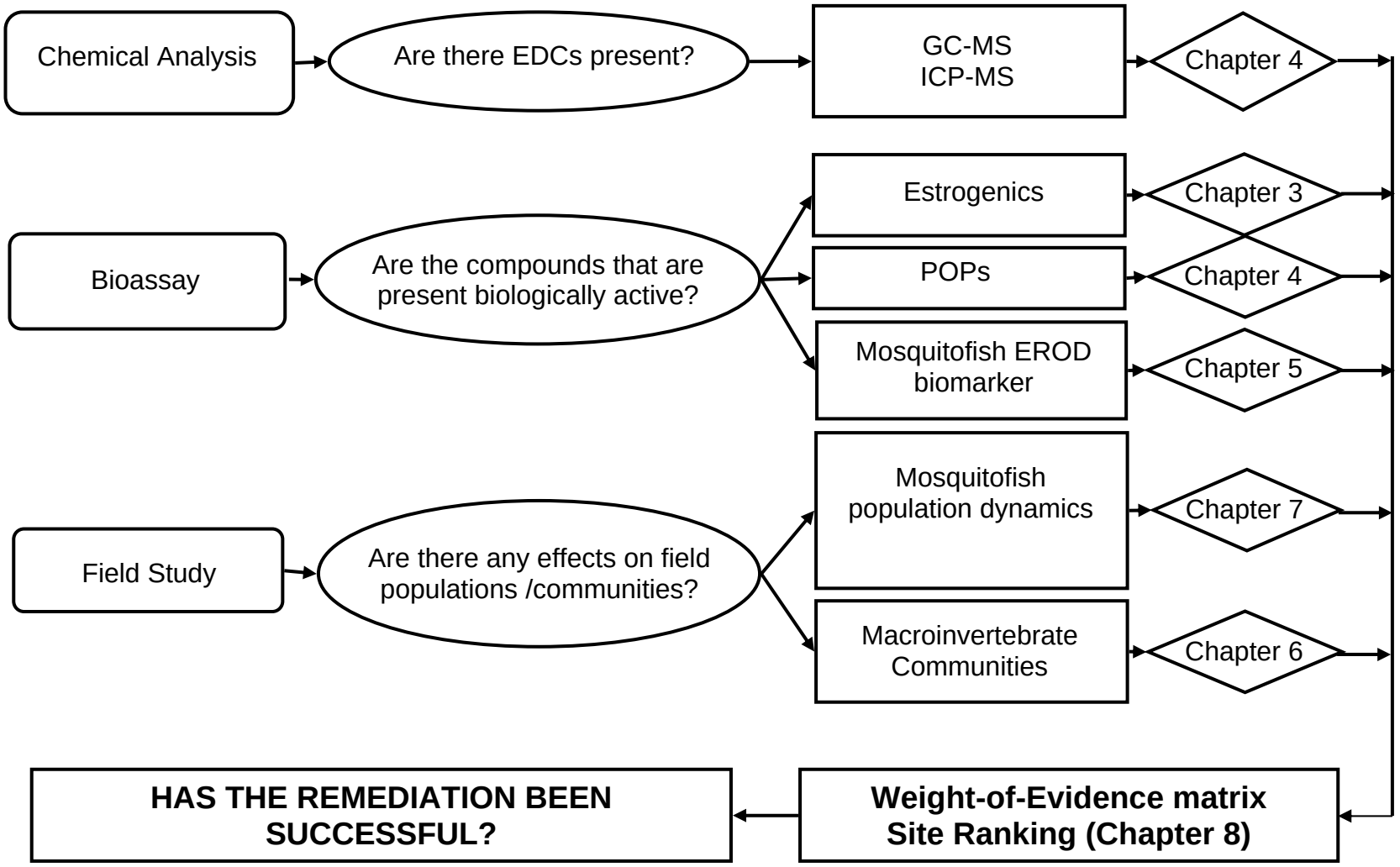


Figure 1-2 Framework used in the current study for the assessment of the study sites used in the current study with the chapters where the material is presented

to evaluate potential influencing factors on the aqueous and sediment TCDDeq. This chapter also describes the results of an experiment simulating the effects of anthropogenic disturbance of contaminated sediment on the potential bioavailability of POPs to aquatic organisms.

Chapter 5 presents the results of an *in vivo* assessment of the exposure of resident populations of mosquitofish at SOP and reference sites to POPs using hepatic CYP1A activity as a biomarker of exposure. The results are presented with reference to a range of urban impacted wetlands around Sydney.

Chapter 6 offers a view of the state of the benthic macroinvertebrate communities in the SOP wetlands. Taxon richness and macroinvertebrate abundance are presented and multiple regression models are used to explain trends with reference to contaminant levels. Differences in benthic community structure between wetlands and factors influencing this variation are analysed using multivariate techniques (nMDS, canonical correspondence analysis).

Chapter 7 describes the life-history traits of mosquitofish populations in SOP over a 14 month period. An assessment of the overall population condition is made and the population dynamics of mosquitofish inhabiting the study sites are used to assess differences between wetlands which may indicate an effect of POP-related contamination. Sex ratio, population condition, male gonado-somatic index and body condition index are used to assess potential differences.

Chapter 8 sythesises the findings of the research using a semi-quantitative weight-of-evidence approach, to rank the study sites from those of least concern to those of most concern. Reference sites are included to validate the method.

Chapter 9 presents the conclusions of the study. Recommendations are made for future monitoring priorities and research, which would be valuable in maintaining and improving the high ecological value of the SOP wetlands.

2 Description of Study Sites

This study examined a number of wetlands from Sydney Olympic Park (SOP) and the urban Sydney metropolitan area. A guide to the use of various study sites in the different studies making up this thesis is provided in Table 2-1. Ideally the number of sites in a field study are equal across the different groups of sites (i.e., study sites = reference sites) enabling a balanced design. This is an appropriate design for studies with only a few endpoints or study matrices (sediment, aqueous, organism). However, this study included a number of different individual studies across a high number of endpoints and it was decided that it was more important that the reference sites cover a broad geographical range within the Sydney metropolitan area and a range of current and historical land-use and analyses appropriate for unbalanced designs be employed.

2.1 Sydney Olympic Park Study Sites

A range of wetlands across SOP was selected as primary study sites. These represent a range of contaminant and remediation histories (Table 2-2) and cover as wide a spatial scale as possible (Figure 2-1).

2.1.1 Narrawang 22

The Narrawang wetlands were created in the north of the Park during the remediation process. Narrawang 22 is located adjacent to the Newington Nature Reserve, a remnant Eucalypt woodland, and is primary wading bird and frog habitat. This wetland is regularly subject to inundation as a result of stormwater amelioration action (Laginestra et al. 2001) and is subject to regular draining in an attempt to control populations of the mosquitofish, *Gambusia holbrooki*. Narrawang 22 is located to the immediate west of the largest contaminated soil containment mound in SOP, Woo-La-Ra. Seepage from the leachate drains from the adjacent waste mound is a possible source of contamination to Narrawang 22. Leachate from this mound has been shown to contain the highest concentrations of POPs of any on the site and to be the most acutely toxic (SKM and EVS 2001).

Table 2-1 Guide to which study sites were used for the different sections of this study.

		E_2eq[a]	Fish[b] Sexual Morphology	Chemical Analysis	TCDD eq	Hepatic CYP1A	Macro-invertebrate Community	Fish[c] Life History Traits
Wetlands in Sydney Olympic Park	Narrawang 22	*	*	*	*	*	*	*
	Boundary Ck.	*	*	*	*	*	*	*
	Lake Belvedere	*	*	*	*	*		*
	EWQCP	*	*	*	*	*	*	*
	SWQCP	*		*	*		*	
	Wharf Stream	*		*	*			
	Nth Water Feature	*		*	*		*	
	Bicentennial Pk.	*		*	*		*	
	Wharf Pond	*		*	*		*	
Homebush Bay and Parramatta R. Sites	Silverwater Bridge	*		*	*			
	Haslams Mouth	*		*	*			
	Newington Wharf	*		*	*			
	Homebush Bay	*		*	*			
Urban Impacted Reference Sites	Upper Colo		*	*	*	*	*	*
	Macquarie Uni	*	*	*	*	*	*	*
	Bardwell Ck.			*	*	*		
	Galston			*	*	*		
	2nd Ponds Ck.			*	*			
	Narrabeen Lake			*	*			
	Moores Ck			*	*			
	Quakers Hill			*	*	*		
	Yarramundi			*	*			
	Eastern Ck.			*	*			
	Kemps Ck.			*	*			
	Chipping Norton Lk			*	*			
	South Ck.			*	*	*		
	Joseph Banks Pk.			*	*	*		
	Oyster Bay			*	*	*		
	Cecil Hills			*	*	*		
	Terrys Ck.			*	*			
	Saltpan Ck.			*	*			
	Nepean River			*	*	*		
	Prospect Ck.				*	*		

[a] 17β-estradiol equivalency. [b] Mosquitofish gonopodial characteristics. [c] Mosquitofish life-history traits.

Table 2-2 Description of Sydney Olympic Park wetlands used in the current study. Catchment area estimated from Laginestra et al. 2001 and Figures 2-1, 2-22.

Wetland	Wetland Type	Catchment Area (km^2)	Catchment Inputs and Current Landuse
Narrawang Wetlands	Remediated/ Created	~1.5	Containment mound, open space, car park, water quality remediation, residential, commercial.
Boundary Ck	Remediated	~2.1	Highly urbanised catchment, commercial (markets), light industry, open space, motorway, water quality remediation.
Lake Belvedere	Remnant	~3	Open space recreation, input from Boundary Ck., recreation, transport.
EWQCP	Water Treatment	~1[a]	Stormwater collection, sedimentation and reservoir for reuse and recycling
SWQCP	Water Treatment	~1[a]	Stormwater collection, sedimentation and reservoir for reuse and recycling
Wharf Pond	Remnant	~5	Remnant in Newington Nature Reserve, adjacent to Parramatta River
Wharf Stream	Remnant	~3	Altered prior to remediation, previously part of navel armaments depot, urban catchment with correctional facility
Northern Water Feature	Constructed	~2	On capped, excavated, landfill site, adjacent to containment mound.
Bicentennial Pk.	Remnant Mangrove	~0.5[a]	Estuarine impacts from Homebush Bay, freshwater impacts from Bicentennial Park, used in educational programs.

[a] EWQCP, SWQCP and Bicentennial Park receive input beyond the immediate catchment.

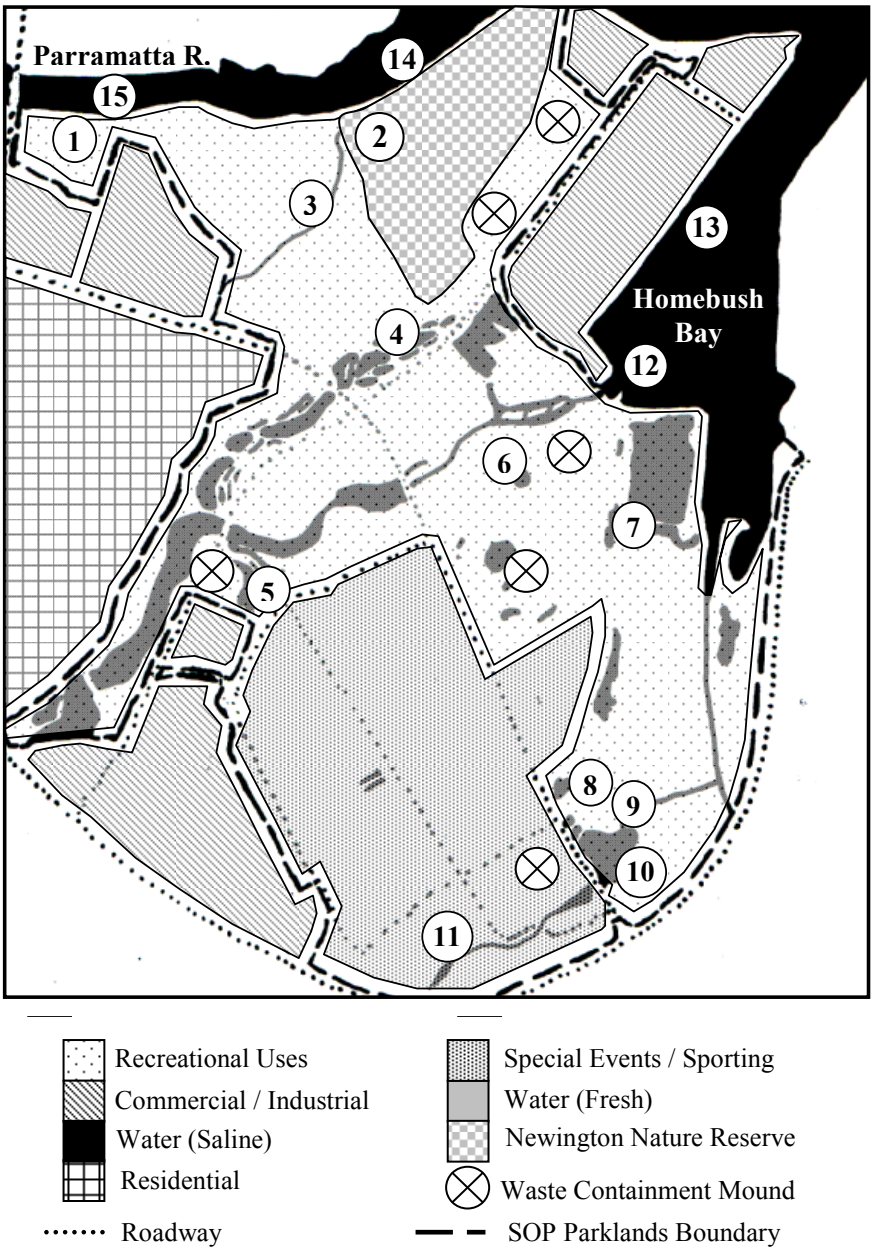


Figure 2-1 Map of Sydney Olympic Park and Homebush Bay surrounds showing water, sediment and fish collection sites. 1.Wilson Park, 2.Wharf Pond, 3.Wharf Stream, 4.Narrawang Wetlands (N22) 5.Northern Water Feature, 6.Eastern Water Quality Control Pond, 7.Bicentennial Park, 8.Southern Water Quality Control Pond, 9.Fishway, 10.Lake Belvedere, 11.Boundary Ck, 12. Haslams Mouth, 13. Homebush Bay, 14. Newington Wharf, 15. Silverwater Bridge.

Mosquitofish from Narrawang 22 have been shown elsewhere to be affected by estrogenic EDCs (Brennan et al. in prep.).

2.1.2 Boundary Creek

Boundary Ck (formerly a concrete channel) was initially remediated in the late 1980s along with Bicentennial Park. The Creek was lined, wastes were removed and the course of the Creek returned to a more natural state with a series of pools and riffles designed to catch storm water and allow settling of contaminated sediment (Laginestra et al. 2001). Rehabilitation of this wetland was further enhanced in 1993 after levels of OCPs (50 μg/kg) and PAHs (3-5 mg/kg) were recorded (Laginestra et al. 2001). During the remediation works contaminated soil and sediment were removed to a containment mound downstream of the current study site.

Boundary Ck has degraded, channelised upstream reaches located in highly urbanised land. It is unlikely that reaches of high ecological quality are present upstream of this wetland. Potential sources of contamination at this wetland are urban storm water runoff and a sewage overflow point located immediately upstream of the sampling point. The wetland is significant bird habitat (Laginestra et al. 2001) and therefore the presence and sources of any contamination require further investigation.

2.1.3 Lake Belvedere

The area surrounding Lake Belvedere was initially remediated in the mid 1980s with a 30 year old landfill capped with 1 to 1.5m of imported fill (Laginestra et al. 2001). Subsequent testing of the Lake in the 1990s indicated significant concern over seepage of contamination from beneath the cap towards surrounding waterways (Laginestra et al. 2001). Leachate drains installed at this time did not halt the seepage and a new drainage system including a leachate evaporation pond was installed in 1999 (Laginestra et al. 2001). Studies in 1997 and 1999 identified significant reproductive impairment, PAH and OCP body burdens and physical malformation (internal and external abscesses) in carp inhabiting the Lake (unpubl. data cited in Laginestra et al. 2001).The Lake now falls within

the boundaries of Bicentennial Park, an area of high recreational amenity. The Lake is surrounded by a walking/ bicycle track and by large areas of open space. Large populations of waterbirds inhabit the surrounds of the Lake.

2.1.4 Eastern Water Quality Control Pond (EWQCP) and Southern Water Quality Control Pond (SWQCP)

These wetlands are stormwater harvesting measures, which receive first flush inputs (Laginestra et al. 2001). They are the initial collection ponds for impermeable surface runoff for a large area of the Park and the catchments do not include any land outside the boundaries of SOP. They are designed to remove pollutants and sediments from the stormwater before its redistribution. From these wetlands, water is reused for irrigation across vegetated areas of the Park, can be pumped to nearby reservoirs and can be recycled through the on-site treatment plant for non-potable uses. The wetlands are lined with macrophytes to aid in bioremediation and are designated bird and frog habitats. They are, therefore, important in the ecological planning of the Park as well as water control measures. These wetlands are not adjacent to any of the waste containment mounds created during the remediation of the Park.

2.1.5 Wharf Stream

The Wharf Stream is a pre-remediation remnant but seems to have been redirected during the initial construction of the naval site through which it now flows. Its catchment has previously contained municipal waste dumps and currently consists of roads, residential and commercial land, a prison and playing fields. During the SOP remediation program, temporary leachate ponds were constructed adjacent to the stream during the construction of the nearby containment mound. Testing of the sediments around these ponds suggested contaminant migration, but where this occurred the sediment was removed and treated. The possibility of some infiltration during this process cannot be ruled out. The area is currently not in high use and the stream flows only at high rainfall events so the possibility of migration or exposure of biota to contaminants at this wetland is low.

2.1.6 Northern Water Feature

The Northern Water Feature was constructed during the remediation project on land that was used for municipal and industrial waste dumping. 2,3,7,8-TCDD concentrations of up to 67.5 μg/kg were reported prior to remediation and necessitated that contaminated soil and sediments were removed (to an adjacent containment mound). The area is built on a clay cap to reduce infiltration. The water feature is designed as a storm water mitigation measure receiving first flush and contains large pools for sedimentation. This is primary bird habitat and habitat for a population of green and golden bell frogs. As the wetland is linked to the water recycling system within SOP and is used for local irrigation there is potential for migration of contaminants at this wetland to other areas of the Park.

2.1.7 Bicentennial Park

This site within the Bicentennial Park wetlands is located in an intertidal expanse of mangrove and saltmarsh. The surrounds of the wetland has been modified for public access with boardwalks and educational stations spread through the wetlands. Water at the study site is fresh to brackish and subject to limited tidal flushing. It is surrounded by *Casuarina* woodland and is a pre-remediation remnant area. It was likely to have been subject to some dumping but contaminant concentrations were below those requiring remediation.

2.1.8 Wharf Pond

Wharf Pond is located within the Newington Nature Reserve, which is closed to public access. It was not subject to any remediated works and there is no evidence to any waste dumping on the wetland in the past. The pond is shallow (<1 m) and fringed with *Casuarina* and *Eucalypt* woodland. It is habitat for a number species of waterbird.

2.2 Reference Sites

A series of reference sites was selected to place the Sydney Olympic Park wetlands in context. Three primary reference sites (Upper Colo, Macquarie University and Homebush

Bay) were selected to represent an expected gradient of contamination from pristine to heavily contaminated wetlands (Table 2-3). A further 17 reference sites from the Sydney metropolitan area were chosen to place the SOP wetlands within a context of urban impact (Table 2-3, Figure 2-2, Figure 2-3).

2.2.1 Upper Colo

Upper Colo is located on the Colo River in a relatively pristine region north-west of Sydney, isolated from the effects of urbanisation. Its catchment is mainly National Park and the possibility of anthropogenic contamination is low. The only potential contaminant sources are long-range aerial deposition and the use of pesticides in the small agricultural holdings in the upper reaches of the catchment. Upper Colo is somewhat different to most other wetlands included in the current study. The Colo River at this point is a wide, shallow, sandy-bottomed stream. Flow is highly dependant on local rainfall and during this study ranged from close to zero to moderate. Water levels dropped over the course of the study and rose suddenly with high rainfall at the conclusion of the study. Riparian vegetation consists mostly of native *Casuarina* and *Eucalypt*.

Upper Colo was selected to approximate a "pristine" reference site meaning that it is outside the influence of urban development in the Sydney metropolitan area (>100km from Sydney Olympic Park). The selection of this site was made as a result of an extensive search and site assessment. In many respects this is not an ideal reference site, (different flow regime and benthic properties), however, it was the best approximation that could be made. In order to overcome some of these problems a number of "non-pristine" reference sites within the Sydney metropolitan area and therefore subject to many of the same influences as Sydney Olympic Park, but without the direct contamination history and remediation efforts were also selected.

Table 2-3 Description of reference wetlands used in the current study.

Wetland	Wetland Type	Catchment Area (km^2)[a]	Catchment Inputs and Current Landuse
Upper Colo	Pristine river	3277.2	National park, recreation, light agriculture
Yarramundi	Lagoon	7.1	Light agriculture
Second Ponds Ck	Degraded stream	6.4	Light agriculture, residential building development site. prison
South Ck	Med flow stream	302.4	Light agriculture, urban and rural residential, sewage treatment plant
Quakers Hill	Urban stream	62.4	Urban residential, sewage treatment plant
Eastern Ck	Urban stream	86.7	Urban residential, motorway overpass
Nepean River	River	726.7	Light agriculture, urban and rural residential
Kemps Ck	Degraded stream	69.7	Light agriculture
Cecil Hills	Created pond	2.8	Urban residential,
Prospect Ck	Channel	10.2	Industrial
Chipping Norton Lk	Upper tidal reaches	418.0	Recreation, residential and racecourse
Oyster Bay	Tidal stream	4.7[b]	Residential and Port Hacking, medium density urban
Georges River	Tidal region of degraded stream	n/a[b]	Tidal region of Georges River
Bardwell Ck	Modified stream	8.9	Residential and light commercial, narrow riparian zones, medium density urban
Joseph Banks Pk	Created pond	1.1	Parklands
Macquarie Uni.	Created	2.8	University, open space, standing pond
Terrys Ck	Urban stream	5.7	Urban residential, no riparian zone, some channelisation
Moores Ck	Urban stream	1.0	Urban residential, narrow riparian zone
Galston	Stream in parklands	42.9	Urban residential, parklands
Narrabeen	Estuarine lagoon	n/a[b]	Estuarine impacts, forested catchment, some residential

[a] Catchment areas are map based approximations. [b] Oyster Bay, Georges River and Narrabeen are tidal sites, so contaminant inputs could be from downstream of this sampling point.

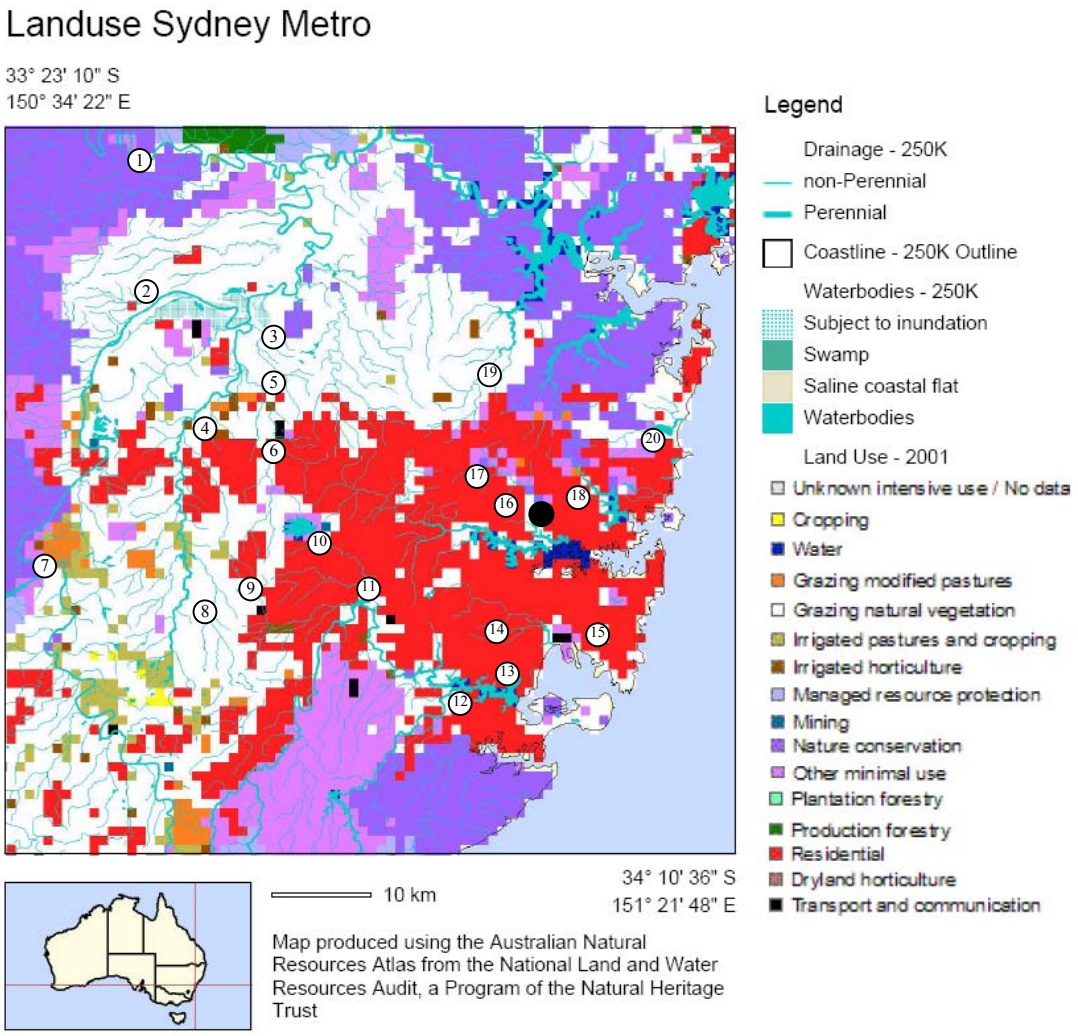


Figure 2-2 Landuse map of greater Sydney showing all urban reference sites used in this study; 1. Upper Colo, 2. Yarramundi, 3. Second Ponds Ck, 4. South Ck, 5. Quakers Hill, 6. 7. Nepean R., 8. Kemps Ck, 9. Cecil Hills, 10. Prospect Ck, 11. Chipping Norton Lake, 12. Oyster Bay, 13. Georges R., 14. Bardwell Ck, 15. Joseph Banks Park, 16. Macquarie Uni, 17. Terrys Ck., 18. Moores Ck, 19. Galston, 20 Narrabeen Lagoon. Solid black circle denotes position of SOP.

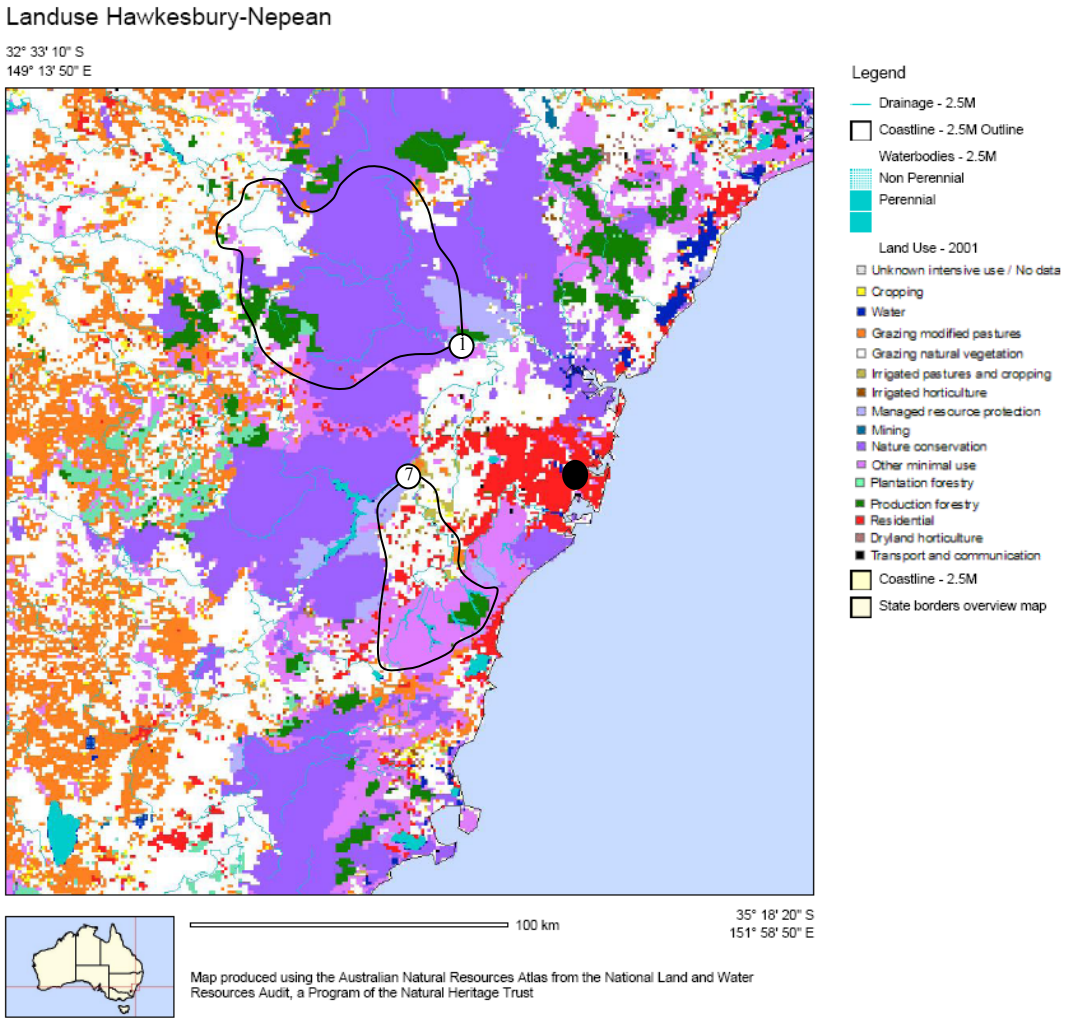


Figure 2-3 Landuse map showing location and catchment size of Nepean R (7) and Upper Colo (1) study sites. Solid black circle denotes position of Sydney Olympic Park.

2.2.2 Macquarie University

Macquarie University is located 17km north-west of the Sydney CBD. Detailed histories of the site prior to occupation by the University in 1964 indicate no evidence of waste dumping or remediation in the last 50 years. The catchment of the wetland studied at Macquarie University is entirely urbanised. The wetland is therefore subject to runoff from 2.8km^2 of residential property and paved surfaces. The Macquarie University wetland studied (hereafter Macquarie Uni) has highly degraded upstream reaches and is unlikely to include healthy aquatic taxon rich regions. The wetland was selected as an urban impacted reference site.

2.2.3 Homebush Bay

There were two sites sampled in Homebush Bay. The first is located near the mouth of Haslams Ck (Haslams Mouth) and the second is located toward the western shore in the central part of the Bay (Homebush Bay). The sediments of these parts of the Bay were neither remediated during the Olympic remediation project nor were part of the current remediation of the sediments along the eastern shore. The Bay deepens away from Haslams Mouth (2-3 m) toward the Homebush Bay sampling site (~5 m). Homebush Bay was subjected to extensive pollution from surrounding industry up to the 1980s and high concentrations of organochlorines and other aromatic hydrocarbons have been consistently measured in sediment from the Bay over many years (McCready et al. 2000, Birch and Taylor 2002).

3. Potential Estrogenicity of the Sediment and Water of Wetlands in Sydney Olympic Park

3.1 Abstract

Sites previously contaminated by industrial and domestic pollution are of concern when investigating potential effects on wildlife. Many human activities produce pollutants that are ligands for different receptors, including the estrogen receptor (ER), the androgen receptor (AR) and the aryl hydrocarbon receptor (AhR). This study investigated the presence of ER ligands in aqueous and sediment phases of wetlands in a site that was identified as significantly polluted and subsequently remediated, Sydney Olympic Park (SOP), and their *in vivo* estrogenic effect on male secondary sexual characteristics (gonopodial development) in the mosquitofish (*Gambusia holbrooki*). While there was no evidence of ER ligands in the water of any of the wetlands studied, 17β-estradiol equivalence (E_2eq) was quantifiable in the sediments of eight of eleven wetlands studied with the highest measured value at the SWQCP (22204 ng/kg). Further, there was no evidence of reduced gonopodial development in male mosquitofish inhabiting SOP wetlands, indicating no estrogenic effect of measured ER ligands. Both adult and juvenile mosquitofish at Boundary Ck had more masculine secondary sexual characteristics than those at other wetlands. Further studies are required to completely characterise the levels of endocrine disruption in the wetlands of SOP, particularly assays focusing on activity mediated through the AhR.

3.2 Introduction

There has been worldwide concern regarding the presence and ubiquitous nature of endocrine disrupting compounds (EDCs) in urban wetlands (Damstra et al. 2002, Schawarzenbach et al. 2006). It has been determined that these are mostly of anthropogenic origin. The remediation of the Sydney Olympic Park (SOP) included the removal, treatment and on-site storage of known and suspected endocrine disrupting compounds (EDCs) (e.g., polycyclic aromatic hydrocarbons (PAHs, e.g., benzo[*a*]pyrene), phenolics, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs, e.g., DDT and its persistent metabolites DDE, DDD) and organophosphate pesticides) (Table 1-2). The leachate collected in the drains surrounding the waste containment mounds now on the site contains concentrations of EDCs including PAHs, (e.g., benzo[*a*]pyrene), DDD and DDE (SKM and EVS 2001).

One of the main mechanisms of action of EDCs is through their interactions with receptors. Estrogen receptor (ER) mediated effects are the most studied and create the highest level of concern due to the reported effects on reproductive fitness and population level endpoints. This link between exposure to estrogenic EDCs and effects on a population level has recently been demonstrated by Kidd et al. (2007) who reported the collapse of a wild fish population, chronically exposed to 17α-ethynylestradiol. Of the contaminants found prior to the remediation at SOP and in the containment mound leachate, PCBs (selected congeners only), DDT (and its metabolites DDE and DDD), are known to compete with the endogenous estrogen, 17β-estradiol (E_2) for estrogen receptor binding sites (Table 1-1).

SOP has high recreational, educational and ecological values with large numbers of visitors each year. Water-sensitive designs have been incorporated into the redevelopment of the area with stored stormwater and sewage collected and treated in an on-site facility being reused for irrigation and other non-potable uses (OCA 2000b). Habitat (particularly aquatic) has been preserved where appropriate and constructed elsewhere to encourage recruitment of waterbirds and aquatic fauna. There is concern that some estrogenic contaminants are still available to the biota in the wetlands of SOP. A previous study by

Brennan et al. (Unpubl. Data) found significant alterations in the secondary sexual characteristics and sexual behaviour in populations of the mosquitofish, *Gambusia holbrooki* inhabiting wetlands within SOP. The authors recommend that a wider study of the estrogenicity of the waters of the Park is necessary. In particular, many of the wetlands of SOP were not included in their above study as they did not support populations of mosquitofish. Many of these, however, support other wildlife including endangered and migratory species (OCA 2000b). It is, therefore, pertinent to examine the potential of these waters to cause endocrine disruption in the wildlife inhabiting them.

Mosquitofish are considered ideal indicators for endocrine disruption as they are strongly sexually dimorphic (Batty and Lim 1999). The male is smaller than the female and possesses an elongated anal fin (the gonopodium) (Figure 3-1), which is used as an intromittant organ during copulation, delivering sperm packets to the gonopore of the female (Collier 1936). Its correct formation and function, is therefore, essential for reproductive fitness. The development of the gonopodium is under androgenic control (Turner 1941) and is delayed by exposure to natural estrogens (Doyle and Lim 2002) and anthropogenic pollutants (e.g., sewage effluent (Doyle et al. 2003)). Exposure to 17β-estradiol delays the development of a gonopodial terminal hook complex (Figure 3-1) (Doyle and Lim 2002), which is the morphological marker for the attainment of sexual maturity.

Competitive binding assays are an efficient method for determining the affinity that certain compounds and/or environmental samples have for cytosolic receptors. They are considered a first tier assay as they determine the ability of compounds to bind to a receptor but do not give information in terms of receptor activation, transcription or translation (Damstra et al. 2002). Their utility is in enabling researchers to refine their searches for the effects of pollution and active constituents of environmental samples. Mammalian estrogen receptors have been used in competitive binding assays for the detection of exogenous hormones in environmental samples (e.g., Leusch et al. 2006). The ER and androgen receptor (AR) are relatively well conserved (Matthews and Zacharewski 2000) and affinity for exogenous ligands is similar across taxa (Leusch et al. 2006).

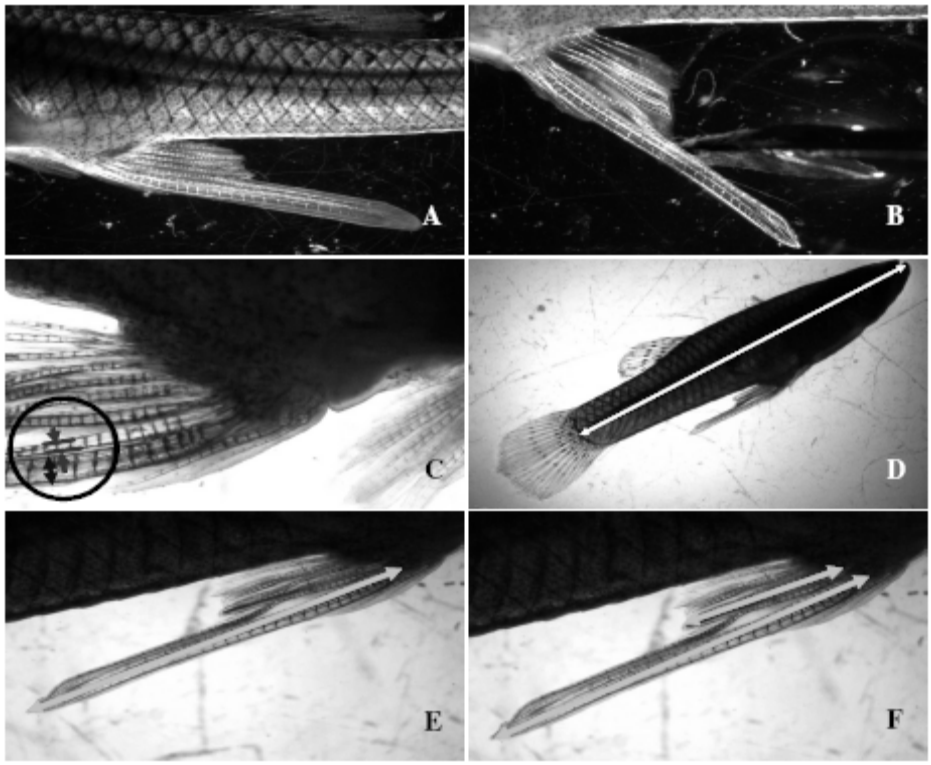


Figure 3-1 Detail of the male mosquitofish. A. Gonopodium of non-sexually mature male (no gonopodial terminal hook complex). B. Gonopodium of sexually mature male (gonopodial terminal hook complex present). C. Detail of gonopodium showing the measurement of the width ratio. D. Measurement taken as the standard length of the fish. E. Detail of the gonopodium showing the measurement of the gonopodium length. F. Detail of gonopodium showing the measurement of the length ratio.

The aim of this study was to investigate the potential estrogenicity of the surface water and sediment of the wetlands of SOP *in vitro*, using an ER radioligand competitive binding assay and *in vivo* assessment of alteration to the male reproductive morphology in resident populations of mosquitofish. Given previous work on the area it was hypothesised that there would be measurable levels of ER ligands in collected samples and that these would be reflected in reduced secondary sexual male characteristics in mosquitofish inhabiting individual wetlands within SOP and compared to those inhabiting external reference sites.

3.3 Materials and Methods

3.3.1 Water and Sediment Sample Collection and Preparation

Water and sediment samples were collected in January 2006. One litre grab water samples were collected using clean (washed and solvent rinsed) amber glass bottles from six wetlands in SOP and three reference wetlands (Table 2-1, Figure 2-1, Figure 2-2) and transported on ice to the laboratory where they were stored at -20^{o}C until extraction. The study sites chosen represent a range of pollution and remediation history (Table 2-2, Table 2-3). Within 48 hours the samples were filtered (1 μm GFC filters) and extracted onto C18 Empore disks through a six station Empore extraction manifold. Disks were twice eluted with 10 ml dichloromethane (DCM) and solvent exchanged under nitrogen to 500 μl DMSO and stored in amber glass vials at -80 ^{o}C.

Composite sediment samples (1 L) were collected using a grab sampler from the same wetlands described above plus four estuarine sites in Homebush Bay and Parramatta River (Figure 2-1). Narrawang 22 is regularly drained in an attempt to control the mosquitofish population. This coincided with the scheduled collection of sediment at this study site so collection was not possible. Sediment collection was also not possible at Lake Belvedere as it consisted almost entirely of particles >6 mm^2. Samples were transported in 1L solvent rinsed glass bottles on ice to the laboratory. Wet sediment samples were transferred into glass jars (500 mL) and freeze-dried for one week. Organic compounds in the freeze-dried sediments were extracted with DCM using a Dionex ASE300 Accelerated Solvent Extractor (ASE). Five grams of each sample was weighed into each extraction cell having a

filter pad at the bottom and the cell was filled up using diatomaceous earth from Dionex. The ASE used an oven temperature of 100 ^{o}C with 10 MPa (1500 psi) pressure. The heat up time was 5 min and the static time was 5 min for 2 cycles. The extracts were concentrated in collection bottles under nitrogen gas using a Dionex SE500 Solvent Evaporator and stored at -80 ^{o}C. Some dirty extracts were further cleaned up using Solid Phase Extraction (SPE) silica cartridges.

3.3.2 Estrogen Receptor Competitive Binding Assay

Cytosolic ER were isolated from sheep uteri, (collected at slaughter from Wellcome Laboratories, Gore Hill NSW), following a protocol adapted from Leusch et al. (2006) and detailed in Appendix A, and stored at -80 ^{o}C until used. All receptor suspensions were characterised using the method of Scatchard (1949) giving a value for maximum binding capacity (B_{max}) and the binding affinity of the receptors (K_d) (see Appendix B for detailed methods). The competitive binding assay method was described earlier in Leusch et al. (2006) (see Appendix C for detailed methods). Briefly 17β-estradiol (E_2) standard curve data were fitted to a four-parameter logistic growth curve (Eqn 3-1, where a = curve minimum, b = curve maximum, c = log(EC50), d = curve slope at EC50, y = radioactive desintegrations per minute, x = E_2 concentation) to evaluate the 50% binding to the ER binding sites of E_2 when in competition with tritiated estradiol (^{3}H-E_2).

$$y = \frac{a + (a - b)}{1 + 10^{((c - \log x) * d)}} \qquad \text{(Eqn 3-1)}$$

Five dilutions of each sample were allowed to compete with ^{3}H-E_2 for ER binding sites overnight (18 hours). Fitting the radioactivity remaining after washing with Dextran T70 coated charcoal (DCC) at each dilution to a four-parameter curve (Eqn 3-1) allows the calculation of the concentration of E_2 required to displace the same concentration of ^{3}H-E_2 from the receptor sites giving a measure of estradiol equivalency (E_2eq) for each sample.

The method was verified using model compounds with known affinity for the ER. The compounds selected were the natural hormone estrone (E_1), the synthetic hormone 17α-ethynylestradiol (EE_2), the pharmaceutical diethylstilbestrol (DES), and the alkylphenols octylphenol and nonylphenol. The model compound concentration at which 50% of the radioligand displaced (EC50) is measured against the EC50 of the natural ligand giving the Relative Binding Affinity (RBA). The RBA does not take into account the properties of the receptor and therefore can be affected by individual experimental conditions and is therefore inappropriate for comparison to published values. The equilibrium dissociation constant (K_i) for binding of the model compound is calculated using the binding properties of the compound and the properties of the receptor (Eqn 3-2) (where K_d = the dissociation constant for the binding of the radioligand) and is therefore more appropriate for comparison to published values.

$$K_i = \frac{\text{EC50}}{1 + \frac{[\text{radioligand}]}{K_d}} \qquad \text{(Eqn 3-2)}$$

3.3.3 Mosquitofish Collection and Processing

Mosquitofish were collected using aquatic dip nets in January 2006. The fish were sacrificed using benzocaine (400 mg/L), digitally photographed and weighed. Morphological measurements were made using the Leica QWIN program. Measurements of the standard length (SL), gonopodium length (GL), gonopodial extension (G_x) gonopodial length ratio (LR) (ratio of the length of the 3rd gonopodial ray to the length of the 6th gonopodial ray) and gonopodial (WR) width ratio (ratio of the width of the 3rd gonopodial ray to the 6th gonopodial ray) were recorded (Figure 3-1). These measures have been demonstrated as appropriate to assess the exposure of male mosquitofish to estrogenic compounds (Doyle and Lim 2002). Fish age was not determined in this study. Previous work using otolith analysis has demonstrated a linear relationship between age and standard length in males of this species (Rawson, unpubl. data) as they are short lived (generally <12 months).

3.3.4 Data Analysis

The gonopodial characteristics, GL and G_x, were analysed using analysis of covariance (ANCOVA) using the SL of the fish as a covariate to account for allometry and fish age. The use of SL as a covariate also accounts for differences due to the age of the fish. The assumption of homogenous regression slopes was tested for each of the analyses. A scatter plot of GL against SL (Figure 3-2A) showed two distinct groups at each study site, which skewed the covariate regression lines violating the ANCOVA assumption of parallel slopes. The analysis was therefore split into sexually mature and juvenile fish based on the presence of the gonopodial terminal hook complex. Angus et al. (2002) and Rawson et al. (2008), analysed gonopodial data in wild *Gambusia* spp. populations by only including those males with gonopodial terminal hook complexes as mature adults. While this method allows an ANCOVA to be performed (all assumptions met) it does not allow the inclusion of males with delayed gonopodial development (sensitive males), a known effect of exposure to estrogenic compounds (Doyle and Lim 2002, 2005). An alternate method of these gonopodial measures includes all male fish with SL greater than that of the smallest morphologically mature male (possessing a gonopodial terminal hook complex) (Game et al. 2006, Brennan et al. in prep.). However, this method does not allow the inclusion of all fish in a single group with homogeneous covariate slopes permitting analysis by ANCOVA (Figure 3-2B). Therefore an ANOVA (single factor: study site) was run using all males larger than the smallest male with a gonopodial terminal hook complex from each wetland using gonopodial data standardised against the standard length of the individual. These data are bimodal (Figure 3-3) and required a Box-Cox transformation (λ=4) to satisfy the ANOVA assumption of homogeneity of variance. The results of both analyses (ANOVA including all males larger than the smallest with a gonopodial terminal hook complex, and ANCOVA using only males with gonopodial terminal hook complexes) are presented to compare the results of the different approaches on the same dataset.

Ratio measures (LR, WR) were examined using ANOVA (single factor: study site) after testing the assumption of homogeneous variances. Males at different stages of development were defined as minimal gonopodial development (Gx < 1 mm), developing gonopodium (Gx > 1 mm but no gonopodial terminal hook complex) and mature male (gonopodium

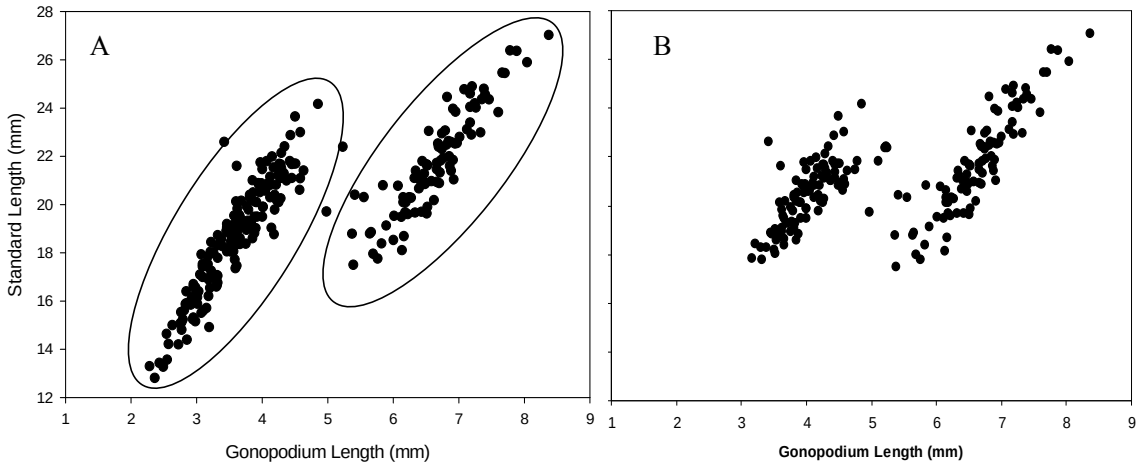


Figure 3-2 Scatter plots of combined gonopodium length vs. standard length of male mosquitofish from SOP and reference sites. A. Relative gonopodial lengths of all males mosquitofish, and B. Relative gonopodial lengths of male mosquitofish larger than the smallest male fish with a gonopodial terminal hook complex.

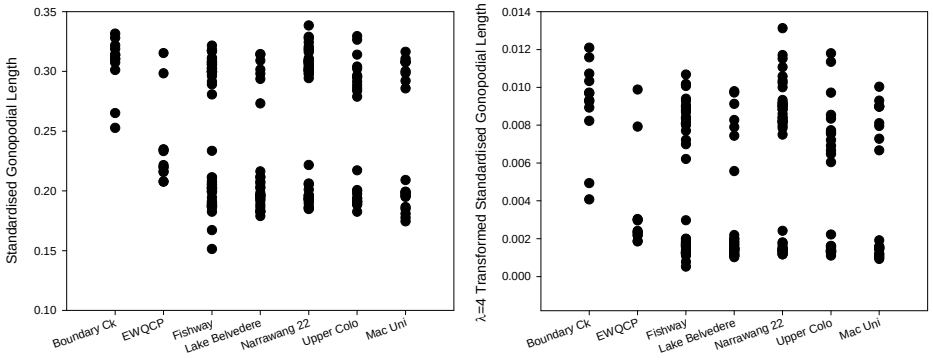


Figure 3-3 Distribution of standardised gonopodial length (left) and Box-Cox (λ=4) transformed standardised gonopodial length (right) of all male mosquitofish at Sydney Olympic Park and reference sites.

terminal hooks complex present). Proportions of each group at each wetland were calculated and analysed graphically.

3.4 Results

The equilibrium dissociation constant (K_i) values obtained for the model compounds are in reasonable agreement with published values (Table 3-1). The method is therefore appropriate for determination of 17β-estradiol equivalence (E_2eq) of the water and sediment extracts. The E_2eq of all water samples was below the detection limit of the assay (6.81 ng/L) (Table 3-2). Sediment E_2eq was above the detection limit (>27.2 ng/kg) at eight of the eleven study sites with the maximum value of 22204 ng/kg (dry weight) recorded at SWQCP (Table 3-2).

There were significant ($p<0.05$) variations in the gonopodial morphology of male mosquitofish inhabiting the study sites (Table 3-3). When analysed using ANCOVA, sexually mature males (those with gonopodial terminal hook complexes) from Boundary Ck and Narrawang 22 had significantly ($p<0.05$) longer gonopodia than those from the "clean" reference site, Upper Colo. All other gonopodial measures were not significantly different ($p\geq0.05$) to those in fish from Upper Colo (Figure 3-4). When males without gonopodial terminal hook complexes but which were larger than the smallest male with a gonopodial terminal hook complex were included in the analysis of gonopodial length (by ANOVA) (Figure 3-4) the results indicate that only the fish from Boundary Ck had significantly ($p<0.05$) longer GL and G_x than those at Upper Colo.

The GL and G_x of the juvenile males (males without gonopodial terminal hook complex) at Boundary Ck were significantly ($p<0.05$) longer than those collected at all other study sites (Figure 3-5). The LR of these fish was also significantly ($p<0.05$) greater than those collected at from the other study sites while the WR was, along with the fish at Macquarie Uni, significantly ($p<0.05$) greater than those in fish from both Upper Colo and Lake Belvedere (Figure 3-5). At Boundary Ck, EWQCP and Lake Belvedere the proportion of

Table 3-1 Measured values for relative binding affinity (RBA) and equilibrium dissociation constant (K_i) for binding to the mammalian estrogen receptor by model estrogenic compounds.

	log RBA	Measured K_i (nM)	Published K_i[1] (nM)
17β-Estradiol	-	0.5	0.4
Ethynylestradiol	-0.22	0.7	0.4
Diethylstilbestrol	0.30	0.2	0.2
Nonylphenol	-4.32	987	672
Octylphenol	-3.85	886	781

[1]Laws *et al*. (2000). Values for binding to estrogen receptors isolated from rat uteri.

Table 3-2 Measured values of 17β-estradiol equivalence (E_2eq) in aqueous and sediment samples. Detection limits for determination of E_2eq in aqueous and sediment samples were 6.81ng/L and 27.24 ng/kg dry weight respectively. n/a = no data collected.

		E_2eq Aqueous (ng/L)	E_2eq Sediment (ng/kg dw)
Wetlands in Sydney Olympic Park	Narrawang 22	<6.81	n/a
	Northern Water Feature	<6.81	<27.24
	Lake Belvedere	<6.81	n/a
	Wharf Stream	<6.81	**217.91**
	Boundary Ck	<6.81	**129.04**
	Bicentennial Park	<6.81	**43.95**
	SWQCP	<6.81	**22203.8**
	EWQCP	<6.81	**30.64**
	Wharf Pond	n/a	<27.24
Reference Sites	Macquarie Uni	<6.81	n/a
	Silverwater Bridge	n/a	<27.24
	Haslams Mouth	n/a	**111.46**
	Homebush Bay	n/a	**167.36**
	Newington Wharf	n/a	**28.63**

Table 3-3 Results of ANCOVA and ANOVA on gonopodial endpoints in juvenile and adult male mosquitofish collected at wetlands within SOP and reference sites. For ANCOVA adult male is defined by the presence of terminal gonopodial hooks. For ANOVA adult male is defined as being larger than the smallest male with terminal gonopodial hooks. ANCOVA data standardised against covariate mean (juvenile = 18.61mm, adult = 21.77mm). n/a = analysis was not appropriate for this group of fish.

		Juvenile Males		Adult Males	
		F-value	p-value	F-value	p-value
ANCOVA	Gonopodium Length	6.93	0.000	3.09	0.013
	Gonopodium Extension	7.30	0.000	1.13	0.350
ANOVA	Gonopodium Length	n/a	n/a	5.48	0.000
	Gonopodial Extension	n/a	n/a	5.03	0.000
	Length Ratio	6.99	0.000	3.92	0.001
	Width Ratio	3.74	0.003	2.49	0.024

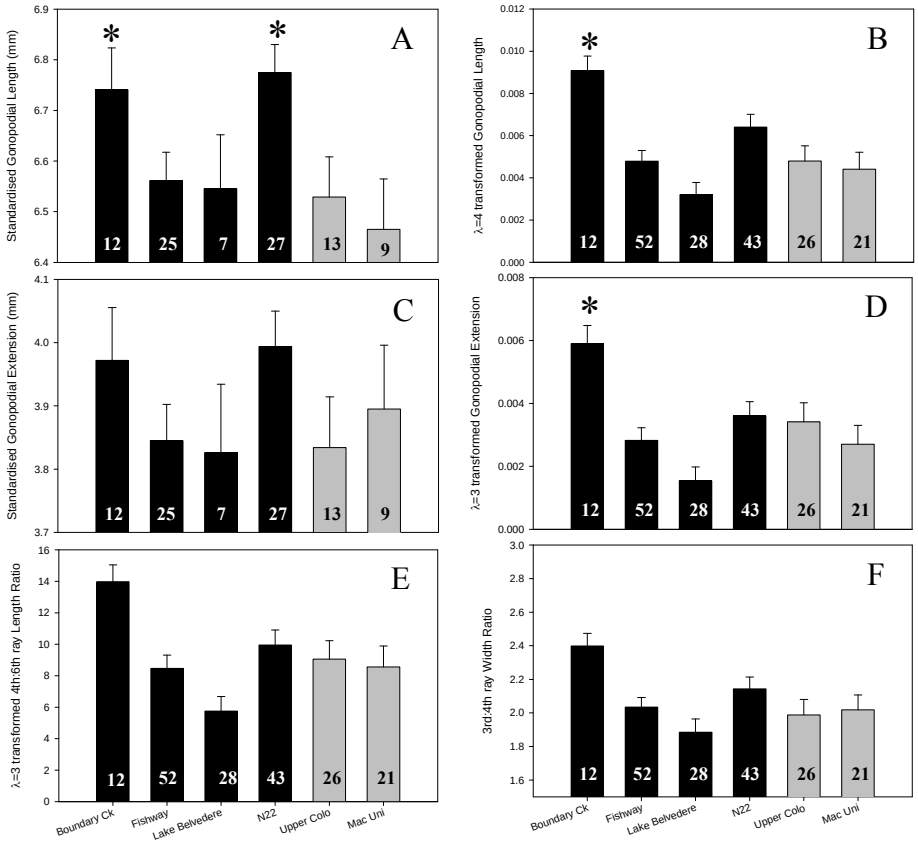


Figure 3-4 Results of analyses of gonopodial characteristics of male mosquitofish from SOP (black bars) and reference sites (grey bars). A. ANCOVA on gonopodium length for males with terminal gonopodial hook complexes only (standardised to covariate mean 21.77mm), B. ANOVA gonopodium length for all males longer than the smallest male with a terminal gonopodial hook complex, C. ANCOVA on gonopodium extension of males with terminal gonopodial hook complexes only (standardised to covariate mean 21.77mm), D. ANOVA gonopodium length for all males longer than the smallest male with gonopodial terminal hook complex, E. ANOVA on Length Ratio and D. ANOVA on Width Ratio. Sample sizes given numerically in each figure. * denotes significantly ($p<0.05$) different to Upper Colo (clean reference site).

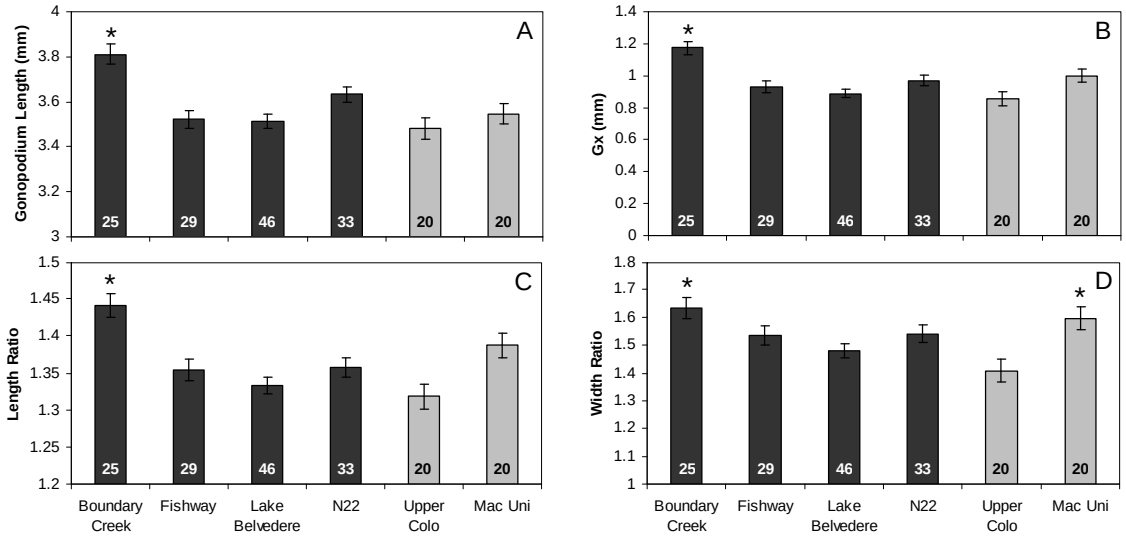


Figure 3-5 Results of ANCOVA on the A. gonopodium Length and B. gonopodium extension (Gx), and of ANOVA on C. Length Ratio and D. Width Ratio of juvenile male mosquitofish collected at wetlands within SOP and reference sites. Sample sizes given numerically in each figure. * denotes significantly ($p<0.05$) different to Upper Colo (clean reference site).

mature male fish was less than that at the pristine reference site (Upper Colo) and the urban impacted site (Macquarie Uni) (Figure 3-6).

3.5 Discussion

The absence of detectable E_2eq in the aqueous phase at the wetlands in SOP using the *in vitro* estrogen receptor competitive binding assay indicate concentrations of bioavailable ER ligands are likely to pose a low risk of estrogenic reproductive impairment in resident fish populations via direct uptake of contaminants. The detection limit of the assay using the methods described above is below the levels that have been shown to cause *in vivo* reproductive morphological abnormalities in male mosquitofish. The results of laboratory exposures presented by Doyle and Lim (2002) and Rawson et al. (2006) suggest significant effects on the reproductive morphology of this species at levels >20 ng/L E_2. The current study suggests that, in the wild, concentrations lower than 6.81ng/L did not cause measurable differences in the same endpoints.

The presence of measurable levels of E_2eq in the sediment at many of the wetlands studied does not appear to be related to the different remediation histories of the study sites. The wetlands with highest E_2eq were not necessarily those with the highest level of remediation. Indeed the study site with the highest E_2eq (SWQCP) is a created wetland not located near a waste containment mound. This suggests that the causative compounds are recent (post-remediation) additions to the sediment. Lai et al. (2000) demonstrated that there is a tendency for natural and synthetic estrogens to partition to sediments and that this is a rapid process taking a matter of hours. Some higher log K_{ow} estrogen mimics (e.g., some PAHs and OCPs) will follow this pathway and may accumulate in the sediment with limited flux with the water column. The results concur with this hypothesis with quantifiable E_2eq in sediments where there is none in the water column (e.g., SWQCP). It is, therefore, suggested that in wetlands subject to inputs of estrogen receptor ligands, the solid compartments (suspended and benthic sediment) are important phases and must be included in any examination for the presence of estrogenic or antiestrogenic activity.

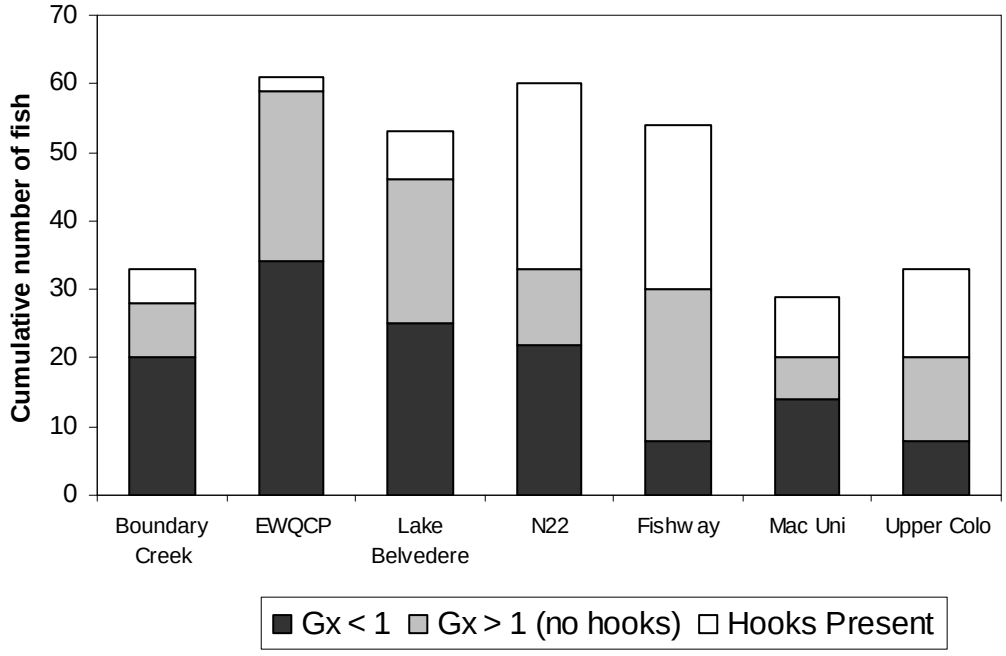


Figure 3-6 Distribution of stages of maturity of male mosquitofish from Sydney Olympic Park and reference study sites. Presence of hooks defines sexual maturity. Gx>1 defines a fish with a developing gonopodium. Gx<1 denotes a morphologically male fish prior to gonopodial development.

The very high E_2eq measured at SWQCP requires further study. This is a storm water receiving wetland and, therefore, will act as a sink for much of the contamination deposited in its catchment. Work on other sites has previously reported E_2eq in sediment of 7.7 pmol E_2/g dw (2.1 μg E_2/kg) (Verslycke et al. 2005), 5.3 ng E2/ kg (Thomas et al. 2004) and 38.4 pmol E_2 /g (10.5 μg E_2/kg) (Legler et al. 2002). The value for SWQCP of 22 μg E_2/kg is, therefore, considered high in the context of other studies. This wetland is habitat for the endangered green and golden bell frog and is used as a feeding and breeding ground for many species of wading bird (Laginestra et al. 2001). While the green and golden bell frog is not in intimate contact with the sediment as an adult, juvenile stages feed on animals that are. Similarly wading birds commonly feed on both benthic invertebrates and juvenile frogs. This raises bioaccumulation and biomagnification concerns.

The relatively high E_2eq measured in the sediments from the two study sites in Homebush Bay (Haslams Mouth and Homebush Bay) were expected given its well documented contamination history. Known ER ligands including organochlorines and other aromatic hydrocarbons have been consistently measured in Homebush Bay sediments over many years (McCready et al. 2000, Birch and Taylor 2002). The fact that samples from nearby Parramatta River (Silverwater Bridge and Newington Wharf) contained very low (just above or below the detection limit) levels of ER ligands further highlights the status of Homebush Bay as highly contaminated.

Both the Wharf Stream and Boundary Ck are wetlands that were not directly remediated immediately prior to the 2000 Olympic Games. Boundary Ck was initially remediated in the early 1990s while the Wharf Stream has been modified but not remediated since the creation of the Naval Armory site on which it now stands. The source of ER ligands at Boundary Ck is likely to be from its highly urbanised catchment. The catchment of the Wharf Stream is now mostly parklands with the Silverwater Correctional Complex upstream. During the remediation, contaminated sediment treatment ponds were located adjacent to the stream and some escape of contaminants was recorded. While these were subsequently cleaned up, the results presented here may suggest that some legacy of these

ponds remains. The cause of the high E_2eq is, therefore, undetermined and further investigation is warranted.

The results of the *in vivo* analysis of male mosquitofish secondary sexual characteristics do not concur with those of Brennan et al (in prep.), finding no evidence of reduced male secondary sexual characteristics. As the males of this species generally survive for only one reproductive season differing results may point to either pollutant pulses and/or gradual removal of the pollutants from the waters of the wetlands. The current study indicates a slight increase in masculine characteristics at Boundary Ck. A variety of compounds (e.g., Tributyltin (Ellis and Pattisina 1990), 17β-trenbolone (Wilson et al. 2002)) and mixtures such as kraft mill effluent (Bortone et al. 1989, Cody and Bortone 1997) can cause androgenic effects; however, none of these have previously been reported in SOP. Furthermore *p,p'*-DDE and some PCB congeners and mixtures are AR antagonists (Kelce et al. 1995, Bonefeld-Jorgensen et al. 2001, Schrader and Cooke 2003) and 2,3,7,8-TCDD causes anti-androgenic effects via the AhR pathway (Jana et al. 1999). These persistent organic pollutants (POPs) are therefore more likely to reduce than enhance male reproductive development than enhance it. This potential androgenic effect requires further examination.

Exposure to estrogenic EDCs is known to delay the attainment of maturity in fish (Doyle and Lim 2002), which may result in the reduction in the proportions of sexually mature males. In this study it was not possible to statistically analyse the proportions of males at different stages of maturity so the discussion here is solely descriptive. Boundary Ck and Lake Belvedere are both subject to off-site catchment influences from nearby residential and commercial areas. EWQCP, as a water quality control pond is designed to receive stormwater runoff from SOP. The three wetlands with reduced proportions of mature male fish are, therefore, those most exposed to hard surface runoff in SOP. However, the urban impacted site, Macquarie Uni, is also subject to hard surface runoff and did not have reduced proportions of mature males. While a decrease in the numbers of reproducing males in a pest species such as mosquitofish can be seen as a positive ecological effect, SOP is habitat for many other species for which such an effect is of concern.

Radioligand binding assays are first tier screening assays, which detect the presence of receptor ligands but provide no information on the effect of ligand binding (e.g., agonism or antagonism). In other studies, concentrations lower than the detection limit in this study, have caused adverse reproductive effects *in vivo*. For example, Purdom et al. (1994) demonstrated that vitellogenin can be induced in male rainbow trout exposed to 0.1 ng/L of the synthetic estrogen 17α-ethynylestradiol and Kidd et al. (2007) showed the collapse of a population of fish exposed over a three year period to concentrations of the 17α-ethynylestradiol as low as 5 ng/L. It is, therefore, possible that concentrations below our detection limit exist in some of the wetlands studied and these may have deleterious effects on biota; however, these concentrations were not sufficient to cause effects in our study species. Alternatively, the lack of *in vivo* effects of the ER ligands in the sediments may suggest that these are ER antagonists. Examination of anti-estrogenic effects was beyond the scope of this study but should be considered in the future.

The different result given by the two different analyses of gonopodial length, based on the inclusion or exclusion of large males without gonopodial terminal hook complexes, demonstrates the importance of correct analysis of this endpoint. Analysis by ANCOVA necessarily eliminates potentially sensitive, reproductive EDC affected males with delayed gonopodial development (a known effect of exposure in this species (Doyle and Lim 2002)) in order to fit the assumption of homogeneity of covariate slopes. Analysis of the gonopodial length (standardised to fish standard length) of all males larger than the smallest male with a gonopodial terminal hook complex by ANOVA, includes these potentially sensitive individuals and provides different results to ANCOVA. Further, the results are more consistent when compared to those from an analysis of juvenile fish. Analysis of mosquitofish gonopodial length by ANOVA using all males larger than the smallest sexually mature males is therefore recommended for future studies.

3.6 Conclusions

The cleanup of the SOP site prior to the construction of the Olympic venues in the 1990s was hailed as a success. The pollutant load was large and included known ER ligands. The results do not suggest that fish populations are free from the risk of the effects of possible pollutants in the waters and sediments of the area. Rather they take an important step in this direction by showing that levels of pollutants that are ligands for the ER, remaining in the wetlands on the site are currently below those that should interfere with normal reproductive function of fish populations. There are, however, other forms of endocrine disruption mediated through a variety of different pathways. Given the discrepancy between the results found in water and sediment samples it is important that the high log K_{ow} estrogenic compounds (e.g., some PAHs, OCPs etc.) be further examined. Many PAHs, PCBs and pesticides and their breakdown products are not ER ligands but may be otherwise toxic. As these results suggest the presence of these classes of compounds (particularly in benthos) it is recommended that studies be undertaken to investigate their presence, concentration and potential toxicity. This should involve chemical analysis and *in vitro* bioanalysis. Further more sensitive examination of possible effects of these compounds on the aquatic fauna of the region should be undertaken.

4. TCDDeq of Sediment and Water of the Wetlands of Sydney Olympic Park

4.1 Abstract

Sydney Olympic Park (SOP), Sydney, Australia was highly contaminated by persistent organic pollutants (POPs) prior to remediation. There are concerns that POPs may still exist within the wetlands in SOP and that these may have ecological impacts. This study investigates the presence of POPs in the sediment and water of wetlands across SOP using the H4IIE rat hepatoma cell line (an *in vitro* assay with an active aryl hydrocarbon receptor (AhR) giving a 2,3,7,8-TCDD equivalency (TCDDeq) for environmental samples) and chemical analysis. Further, it investigates whether disturbance of these sediments is likely to mobilise ligands for this receptor from the sediments into the water column. TCDDeq in water samples ranged from 0.013 to 0.057 pM, which was significantly ($p<0.05$) less that that at urban reference sites and these concentrations were not correlated to physical or chemical characteristics of the wetlands. In the SOP sediments, TCDDeq ranged from 0.0016 to 8.16 μg/kg and these were not significantly ($p\geq0.05$) different to TCDDeq measured in sediment from urban reference sites. Sediment TCDDeq was correlated to sediment ΣPAH concentration in 2006 (r^2 = 0.80) and sediment ΣPCB, ΣDDT concentrations and fine sediment grain size in 2005 (r^2 = 0.95). Agitation of small quantities of sediment in water samples significantly ($p<0.05$) increased the levels of TCDDeq measured in the water. This increase was correlated with medium sand grain size (r^2 = 0.968). This is due to the loose binding of organic components to these size classes. It is concluded that the levels of TCDDeq in the wetlands of SOP are low, relative to urban reference sites but that disturbance of the sediments may increase the exposure of aquatic organisms to biologically active concentrations of POPs.

4.2 Introduction

Prior to the remediation of Sydney Olympic Park (SOP) for the Sydney 2000 Olympic Games, studies were conducted to describe and quantify the contamination present (summarised and reviewed by Laginestra et al. 2001). Amongst the compounds present were persistent organic pollutants (POPs), a group of known carcinogens or pro-carcinogens, including organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs or dioxins) and polychlorinated dibenzofurans (PCDFs or furans) (Table 1-2). Also found were concentrations of known carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Table 1-2). As part of the remediation process, soils and sediments identified as "scheduled chemical waste" (containing one or more scheduled compounds (including OCPs and PCBs) where the total concentration of these chemicals is greater than 2 ppm), were treated by thermal desorption and base catalysed decomposition in an on-site facility.

Four hundred tonnes of scheduled chemical waste were excavated, treated and combined with non-treated excavated material in large containment mounds. The containment mounds were clay-capped and geotextile lined to avoid infiltration and leaching of contaminants to the groundwater (OCA 1997, OCA and ADI 1999). Leachate drains were installed and their contents can contain measurable levels of POPs (e.g., SKM and EVS 2000). Given the current high ecological value of SOP, it is essential that the monitoring of the presence of POPs is continued.

PCBs, PAHs, PCDD/Fs and some OCPs are ligands for the endogenous aryl hydrocarbon receptor (AhR) (Sierra-Santoyo et al. 2000, Fent 2001, Oropeza-Hernandez et al. 2003). The AhR / ligand complex modulates the 1A family of cytochrome P450 mRNA (CYP1AmRNA), which translates for CYP1A (an endoplasmic reticulum associated mixed function oxygenase) and has been isolated from many taxa (Hahn 1998). This pathway is associated with xenobiotic metabolism (reviewed by Schmidt and Bradfield 1996). The induction of CYP1A above background levels has been used as an early biomarker of exposure to POPs (both *in vivo* and *in vitro*) (reviewed by Whyte et al. 2000, 2004).

Chemical analysis of large numbers of samples for a large range of compounds such as those described above is costly and can have a long turnaround time. A way to address both these logistical issues is to use bioassays, which can give an idea of the ecologically and physiologically important fraction of contamination. The H4IIE rat hepatoma cell line produces CYP1A predictably and repeatably in response to exposure to AhR ligands (Whyte et al. 2004). It has been widely used to evaluate the equivalent levels of 2,3,7,8-TCDD present in environmental aqueous and sediment samples (Tillitt et al. 1991, Khim et al. 2000b, Coady et al. 2001, Giesy et al. 2002a, Koh et al. 2005). The assay does not identify causative compounds but gives a total 2,3,7,8-TCDD equivalency (TCDDeq) in a given sample. While the assay uses a terrestrial vertebrate (rat) AhR this study is focused on aquatic habitats. An alignment of AhR amino acid sequences (Figure 4-1) indicates that it is a well conserved protein and it is, therefore, reasonable to expect that the ligands for the rat AhR contributing to the TCDDeq of the samples will also be ligands for AhR in other taxa, including aquatic taxa.

A common method for the remediation of contaminated sediment is removal by dredging. Previous work has suggested the potential for migration and increased bioavailability of contaminants as a result of such disturbance (Rice and White 1987, Latimer et al. 1999). Wetlands in SOP (e.g., Boundary Ck) designed to intercept urban contaminated sediments (by settlement in ponded areas) may require future alterations to deal with reductions in stream / pond depth. Further, the eastern shore of Homebush Bay, a heavily POP contaminated site (Birch et al. 2007), will be subject to remediation by dredging of sediments and subsequent treatment by thermal desorption in the near future (Standing Committee on State Development 2002). It is important that the potential of these processes to increase the bioavailability of sediment-bound contaminants be assessed in order that their impact may be minimised.

By determining concentrations of POPs and PAHs and quantifying the levels of bioactive POPs (by AhR binding) as a TCDDeq in aqueous and sediment fractions of the wetlands in SOP this study aimed to assess the success of the remediation of the site in returning the

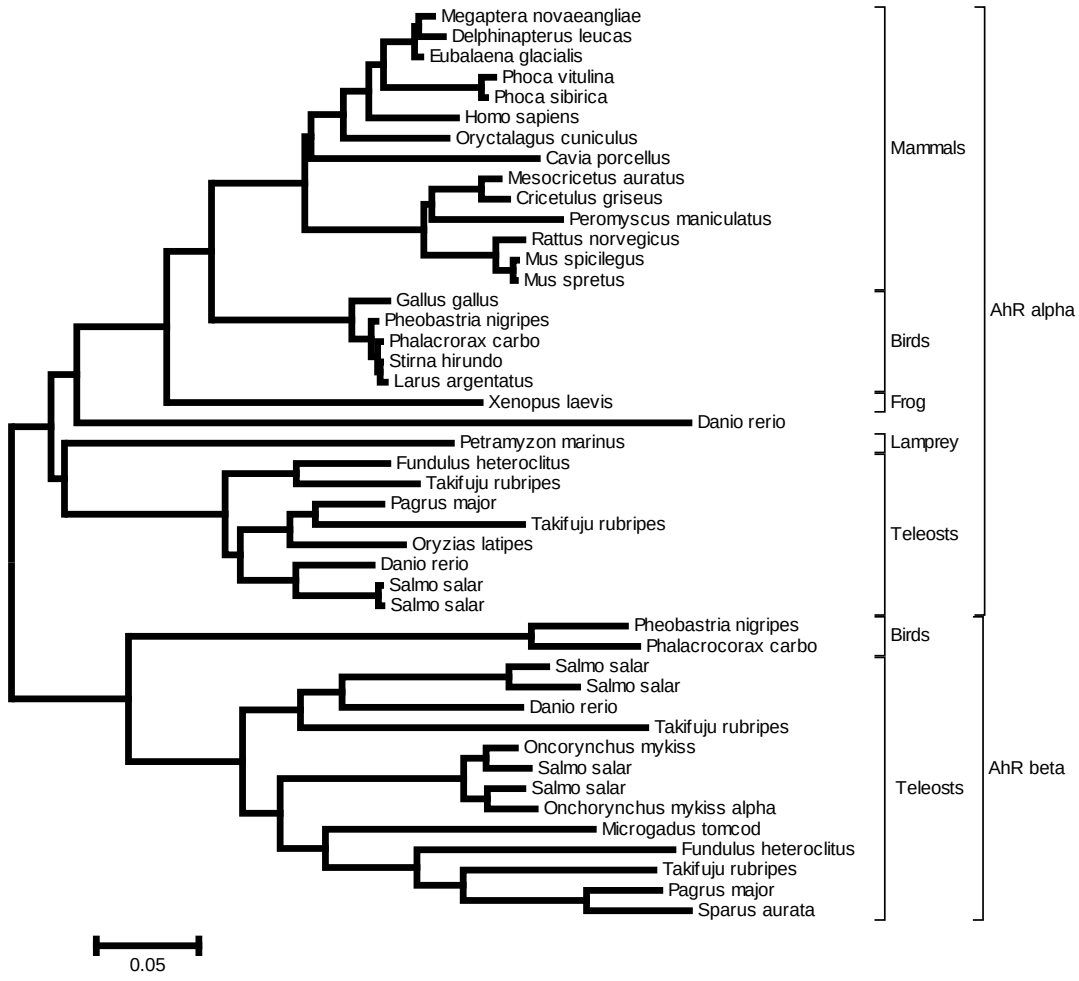


Figure 4-1 Phylogenetic tree showing evolutionary relationships of the aryl hydrocarbon receptor (AhR) protein (45 protein sequences from 32 taxa). The scale bar represents 5% divergence. The tree was constructed by neighbour joining (Saito and Nei 1987) based on protein sequence alignment of 376 positions. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

wetlands to within the chemical bounds expected for an urban impacted wetland in Sydney. It also aimed to simulate the effects of anthropogenic disturbance of POP contaminated sediments on their bioavailability through the aqueous phase. It was hypothesized that; 1. there would be differences between the concentrations of POPs in the water and sediments at the Sydney Olympic Park wetlands and those at the reference sites; 2. between site differences in the measured concentrations of POPs would result in differences in the activation of the AhR in the H4IIE bioassay; and 3. disturbance of contaminated sediments would result in increased aqueous concentrations of POPs as measured by AhR activation in the AhR.

4.3 Materials and Methods

4.3.1 Study Sites

Two sets of study sites were selected for this study: 1) SOP wetlands were selected to cover a range of contamination and remediation histories (Table 2-2, Figure 2-1); 2) a geographically diverse range of wetlands across the Sydney metropolitan area designated as "urban reference sites" (Table 2-3, Figure 2-2). These reference sites were not selected as "clean" sites but rather to represent wetlands that are subject to urban impacts such as aerial deposition of industrial products and surface runoff from their catchments.

4.3.2 Sample Collection

Water samples were collected in clean (washed and solvent rinsed) amber glass bottles (1 L) and transported on ice to the laboratory where they were stored at 4^{o}C for no more than 48 hours prior to extraction. Samples were collected from approximately 20 cm depth to avoid any surface layer contamination. Duplicate samples were collected from different parts of the wetland.

Composite sediment grab samples totaling 1 L were collected from each study site. At least 10 grab samples from the top 5 cm (estimated to be the average depth of the oxic layer) over an area of approximately 225 m^2, were collected using a 10 cm diameter grab sampler.

Collected sediment was sieved and mixed on-site and stored in clean (washed and solvent rinsed) 1 L glass jars on ice for transport to the laboratory. Samples were overnight couriered to the CSIRO Land and Water Adelaide laboratories for extraction.

4.3.3 Agitation Experiment

Seven study sites were selected for use in this set of experiments (i.e., Boundary Ck, EWQCP, SWQCP, Northern Water Feature, Bicentennial Pk., Narrawang 22 and Macquarie Uni). For each of these wetlands water/sediment preparations were made using both field and reverse osmosis (RO) water. Approximately 30 g sediment was added to each of 4x1L water samples for each study site (2 field water, 2 RO water). These were allowed to settle for 1 week and then shaken in an orbital shaker for 48 hours. Samples were centrifuged at 1710 x g for 20 minutes and the supernatant collected for extraction.

4.3.4 Sample Extraction and Elution

Aqueous samples were passed through 2.7 μm and 1.2 μm GFC filters prior to extraction to remove suspended particulate matter. Filtrate was extracted onto C18 Empore disks through a six station Empore extraction manifold. Disks were air dried (1 hr) and stored at -20^{o}C for a maximum of 28 days before elution. Prior to elution the disks were thawed and dried on a drying plate for 1 hour at 40^{o}C. They were twice eluted with 10 ml dichloromethane (DCM) and solvent exchanged under a gentle nitrogen gas stream to 500 μL DMSO for storage at -80^{o}C in amber glass vials.

Wet sediment samples were transferred into glass jars (500 mL) and freeze-dried for one week. POPs in the freeze-dried sediments were extracted with DCM using a Dionex ASE300 Accelerated Solvent Extractor (ASE). Five grams of each sample was weighed into each extraction cell having a filter pad at the bottom and the cell was filled up using diatomaceous earth from Dionex. The ASE used an oven temperature of 100^{o}C with 10 MPa (1500 psi) pressure. The heat up time was 5 min and the static time was 5 min for 2 cycles. The extracts were concentrated in collection bottles under nitrogen gas using a Dionex SE500 Solvent Evaporator. Final extracts were re-dissolved in 1 mL of hexane.

Some dirty extracts were further cleaned up using Solid Phase Extraction (SPE) silica cartridges. Extracts were stored at -80^{o}C. Pore water was extracted by centrifuging wet sediment followed by liquid-liquid extraction with hexane.

4.3.5 H4IIE Bioassay

Tissue culture and H4IIE bioassay methods were adapted from a comparative review of published protocols by Whyte et al. (2004) and are summarised below (detailed protocols are presented in Appendices D, E and F). Vials of the H4IIE rat hepatoma cell line were obtained from Landcare Research, Lincoln, New Zealand frozen in 10% DMSO, 90% Dulbecco's Modified Eagles Media (DMEM = 500 ml; DMEM (Gibco), 25 ml Foetal Bovine Serum (Gibco), 10 ml L-Glutamine (Gibco), 1.25 ml Gentamycin (Gibco), 0.5 ml 2.5 M HEPES (Sigma-Aldrich)) at 1x10^{6} cells/ml. The cells were grown to 80-100% confluence at 37^{o}C and 5%CO_2. When necessary excess cells were refrozen in 10% cryo-preservant (DMSO) at a concentration of 1x10^{6}cells/ml.

Appropriate dilutions of samples were made up in glass amber vials and stored at -80^{o}C. Range finding sample dilutions were made up as 10x dilutions from 100% to 0.01% and definitive test dilutions were made up as 2x dilutions from the highest non-cytotoxic concentration determined by the range finding test. H4IIE cells were added to 96 well tissue culture treated plates at a concentration of 2x10^{4} cells/well. The plates were incubated for 24 hours (37^{o}C and 5%CO_2) or until wells were >80% confluent. Sample dilutions (1 μL) were added to each well and incubated for a further 24 hours (37^{o}C and 5%CO_2). Standard curves were run on each plate with 1 μL aliquots of 2,3,7,8-TCDD (AccuStandard: Lot B4010226) ranging in concentration from 5 to 0.02 nM added to appropriate wells.

After 24 hours the media was removed from the wells and the plates washed three times with phosphate buffered saline. A 100 μL aliquot of 7-ethoxyresorufin (7-ER) (Sigma-Aldrich) (2 μM in methanol) was added to each well and the rate of conversion to the fluorescent endpoint, resorufin, was read using a fluorescence plate reader (excitation = 540

nM, emission = 590 nM) taking continuous readings over 30 mins. After the plates were read the contents were removed and 100 μL ultrapure water added to each well and the plates stored at -80^{o}C for 24 hours. The maximum rate of resorufin production was calculated using a resorufin standard curve and the protein content of each well was quantified using a bicinchoninic acid protein assay (Pierce Micro-BCA Protein Kit). Each EC50 value was converted to appropriate units (pmol resorufin/min/mg protein) and compared with that of the TCDD standard curve giving a value for TCDD equivalency (TCDDeq). This value was interpolated in a standard curve fitted with a four parameter logistic growth equation to give a value for TCDDeq (see Eqn 3-1) (see Appendix F for detailed methods).

4.3.6 Chemical Analysis

Sixteen PAHs (acenaphthene, acenaphthylene, anthracene benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene), eleven PCB congeners (28, 52, 77, 81, 101, 118, 126, 138, 153, 169, 180) and ΣDDT (DDE, DDD, DDT) were analysed by gas chromatography-mass spectrometry (GC-MS, Agilent 6890GC-5973N mass detector) according to USEPA standard methods (USEPA Method 8260C) after some modification. Target compounds were separated on a capillary column (HP-5MS, 30 m × 0.25 mm, 0.25 μm thick stationary phase) with helium as the carrier gas at a constant flow rate of 1.1 mL/min. Quantitative mass spectrometric analyses were conducted by using electron ionisation source (EI) and selected ion monitoring mode (SIM).

Trace elements (Cr, Cu, Pb, Zn, Ni, Cd, As) in pore water and sediment samples were analysed by ICP-MS using USEPA standard methods (USEPA method 6020) with modifications and are presented and included in the analyses as summed metals.

4.3.7 Sediment Analysis

A 20ml 1:5 (w/v) sediment suspension in water was made up in a 30 ml plastic sample container. This was placed in a shaker for 1 hour after which the pH was read using a desktop pH probe (WTW SenTix 61). The conductivity of the sediment was measured using the sediment suspension described above with a portable conductivity probe (WTW LF330i). A 20 ml 1:5 (w/v) sediment suspension in 1 mM $CaCl_2$ was made up in a 30 ml plastic sample container. This suspension was placed in a shaker for one hour after which the pH was read using a desktop pH probe (WTW SenTix 61). Pore water pH was measured by inserting a probe (WTW pH 330) into intact sediments and allowing 30 mins for equilibration. Redox potential was measured by gently inserting a redox electrode (Activon Ag/AgCl) into the sediment and allowing it to equilibrate for 30 mins.

Sediment grain size composition was measured by dry sieve. Dried sediments (24hrs at 100^{o}C) were hand ground using a mortar and pestle until grains were observably discrete under a dissection microscope. Ground sediments were placed in the top of a sequential sieve tower (mesh sizes 2000, 1000, 500, 250, 187.5, 125, 93.5 and 46.75 μm) and mechanically shaken for 10 minutes. The contents of each sieve were weighed and the proportional contribution made to the mass of the sample by each grain size class was recorded.

Carbon content of the sediment was calculated using loss on ignition (LOI) techniques (Heiri et al. 2001). Triplicate samples were ground, weighed and placed in ceramic crucibles in a muffle furnace. Samples were sequentially heated to 375 ^{o}C, 550 ^{o}C and 950^{o}C. Between each heating cycle the samples were cooled to room temperature in a desiccator and weighed. Mass lost at 375, 550, 950 ^{o}C and were recorded as "Black Carbon" (Simpson et al. 2005), organic carbon and total carbon, respectively. Inorganic carbon was calculated by Eqn 4-1.

$$\text{Inorganic Carbon} = \text{mass lost at } 950^{o}\text{C} - \text{mass lost at } 550^{o}\text{C} \qquad \text{(Eqn 4-1)}$$

Water content was evaluated as the mass difference before and after heating a wet sediment sample at 100 ^{o}C. Heating was continued until the mass lost over 24 hours was less than 0.005 g.

4.3.8 Data Analysis

Much of the data presented in this chapter is in the form of chemical measurement with limited replication due to logistical constraints. This lack of replication precludes the use of standard analysis of variance techniques (either single or multi factor). In this chapter the only such analysis is between the TCDDeq at sites inside SOP and at reference sites. Other analyses incorporate the large volume of data in the most appropriate form, attempting to explain the differences between sites using multiple regression techniques.

Multiple regressions were run on the data using the SPSS statistical package. The variables of TCDDeq in water and in sediment were run using predictors of the physical and chemical characteristics of the sediment (concentrations of ΣPCB, ΣPAH, ΣDDT and metals, pH, $CaCl_2$ pH, conductivity, redox potential, grain size, organic carbon composition). The aqueous TCDDeq measured after disturbance with sediment from the study sites were analysed using the same set of predictors as listed above with the addition of aqueous and sediment TCDDeq.

The TCDDeq of the water samples attained after agitation with sediments were analysed using the same predictors but with the addition of water and sediment TCDDeq. Differences between groups of study sites (SOP wetlands and reference sites) were analysed using single factor ANOVA and the assumption of equal variances was met in.

4.4 Results

In general, the highest levels of measured POP contamination were found in the estuarine Homebush Bay, Silverwater Bridge, Newington Wharf and Haslams Mouth sites (Table 4-1). At these sites the levels of ΣPAHs were regularly greater than twice those measured at

Table 4-1. Results of chemical analysis on sediments in Sydney Olympic Park and Parramatta River study sites in 2005 and 2006. All values for sediment concentrations are expressed per gram dry weight. n/a = no data collected.

		2005					2006				
		ΣPAHs (ng/g)	**ΣPCBs (ng/g)**	**ΣDDT (ng/g)**	**Metals (μg/g)**	**Pore Water Metals (μg/L)**	**ΣPAHs (ng/g)**	**ΣPCBs (ng/g)**	**ΣDDT (ng/g)**	**Metals (μg/g)**	**Pore Water Metals (μg/L)**
Wetlands in Sydney Olympic Park	Wharf Pond	2550	15	12	412	36.64	1704	14	25	366	35.63
	Bicentennial Pk	5336	14	13	449	10.19	4309	11	19	441	4.53
	EWQCP	14461	21	19	267	11.18	4269	20	29	429	7.05
	Wharf Stream	2905	24	13	601	29.95	n/a	n/a	n/a	n/a	n/a
	Nth Water Feat.	1960	14	15	204	22.06	2808	12	21	534	15.55
	SWQCP	1146	23	4	403	7.83	804	14	98	489	5.23
	Boundary Ck	3409	33	24	862	1014.64	1516	47	45	825	19.87
	Narrawang 22	n/a	n/a	n/a	n/a	n/a	272	11	25	148	25.56
	Fishway	n/a	n/a	n/a	n/a	n/a	1265	5	20	225	9.65
Parramatta River Sites	Homebush Bay	11714	112	187	419	246.63	7628	175	55	1148	71.81
	Haslams Mouth	13085	124	109	656	61.49	7881	108	10	424	35.59
	Silverwater Bdg.	3701	14	31	264	121.87	19358	62	53	833	29.00
	Newington Wf.	10251	62	106	1152	268.60	1256	16	43	241	83.99

the wetlands in SOP (Table 4-1). High sediment ΣPAH concentrations were found in SOP wetlands at EWQCP in 2005 (14461 ng/g) and 2006 (4269 ng/g) and in Bicentennial Park in 2006 (4309 ng/g). ΣPCB levels in SOP wetlands ranged between 14 and 33 ng/g in 2005 and 11 and 47 ng/g in 2006. ΣDDT concentrations in the SOP wetlands were below those in the estuarine sites except in SWQCP and Boundary Ck in 2006 (98 and 45 ng/g, respectively). In general, the concentrations of POPs in the sediments of the SOP wetlands were within the range measured at urban impacted reference wetlands. EWQCP more than twice the concentration of ΣPAHs (14461 ng/g) as the highest measured concentration in the urban impacted reference wetlands (6608 ng/g) in 2005 but the concentration measured in 2006 was far less (4269 ng/g) (Table 4-2). In 2006 sediments from both SWQCP and Boundary Ck had higher (98 and 45 ng/g respectively) ΣDDT concentrations than all urban impacted reference wetlands (maximum concentration 44 ng/g) (Table 4-2). The sediments and pore water in Boundary Ck had higher concentrations (862 and 1015 ng/g in 2005) of metals than all urban impacted reference sites (Table 4-2).

Pore water metal concentrations were generally lower in the SOP wetlands than in the Parramatta River and Homebush Bay sites (Table 4-1). Metal concentrations in the sediment from six SOP wetlands were higher than in the lowest concentration measured in the Parramatta River and Homebush Bay sites in both 2005 and 2006 (Wharf Pond, Bicentennial Park, EWQCP, SWQCP, Boundary Ck) (Table 4-1). Sediments in Boundary Ck had higher metal concentrations than the average measured in Parramatta River and Homebush Bay sites in both 2005 (623 mg/kg) and 2006 (661 mg/kg) (Table 4-1). The study sites varied with respect to many sediment physico-chemical parameters. Most wetland sediments were dominated by fine to very fine sands (62.5 – 250 μm) (Figure 4-2). Nepean River, Oyster Bay, Narrabeen, Joseph Banks Park, Bardwell Ck, Galston, Moores Ck, Chipping Norton had greater than 60% grains (by weight) >250 μm and sediments at Terrys Ck were dominated by coarse sand to gravel (>500 μm). SWQCP, Boundary Ck., Cecil Hills, Haslams Mouth and Kemps Ck, were high in very fine sands and silt (<125 μm). The pore water pH measured by probe insertion varied little between wetlands while

Table 4-2 Results of chemical analysis on sediments in urban impacted reference sites in the Sydney metropolitan area in 2006. All values for sediment concentrations are expressed per gram dry weight.

	ΣPAHs (ng/g)	**ΣPCBs (ng/g)**	**ΣDDT (ng/g)**	**Metals (μg/g)**	**Pore Water Metals (μg/L)**
Bardwell Ck	6608	17	40	573	15.43
Galston	84	8	21	28	21.60
Second Ponds Ck	2343	32	44	205	26.15
Narrabeen	218	8	16	85	34.76
Moores Ck	2514	18	17	291	18.34
Quakers Hill	312	12	17	259	18.71
Yarramundi Lagoon	4590	19	30	137	32.04
Eastern Ck	988	20	16	288	22.47
Kemps Ck	107	9	15	166	19.55
Chipping Norton Lake	38	13	6	17	29.49
South Ck	555	17	37	267	18.03
Joseph Banks Park	34	4	10	2	25.73
Oyster Bay	1212	10	23	244	28.97
Cecil Hills	327	5	21	123	6.42
Terrys Ck	645	16	31	291	8.89
Georges River	1898	85	25	699	25.97
Macquarie Uni	452	14	33	295	5.75
Nepean River	1246	3	10	170	19.29

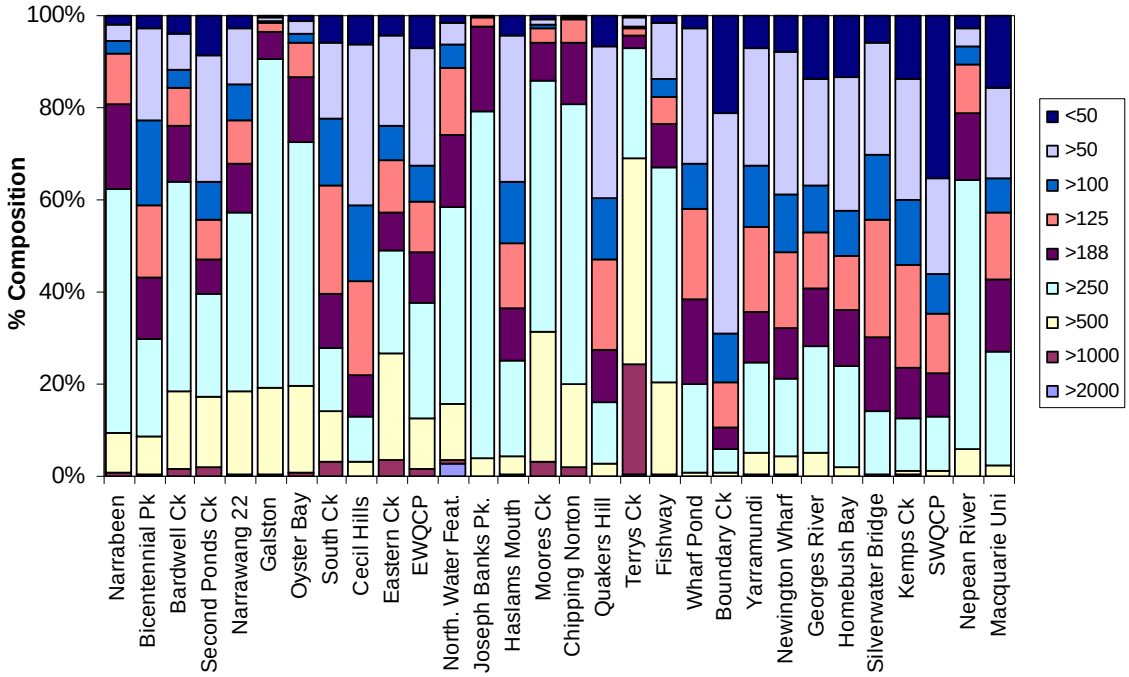


Figure 4-2 Percentage contribution (by dry weight) to sediment by grain size classes (μm) at Sydney Olympic Park, Parramatta River and urban impacted sites.

the pH measured in sediment suspension varied between 4.28 and 8.11 (Table 4-3). The $CaCl_2$ pH ranged from 4.15 to 7.08 (Table 4-3). The average total carbon content of the sediments was 8.23% but some wetlands exhibited carbon contents which were up to three times this value (SWQCP and Georges R.) (Table 4-3). Sediments from SOP wetlands had a high contribution (>70%) to the carbon content by organic carbon. Black carbon (soot) was particularly high in two SOP wetlands (SWQCP and Bicentennial Park) (Table 4-3).

TCDDeq of the aqueous samples from SOP ranged from 0.0140 pM (Narrawang 22) to 0.058 pM (Boundary Ck.) (Table 4-4). In both 2005 and 2006, the aqueous TCDDeq values were significantly ($p<0.05$) lower in SOP wetlands than in urban reference sites (Figure 4-3). In the reference sites aqueous TCDDeq values ranged up to 0.099 pM at South Ck (Table 4-4). Sediment ΣPCB concentrations were positively correlated and explained over 85% of the variation (i.e., $r^2 = 0.867$) in aqueous TCDDeq in 2005 (Table 4-5). In 2006 and when all collections were combined, differences in TCDDeq were not significantly ($p>0.05$) correlated to physical characteristics of the water, or any physical or chemical characteristics of the associated sediment (Table 4-5).

The TCDDeq of the sediment samples taken from SOP ranged from 0.0158 μg/kg (SWQCP) to 7.06 μg/kg (Bicentennial Park) (Table 4-4). The Homebush Bay and Parramatta River sites had generally higher TCDDeq values than those at the SOP sites (up to 8.16×10^{-5} g/kg) (Table 4-4). Sediment TCDDeq values from SOP wetlands were within the range measured in sediments from urban reference sites (Table 4-4). Multiple regression showed high correlations between TCDDeq in the sediment and sediment concentrations of ΣPCBs and ΣDDT (negative relationship) and the amount of fine (<0.63 μm) sediment grains, which explained 95% of the variation in 2005 and with sediment ΣPAH concentration which explained 80% of the variation in 2006 (Table 4-5). When the two collections were considered together the sediment ΣPAH concentration accounted for 44.1% of the variation in TCDDeq across the study sites (Table 4-5). There was no significant ($p\geq0.05$) difference in the TCDDeq after sediment agitation between agitation in site and laboratory water; these were subsequently combined. There was a significant ($p<0.05$) increase in aqueous TCDDeq after agitation

Table 4-3 Sediment characteristics at Sydney Olympic Park, Parramatta River, Homebush Bay and urban impacted study sites.

		pH (insertion)[a]	Redox Potential insertion	Water Content (%)	Black Carbon (%)	TOC[b] (%)	TC[c] (%)	TIC[d] (%)	Cond[e] (µS/cm)	pH (susp.)[f]	$CaCl_2$ pH (susp.)[f]
	Bicentennial Park	7.33	60	58.78	10.52	15.15	17.85	3.17	1226.67	7.05	6.43
	Boundary Ck	7.25	110	50.96	6.25	9.99	12.25	2.51	1061.00	6.62	6.17
	Fishway	7.42	28	21.82	1.37	2.39	3.35	0.99	437.67	5.85	5.95
Wetlands in Sydney Olympic Park	Wharf Pond	7.23	-44	29.91	3.09	4.84	6.32	1.48	498.67	6.25	5.91
	SWQCP	6.96	-92	69.86	18.17	22.40	25.65	4.17	1830.33	6.22	6.03
	North. Water. Feat.[g]	6.72	-135	29.59	1.77	2.59	3.21	0.63	1019.67	4.28	4.15
	EWQCP	7.13	-46	39.74	4.05	7.08	8.79	1.84	412.00	6.63	6.27
	Narrawang 22	7.20	-31	29.19	1.89	3.48	4.39	0.86	457.67	6.50	6.25
	Silverwater Bridge	7.72	-84	36.38	2.97	6.50	8.07	1.68	6593.33	8.11	6.96
Parramatta River and Homebush Bay Sites	Haslams Mouth	7.86	-82	43.05	4.17	7.76	11.51	3.88	9976.67	7.97	7.08
	Newington Wharf	7.32	3	50.24	4.32	7.69	10.36	2.90	9583.33	8.01	7.05
	Homebush Bay	7.05	19	52.43	5.48	11.50	14.06	2.88	14706.67	7.34	6.76
	Bardwell Ck	7.05	-63	47.10	6.80	8.72	9.58	0.94	668.00	6.72	6.15
	Galston	7.52	-70	21.60	0.92	1.17	1.43	0.27	142.77	6.67	6.52
	Second Ponds Ck	7.01	21	32.35	4.58	7.75	9.83	2.25	158.00	7.20	6.21
	Narrabeen	7.00	-174	28.24	3.34	4.74	5.52	0.82	5733.33	6.17	6.10
	Moores Ck	6.98	-37	22.58	1.96	2.70	3.12	0.43	476.00	6.64	6.16
	Quakers Hill	7.14	-20	33.20	3.73	7.11	8.62	1.62	344.33	5.91	6.00
	Yarramundi Lagoon	6.97	26	34.70	5.73	7.23	8.07	0.90	119.63	6.29	5.71
	Eastern Ck	7.14	3	24.64	3.23	5.42	6.84	1.50	112.53	7.01	6.65
Urban Impacted Reference Sites	Kemps Ck	6.88	103	33.20	4.34	6.91	8.49	1.69	367.00	7.14	6.25
	Chipping Norton Lake	7.22	-107	15.83	0.29	0.48	0.66	0.19	1021.67	6.68	6.27
	South Ck	7.23	-2	33.31	3.84	5.23	6.25	1.08	214.03	6.48	6.12
	Joseph Banks Park	6.95	301	15.80	0.40	0.55	0.72	0.17	146.47	6.31	5.90
	Oyster Bay	7.10	-172	34.70	1.89	3.00	3.58	0.60	4650.00	5.66	5.68
	Cecil Hills	7.12	-24	34.78	5.78	7.61	8.76	1.25	220.43	6.16	5.75
	Terrys Ck	7.31	-58	20.78	1.30	2.25	2.89	0.55	160.50	7.01	6.79
	Georges River	6.59	78	59.06	12.59	20.58	23.73	3.98	19333.33	6.95	6.41
	Macquarie Uni	6.62	68	33.36	6.78	8.97	10.07	1.20	197.27	6.20	5.16
	Nepean River	7.35	-13	20.27	1.47	2.29	2.92	0.87	146.87	6.82	6.21

[a] pH measured by probe insertion. [b] Total Organic Carbon. [c] Total Carbon. [d] Total Inorganic Carbon. [e] Conductivity. [f] measured in 1:5 sediment:solution suspension.
[g] Northern Water Feature.

Table 4-4 TCDDeq values for surface water and sediments of Sydney Olympic Park and Homebush Bay and urban impacted wetlands measured using the H4IIE cell line bioassay in 2005 and 2006. n/a = no data, BDL = below assay detection limit.

		2005		2006	
		Water TCDDeq (pM)	Sediment TCDDeq (μg/kg)	Water TCDDeq (pM)	Sediment TCDDeq (μg/kg)
Wetlands in Sydney Olympic Park	Wharf Pond	n/a	0.0228	n/a	1.39
	Bicentennial Park	0.0226	2.22	0.0157	7.06
	EWQCP	0.0213	0.402	0.0218	4.58
	Wharf Stream	n/a	0.689	n/a	n/a
	North. Water Feat.[a]	0.0145	1.63	0.0193	4.21
	SWQCP	0.0401	0.0158	0.0267	5.20
	Boundary Ck	0.0576	3.52	0.0334	5.01
	Narrawang 22	0.0140	n/a	0.0320	0.809
	Fishway	0.0535	n/a	0.0473	2.26
	Lake Belvedere	0.0147	n/a	0.0157	n/a
Parramatta River Sites	Newington Wharf	n/a	6.57	n/a	5.01
	Homebush Bay	n/a	0.313	n/a	22.0
	Haslams Mouth	n/a	81.7	n/a	4.89
	Silverwater Bridge	n/a	0.210	n/a	80.9
Urban Impacted Reference Sites	Bardwell Ck	n/a	n/a	0.0564	9.16
	Galston Ck	n/a	n/a	0.0348	0.359
	Second Ponds Ck.	n/a	n/a	0.0094	10.5
	Narrabeen Lake	n/a	n/a	0.0245	1.23
	Moores Ck	n/a	n/a	0.0548	12.9
	Quakers Hill	n/a	n/a	0.0677	2.25
	Yarramundi Lagoon	n/a	n/a	0.0086	4.95
	Eastern Ck	n/a	n/a	0.0444	2.89
	Kemps Ck	n/a	n/a	0.0499	0.131
	Chipping Norton Lk	n/a	n/a	0.0497	0.00479
	South Ck	n/a	n/a	0.0968	3.47
	Joseph Banks Park	n/a	n/a	0.0263	0.0543
	Oyster Bay	n/a	n/a	0.0706	8.94
	Cecil Hills	n/a	n/a	0.0347	0.525
	Terrys Ck	n/a	n/a	0.0267	4.29
	Georges River	n/a	n/a	0.0367	8.96
	Nepean River	n/a	n/a	0.0167	0.266
	Prospect Ck	n/a	n/a	0.0954	n/a
	Macquarie Uni	0.0384	n/a	0.0312	6.65
	Upper Colo	0.0070	n/a	0.0056	BDL

[a]Northern Water Feature.

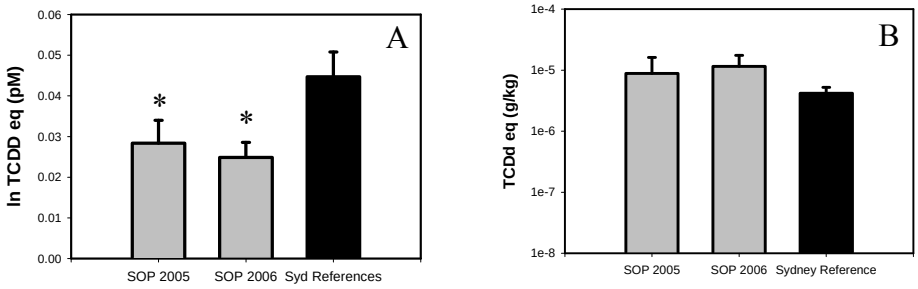


Figure 4-3 TCDDeq (measured by the H4IIE cell line bioassay) for A: surface water and B: sediments at Sydney Olympic Park and urban impacted reference sites (Syd References) at different collection times. The Sydney reference collection was collected in 2006 * denotes significantly ($p<0.05$) different from reference sites.

Table 4-5 Results of multiple regressions between TCDDeq values (measured using the H4IIE cell line bioassay) and water and sediment chemical and physico-chemical characteristics.

		F	Sig. Level	Predictors	Correlation Type	r^2
Water	2005	19.62	0.021	ΣPCB	+	0.867
	2006	0.51	0.883	NSP[a]		
	2005 + 2006	0.53	0.806	NSP[a]		
Sediment	2005	38.31	0.000	ΣPCB ΣDDT Grain size <0.63 μm	+ - +	0.950
	2006	108.80	0.000	ΣPAH	+	0.795
	2005 + 2006	29.17	0.000	ΣPAH	+	0.441
Agitated Sediment	2006	30.21	0.004	Grain size 1 – 2 mm Grain size 0.25–0.5 mm	+ -	0.968

[a] NSP = no significant predictors in model.

(Figure 4-4) and multiple regression indicated that differences in sediment grain size (medium to coarse sand) accounted for 96.8% of the variation in TCDDeq (Table 4-5).

4.5 Discussion

The concentrations of POPs in the Parramatta River and Homebush Bay sites were generally much higher than those in the adjacent freshwater wetlands. The SOP remediation program did not include the sediments of the adjacent Parramatta River and Homebush Bay. It was estimated that 160,000m^3 of sediment from Homebush Bay may require remediation should the project be undertaken (Rubenstein and Wicklund 1991). Therefore, it was expected that POP concentrations at these study sites would represent the upper end of the range of contamination measured in this study. These high values were not measured in the estuarine reference sites (Georges River and Oyster Bay). The high concentrations of POPs at the Parramatta River and Homebush Bay study sites were reflected in the high TCDDeq values measured by the H4IIE bioassay. The TCDDeq values in these study sites ranged from being similar to those measured in the sediments of the adjacent wetlands and reference estuarine sites to being an order of magnitude higher.

In previous studies conducted around the world, very wide ranges of ΣPAH, ΣPCB and ΣDDT concentrations in sediments have been reported (Table 4-6). The concentrations of ΣPAHs measured in the sediments of the SOP wetlands fell in the mid-range of reported values. The ΣPAH concentrations for SOP wetlands in the current study are similar to the average concentrations for estuaries in the US (3200 ng/g) and those measured at the Parramatta River and Homebush Bay study sites fall into the higher end of the ranges reviewed by NOAA (1987). Measured concentrations of ΣPCBs in the current study are low compared to published values (Table 4-6). The maximum value reported here (175 ng/g) is exceeded by most reported levels. Of particular note is the level reported for a remediated lake in Sweden (31000 ng/g before remediation reduced to 54 ng/g after remediation). This remediated level was not exceeded in the SOP wetlands in the current study and by only one reference site (Georges River, an estuarine site in the South of Sydney). While ΣDDT concentrations in the current study also fell in the lower end of

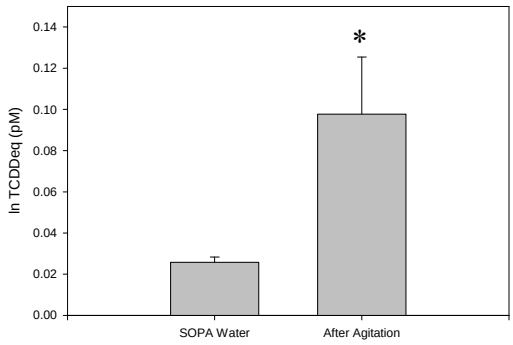


Figure 4-4 TCDDeq values (measured by the H4IIE cell line bioassay) of water from study sites before agitation and of water (combined field and laboratory) after agitation with sediments. * denotes significant ($p<0.05$) difference.

Table 4-6 Values for sediment ΣDDT, ΣPAH and ΣPCB and TCDDeq concentrations reported elsewhere. This list represents a range of reported values and is not meant as an exhaustive review.

	Location	Concentration (ng/g)	Reference
ΣDDT	**Daya Bay, China**	**20.2**	**(Zhou et al. 2001)**
	Brest, France	**52.2**	**(Fowler 1990)**
	Upper Steele Bayou, MS, USA	**110 – 160**	**(Ford and Hill 1991)**
	Hudson River, USA	**739**	**(Fowler 1990)**
	Aegean Sea	**893**	**(Fowler 1990)**
	Palos Verde, Ca, USA	**100000**	**(Fowler 1990)**
ΣPCB	**Daya Bay, China**	**27.4**	**(Zhou et al. 2001)**
	Unnamed Lake, Sweden, post-remediation	**54**	**(Bremle and Larsson 1998)**
	Placentia Bay, Newfoundland	**<100**	**(Khan 2003)**
	Baltic Sea	**212**	**(Fowler 1990)**
	Hudson River, USA	**1400**	**(Feng et al. 1998)**
	Osaka Bay, Japan	**2000**	**(Fowler 1990)**
	Brest, France	**2100**	**(Fowler 1990)**
	New York Bight, NY, USA	**2200**	**(Fowler 1990)**
	Palos Verde, CA, USA	**7420**	**(Fowler 1990)**
	New Bedford Bay, USA	**8400**	**(Fowler 1990)**
	Unnamed Lake, Sweden, pre-remediation	**31000**	**(Bremle and Larsson 1998)**
	Escambia Bay, USA	**480000**	**(Fowler 1990)**
ΣPAH	**Adriatic Sea**	**527**	**(Guzzella and DePaolis**
	Average US estuaries	**3200**	**(NOAA 1987)**
	Penobscot Bay, USA	**8794**	**(Johnson and Larsen 1985)**
	East Long Is. Sound, NY, USA	**48500**	**(NOAA 1987)**
	Placentia Bay, Newfoundland	**>50000**	**(Khan 2003)**
TCDDeq	**Thunder Bay, Michigan, USA**	**0.014**	**(Jones et al. 1993)**
	Korean Coastal Sediment	**0.28**	**(Koh et al. 2005)**
	Ulsan Bay, Korea	**0.49**	**(Khim et al. 2000a)**
	Bayou Meto, Arkansas, USA	**1.8[a]**	**(Lebo et al. 1995)**
	Czech Republic	**18**	**(Hilscherova et al. 2000)[b]**
	Grand Calumet R., Indiana,	**138[c]**	**(Hoke et al. 1994)**

[a]Time-integrated water sample from SPMD. [b] Cited in (Whyte et al. 2004), [c]Average value from a large range (4.7-565 ng/g).

values reported elsewhere (Table 4-6) some regard must be taken of the context of these reports. The Upper Steele Bayou Watershed reportedly received large quantities of DDT and other OCPs in the past. In 1991, sediment DDT concentrations of 110-160 ng/g were accompanied by concentrations in fish which were above US FDA food limits (Ford and Hill 1991). Measured concentrations in the current study of up to 98 ng/g are, therefore, of ecological concern given the presence of high trophic consumers (fish and birds).

There are still measurable levels of POPs in the SOP wetland sediments (including wetlands created with "clean fill"). It unlikely that this is "legacy" contamination from the pre-remediation period given that the concentrations are similar to those measured in urban reference sites around Sydney. The contaminants could have been brought on-site during the creation of the wetlands as it appears that the pollutant loads are consistent with any sediment from the Sydney area. Similarly, the pollutants could be imported through catchment processes given that the catchments of many of the wetlands lie at least partially outside SOP. There is a significant body of literature describing the aerial deposition of POPs (especially PCBs) in urban areas (e.g., Eisenreich et al. 1981, Franz et al. 1988, Lohman and Jones 1988, Wania 2003, Tasdemir and Holsen 2005). It is likely that, given the industrialised nature of the city, all wetlands in the Sydney metropolitan area will be subject to aerial deposition of contaminants. It is important that the sources of POPs in newly created waterways (such as those in SOP) be investigated and ameliorated to the greatest extent possible. The possibility of deposition on reservoirs of town water and the concomitant human health impacts must also be considered. Newly created wetlands provide a unique opportunity for the study of novel POP inputs (either from aerial deposition or catchment influences) and should form the basis of further investigation.

The Australian water quality guidelines (ANZECC and ARMCANZ 2000) do not set a criteria value for PCDDs for the protection of aquatic biota. Nor is there any water quality criterion set by the USEPA (USEPA 2006). The current USEPA National Primary Drinking Water Standard for 2,3,7,8-TCDD is 30 pg/L (0.09 pM) (USEPA 2003) which is higher than any of the aqueous TCDDeq values measured in the current study (Table 4-4). Given that drinking water guideline criteria are generally many orders of magnitude lower than

criteria for protection of aquatic biota (e.g., for pentachlorophenol USEPA WQG = 19 g/L and USEPA DWG = 0.001 mg/L) the aqueous TCDDeq values for the SOP wetlands could be considered of low risk. However, dioxin-like POPs in the dissolved phase may be bioconcentrated by aquatic biota across skin and gill membranes (Verweij et al. 2004) and biomagnified through the food chain. As many of the wetlands examined in the current study are habitat for native species, including the endangered green and golden bell frog (*Litoria aurea*), and are primary habitat for many wading birds and occasionally larger birds of prey (e.g., white bellied sea eagle) (OCA 2000d) the potential for toxic effects at these higher trophic levels necessitates further investigation. TCDDeq in the water samples was significantly lower in the SOP wetlands than in the urban reference sites. Since there were no overall strong physical or chemical predictors of TCDDeq in the water sources, these are likely to be more closely related to catchment influences.

There were measurable levels of TCDDeq in the sediment collected at all the study sites. There was a much larger relative range measured in the sediment than in the water column reflecting the fact that these hydrophobic compounds preferentially partition to sediment. The high levels measured at the mouth of Haslams Ck and Homebush Bay reflect the known history of uncontrolled dumping of chemical manufacturing wastes in and around the Bay. These results are also consistent with the high levels of organic contaminants previously reported in this region (Birch and Taylor 2002, Birch et al. 2007). In a United Kingdom contaminated lake, Sanders et al. (1992) showed that anoxic conditions can have high concentrations of PCBs and DDT in sediments, which reflect historical inputs. It is likely that, in Parramatta River and Homebush Bay, higher concentrations of contaminants could be measured deeper in the sediment profile as much of the contamination was the result of uncontrolled dumping prior to the 1960s (Rubenstein and Wicklund 1991). This legacy material is unlikely to be of ecological concern unless it is remobilised by disturbance (anthropogenic or natural).

The differences in sediment TCDDeq values measured in 2005 and 2006 may point to the patchiness of the concentrations of POPs in the sediment. Spatial patchiness in sediment contamination has been described over large (e.g., Johnson and Larsen 1985, Feng et al.

1998) and small (Koh et al. 2004) spatial scales. Similarly, differences in contaminant concentrations measured on resampling the same sediments have been reported (e.g., Huggett et al. 1998). The sampling regime employed in the current study aimed to account for spatial heterogeneity by collecting composite samples over a wide area and resampling the same area at different times. However, it is possible that more contaminated pockets of sediment may be collected and/or missed between sampling times. Therefore, differences TCDDeq may reflect certain changes in the sediment characteristics over the time frame measured in the current study. Alternatively, these differences could be due to contaminated sediment migration to and/or from the study site by the effects of wind, or increased flow (e.g., tides or stormwater).

Although both the TCDDeq measured at the SOP wetlands and the significant predictor variables of TCDDeq (by multiple regression) varied between the two sampling times, the average TCDDeq across the sites remained within the same range. Further, this range was within that measured at the urban reference sites. High levels of sediment TCDDeq at the SOP wetland Boundary Ck are of greater concern. Prior to the remediation of this wetland high concentrations of OCPs (50 μg/kg) and PAHs (3-5 mg/kg) were measured in the sediments (Laginestra et al. 2001). The concentrations of ΣDDT (47 μg/kg) and ΣPAHs (3.4 mg/kg) measured in the current study, eight years after the completion of the remediation, are similar to these pre-remediation concentrations. During the remediation works contaminated soil and sediments were removed to a containment mound downstream of the current study site. Due to its position, leachate from this mound is unlikely to be contributing new contaminants to the wetland and it is unlikely that materials were left behind as the stream was geotextile lined to prevent possible infiltration. Likely sources of contamination at this wetland are storm water from a highly urbanised catchment and potentially a sewage overflow point that is located immediately upstream of the sampling point. The wetland is designed to remove potentially contaminated suspended sediment from the stream (by sedimentation) and prevent its entry to the rest of the Park. The high concentrations reported here may indicate the success of this design; however, the wetland is a significant bird habitat and, therefore, the presence and sources of POP contamination requires further investigation.

The high sediment TCDDeq in EWQCP and SWQCP in 2006 (4.58 and 5.2 μg/kg, respectively) also requires further investigation. These wetlands were created during the remediation program and are not adjacent to any waste containment mound. They are however, subject to first flush stormwater inputs (including impermeable surface runoff). Catchment inputs are, therefore, the likely source of AhR ligands at these wetlands. They are designated as primary habitat for the endangered green and golden bell frog and also for a number of species of water bird. Together with the Northern Water Feature (sediment TCDDeq = 4.21μg/kg), these wetlands are linked to the water recycling system within SOP, which distributes treated water from these sites for irrigation and other non-potable uses within the Park. There is, therefore, potential for contaminants at these wetlands to migrate to other areas of the Park.

The other SOP wetland showing high levels of contamination is the Wharf Stream. This wetland is a pre-remediation remnant but water flow seems to have been redirected during the initial construction of the naval site on which it now stands. Its catchment previously contained municipal waste dumps and now consists of roads, residential and commercial land, a prison and playing fields. These are all potential sources of contamination measured in the current study. Temporary leachate ponds were constructed adjacent to the stream during the creation of the nearby containment mound. Testing of the sediments around these ponds suggested that there was migration of the leachate from the ponds but where this occurred the sediment was removed and treated (Laginestra et al. 2001). The possibility that infiltration occurred during this process cannot be ruled out. The area is currently not in high use and the stream itself flows only at times of high rainfall so the possibility of migration or exposure of biota to contaminants at this wetland is low.

Anthropogenic disturbance can mobilise contaminated sediment (Latimer et al. 1999) and can increase the bioavailability of organic contaminants (Rice and White 1987). The increase in TCDDeq in water samples through agitation of sediments in the current study reflects this and has implications for any further remediation in the area, particularly in Homebush Bay. In February 2006, fin fishing and prawn trawling was banned in the Port

Jackson estuary due to high levels of dioxins found in commercial and recreational fish species , which were linked with contamination of sediment in Homebush Bay (Birch et al. 2007). If the highly POP contaminated sediments in Homebush Bay were disturbed by dredging (suggested as the most efficient way to remediate the sediments) the bioavailability of these POPs and their concentration in body tissues of higher trophic level consumers will increase dramatically. They are also likely to cause increased transportation of contaminants throughout the Port Jackson estuary for some time. Bergen et al. (1998) showed that dredging can cause increased transportation of PCBs for up to 6 months after dredging stops. Equally, there are implications for management of the wetlands of SOP. Disturbance of the wetland sediments should be avoided at times during which sensitive organisms are present (e.g., frog and wading bird breeding season). This should also apply to the disturbance of sediments from other urban impacted wetlands.

4.6 Conclusions

This study showed that the remediation program has reduced POP contamination of most of the Sydney Olympic Park wetlands to within the chemical bounds expected from typical urban wetlands in Sydney, Australia, and indicates the heavy influence of catchment based contamination sources. There is a particular need for the ongoing monitoring of the concentrations of POPs in Boundary Creek, EWQCP, SWQCP and the Northern Water Feature. The importance of avoiding the disturbance of wetland sediments, thereby minimising mobile and bioavailable contaminants is stressed. Measurable concentrations of POPs in any aquatic environment are cause for concern given the widely reported effects on individuals, populations, communities and ecosystems. It is important to investigate these higher order effects in order to fully quantify the ecological risk POPs pose in the study system. This must involve some investigation of their bioavailability (both *in vitro* and *in vivo*) and any effects on populations and communities exposed to them.

5. CYP1A in Mosquitofish (*Gambusia holbrooki)* in Sydney Olympic Park

Part (A) – Method Development

5(A).1 Abstract

The evaluation of cytochrome P4501A (CYP1A) induction by ethoxyresorufin-*O*-deethylase (EROD) activity is a common tool for quantifying environmental exposure to aryl hydrocarbon receptor (AhR) ligands. This pilot study aimed to adapt previously published methods for an EROD bioassay for use with mosquitofish, *Gambusia holbrooki,* in Australia. Livers, gills and gonads were excised from adult male, juvenile male, adult female and juvenile female mosquitofish collected from study and reference sites from the Sydney metropolitan area. The S9 fraction was extracted from the tissues and reagent concentrations adjusted to maximise the fluorescence signal. It was not possible to evaluate gill and gonadal EROD activity but hepatic EROD was quantifiable. There was a significant interaction between the developmental stage and the sex of the fish. It was, therefore, recommended that only adult male livers be used to conduct the EROD activity assay in mosquitofish.

5(A).2 Introduction

CYP1A is a member of the cytochrome P450 family of membrane (endoplasmic reticulum) associated mixed function oxygenases (MFOs). The induction of CYP1A above background levels can be used as a biomarker for the exposure of an animal to halogenated aromatic molecules (see Whyte et al. 2000 for review). In this bioassay, CYP1A hydroxylates 7-ethoxyresorufin (7-ER) to the fluorescent product resorufin and the rate of reaction is measured as EROD (ethoxyresorufin – *O* – deethylase) activity.

The aim of this pilot study was to develop repeatable, appropriate methods for evaluating EROD activity in the mosquitofish, *Gambusia holbrooki*. It was necessary to identify appropriate groups of fish (based on developmental stage and sex), tissues and reagent concentrations to be used in a wider scale study of EROD activity in fish inhabiting Sydney Olympic Park and urban reference sites (Chapter 5B).

5(A).3 Materials and Methods

Methods for determining EROD activity were adapted from the Eggens and Galgani (1992) modifications to the methods of Burke and Mayer (1974) for high sample throughput using a fluorescence plate-reader.

5(A).3.1 Fish Collection and Processing

Mosquitofish populations in wetlands in Sydney Olympic Park (SOP) and reference sites were sampled in May 2006. The fish were collected using an aquatic dip net and transported to the laboratory in aerated plastic bags and maintained in site water in the laboratory until processing (<24 hrs). The fish were weighed and photographed using a digital camera (Leica DFC 320) mounted on a dissecting microscope. The external morphology of each individual was used to classify it as adult male (fully developed gonopodium with a gonopodial terminal hook complex), juvenile male (evidence of gonopodial development but no gonopodial terminal hook complex), adult female (presence of lateral abdominal black spot) or juvenile female (absence of gonopodial

development and absence of lateral abdominal black spot) (Figure 5-1). Fish were sacrificed by immersion in benzocaine (400 mg/L) and gill, liver and gonadal (adult male only) tissue removed. Excised tissue was stored in microfuge tubes at -80^{o}C until being processed.

5(A).3.2 S9 Fraction Preparation

Tissues were thawed on ice and mechanically homogenised in phosphate buffered saline by repeatedly drawing up and expelling through a flat-based syringe, which had been primed with phosphate buffer. Homogenates were centrifuged at 10,000 X g (4^{o}C for 20 minutes) and the supernatant (the S9 fraction) pipetted into a separate, chilled microfuge tube. Most published methods (e.g., Burke and Mayer 1974, Eggens and Galgani 1992) call for a further centrifugation step to isolate a microsomal pellet. This microsomal fraction yields higher EROD activity than the S9 fraction but induction relative to basal levels is similar (Whyte et al. 2000). It was decided that analysis of the S9 fraction was sufficient as the study was concerned with the induction above background levels in field sites rather than absolute values.

5(A).3.3 Mosquitofish EROD Assay Development, and Verification

This assay is generally used to evaluate the EROD activity in larger taxa. It was necessary to adjust the assay for use with a small species such as mosquitofish. Standard methods (Eggens and Galgani 1992) were used to run the assay a number of times using larger species (Murray rainbowfish, *Melanotaenia fluviatilis,* and silver bream, *Acanthopagrus australis*) to ensure measurable and repeatable results. With low tissue quantities obtained from mosquitofish (maximum liver weight was approximately 0.01g) it was necessary to use the entire tissue (liver, gill and gonad) of each individual.

While CYP1A is found in gill, gonad and liver tissues in other species (Whyte et al. 2000), in mosquitofish EROD activity could not be reliably measured in gill or gonadal tissue, even with very high reagent concentrations. Hence, only hepatic EROD activity was

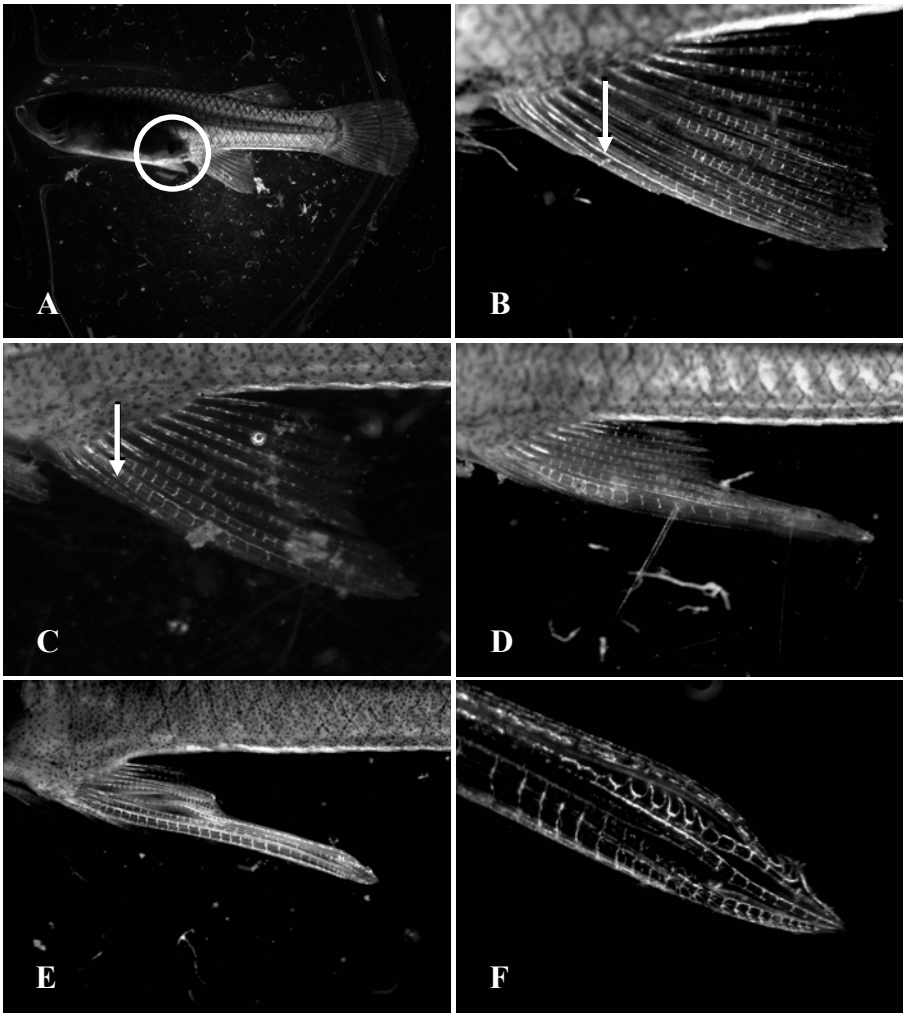


Figure 5-1 Details of mosquitofish anal fins used for sex and developmental stage identification. A. Adult female. Black abdominal spot circled; B. Adult female anal fin. Note long first segment on 3rd anal fin ray (denoted by arrow); C. Early stage juvenile male. Note shortened and thickened 1st rays of anal fin (denoted by arrow); D. Mid stage juvenile male. Note elongated 3rd, 4th and 5th anal fin rays; E. Adult male with fully developed anal fin (gonopodium); F. Detail of gonopodial terminal hook complex on gonopodium denoting sexual maturity.

investigated in the current study. The signal obtained was close to the detection limit of the fluorescence plate reader using reagent concentrations cited in standard methods for larger species (rats (Pohl and Fouts 1980)). Incrementally increasing the well concentrations of 7-ethoxyresorufin (7-ER) and NADPH increased the measured signal for individual samples. Measurable EROD activity from 90% of liver samples was obtained at 7-ER and NADPH well concentrations of 1.50 μM and 24.7 μM, respectively. The result was an assay protocol that gave acceptable results for mosquitofish with a hepatic tissue weight around 0.001g.

5(A).3.4 Mosquitofish EROD Assay

Detailed methods for the mosquitofish EROD assay can be found in Appendix 1. Briefly, homogenised and fractionated (S9) adult male mosquitofish liver samples (25 μl) were added in quadruplicate to a black 96 well plate along with 75 μl HEPEs buffer (0.12 M HEPEs, 5 mM $MgCl_2.6H_2O$, pH = 7.8) and 80 μl of 7-ER (3.8 μM). Immediately prior to reading 20 μl NADPH (247 μM) was added to each well of the plate to commence the reaction. The maximum rate of generation of the fluorescent product, resorufin, was measured in a BMG Fluostar Optima fluorescence plate reader (excitation: 512 nm, emission: 596 nm, 37^0C, 30 mins). The conversion of 7-ER to resorufin is catalysed by CYP1A and the rate of reaction (generation of the fluorescent endpoint) is proportional to its concentration in the sample. Measured fluorescence units were converted to molar concentrations using a resorufin standard curve run with each assay. The EROD activity of each sample was standardised against its protein concentration, measured using a bicinchoninic acid protein assay (Pierce BCA Protein Kit). The result for each sample was a value that was the maximum rate of formation of resorufin expressed as the moles of resorufin formed per minute per mg protein in the sample (pmol res/min/mg protein).

5(A).3.5 Data Analysis

A three factor ANOVA (study site*sex*developmental stage, α=0.05) was run on the data for hepatic EROD activity using the SPSS statistical software (version 14.1, 2005) having

first tested the assumptions of homogeneity of variances. Significant interactions were investigated graphically.

5(A).4 Results

There was a significant ($p<0.05$) interaction in hepatic EROD induction between the sex and developmental stage (Adult male, adult female, juvenile male and juvenile female) of the fish (Table 5-1). A plot of this interaction shows that, while the levels of CYP1A in immature fish were similar for males and females, that of males increased to maturity while that of the mature females decreased greatly (Figure 5-2).

This is believed to be due to an interaction between endogenous estradiol (17β-estradiol) and the AhR (Navas and Segner 2000). As it is difficult to determine the stage of the reproductive cycle of an adult female mosquitofish (the reproductive season lasts almost the entire year in temperate climates) and can be difficult to determine when a female fish reaches maturity, the mosquitofish EROD assay is restricted to use with sexually mature male (possession of a well developed gonopodium with a gonopodial terminal hook complex) mosquitofish.

5(A).5 Discussion

EROD activity has been measured previously in small fish species (e.g., Japanese medaka (Chen and Cooper 1999, Carlson et al. 2004)) including poeciliids (e.g., *Poecilia reticulata* (Larsson et al. 2002). We have shown that it was possible to measure hepatic CYP1A in wild sexually mature mosquitofish suggesting these are appropriate organisms in which to investigate exposure to POPs via EROD induction in Australian freshwater wetlands.

Modulation of the CYP1A response to AhR ligand binding and activation (dimerisation with the aryl hydrocarbon nuclear translocator (ARNT)) by steroid and thyroid hormones has been widely studied (reviewed by Carlson and Perdew 2002). In particular, interactions between the estrogen receptor (ER) and the aryl hydrocarbon receptors have been well

Table 5-1 Results of a three factor ANOVA on the hepatic EROD activity in mosquitofish collected from Sydney Olympic Park and reference study sites. The factor maturity, has two levels (sexually immature and mature). * Significant at $\alpha = 0.05$, *** significant at $\alpha = 0.001$.

	Sum of Squares	Degrees of Freedom	F-Value	Significance Level	
Sex	**21.23**	**1**	**30.094**	**0.000**	***
Maturity	**4.35**	**1**	**6.160**	**0.014**	*
Study Site	**30.37**	**5**	**8.608**	**0.000**	***
Sex * Maturity	**16.00**	**1**	**22.679**	**0.000**	***
Sex * Study Site	**0.51**	**5**	**0.144**	**0.982**	
Maturity * Study Site	**3.7**	**5**	**1.049**	**0.990**	
Sex * Maturity * Study Site	**3.41**	**5**	**0.967**	**0.439**	

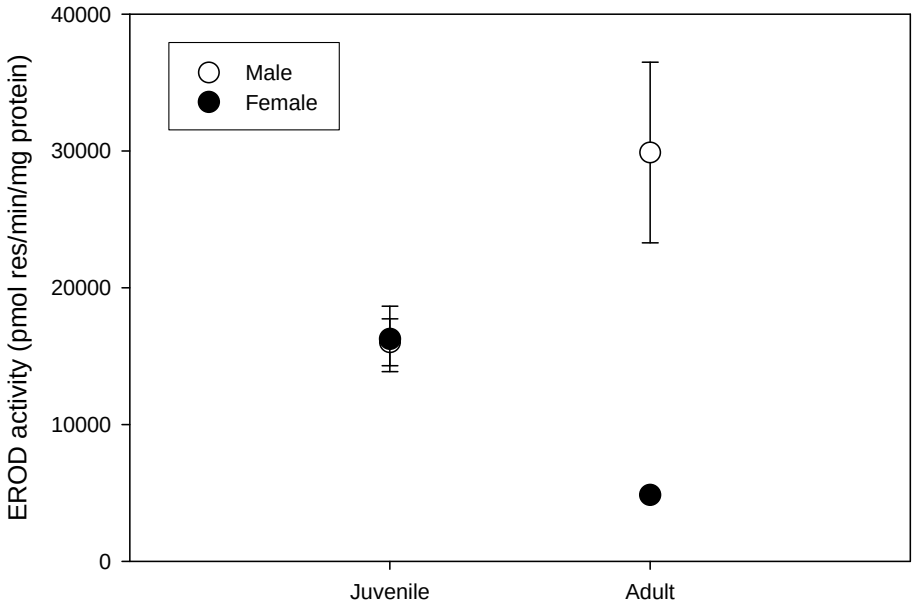


Figure 5-2 The interaction between sex and developmental stage of mosquitofish on the induction of CYP1A activity (EROD).

documented and have been used in the study of hormonally mediated human cancers (e.g., Safe et al. 1999, Safe and MacDougal 2002). AhR activation results in reductions in ER expression and activation and increases in 17β-estradiol metabolism indicating an antiestrogenic effect (Safe et al. 1998). Conversely, increased 17β-estradiol decreases the CYP1A expression, (Ricci et al. 1999) and EROD activity *in vitro* (Lai et al. 2004). Hence *in vivo* EROD activity in reproductively active mature females (compared to mature males) is reduced (Goksoyr and Larsen 1991, Elskus et al. 1992, Larsen et al. 1992, Lindstrom-Seppa and Stegeman 1995, Devaux et al. 1998). The results described above concur with these previous studies. The EROD activity in mature female mosquitofish was markedly less than that in mature and immature males and immature females. It is suggested that, in order to reduce variability and increase fluorescence signal, only mature male mosquitofish should be used for EROD determination.

Part (B) – Hepatic CYP1A Activity of Mosquitofish in Sydney Olympic Park

5(B).1 Abstract

Cytochrome P4501A (CYP1A) is a mixed function oxygenase induced by ligand binding to the aryl hydrocarbon receptor (AhR). While certain endogenous compounds have been identified as AhR ligands the majority of identified ligands are exogenous aromatic compounds (e.g., polychlorinated dibenzodioxins (PCDDs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs)). The concentration of hepatic CYP1A (measured as 7-ethoxyresorufin-*O*-dethylase (EROD) activity) has been widely used as a biomarker of exposure for these persistent organic pollutants (POPs). The current study evaluated the presence of POPs in wetlands in the remediated Sydney Olympic Park (SOP) site by measuring hepatic EROD activity in sexually mature male mosquitofish, *Gambusia holbrooki*. While fish at some SOP wetlands had hepatic EROD activity elevated above the estimated basal level for this species, these were toward the lower end of the range measured in urban impacted, non-remediated wetlands. There were differences between the study sites in terms of EROD activity after rainfall. This difference points to catchment or in-stream sources of POPs. EROD activity was positively correlated with the sediment ΣPCB load and aqueous 2,3,7,8-TCDD equivalence and there was a geographic trend suggesting decreased POP concentrations toward the outskirts of the Sydney metropolitan area (i.e., away from the highly residential areas). Increased catchment size was correlated with increased EROD activity suggesting an even spread of POPs throughout the residential areas of the Sydney metropolitan area. The EROD activity in resident fish suggests that bioavailable AhR ligands (POPs) in the SOP wetlands are within the range expected for an urban impacted wetland and, therefore, exposure to legacy contamination is not high.

5(B).2 Introduction

Endocrine disruption describes interference at any point in the endocrine system of an animal as a result of exposure to an exogenous agent. While the term is usually associated with interference with the normal function of the system of steroid hormones and their receptors, endocrine disruption via the aryl hydrocarbon receptor (AhR) has recently received close attention. While limited numbers of endogenous AhR ligands have been identified (Rannug et al. 1987, Andreola et al. 1997) the AhR promiscuously binds many compounds (Denison and Heath-Pagliuso 1998). High affinity ligands are generally planar, aromatic and hydrophobic (Denison 1991) and include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), some polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and some organochlorine pesticides (OCPs) (Sierra-Santoyo et al. 2000, Fent 2001, Oropeza-Hernandez et al. 2003). Exposure to these persistent organic pollutants (POPs) can cause major physiological damage. POPs are variously carcinogenic, teratogenic, genotoxic and can cause reproductive impairment at low concentrations (reviewed by Delzell et al 1994a,b,c).

AhR binding can result in upregulation of xenobiotically active enzymes, which contribute to detoxification (particularly of PAHs) and subsequent elimination from the body (Denison 1991). However, it may lead to subsequent toxic effects such as carcinogenesis (Schmidt and Bradfield 1996). The mechanism of AhR mediated toxicity is not well understood but may involve metabolisation of procarcinogens to more toxic metabolites (Huff et al. 1994). AhR and its dimerisation partner ARNT (aryl hydrocarbon receptor nuclear translocator) bind to the AhR response element (ARE), leading to the translation of cytochrome P4501A (CYP1A) in response to halogenated aromatic hydrocarbons such as TCDD in a dose-dependent manner (Hahn 1998). CYP1A is a member of the cytochrome P450 family of membrane (endoplasmic reticulum) associated mixed function oxygenases (MFOs). While the direct toxicological implications of small increases in CYP1A concentrations are relatively minor (e.g., CYP1A metabolism of xenobiotics can lead to oxidative stress (Nebert et al. 2000)), the induction of CYP1A above background levels can be used as a biomarker for the exposure of an animal to halogenated aromatic molecules

(see Whyte et al. 2000 for review). Further, CYP1A induction above basal levels has been strongly correlated with toxic effects including carcinogenicity, embryotoxicity, and immunotoxicity, indicating the utility of this endpoint as an early warning marker for toxicity (Whyte et al. 2000). In the bioassay, CYP1A hydroxylates 7-ethoxyresorufin (7-ER) to the fluorescent endpoint resorufin and the rate of reaction is measured as EROD (ethoxyresorufin – *O* – deethylase) activity.

Prior to remediation, there were significant concentrations of persistent organic pollutants (POPs) in the soils and sediments of Sydney Olympic Park (SOP) (Laginestra et al. 2001). Their sources were industrial processes including chemical manufacture (Rubenstein and Wicklund 1991) and uncontrolled industrial and municipal dumping (Waste Services, NSW 1994). In particular, the herbicides 2,4-D and 2,4,5-T (the active ingredients of Agent Orange of which 2,3,7,8-TCDD is both a by-product and contaminant (Barsotti et al. 1979)), PCBs and phenols were manufactured at this site (Rubenstein and Wicklund 1991). During remediation, landfill with low levels of contamination and treated fill (previously with high concentrations of POPs) were consolidated in clay-capped, geotextile lined on-site containment mounds (OCA 2000c). Each mound was fitted with an extensive system of drains designed to intercept leachate to prevent infiltration to groundwater. A leachate monitoring program (SKM and EVS 2001) found detectable levels of dioxins (HpCDD, OCDD and ΣTCDD), and some PAHs (naphthalene, fluoranthene, pyrene) in samples collected from containment mound leachate drains. It is possible that the collection of leachate in containment mound drainage systems is not complete resulting in release of POPs to the wetlands of the Park.

A previous study (Chapter 4) showed that there are measurable levels of POPs in the sediments of the wetlands in SOP. In particular, some wetlands were high in ΣPAHs and ΣDDT and TCDDeq. The aim of the current study was to investigate the bioavailability of measured concentrations of POPs to the hepatic AhR of sexually mature male mosquitofish, (*Gambusia holbrooki*) inhabiting the wetlands of SOP using hepatic EROD activity as a biomarker of exposure. Poeciliids, including mosquitofish, have been widely used to investigate the *in vivo* effects of estrogenic compounds. They are less commonly

used in the investigation the effects and exposure to POPs. POP exposed poeciliids bioconcentrate TCDD (Yockim et al. 1978, Gobas 1990) and certain PCB congeners (Freidig et al. 1998) but Ford et al. (1991) found minimal bioconcentration of OCPs. Their small size, ease of capture and opportunistic feeding regime (Pen et al. 1993) recommends them to this use.

5(B).3 Materials and Methods

5(B).3.1 Fish Collection and Processing

Five collections of adult male mosquitofish were made between May 2006 and February 2007 from the wetlands in Sydney Olympic Park (SOP). Approximately 100 mosquitofish were collected from four wetlands within SOP (Boundary Ck, Narrawang 22, Lake Belvedere and EWQCP) and two reference sites (Macquarie Uni and Upper Colo). The selected wetlands represent a range of current and historical pollution, and remediation works (Table 2-2, Table 2-3). In order to place the SOP wetlands in a broader urban context, mosquitofish were collected from a further nine study sites across the Sydney metropolitan area during the final sampling period (February 2007) (Figure 2-2). The fish were collected with an aquatic dip net, transported to the laboratory in aerated plastic bags and maintained in site water in the laboratory until processing (<24 hrs). Fish were weighed and photographed using a digital camera (Leica DFC 320) mounted on a dissecting microscope. Livers were excised and stored at -80^{o}C until processed (<two months).

5(B).3.2 S9 Fraction Preparation

Tissues were thawed on ice and mechanically homogenised in phosphate buffered saline by repeated drawing up and expelling through a flat-based syringe, which had been primed with phosphate buffer. Homogenates were centrifuged at 10,000 X g (4^{o}C for 20 minutes) and the supernatant (the S9 fraction) pipetted into a separate, chilled microfuge tube.

5(B).3.3 Mosquitofish EROD Assay

To verify the reliability of the methodology and the machinery and, therefore, the comparability of the results, silver bream (*Acanthropagrus australis*) liver samples were analysed for EROD activity prior to the conduct of each assay run. Silver bream are marine to estuarine fish measuring to 40 cm in adulthood. Five silver bream livers (4.5 – 6 g) were stored in liquid nitrogen for the duration of the study. Prior to each assay run 5 small central sections of each liver were excised and examined for hepatic CYP1A activity. The measured EROD activity in these reference livers did not vary statistically ($p \geq 0.05$) from prior to the first assay run to prior to the final assay run. It was, therefore, valid to compare the mosquitofish livers across all collections.

Homogenised and fractionated (S9) adult male mosquitofish liver samples (25 µl) were added in quadruplicate to a black 96 well plate and 80 µl of 7-ethoxyresorufin (7-ER) (3.76 µM) was added to each well. Immediately prior to reading, 25 µl NADPH (247 µM) was added to each well of the plate. The maximum rate of generation of the fluorescent product, resorufin, was measured in a BMG Fluostar Optima fluorescence plate reader (excitation: 512 nm, emission: 596nm, 37^0C, 30 mins). The conversion of 7-ER to resorufin is catalysed by CYP1A and the rate of reaction (generation of the fluorescent endpoint) is proportional to its concentration in the sample. Measured fluorescence units were converted to molar concentrations using resorufin standard curves run with each assay. The EROD activity of each sample was standardised against its protein content using a bicinchoninic acid protein assay (Pierce BSA Protein Kit). The result for each sample was a value that was the maximum rate of formation of resorufin expressed as moles of resorufin formed per minute per mg protein in the sample (pmol res/min/mg protein). Detailed methods for the mosquitofish EROD assay can be found in Appendix G.

5(B).3.4 Data Analysis

All statistical analyses were conducted with the SPSS statistical software (version 14.1, 2005). EROD activities were compared using a 2-factor ANOVA (study site * collection, $\alpha = 0.05$). The EROD activity in mosquitofish collected from SOP and reference wetlands in

February 2007 were compared using a single factor ANOVA (study site). In each case the assumption of equal variances was tested and met. All tests were run at a significance level of 5%. Where necessary, Tukeys pairwise comparisons were run as *post-hoc* tests.

Multiple regressions were run to investigate site-specific environmental factors, which may influence the EROD activity in the fish. One regression model included measured chemical characteristics of the wetlands (water and sediment TCDDeq (H4iiE), ΣPCB, ΣDDT, ΣPAH, sediment and pore water metal concentrations, organic carbon, inorganic carbon and black carbon) as independent variables. Another model included the physical characteristics (sediment grain size, pH, conductivity, redox potential average water temperature and diurnal temperature range). For each regression a lack of collinearity between independent variables was ensured. These analyses were split to avoid problems associated with overfitting the models.

5(B).4 Results

Estimated basal EROD activity for mosquitofish in the Sydney region was 2308 pmol res/min/mg protein. This estimate was based on average measured levels in the pristine reference site, Upper Colo. Four of the five mosquitofish collections were made during dry weather periods in Sydney. One collection was made during an extended high rainfall period. At this time it was not possible to collect fish from Boundary Ck as the water level was too high. All other wetlands were sampled at all other sampling times.

There was a significant ($p<0.05$) interaction between the effects of study site and sampling time on the EROD activity (Table 5-2). A plot of this interaction (study site vs. sampling) (Figure 5-3) indicated that this interaction could be driven by either the absence of data for one study site (Boundary Ck.) at one sampling time (collection 2) or by the spike in readings during this collection at Macquarie Uni. Given that this collection was made during a rain event, while all other collections were made without this influence it was decided that this collection should be removed from this part of the analysis. Re-analysing the EROD activity data without this collection removes the significant interaction term and

Table 5-2 ANOVA table for 2 factor analyses of EROD activity across Sydney Olympic Park and reference sites and times of collection. *** Significant at $\alpha = 0.001$, ** significant at $\alpha = 0.01$.

	Source	Degrees of Freedom	F-Value	Significance Level	
All Collections	Study Site	5	5.614	0.000	***
	Collection	4	0.699	0.593	
	Study Site*Collection	19	2.023	0.007	**
Rain affected collection removed	Study Site	5	5.300	0.000	***
	Collection	3	0.548	0.626	
	Study Site*Collection	15	1.054	0.401	

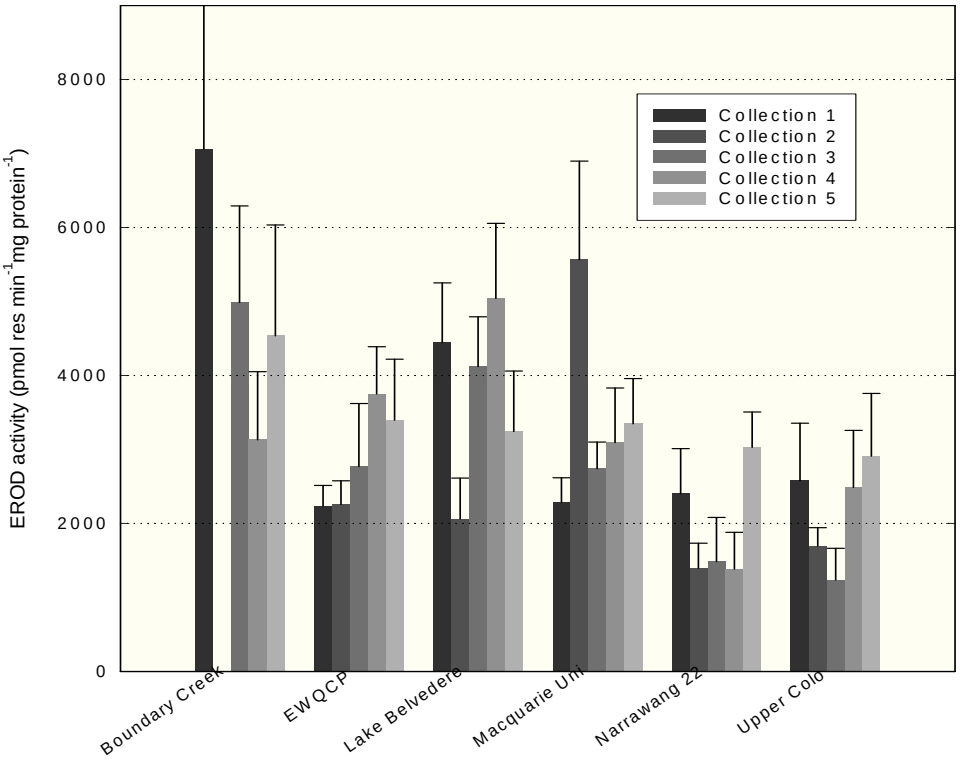


Figure 5-3 Mean (± standard error) of EROD activity in mosquitofish from Sydney Olympic Park and key reference study sites across collection times.

shows that the time of collection did not affect the hepatic CYP1A activity. There was, however, a significant ($p<0.05$) impact of the study site. Fish from only one SOP wetland (Boundary Ck) had significantly ($p<0.05$) greater hepatic EROD activity than the estimated basal levels (Figure 5-4). Fish from Lake Belvedere had significantly ($p<0.05$) greater CYP1A levels than those from the created habitat pond, Narrawang 22 (Figure 5-4). Compared to the nine urban reference sites, the EROD activity in fish sampled from SOP wetlands were toward the lower end of the measured range (Figure 5-5) with only fish from Joseph Banks Park and Cecil Hills having CYP1A levels not significantly ($p\geq0.05$) greater than those in Upper Colo.

The only physical characteristic of the wetlands examined which was significantly ($p<0.05$) correlated to the EROD activity in the fish was the percentage sediment composition of the 1000 μm grain size class (positive relationship; $r^2 = 0.477$) (Table 5-3). The aqueous TCDDeq was significantly ($p<0.05$) correlated to hepatic EROD activity in mosquitofish. However, an analysis of the power in this regression suggested it was too low (0.61). Changing the significance level for exclusion from the model ($\alpha = 0.1$) gave a model (power > 0.9) with significant ($p<0.1$) positive relationships between aqueous TCDDeq and sediment ΣPCB load ($r^2 = 0.787$) and mosquitofish EROD activity (Table 5-3).

In general, populations of fish with high levels of hepatic EROD activity were located in wetlands with large catchment areas. The exceptions to this were the two study sites with the largest catchment area. These sites were located on the outskirts of Sydney and had comparatively low residential landuse. The Nepean River catchment is a mix of minimal land use and natural vegetation used for grazing while the Upper Colo catchment is almost entirely National Park (Figure 2-3). There was a positive relationship between catchment size and EROD activity based on the study sites with high residential landuse (Figure 5-6). In terms of location, the study sites with fish with the highest hepatic EROD activity were located closer to the geographic centre of the Sydney metropolitan area (Figure 5-7). Fish from study sites located closer to the coast and those on the outskirts of the city had lower EROD activity.

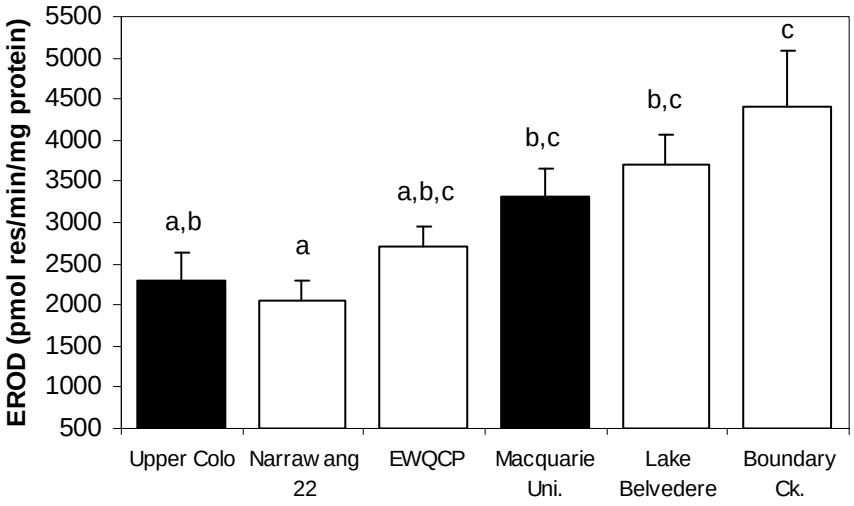


Figure 5-4 Mean (± standard error) of EROD activity in male mosquitofish inhabiting Sydney Olympic Park wetlands (white bars) and reference sites (black bars). Different letters represent significantly different homogeneous subsets at $\alpha = 0.05$.

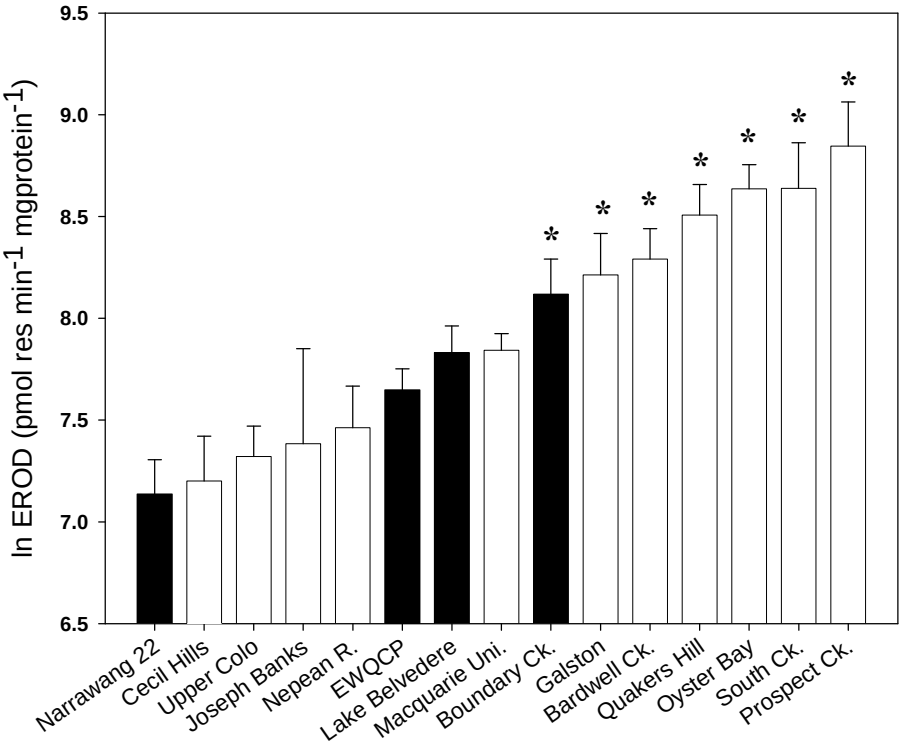


Figure 5-5 Mean (± standard error) for EROD activity in mosquitofish collected in February 2007 from SOP wetlands (black bars) with reference to those from other urban impacted sites around Sydney (white bars). * Significant difference from estimated basal hepatic EROD activity (Upper Colo = 2308 pmol res/min/mg protein) at $\alpha = 0.05$.

Table 5-3 Results of multiple regressions to show sediment and water physical and chemical characteristics which significantly influence the induction of CYP1A (EROD) in mosquitofish from Sydney Olympic Park and reference sites.

Predictor Set	F-value	Significance Level	Predictors included in model	r^2
Contaminant load	14.773	0.002[a]	TCDDeq (Water) Sediment ΣPCB	0.787
Sediment grain size	8.208	0.019	1000μm	0.447
Sediment physico-chemical parameters	-	-	Nil	-

[a] $\alpha = 0.1$ due to lack of power.

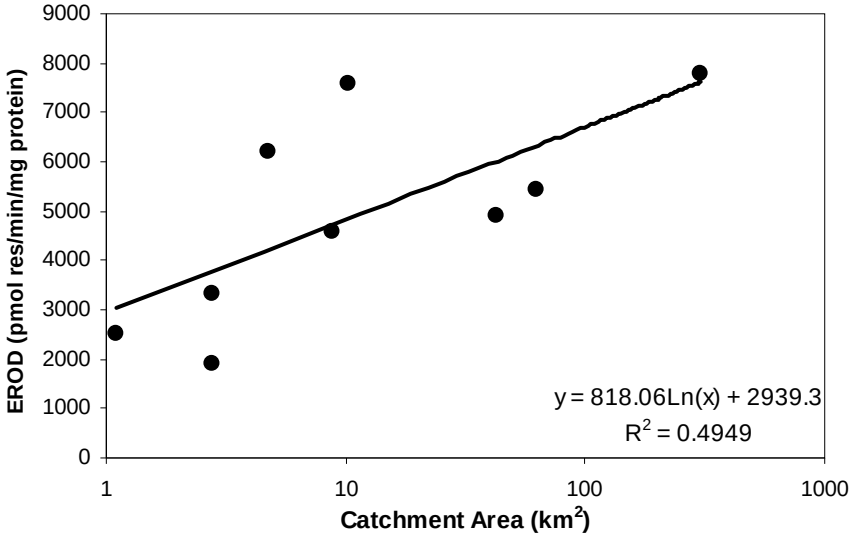


Figure 5-6 Relationship between catchment size and EROD activity in adult male mosquitofish at urban impacted reference sites with linear regression equation and coefficient of variation.

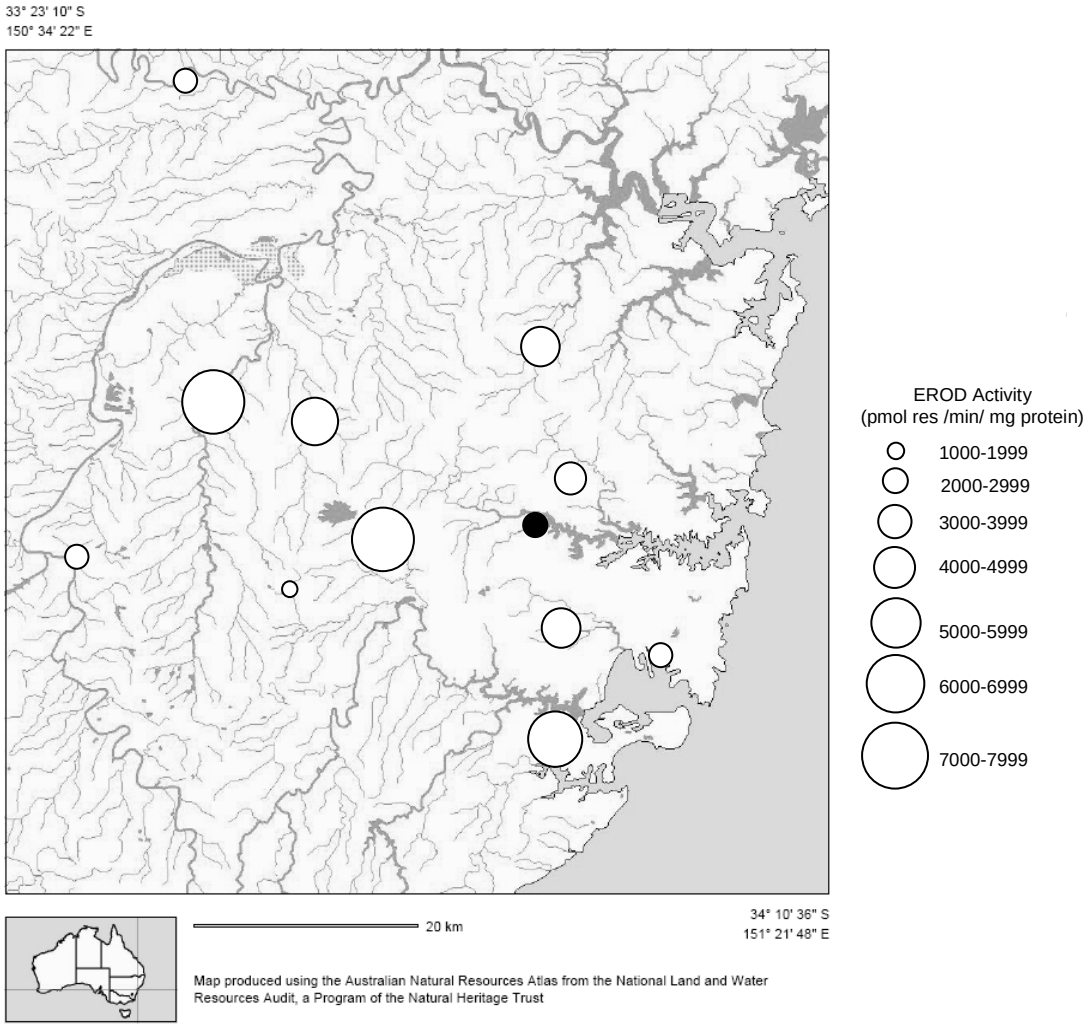


Figure 5-7 Location of urban reference sites with categorical indication of adult male mosquitofish hepatic EROD activity. Black marker denotes the position of Sydney Olympic Park.

5(B).5 Discussion

The AhR appears to play important developmental and physiological roles (Gonzalez and Fernandez-Salguero 1998). While AhR activation appears to result in cellular oxidative stress (reviewed by Dalton et al. 2002) and cause sensitivity to cell apoptosis (reviewed by Nebert et al. 2000), neither the acute nor the chronic effects of increased CYP1A concentrations are well understood. It is necessary, therefore, to view the elevated CYP1A levels reported here as a biomarker of exposure rather than a biomarker of effect. Increased EROD activity (through increased CYP1A concentration) has been widely used as an indication of exposure to halogenated aromatic hydrocarbons (reviewed by Whyte et al. 2000). Whyte et al. (2000) pointed out the importance of understanding and considering the mechanisms behind CYP1A induction via the AhR pathway when designing tests using the assay and drawing conclusions based on the results. The current study aimed to examine the EROD activity while taking factors such as sex, developmental stage and season into account. The estimated basal EROD activity in mosquitofish in the Sydney region (based on levels at the pristine Upper Colo site) was towards the upper end of basal levels reported elsewhere (reviewed by Whyte et al. 2000). This may reflect recruitment from urban impacted sites and/or the hardiness of the species with its ability to effectively metabolise relatively high concentrations of POPs.

Previous studies have investigated the effects of common exogenous estrogen receptor ligands on the expression of CYP1A and EROD activity. Navas and Segner (2000) described a significant reduction in EROD activity in the presence of 17β-estradiol (100 nM) but no inhibition at high (100 μM) concentrations of the weakly estrogenic octylphenol. The synthetic estrogen 17α-ethylnylestradiol did not alter induced CYP1A (0.1 μg/L) (Aubry et al. 2005). Lai et al. (2004) report a significant inhibition of EROD activity by 17β-estradiol and the strongly estrogenic synthetic drug diethylstilbestrol (DES). Results described in Chapter 3 showed that unidentified estrogen receptor ligands are present in the sediment (but not in the water) at some study sites. In particular, in terms of 17β-estradiol equivalence Boundary Ck>Lake Belvedere>EWQCP (Table 3.2). This is the same ranking of wetlands obtained from the investigation of EROD activity (presence

of AhR ligands). This suggests that the estrogen receptor ligands described in Chapter 3 are either not bioavailable (by direct uptake or biomagnification) to male mosquitofish or are not sufficiently available to cause estrogenic effect, and therefore inhibit EROD activity.

The lack of influence of season, and by implication the stage of the reproductive cycle on mature male EROD activity, supports the species applicability as a model organism. The only difference in EROD activity through time occurred where there was high rainfall. As CYP1A induction is a rapidly induced response to contaminant exposure (Kloepper-Sams and Stegeman 1992, Levine and Oris 1999, Rees et al. 2003) and is rapidly reduced when the animal is removed from the contaminated environment (Opperhuizen et al. 1986, Kloepper-Sams and Stegeman 1989) sampling during and immediately following suspected contaminant influx should be considered separately from the chronic exposure through the food chain measured during steady state.

The significant interaction term in the analysis prior to the removal of the second collection is of biological significance. The results suggest that the fish were exposed to an increased level of POPs after rain events. This is consistent with the potential of urban stormwater to contain a variety of contaminants such as POPs depending on the surface and landuse around the catchment (Eriksson et al. 2007). In mosquitofish from three of the five wetlands sampled at this time there was a decrease in the CYP1A levels while at EWQCP there was no change. At Macquarie Uni, however, there was a large increase in hepatic CYP1A in the fish. Since changes in CYP1A levels represent recent changes in organic pollutant load, differences at the study sites reported here represent changes in the short-term availability of POPs to the fish. It is unlikely that these changes were due to dietary uptake as this would presumably take some time to show effect. The catchment runoff to the Macquarie Uni wetland may therefore be high in available POPs. Whether these are in the dissolved fraction or loosely bound to the particulate matter load requires investigation. The catchment of this wetland is (with the exception of Boundary Ck) more directly impacted by impermeable surfaces (especially roads) and urban development than the SOP wetlands considered here. This may be contributing contaminated runoff to the wetland under rain conditions and, therefore, causing the reported increase in EROD in the

inhabitant fish. The decrease in EROD activity (in fish from Lake Belvedere, Narrawang and Upper Colo) upon rain input suggests catchment runoff is relatively free of bioavailable POPs and may provide some dilution or flushing effect to the wetlands.

The two SOP wetlands (Boundary Ck and Lake Belvedere) with levels of hepatic EROD activity higher than that at Narrawang 22 represent different remediation histories and current catchment landuse. Boundary Ck (formerly a concrete channel) was initially remediated in 1993. The Creek was lined, wastes were removed and its course returned to a more natural state with a series of pools and riffles designed to catch storm water and allow settling of catchment-contaminated sediment (Laginestra et al. 2001). Mosquitofish populations at Boundary Ck had the highest EROD activities compared with fish from other SOP wetlands, but it seems unlikely that the causative contaminants are derived from the Park itself given the remediation of this wetland and the fact that the closest containment mound is downstream of the sampling point. It is more likely that the contamination of this wetland originates in its highly urban catchment and is transported as stormwater runoff. The high sediment contaminant load (Chapter 4) and the high EROD induction in fish suggest that this wetland is subject to sedimentation; however, migration off-site in the form of bioconcentration in birds or downstream in the dissolved organic carbon fraction must be considered and avoided. Further, regular testing of the sediment and removal of contaminated sediment is recommended to reduce the exposure of resident biota to POP contamination.

The area surrounding Lake Belvedere was initially remediated in the mid 1980s with 30 year-old landfill capped with 1 to 1.5m thick imported fill (Laginestra et al. 2001). Subsequent testing of the site in the 1990s indicated significant concern over seepage of contamination from beneath the cap toward surrounding waterways (Laginestra et al. 2001). Leachate drains installed at this time did not halt the seepage and a new drainage system including a leachate evaporation pond was installed in 1999 (Laginestra et al. 2001). The elevated levels of EROD activity in fish inhabiting the Lake may indicate that previously identified problems with seepage has contaminated the sediment of the Lake. While the secondary remediation effort in the immediate surrounds of the Lake suggests

that it is unlikely, continued leaching of the contained waste remains a possibility. Studies in 1997 and 1999 identified significant reproductive impairment, PAH and OCP loads and physical malformation (internal and external abscesses) in carp inhabiting the Lake (unpubl. data cited in Laginestra et al. 2001) and it is possible that this is linked to the current result. The Lake is a designated waterbird refuge so increased exposure to AhR ligands (most of which have high bioaccumulation and biomagnification factors) should be of concern. The upstream catchment of the Lake includes Boundary Ck (the SOP wetland in which mosquitofish had the highest EROD activity) and further upstream is highly urbanised. It is possible that the measured EROD activities in fish from Lake Belvedere are elevated due to contaminant influx from Boundary Ck or higher in the catchment. Rapid dilution of high log K_{ow} compounds in both the environment (e.g., PCBs (Imamoglu and Christensen 2002)) and biological tissue (e.g., TCDD (Crunkilton et al. 1987), PCBs (Southworth 1990)) as well as associated biomarkers (e.g., EROD (Wilson et al. 2000)) have been reported downstream of point sources due to rapid binding to sediments. Decreases in the EROD activity between Boundary Ck and Lake Belvedere may reflect a dilution effect or the sedimentation of dissolved organic carbon and particulate matter, which carry POPs and be immediately bioavailable to the fish.

Low EROD activity in fish from Narrawang 22 is particularly encouraging given its proximity to the largest containment mound in the Park and the use of this wetland by wading birds and as primary frog habitat for the green and golden bell frog. The results from Upper Colo were as expected as it is located in a relatively pristine region north-west of Sydney isolated from the effects of urbanisation. Its catchment is mainly National Park and the possibility of anthropogenic contamination is low with the only possible source being aerial deposition and potentially the use of pesticides in the few agricultural land parcels in the region. The fact that there is no evidence of increased EROD activity in fish from EWQCP suggests that the POP contamination of stormwater runoff collected at this site is not available to resident fish populations (relative to the Boundary Ck catchment).

The catchments of the urban reference sites chosen for this study represent a range of landuses. Hoffman et al. (1984) found that wetlands affected by industrial zones and

highways had greater PAH inputs (loading factors) than those affected by residential and commercial zones. In the current study, the immediate landuse is variously highly residential (Bardwell Ck, Quakers Hill) to light agricultural (South Ck.). The wetland with the highest EROD activity (Prospect Ck) is a degraded stream with an entirely industrial catchment. Of interest are the levels at Galston, which are significantly higher than those in the pristine reference site. While this study site is located adjacent to a medium use roadway, it is in parklands and five km downstream of residential landuse. Contaminants, may, therefore be transported some distance from their source in the dissolved fraction or adsorbed to fine particulates

Previous studies have suggested that elevated CYP1A induction may be relatively localised to the source. CYP1A inducers decreased downstream (with increasing catchment size) in the Delaware River, away from the severely impacted upper catchment (McCoy et al. 2002). In a study of EROD activity in a marine fish (*Platycephalus bassensis*) in Port Phillip Bay, Australia, Gagnon and Holdway (2002) found activities in the fish from locations close to the city of Melbourne were generally higher than those from locations in the rest of the Bay. The current results show a positive relationship between hepatic EROD activity and catchment size in the mainly urban impacted sites. It is concluded that through the studied catchments the sources of CYP1A inducers are evenly distributed and likely to be the result of the phenomenon of urban development rather than a series of point sources of contamination.

Urban wetlands are generally higher in CYP1A inducers than those whose catchment is mainly agricultural and natural vegetation. Cavanagh et al. (2000) reported that pikey bream (*Acanthropagrus berda*) from intensively farmed (sugarcane) regions had 2.5 times the EROD activity than those from undisturbed sites, but a nearby urban impacted site had double the activity of the sugarcane sites. In the current study, wetlands fed by highly urban catchments had fish with greater hepatic EROD activity than catchments with mainly natural vegetation or light agriculture. This indicates the importance of urban development in causing organic contamination of waterways. The sources of POPs in urban waterways may be aerial deposition (Odabasi et al. 1999) and/or catchment runoff (Gavens et al. 1982,

Ellis et al. 1985). The current results showing increasing exposure to POPs with increasing urban catchment size concur with the studies described above, indicating that the impact of agricultural chemicals appears to be lower than that of industrial processes and high population densities.

There were two significant predictors of hepatic EROD activity in the mosquitofish. Aqueous TCDDeq was generally low across all study sites (Chapter 4) yet appears to influence EROD activity. Most AhR ligands have a high log K_{ow} and preferentially bind to the organic fraction of sediments (Gustafsson et al. 1997). Some slightly soluble PAHs and PCB congeners are directly available to aquatic biota across gill surfaces and could be a source of CYP1A inducers here. Alternatively, some study sites were high in fine suspended particulate matter, which may contain bound POPs.

Sediment ΣPCB concentration was also significantly correlated with hepatic EROD activity. This indicates either flux between sediment and aqueous compartments (usually low for PCBs (Achman et al. 1996)) or a biomagnification/dietary effect. Mosquitofish were introduced to Australia to control mosquito populations and have superior mouths suggesting a surface feeding habit. The stomach content of individuals indicates that they are far more opportunistic feeders than this would suggest. Common food items are terrestrial insects, chironomid larvae, dipteran larvae and pupae, ephemeropteran larvae, cladocerans and copepods (Pen et al. 1993). While there is conflicting evidence on the ability of poeciliids to bioconcentrate POPs (Yockim et al. 1978, Gobas 1990, Ford and Hill 1991, Freidig et al. 1998) it is possible that they are exposed to them through dietary mechanisms where sediment ΣPCB concentrations are elevated. The TCDDeq in the sediment was not correlated with the hepatic EROD activity suggesting that while some AhR ligands are bioavailable to the fish (through flux or dietary uptake) others may be either unavailable to benthic biota or do not bioconcentrate in them and, therefore, do not enter the food chain. In the current study, sediment POP concentrations were generally low compared with globally reported levels so these correlative results should not be seen as an indication of the state of the studied wetlands but rather as an indication of the utility of the

hepatic EROD activity of the mosquitofish in examining the contaminant (particularly PCBs) load of wetlands.

POPs preferentially bind to fine sediments with high organic content. In the current study there was no correlation between EROD activity and the organic carbon content in the sediments. Increased EROD activity was, however, correlated to the increased proportion of sediment grain size of 1000 μm. Results presented in Chapter 4 suggest that mechanical agitation of sediments released AhR ligands into the water and that this was also correlated to mid-size sediment grains. While sediment grains of this size may possess a similar organic content to other sediments (neither result was correlated with organic carbon content) POPs may be less tightly bound than to finer particles. This indicates the importance of combining organic carbon content with sediment grain size composition when considering the bioavailability of organic contaminants.

There is no data for the hepatic CYP1A levels in fish inhabiting SOP wetlands prior to the remediation (many of the wetlands in the current study did not exist) making direct comparisons impossible. Given the extremely wide range of basal EROD activity reported in the literature (Whyte et al. 2000) and the species specific induction of CYP1A it is difficult to make direct comparisons with other remediated sites based on this endpoint. The most efficient means of comparison is to compare the proportional increase above basal level. In the current study a four-fold increase above the estimated basal level is maximal. In one of the only other reported studies on this endpoint in a remediated site, Brammell et al. (2004) similarly found a four-fold increase in EROD activity in a remediated site compared to a reference site. In the absence of other literature it appears that, in terms of this endpoint, the remediation has been adequate. It should be noted that laboratory-based studies have described far greater induction of EROD activity above basal levels. EROD activity in rainbow trout can be increased 172 fold by the PAH β-naphthoflavone (100 mg/kg by i.p. injection) and up to 600 fold by 2,3,7,8-TCDD (0.002 mg/kg by i.p. injection) (reviewed by Whyte et al. 2000). These studies do not extrapolate this induction to any physiological effect and caution must be exercised in predicting toxic effects from this endpoint. However it remains a useful biomarker of exposure to many POPs.

EROD activity can be modulated by factors not included in this study. It has been shown that nutritional status can influence the induction of CYP1A. The hepatic EROD activity of rats fed with soy protein was less than half that of rats fed with casein (Rowlands et al., 2001). In addition, Jimenez et al. (1998) investigated the interactive effect of feeding regime and temperature on EROD activity in bluegill sunfish (*Lepomis macrochirus*) showing that feeding increased EROD, particularly at higher temperature. These and other authors (e.g. Anderson and Koivusaari, 1985) showed that temperature can have an effect on EROD activity. Other environmental contaminants can increase or decrease EROD activity through nonspecific pathways (e.g., copper can decrease EROD induction while cadmium enhances it). It is, therefore, possible that the increase observed here may be in some part be due to factors other than exposure to POPs.

5(B).6 Conclusions

It is important to note that while the levels of hepatic CYP1A induced in fish from some of the SOP wetlands are statistically higher than that at a pristine non-urban impacted wetland they are not higher that that expected from an urban wetland in Sydney. Wetlands in large cities are notoriously high in organic contamination from aerial deposition and runoff. The fact that EROD induction above basal levels are not exceptionally high in mosquitofish populations from the SOP wetlands studied suggests that, while the causative agents should be identified and an attempt made to avoid the possibility of biomagnification to native species using the waters (e.g., wading birds and frogs), the remediation of the Park has been moderately successful in closing the organic contaminant pathways into resident fish populations. Given the presence of POPs in the sediments of the study sites and evidence of the exposure of fish populations to POPs it is important to investigate any associated ecological effect. Examination of population and community level responses will provide highly ecologically relevant data and provide a logical next step in the assessment of the ecological health of the wetlands of SOP post-remediation.

6. Benthic Macroinvertebrate Assemblages in Wetlands of Sydney Olympic Park

6.1 Abstract

To investigate potential higher organisational level impacts of persistent organic pollution in the wetlands in the Sydney Olympic Park (SOP) remediated site, the benthic macroinvertebrate assemblages of seven wetlands within SOP and two off-site reference wetlands were examined. Sediment cores were collected, stained and preserved from each study site and the macroinvertebrates from each core were identified to the appropriate level (Class, Order, Family, Subfamily). Data were analysed in terms of taxon richness and macroinvertebrate abundance and multivariate techniques were used to identify chemical/physical characteristics of the sediment, which were important influences on the differences in the assemblage between study sites. Macroinvertebrate abundance was highly variable between study sites and taxon richness was low across all wetlands. Oligochaetes, nematodes, ostracods and chironomids were the most common taxa found and were the most important in influencing differences between the macroinvertebrate assemblages at the study sites. Sediment grain size and chemical characteristics of the sediments (ΣPAH, ΣPCB, TCDDeq, metals) were important in separating the study sites based on taxon richness and abundance. Canonical correspondence analysis separated the macroinvertebrate assemblages at Narrawang 22 and the Northern Water Feature from those at other study sites. This separation was along an axis influenced by decreasing 2,3,7,8-TCDD equivalence and decreasing concentrations of ΣDDT and total organic carbon in the sediment. The assemblages at these two sites were high in rare taxa and relatively low in pollution tolerant taxa (e.g., oligochaetes). The remediation has established wetlands whose benthic communities are representative of those expected in an urban impacted wetland.

6.2 Introduction

Freshwater macroinvertebrates fulfill vital roles in lentic and lotic food webs (Fry 1991), acting as detritivores, algal and macrophyte grazers, and low level predators. In turn, they contribute a large dietary component to higher level consumers such as larger invertebrates, fish and wading birds. Benthic macroinvertebrates are important in providing essential ecosystem services, particularly nutrient cycling (reviewed by Vanni 2002), sediment mixing (Kresoski et al. 1978) and food web energy flow (Lindegaard 1994). The absence of even a single (keystone) benthic macroinvertebrate species may alter decomposition rates and nutrient cycling (Covich et al. 1999). Previous work has described functional feeding groups within macroinvertebrate assemblages. Cummins and Klug (1979) describe three feeding groups (shredders, collectors and scrapers) which are intimately involved with nutrient cycling, many examples of which are benthic in habit.

Complex interactions between benthic macroinvertebrates, microbes, primary producers and physico-chemical characteristics (light, pH etc.) ensure a balance between nutrient retention and recycling in the sediment and dissolved nutrients, fine particulate organic matter (FPOM) and coarse particulate organic matter (CPOM) in the water column (Boulton and Brock 1999). Benthic invertebrates release dissolved nutrients, which are readily available to primary producers (aquatic plants) and microbes, which in turn provide food for herbivores and thence higher trophic levels (Covich et al. 1999). It follows that if the nutrient equilibrium is shifted by a decrease in nutrient cycling (e.g., by reduced benthic invertebrate abundance and/or diversity), primary productivity and microbial activity will be altered and may precipitate a bottom-up driven trophic cascade.

Fine and coarse scale changes to a macroinvertebrate assemblage can indicate changes in the health of a wetland. Decreased overall taxon richness (or simply decreased richness in the sensitive orders Ephemeroptera, Plecoptera and Trichoptera (the EPT index)) may result from physico-chemical stress (e.g., thermal stress (Hodkinson and Jackson 2005), acidification (Sandin and Johnson 2000)) , chemical stress (organic pollutants (Wallace et al. 1996), metals (Hickey and Clements 1998), eutrophication (Sandin and Johnson 2000))

or the introduction of exotics (Stenroth and Nystrom 2003). Decreased macroinvertebrate abundance may be associated with seasonal effects (Boulton et al. 1992), site-specific physical characteristics (e.g., sediment type (Quinn and Hickey 1990)), severe pollution (e.g., by sewage effluent (Burt et al. 1991) or metals (Hirst et al. 2002)) or low ecosystem energy flow.

Exposure of benthic organisms to persistent organic pollutants (POPs) such as PCBs, PAHs and PCDDs is an important ecological process. POPs rapidly bind to fine sediment (Gustafsson et al. 1997) (predominantly isolated to lotic systems and lentic systems with low flow) and are slow to flux with surficial waters (Achman et al. 1996, Persson et al. 2005). The benthos and pore water are, therefore, likely sites for exposure of these contaminants to biota. POPs have high bioconcentration (Neely et al. 1974) and biomagnification factors (Sijm et al. 1992). Bioconcentration in benthic organisms is usually high (Schrock et al. 1997, Thoman and Komlos 1999, Magnusson et al. 2006) and biomagnification, therefore, likely. Apart from incorporation and transfer to higher trophic levels, the acute toxicity of some POPs to benthic organisms is high. This is the case for both pollutants with invertebrate-targeted effects (e.g., organochlorine insecticides (Swartz et al. 1994, Phipps et al. 1995)) and non-targeted products of industrial or other processes (e.g., PCBs (Mayer et al. 1977, Reynoldson 1987) and PAHs (Boese et al. 1998)). High benthic concentrations of these compounds may, therefore, lead to the exclusion of certain taxa or feeding groups from a macroinvertebrate assemblage.

Assessment of benthic macroinvertebrate communities has historically been a useful tool for the assessment of the ecological health of an aquatic system (reviewed by Cairns and Pratt 1993). The macroinvertebrate assemblages of both lentic and lotic systems have been widely studied and the expected assemblages vary greatly between the two (Brabec et al. 2004). This may be ascribed to differences in sedimentation or the need for morphological and behavioural adaptations to deal with flowing waters (Hynes 1970). The analysis of benthic macroinvertebrate communities has also been used in the assessment of remediated sites and restoration projects. In particular, the rate of recruitment of macroinvertebrate taxa to a wetland in the post-remediation period has been used to assess continuing remediation

success (e.g., Nelson and Roline 1996, LeFevre and Sharpe 2002, Simon et al. 2006). Tolerant species recolonise created and remediated wetlands prior to development of a taxon rich macroinvertebrate community (den Besten and van den Brink 2005).

Sydney Olympic Park (SOP) is a remediated site situated in an urban residential and light commercial area of Sydney. Prior to remediation of the site, soils and sediments contained high OCP, PAH, TCDD, and PCB concentrations (Laginestra et al. 2001). Many wetlands were created on the site during an intensive remediation process between 1992 and 1999. Chemical analysis, an *in vitro* assay and an *in vivo* assay have suggested current POP contamination in some of the wetlands within SOP. There were measurable concentrations of ΣPAHs, ΣPCBs, TCDDeq and metals in the wetlands of the Park but these were generally toward the lower end of concentrations measured at remediated sites elsewhere in the world (Chapter 4). Mosquitofish, *Gambusia holbrooki*, from the wetlands on the site showed EROD activity (a biomarker of exposure to POPs) that, while high compared to other species (Whyte et al. 2000), were elevated above estimated basal levels for mosquitofish in Sydney at only one SOP wetland (Boundary Ck.) and were lower than many urban impacted reference sites around the Sydney metropolitan area (Chapter 5). As mosquitofish feed opportunistically (including on invertebrates) (Morton et al. 1988, Pen et al. 1993, Dreze et al. 1998) the increased EROD activity at Boundary Ck. may indicate some biomagnification effect.

The current study aimed to investigate effects of this contamination at a higher level of organisation by examining benthic macroinvertebrate community structure. It was hypothesised that wetlands with high concentrations of sediment contamination would have depauperate benthic communities with low taxon diversity.

6.3 Materials and Methods

6.3.1 Study Sites and Sample Collection

Five wetlands were studied within Sydney Olympic Park (SOP) and two reference sites were chosen outside the Park (Figure 2-1, Figure 2-2). Five sediment cores (75 cm^2) were

collected from each study site in February 2007. The top 15 cm of each core was excised and placed in a plastic bag and preserved with formalin (buffered with borax to maintain calciferous integrity in molluscs) containing 5 ml/L Rose Bengal stain. These were transferred on ice to the laboratory and stored at $4^{O}C$. Cores from certain study sites had high organic content necessitating the removal of over-lying liquid after two days and replacement with fresh formalin/Rose Bengal/borax solution.

Physico-chemical parameters of sediment (pH, redox potential, conductivity) and over-lying water (pH, DO, conductivity) were measured for each study site. Sediment grain size composition was evaluated for each study site and chemical analysis conducted for ΣPAH, ΣPCB, ΣDDT, TCDDeq, sediment metals and pore water metals. Detailed methods and results of these analyses are presented in Chapter 4.

6.3.2 Sample Processing

Each sample was rinsed thoroughly in a fume cupboard to ensure removal of as much formalin as possible. The sediment sample was washed through a stacked 1mm mesh sieve and a 250μm mesh sieve to retain sediments and animals greater than these mesh sizes. The animals retained from each mesh size sub-samples were removed and preserved in 70% ethanol. The sample was further examined under a dissecting microscope with the aid of a Bogorov plate and the remaining animals placed in 70% ethanol. Preserved animals were first sorted into coarse taxonomic groups before identification to lower levels. All arthropods were identified to Family level while other non-arthropod groups were identified to the appropriate taxonomic level. Resh and McElvray (1993) reviewed the taxonomic level of identification used in a number of articles on both lentic and lotic systems. They point out that while species level taxonomic identifications are preferable in some instances they are often neither cost nor time efficient. Identification to higher taxonomic levels can be just as useful where the purpose of the study is to investigate broad differences in the benthic community (Resh and McElvray 1993). In the current study identification to Family level for all arthropods was considered sufficient to detect such differences. Further, with very low taxon richness at this level for most study sites, further

taxonomic identification of individuals was not considered advantageous. Taxon richness and overall macroinvertebrate abundance were calculated for each study site based on the average content of all five sediment cores.

6.3.3 Data Analysis

Differences between study sites based on (log-transformed) taxon richness and abundance (per m^2) were examined using a single factor ANOVA (after confirming that the data fit the assumption of homogeneity of variances). A similarity matrix based on the Bray-Curtis similarity index (4^{th} root transformed data) was constructed and a multivariate analysis of similarity (ANOSIM) used to investigate differences between macroinvertebrate assemblages noted under examination with a non-metric multidimensional scaling ordination (PRIMER v6). A hierarchical clustering procedure was run to show within and between study site similarities and similarity profile permutation (SIMPROF) tests were run to determine the similarity between samples above which samples cannot be significantly differentiated (PRIMER v6). A SIMPER analysis was used to examine which species were important in driving the differences between macroinvertebrate assemblages. All multivariate analyses were conducted using the PRIMER statistical software (PRIMER v6). Canonical correspondence analysis (CCA) was run to investigate the influences of selected environmental variables (pH, Total Organic Carbon (TOC), ΣDDT, ΣPCB, ΣPAH, sediment 2,3,7,8-TCDD equivalence (TCDDeq) and sediment metals) on individual taxa and benthic macroinvertebrate assemblages at the study sites (MSVP version 3.13p).

To examine whether environmental factors influenced taxon richness or abundance a multiple regression was conducted including the potential predictors TOC, inorganic carbon, sediment grain size composition, sediment pH, sediment conductivity and sediment concentrations of ΣPAHs, ΣPCBs, metals and ΣDDT.

6.4 Results

Thirty two macroinvertebrate taxa were identified in the sediment cores from the study sites. Of these four were benthic infauna and six were benthic epifauna (Table 6-1). A further 18 taxa may spend some time in intimate contact with the benthos (e.g., benthic foraging, detrital feeding) and were probably epifaunic at the time of collection (Table 6-1). Three taxa are generally not considered to be in intimate contact with the benthos but have been included in the analysis as they were likely to have been foraging on the surface of the benthos at the time of collection.

The study sites were significantly ($p<0.05$) different from each other in both macroinvertebrate abundance and taxon richness (Figure 6-1). This was apparent even over the small spatial scale of the study sites within Sydney Olympic Park (SOP). Macroinvertebrate abundance was variable both between and within study sites. The largest variability was at EWQCP where abundance was between 2800 and 104133 animals.m^{-2} (a 37 fold difference). The lowest variability measured was at Upper Colo, the pristine reference site. The highest average abundance was at Macquarie Uni (121366 animals.m^{-2}) and the lowest was at Upper Colo (10399 animals.m^{-2}) (Figure 6-1). At Upper Colo, overall abundance was significantly ($p<0.05$) less than that at Macquarie Uni, Boundary Ck., Narrawang 22 and the Wharf Pond and at SWQCP abundance was significantly ($p<0.05$) less than that at Macquarie Uni (Figure 6-1).

Macroinvertebrate taxon richness was low across all study sites ranging from an average of 4.5 taxa at Boundary Ck. to 12 taxa at Narrawang 22. Taxon richness was significantly ($p<0.05$) higher at Narrawang 22 than at Boundary Ck., Bicentennial Park, Macquarie Uni, Upper Colo and SWQCP. EWQCP, Northern Water Feature and Wharf Pond all had intermediate taxon richness (Figure 6-1).

A multiple regression model for predicting macroinvertebrate taxon richness in the study sites gave negative relationships ($r^2 = 0.999$) for sediment TCDDeq and ΣPCB concentrations and sediment grain sizes between 187 and 250 μm (Table 6-2).

Table 6-1 Macroinvertebrate taxa collected at SOP and reference sites. Average density (m^{-2}) shown: * < 1000, ** <10000, *** > 100000. BC = Boundary Ck., EWQ = EWQCP, NWF = Northern Water Feature, N22 = Narrawang 22, BP = Bicentennial Pk., WP = Wharf Pond, MCU = Macquarie Uni, UPC = Upper Colo, SWQ = SWQCP, H = Habitat Classification.

Phylum	Class	Order	Family	BC.	EWQ	NWF	N22	BP	WP	MCU.	UC	SWQ	H[a]
Nematoda				**	***	**	***	**	*	***	*	*	A
Platyhelminthes	Temnocehpalidea										*		C
	Turbellaria				*	*		*	*	*			C
Mollusca	Gastrapoda	Pulmonata	Lymnaeidae		*		*		*	*			C
			Physidae				*		*			*	C
Annelida	Hirudinea	Rhychobdellida	Glossiphoniidae	*		*				*	*		C
	Oligochaeta			***	***	***	**	**	***	***	*	**	A
Arthropoda	Maxilliopoda	Cyclopoida			*	*	**	*	**	*			A
	Branchiopoda	Cladocera			*	*	*		*		*		B
	Ostracoda			**	***	*	**	**	**	***	*	**	B
	Malacostraca	Isopoda					*						C
	Arachnida	Acarina					*						D
	Insecta	Ephemeroptera	Baetidae				**						C
			Caenidae				*						B
			Leptophlebidae			*	*						B
		Coleoptera	Berescidae						*				A
			Hydrophilidae						*				C

Table 6-1 (cont.)

Phylum	Class	Order	Family	BC	EWQ	NWF	N22	BP	WP	MCU	UPC	SWQ	H[a]
Arthropoda	Insecta	Diptera	Chironominae[2]	**	**	***	***	**	**	***	**	*	B
			Orthocladiinae[2]				*						C
			Tanypodinae[2]		*	*	*	*	*		*	*	B
			Ceratopogonidae		*		*	*			*	*	A
			Culicidae				*						C
			Syrphidae			*							C
		Odonata	Corduliidae			*				*			C
			Coenagrionidae			*							C
		Tricoptera	Hydroptilidae				*					*	C
			Leptoceridae		*		**				*		C
			Odontoceridae				*						C
			Unknown 1			*							C
			Unknown 2		*								C
		Hemiptera	Notonectidae			*		*					D
			Corixidae						*				D

[1]Suborder, [2]Subfamily of Chironomidae.

[a]A = Benthic infauna, B = may be either benthic infauna or epifauna at different life-stages, C = may spend time as epifauna, D = Rarely in direct contact with sediment (from information in Williams 1980 and Gooderham and Tsyrlin 2002).

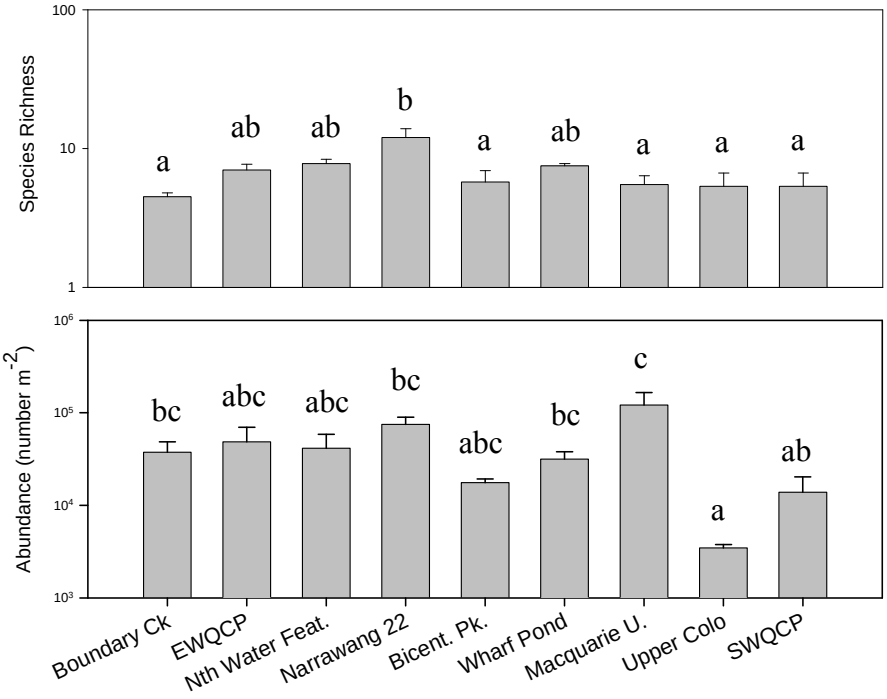


Figure 6-1 Species richness (top) and abundance (bottom) of benthic macroinvertebrates (m^{-2}) at Sydney Olympic Park and key reference sites. Letters represent significantly different homogeneous subsets at $\alpha = 0.05$.

Table 6-2 Results of multiple regressions on macroinvertebrate community data using physical and chemical characteristics of the sediments as predictors. Individual taxa are those which regularly influenced the pairwise differences between the sites (SIMPER). The direction of the relationships is denoted as + (positive relationship) and – (negative relationship).

	F	p-value	Predictors	r^2
Abundance	**3551.5**	**0.000**	**Grain size <1000µm, >500µm (+)** **Pore Water Metals (+)** **Sediment Metals (-)** **ΣDDT (+)** **ΣPAH (-)**	**1.000**
Taxon Richness	**948.63**	**0.000**	**Sediment TCDDeq (-)** **ΣPCB (-)** **Grain size <250µm, > 187.5µm (-)**	**0.999**
Chironomidae	**24.45**	**0.03**	**Grain size <250µm, >187.5µm (+)** **ΣPAH (-)**	**0.952**
Nematoda	**58.931**	**0.000**	**Sediment TCDDeq (-)**	**0.959**
Oligochaeta	**37.255**	**0.001**	**Sediment TCDDeq (+)** **Grain size <1000µm, >500µm (-)**	**0.937**
Ostracoda			**No significant Predictors**	

The model for predicting of benthic macroinvertebrate abundance included positive coefficients for sediment grain sizes between 500 and 1000 μm, pore water metal concentration, sediment ΣDDT concentration and negative relationships for sediment bound metals and ΣPAH concentration ($r^2 = 1.000$) (Table 6-2).

The nMDS ordination indicates that the macroinvertebrate assemblages at Narrawang 22, Upper Colo and the Northern Water Feature are somewhat separated from the other study sites on the basis of a Bray-Curtis similarity index (Figure 6-2). However, the clustering procedure (Figure 6-3) suggests significant overlap between these groups. Further, the similarity profile permutation (SIMPROF) tests suggest that above a similarity distance of 46.5% macroinvertebrate assemblages from individual cores were not significantly differentiated. This distinguished three groups of individual cores; Narrawang 22 was separated from all study sites while Upper Colo was linked with one core each from the Northern Water Feature and SWQCP. Analysis of Similarity (Table 6-3) indicates that macroinvertebrate assemblages were significantly ($p<0.05$) different from each other but that the pattern was not obvious. Macroinvertebrate communities at Narrawang 22 and Upper Colo were separated from all other study sites. The macroinvertebrate community at Macquarie Uni was significantly ($p<0.05$) different from that at Narrawang 22, Upper Colo and SWQCP. SIMPER analysis indicating the contribution of individual taxa to the dissimilarity between macroinvertebrate assemblages reveals the importance of four common taxa and a number of rare taxa (Table 6-4). In particular, the lack of common taxa (Oligochaeta, Chironominae, Ostracoda, Nematoda) separated the assemblages at Upper Colo from those at other study sites and the presence of rare taxa (including Baetidae, Caenidae, Leptophlebidae, Cladocera and Ceratopogonidae) at Narrawang 22 separated the assemblages at this wetland.

As previously stated Upper Colo is not an ideal reference site has some properties which make it inherently different to the Sydney Olympic Park study sites (e.g., sediment grain size, and flow regime) flow. These differences mean that its inclusion in the canonical correspondence analysis (CCA) of macroinvertebrate assemblages is not valid. A site (Macquarie University) within the Sydney metropolitan area (i.e., exposed to urban

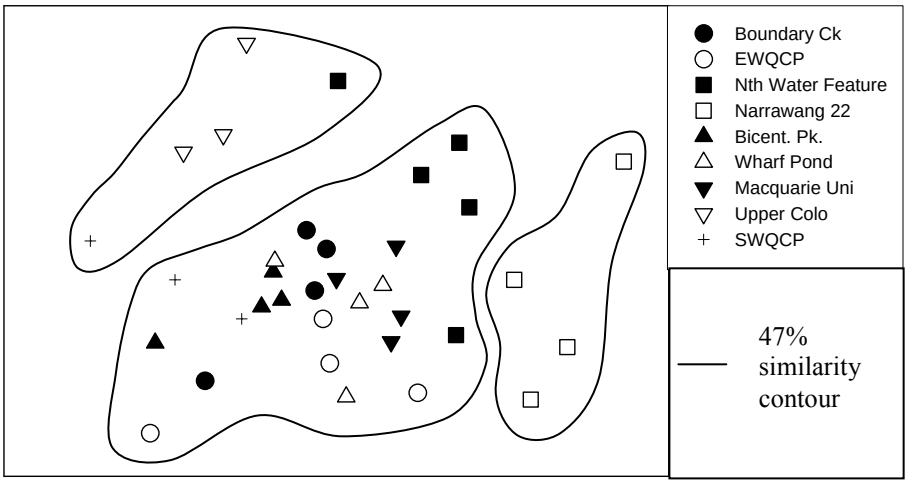


Figure 6-2 Non-metric multi dimensional scaling (nMDS) ordination plot of SOP and reference sites using all benthic macroinvertebrate data. Contours show significantly ($p<0.05$) similar groups as defined by similarity profile permutations (SIMPROF).

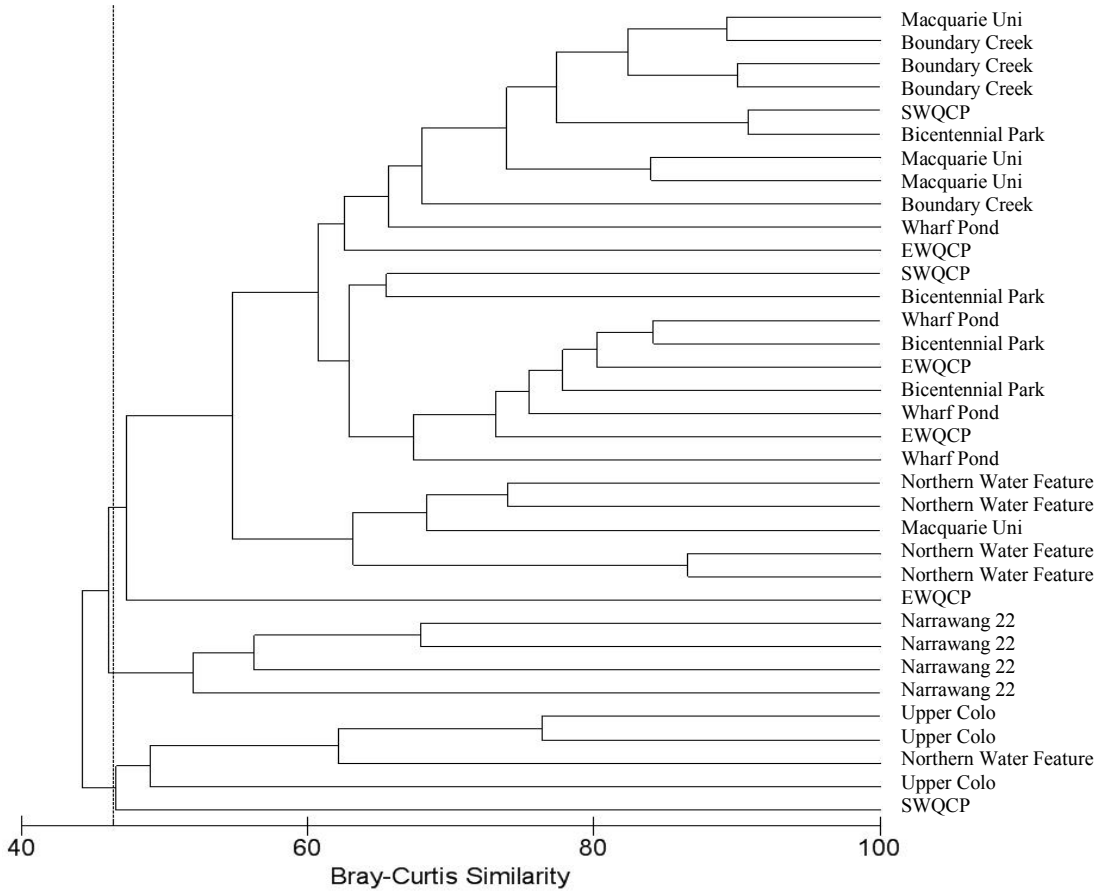


Figure 6-3 Dendogram of similar samples based on benthic macroinvertebrate assemblage. Bray-Curtis similarity. Fourth-root transformed data. Vertical line represents point above which divergence is not significant as determined by similarity profile permutation tests (SIMPROF) at $\alpha = 0.05$.

Table 6-3 Matrix of pairwise comparisons of macroinvertebrate assemblages at SOP and reference study sites using ANOSIM. Significantly ($p<0.05$) different values in boldface. BC = Boundary Ck., NWF = Northern Water Feature, N22 = Narrawang 22, BP = Bicentennial Pk., EWQ = EWQCP, WP = Wharf Pond, MCU = Macquarie Uni, UPC = Upper Colo, SWQ = SWQCP.

	BC	EWQ	NWF	N22	BP	WP	MCU	UPC	SWQ
Boundary Ck		**0.03**	**0.02**	**0.03**	**0.02**	**0.03**	0.06	**0.03**	**0.03**
EWQCP			**0.03**	**0.03**	0.11	0.09	0.06	**0.03**	0.20
Nth Water Feat				**0.02**	**0.01**	**0.02**	0.06	**0.01**	**0.02**
Narrawang 22					**0.03**	**0.03**	**0.03**	**0.03**	**0.03**
Bicent. Pk						**0.03**	0.06	**0.03**	0.11
Wharf Pond							0.06	**0.03**	**0.03**
Macquarie Uni								**0.03**	**0.03**
Upper Colo									0.10
SWQCP									

Table 6-4 Relative contribution of the main taxa influencing pairwise dissimilarity between study sites. All pairs of study sites represented are significantly ($p<0.05$) different from each other. Contribution to dissimilarity is represented categorically; + signifies greater abundance of taxa in study site (a), - signifies greater abundance of taxa in study site (b). Contribution dissimilarity is represented categorically; 1 symbol > 5%, 2 symbols >6%, 3 symbols >7%, 4 symbols >8%, 5 symbols >9%, 6 symbols >10%. Boundary Ck is included as a representative of the main group of study sites separated by similarity profile permutations (SIMPROF).

Study Site (a) Study Site (b)	Narrawang 22 → Upper Colo	Upper Colo → Boundary Ck	Narrawang 22 → Boundary Ck
Average Dissimilarity (%)	65.72	46.9	56.88
Chironominae	+ + + + +		+ + + +
Ostracoda	+ + + +	- - - - - -	+ +
Oligochaeta	+ + +	- - - - - -	- - - - -
Nematoda	+ + + + +	- - - - - -	+
Leptophlebidae	+ +		+ +
Cladocera			+
Caenidae	+		+ +
Baetidae	+ +		+ +
Copepoda	+ +		+ + +
Tanypodonidae		+ + + + + +	
Ceratapogonidae		+	+ +
Hirudinea		- - - -	

impacts) was included in this analysis to provide a reference condition. The CCA separated the macroinvertebrate assemblages at Narrawang 22 and Northern Water Feature from those at other study sites (Figure 6-4). The taxon centroids indicate that these wetlands had higher numbers of rare taxa while the other wetlands lacked these taxa. The environmental variables with the greatest influence on the analysis were ΣDDT, TOC and TCDDeq in the sediment (Table 6-2).

Multiple regression indicated that the abundance of Chironominae was negatively affected by sediment ΣPCB concentrations (and positively affected by sediment grain sizes between 250 and 500 μm) (Table 6-2). Differences in nematode abundance were due to sediment TCDDeq and oligochaete abundance was positively affected by sediment TCDDeq (and negatively by sediment grain sizes between 1000μm and 5000μm). There were no significant predictors for the abundance of ostracods (Table 6-2).

6.5 Discussion

Urban wetlands generally have reduced benthic macroinvertebrate taxon richness (Shutes 1984, Hall et al. 2001) represented by taxa that are tolerant to urban contaminants (Whiting and Clifford 1983). Compared to similar pristine wetlands, taxa commonly excluded include odonates, trichopterans, ephemeropterans and plecopterans, with oligochaetes, nematodes and chironomids often dominating the benthic communities (Lenat and Crawford 1994, Hall et al. 2001). The common taxa found in this study were those expected for degraded urban habitats with all wetlands having abundant tolerant taxa. However, a few wetlands contained pollution sensitive taxa such as trichopterans and ephemeropterans. Only Narrawang 22 and Northern Water Feature within SOP contained odonates and ephemeropterans but in very low numbers while trichopterans were moderately abundant only at Narrawang 22. While these are not strictly benthic dwellers, they can be benthic foragers and are, therefore, intimately associated with the benthos.

Macroinvertebrate recruitment times to created and newly remediated wetlands depend on a number of factors including organism life-history (e.g., generation time and dispersal

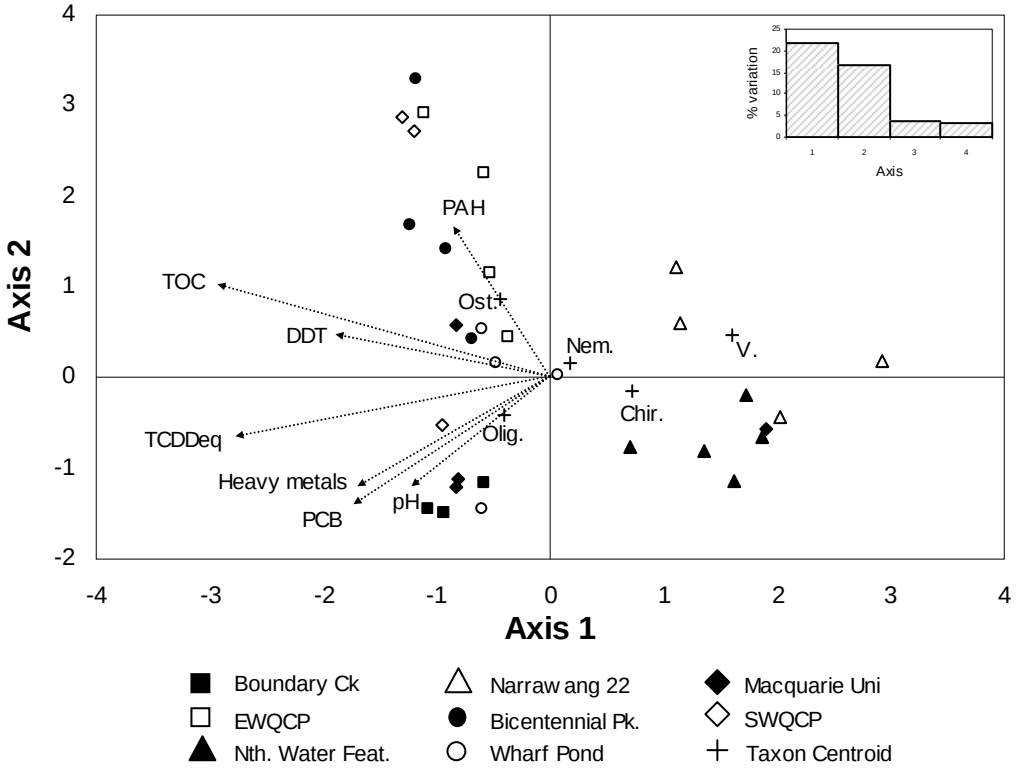


Figure 6-4 Canonical correspondence analysis ordination for macroinvertebrate assemblages at SOP and reference study sites. Vectors representing environmental variables are scaled x3. TOC = Total Organic Carbon. PCB, DDT and PAH values are summed anayte concentrations. + denotes centroids for major taxa influencing the significant dissimilarity between Narrawang 22 and other wetlands. Nem. = Nematoda, Ost. = Ostracoda, Olig. = Oligochaeta, Chir. = Chironominae, V. = a group of rare taxa including Baetidae, Caenidae, Culicidae, Odontoceridae, Orthocladinae.

strength) and the distance to a source of recolonisation (Niemi et al. 1990). In lotic systems drift is an important source for recruitment (Nelson and Roline 1996) and increases in taxon richness after remediation can occur quickly if there are upstream seeding sites (Simon et al. 2006). Slow recruitment to lotic wetlands may result from the absence of taxon rich upstream sites, or low habitat quality (contamination, benthos etc.) at the study site. The two urban lotic sites included in the current study (Macquarie Uni and Boundary Ck) have highly degraded upstream reaches located in highly urbanised land. It is unlikely that taxon rich regions exist upstream of either wetland. This will contribute to decreased taxon richness at these wetlands.

In lentic sites, the proximity to undisturbed macroinvertebrate habitat is also important (Niemi et al. 1990). Only highly aerially dispersive organisms (dipterans, odonates and ephemeropterans) are likely to recolonise these wetlands quickly. SOP is located in the highly urbanised Sydney metropolitan area and proximate, undisturbed wetlands are, therefore, few. The wetland with the highest taxon richness (Narrawang 22) is located adjacent to the Newington Nature Reserve, a remnant bushland which may contain refugia for rare taxa. In their meta-analysis of post-disturbance recovery times Niemi et al. (1990) broadly described that (in lotic systems) dipterans were the first insect colonisers, followed by ephemeropterans, then trichopterans and finally plecopterans. The lack of the latter order in the current study may indicate slow recruitment to newly remediated wetlands.

In general, the time taken for recovery to pre-disturbance taxon richness in lotic wetlands is within 12 months (Simon et al. 2006). There are reported cases of recovery from press disturbance (e.g., channelisation, nutrient enrichment) not completed in as long as 18 years (see Niemi et al. 1990 for review). Given the highly disturbed nature of the catchment of some of the wetlands in the current study it is possible that the recovery process is incomplete and recruitment of new taxa will continue over many years.

In a study of post-remediation recolonisation, den Besten and van den Brink (2005) found that the first colonisers at two nearby but independent sites were nematodes, oligochaetes and chironomids but that the relative abundance of these groups was different at each site.

The authors suggest that differences between the sites are likely due to differences in sediment characteristics. In the current study, sediment grain size was important in predicting chironomid and oligochaete abundances (and total macroinvertebrate abundance). Sediment grain size is, therefore, likely to be important in the between study site differences in the abundances of these tolerant taxa, which in certain study sites contribute strongly to total abundance.

Benthic habitat is dependent on interstitial pore size and, therefore, sediment grain size. Most of the wetlands in the current study had sediment which was dominated by mid size ranges (fine sand to silt) the exception being Upper Colo which had a larger proportion of sand (Chapter 2). This may be reflected in the low macroinvertebrate abundance at this study site but may also be due to the low organic carbon content at this site (Chapter 2). Some of the main taxa identified in the current study feed by ingestion of sediment (e.g., oligochaetes). Where organic carbon is low these animals will be rare. In the current study few oligochaetes were found at Upper Colo relative to other study sites where they were common to very common.

Within study sites assemblage patchiness was high, particularly in terms of abundance. This highlights the need to ensure sufficient sample replicates in such a study. In the current study, replication was sufficient to detect differences in macroinvertebrate abundance. It is unclear whether the patchiness recorded in this study was due to habitat patchiness (often high (Downes et al. 1993, Heino et al. 2004)), or chemical pollutant patchiness (also often high (Johnson and Larsen 1985, Swartz et al. 1989, Feng et al. 1998, Koh et al. 2004)). Correlation between contamination and benthic community measures in a patchy environment has been recorded over very small spatial scales (<500m) (Stark et al. 2005) and thus the effects of contaminant heterogeneity cannot be ruled out here.

Higher TCDDeq in the sediment negatively influenced benthic invertebrate taxon richness. While the acute toxicity of these AhR ligands to benthic invertebrates is not particularly high (West et al. 1997), there is evidence to suggest they can cause significant chronic effects in even the most pollution tolerant taxa (Lotufo 1998b, Hwang et al. 2004) and may

reduce taxon richness in the long-term by excluding sensitive taxa. Many studies have shown the tendency of TCDD and other AhR ligands to bioaccumulate in benthic organisms (e.g., West et al. 1997, Froese et al. 1998, Timmermann and Andersen 2003, Lotufo 1998a). These two processes (reduction of taxon richness and bioaccumulation of toxicants) will have negative impacts on the health of vertebrate consumers (e.g., fish and birds) and must be considered together.

Multivariate analysis (ANOSIM and SIMPER) suggests that the macroinvertebrate assemblage at some study sites were quite distinct. In particular, the assemblage at the pristine reference site Upper Colo had low abundances of common taxa. This is likely due to its geographic separation from the other study sites (about 100 km) and its different wetland characteristics (a lotic system) with concomitant sediment differences (dominated by sand as opposed to silts). While taxon richness was similar, the abundance was much lower than that at other study sites. The difference between the assemblage in the created wetland, Narrawang 22 and that in the rest of the wetlands studied was influenced by an increased abundance of both common and rare taxa. This wetland is less affected by organic contamination than others (Chapter 4) suggesting that the rare taxa are sensitive to this type of pollution.

The assemblage in the Northern Water Feature appears on the nMDS ordination as an intermediate study site which is separated on the basis of some samples. At this wetland the assemblage contained taxa rarely or not found at other study sites (e.g., Odonata and Sypherinidae (Diptera)). The abundance and taxon richness at this wetland was, however, similar to those at other study sites. The assemblages in the main group of study sites varied in macroinvertebrate abundance but all had similar taxa (including Macquarie Uni, an urban impacted reference site).

According to CCA variation between the macroinvertebrate assemblages (at wetlands other than Northern Water Feature and Narrawang 22) is mainly along an axis which poorly correlates with the contamination variables included in the analysis. This suggests the importance of other factors (e.g., physico-chemical characteristics, habitat variety) in

influencing the macroinvertebrate assemblages in these wetlands. CCA however, separated the macroinvertebrate assemblages at Narrawang 22 and Northern Water Feature wetlands along an axis, which was influenced by a gradient of contamination (sediment TCDDeq, ΣDDT, TOC). The strength of the influence of TCDDeq in the sediments suggests that contamination by persistent organic pollutants is likely to affect the macroinvertebrate assemblages. Similarly the influence of the pesticide DDT and its metabolites (ΣDDT) was to separate the two study sites with highest taxon richness (Narrawang 22 and Northern Water Feature). The presence of rare taxa at these study sites suggests that these are pollution sensitive taxa, which are excluded from sites further along the contamination gradient and that the assemblages at these wetlands consist of pollution tolerant taxa (oligochaetes and ostracods). TOC is usually high in wetlands affected by urban catchments and catchment land-use can be an important predictor of sediment contaminant load (Hoffman et al. 1984). The strength of the influence of TOC on the macroinvertebrate assemblages in the study sites indicates that catchment input is important in restricting the occurrence of some taxa.

Pratt et al. (1981) described in detail the disruption to macroinvertebrate community due to runoff from urban catchments. Lenat and Crawford (1994) showed that the taxon richness within the orders Ephemeroptera, Plecoptera and Trichoptera is reduced in urban affected wetlands. Taxon richness within these orders (the ETP index) is considered sensitive to changes in water quality and relatively insensitive to natural disturbance (e.g., changes in flow regime) and is widely used as an indicator of wetland health. Taxa within these orders are sensitive to a range of stressors. Wallace et al. (1996) describe a dramatic drop in EPT taxon richness in response to a three-year pesticide treatment (methoxychlor) and recovery to pre-treatment levels in two years. Whiting and Clifford (1983) attribute decreases in taxon richness (especially Ephemeroptera and Trichoptera) in an urban wetland to organic contamination. In the current study no Plecoptera were identified. Only three families of Ephemeroptera and five families of Trichoptera were found and, in general, these were at the wetlands which were least subject to urban catchment inputs (e.g., stormwater runoff) (Narrawang 22 and Northern Water Feature). The cause of the paucity of EPT taxa at

certain study sites is likely to be a combination of many factors (measured and unmeasured) but is evidence of the importance of catchment input on the macroinvertebrate assemblage.

It is possible that a contaminant driven taxon succession is observable in the current data. As the concentrations of AhR ligands (ΣPAH, TCDDeq) in the sediment decreased the abundance of both nematodes and chironomids increased. Conversely, the concentration of oligochaetes in the sediment increased as TCDDeq increased. Oligochaetes may not only be more tolerant of high concentrations of organic pollutants but may also benefit from the absence of other tolerant taxa (chironomids and nematodes).

The SOP wetlands were subject to a variety of remediation histories. Some were remnant (Wharf Pond), others were remediated either pre-1991 (Lake Belvedere, Boundary Ck.) or created post 1991 (e.g., EWQCP) (Laginestra et al. 2001). There was no clear trend between remediation history and macroinvertebrate community. EWQCP, SWQCP, Northern Water Feature and Narrawang 22 were all created during the remediation of the Park and currently have different macroinvertebrate assemblages. Nor was there a trend between contamination history and macroinvertebrate assemblage. Northern Water Feature is situated on land previously contaminated with PCBs and dioxins (Laginestra et al. 2001) while SWQCP is situated on land that did not require remediation. Yet Northern Water Feature is separated from SWQCP based on the presence of rare taxa. It is, therefore, unlikely that differences between the macroinvertebrate assemblages are the result of incomplete or variation in remediation.

Benthic invertebrates play important ecological roles in the mixing of sediment (bioturbation) (Kresoski et al. 1978) resulting in sediment resuspension (Davis 1993). This can release bound organic contaminants, which may make them more available to higher trophic levels (Ciarelli et al. 1999). Based on this argument, a lack of benthic biota could be seen as an ameliorative process in highly contaminated sediment by enabling slower contaminant flux between the sediments and surficial waters. In a site of high ecological value such as SOP, however, this is outweighed by the positive impact of biodiversity at this important trophic level.

There were observed differences in habitat variety at the study sites. At Boundary Ck there is a stand of emergent macrophytes (*Phragmites australis*) and there is no riparian vegetation or submerged macrophytes. SWQCP and EWQCP are surrounded by a significant littoral zone (*Casuarina* spp.) with emergent macrophytes (*Baumea articulata*, *P. australis*) and Narrawang 22 has a benthic cover of submerged macrophytes, emergent macrophytes (*B. articulata*) in the littoral zone and some riparian vegetation. While sampling at each study site attempted to cover a representative portion of the wetland to account for small-scale patchiness, the absence of healthy riparian, littoral and submerged vegetation at many of the study sites may contribute to a reduction of variety in the benthic habitat. This may influence the macroinvertebrate assemblages present at the study sites particularly in terms of taxon richness.

In the current study the highest taxon richness was in the created wetland Narrawang 22 indicating that sufficient time has passed for enough recruitment to allow effective monitoring of the benthic community. As Narrawang 22 is primary wading bird and frog habitat it is essential that a healthy invertebrate population is maintained. This area is regularly subject to inundation as a result of stormwater amelioration action (Laginestra et al. 2001) allowing some recruitment from upstream. However, it is also regularly drained in an attempt to control mosquitofish populations in the wetland. The current results suggest that inundation is sufficient to allow recruitment and drainage does not cause depauperation of the benthic community. A possible source of POPs to Narrawang 22 is incomplete capture of leachate from the adjacent waste mound. Leachate from this mound (the largest in the Park) has been shown to contain the highest concentrations of POPs and to be the most acutely toxic (SKM and EVS 2001). The high taxon richness at Narrawang 22 compared to the that in rest of the SOP wetlands (and compared to that in Sydney metropolitan urban reference sites) suggests that the effects of this waste mound on macroinvertebrate communities can be considered negligible (a finding supported by previous data (Chapters 4 and 5) indicating that this is a clean wetland). This wetland should be designated as a reference site to benchmark taxon richness in future monitoring of macroinvertebrate communities in SOP wetlands.

6.6 Conclusions

The benthic macroinvertebrate communities inhabiting the wetlands of SOP are consistent with those expected in urban wetlands. This is reflected by low taxon diversity and an abundance of tolerant taxa. Only one wetland (Narrawang 22) had noticeably high taxon richness and this study site was adjacent to remnant bushland. It was also the wetland with the lowest sediment contaminant load. While it is possible that the SOP wetlands are still undergoing the establishment of a healthy benthic community following their remediation or creation, this is considered unlikely given the time that has passed (eight years) since the remediation. There may also be an influence of differences in habitat diversity between the wetlands, which could affect taxon richness. However, given the strength of the relationship (CCA) it is concluded that increased sediment POP contamination (particularly as measured TCDDeq and ΣDDT) is a likely contributor in excluding pollution sensitive taxa and, therefore, alterations to benthic macroinvertebrate assemblages. Further, the influence of TOC suggests the significance of catchment inputs in contributing to changes in macroinvertebrate assemblage.

7. Population Condition and Life-History Traits of Mosquitofish (*Gambusia holbrooki*) in Sydney Olympic Park

7.1 Abstract

Fish population responses to environmental conditions can vary over small, medium and large spatial and temporal scales. Contamination of waterways can alter population characteristics acutely or chronically. This study aimed to describe similarities and differences in overall population condition and life-history traits of isolated populations of mosquitofish, *Gambusia holbrooki,* over small and medium spatial scales between October 2005 and March 2007 in the Sydney Olympic Park (SOP) remediated site and remote reference sites. Sex ratio, the proportion of males which were mature and overall population condition varied between sites over small spatial scales (within SOP). There was no relationship between the contamination (2,3,7,8-TCDD equivalency, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, ΣDDT or metals) of the sites and either the overall population condition or the sex ratio. At the onset of the breeding period (October) the proportion of males, which were sexually mature was negatively correlated with variation in ΣDDT (r^2 = 0.886). This may suggest an estrogenic or anti-androgenic effect and indicates the importance of future monitoring for persistent organic pollutants (POPs) at these wetlands. CYP1A activity (EROD) was not correlated with either the body condition or the gonado-somatic indices indicating no general toxic effect of the level of exposure to POPs experienced at the study sites. Differences in the life-history-traits in the mosquitofish populations studied are likely to be due to a complex combination of biotic and abiotic factors and cannot be attributed to contamination alone.

7.2 Introduction

Spatial and temporal changes in freshwater fish populations can be used to describe differences between populations. Differences in life-history traits such as population size distribution, breeding time, sex ratio and population density have been reported over small (Olsen and Vollestad 2005) and large (L'Abee-Lund et al. 1989, Blanck and Lamouroux 2007) spatial and temporal (Quinn and Bloomberg 1992, Humphries et al. 2002) scales. Differences can be attributed to biotic and/or abiotic variation between habitats. Water temperature and photoperiod tend to influence reproductive timing (Dreze et al. 1998, Koya and Kamiya 2000), food availability can govern fecundity and population density (McFadden et al. 1965, Rozas and Odum 1988) and predation can cause significant variation between populations in a number of life-history traits (reviewed by Endler 1995).

Changes in life-history parameters can been used to indicate the presence of contamination in an ecosystem and, hence, the overall health of the system (Munkittrick and Dixon 1989). While population size is the ultimate response in a heavily contaminated system, less acute responses have been reported in juvenile survivorship (Munkittrick and Leatherland 1984), sex ratio (Larsson et al. 2000) and indices describing body condition (Pyle et al. 2005) and breeding status (e.g., gonado-somatic index) (reviewed by Kime 1995).

Sydney Olympic Park (SOP) was highly contaminated with domestic, commercial and industrial wastes prior to remediation in the 1990s (Laginestra et al. 2001). Metals, organic and inorganic contaminants were in high concentration in the soils and sediments of the Park and were cleaned up on-site (OCA and ADI 1999) (Chapter 1). Highly contaminated material was treated and the resultant material consolidated in mounds (OCA 2000a), which now form part of the visual and recreational amenity of the Park (Chapter 1). The remediation program included the construction of a number of wetlands in the site, which are now habitat to a variety of wildlife including migratory waterbirds that are protected under international treaties, and amphibians including the endangered green and golden bell frog) (OCA 2000d). The wetlands have also attracted invasive pest species including *Salvinia* (an aquatic weed) and the mosquitofish, *Gambusia holbrooki*. Mosquitofish are

ubiquitous inhabitants of urban wetlands around Sydney, are thought to pose a threat to frog (including green and golden bell frog) populations (Hamer et al. 2002) as they may feed on their eggs (Pen et al. 1993, Komak and Crossland 2000).

A previous study described concentrations of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), ΣDDT and 2,3,7,8-TCDD equivalence (TCDDeq) in the sediments of the SOP wetlands (Chapter 4). Other research has shown significantly increased hepatic CYP1A in mosquitofish from one SOP wetland (Boundary Creek) indicating the exposure of these fish to persistent organic pollutants (POPs) (Chapter 5) and the influence of sediment bound POPs on the macroinvertebrate communities in SOP wetlands (Chapter 6). Given these results and the potential for population level impacts in fish from both contamination (Kidd et al. 2007) and decreased food availability (Lobon-Cervia et al. 1997), it is pertinent to examine the health of fish populations within SOP wetlands.

This study investigates the differences and similarities in the life-history traits of mosquitofish populations inhabiting SOP wetlands with different levels of POP contamination. Of particular interest is whether differences between isolated populations (no inter-site migration) were due to the presence of contamination or simply natural variation over a small spatial scale. This species was chosen for its ease of capture, ubiquitous nature and well-characterised biology. It was not intended that this species should represent native species inhabiting the wetlands of the Park, but that its study could provide insights into the potential population level impacts of the previously measured POP contamination in the SOP wetlands. Given that this species is known to be tolerant to a range of environmental toxicants it is possible that the results of the current study may under-estimate the potential for impacts on native species.

7.3 Materials and Methods

7.3.1 Study Sites

Four wetlands within Sydney Olympic Park (SOP) (Boundary Ck, Lake Belvedere, EWQCP and Narrawang 22) were chosen for the study. These wetlands were chosen to represent a range of contamination and remediation histories (Table 2-2) and for ease of access for repeated sampling and presence of substantial, permanent populations of mosquitofish. Two off-site reference wetlands, representing a pristine site (Upper Colo) and an urban impacted site (Macquarie Uni) (Figure 2-2), were selected to give both an urban context and a wide spatial scale to the study. *iBcod* (Alpha Mach Inc.) temperature loggers were deployed at each study site between October 2006 and August 2007 to record Summer and Winter daily and seasonal temperature fluctuations.

7.3.2 Fish Collection and Processing

Mosquitofish were collected every six to eight weeks from October 2005 to March 2007. Each collection continued until 100 fish were caught or collection time reached one hour. Fish were caught using aquatic dip nets and were transported to the laboratory in large aerated plastic bags. They were maintained in water from the study site and not fed until processing (<48 hours). Fish were sacrificed in benzocaine (400 mg/L) and photographed using digital a camera (Leica DFC 320) mounted on a dissecting microscope. Photographs were taken of the whole animal, the anal fin and (for males with a developed gonopodium) the tip of the anal fin to determine the presence or absence of a gonopodial terminal hook complex (a morphological marker for male sexual maturity) (Figure 5-1). The animals were weighed individually (wet weight) and separated into juvenile males, adult males, females, and undifferentiated juveniles based on external morphology (Figure 5-1). Separation of females into juvenile and mature individuals is not possible without histological examination of the ovaries so was not included in this study. The testis was removed from ten randomly selected sexually mature male fish (fish with a gonopodial terminal hook complex) from each study site and weighed. All fish were preserved in 70% ethanol.

7.3.3 Morphology

Morphological measurements were made using the Leica IM500 image analysis software package (Leica Microsystems 2004). Standard length (SL) was measured for all fish and was defined as the distance from the anterior tip of the fish to the caudal plate (i.e., excluding the caudal fin) (Figure 3-1). The gonopodium length (Gp) was measured as the length of the 3rd ray of the gonopodium (Figure 3-1) and was standardised against SL (Eqn 7-1).

$$\text{Standardised Gonopodial Length (SGp)} = \frac{\text{Gp (mm)}}{\text{SL (mm)}} \qquad \text{(Eqn 7-1)}$$

7.3.4 Population Condition, Fulton's K and Gonado-Somatic Index

Overall population condition was calculated as the slope of the linear regression of log-length vs. log-wet weight. A higher value for overall population condition suggests a general trend to larger fish being heavier. Body condition was examined using a widely used body condition index (BCI), Fulton's K (e.g., Thetmeyer et al. 1999, Meka and McCormick 2005, Hoeinghaus et al. 2006) (Eqn 7-2, where n = a multiplier to allow the BCI to be measured between 1 and 10 for ease of presentation).

$$\text{BCI (Fulton's K)} = \frac{[\text{Wet Weight (g)}] \times 10^{n}}{[\text{Standard Length (mm)}]^{3}} \qquad \text{(Eqn 7-2)}$$

The gonado-somatic index (GSI) of mature males and females increases as an individual comes into breeding season and is calculated as the ratio of gonadal wet weight to somatic wet weight (Kime 1995). The male poeciliid testes are fused into a single organ, the testis, at maturity (Constantz 1988). GSI was only calculated for sexually mature males (i.e., males with a gonopodial terminal hook complex) as prior to attaining maturity the testis is unconsolidated and unfused and, therefore, difficult to accurately extract, weigh and preserve. The species is live-bearing and pregnancy artificially increases the GSI so female GSI was not calculated.

7.3.5 Data Analysis

Log-log plots of standard length against wet weight were analysed for differences in the slope (overall population condition) of standardised major axis (SMA) regressions using the (S)MATR line fitting software (Falster et al. 2006). SMA was considered the most appropriate analysis given that the standard length (x-axis) is a continuous random variable with the possibility of error about the values (Quinn and Keough 2002). Proportions of male, female and undifferentiated fish were calculated and plotted as were proportions of sexually mature adult males with gonopodia but with no gonopodial terminal hook complex and juvenile fish. Mean BCI and GSI were calculated for each collection time at each study site. BCI was separated into male and female fish since gravidity has a positive influence on BCI and could provide biased overall data. BCI and GSI were analysed by ANCOVA (body weight with length as covariate and gonad weight with body weight as covariate respectively) but the data failed to meet the assumption of homogeneity of covariate slopes. These endpoints were therefore analysed using two-factor ANOVA (site, time of collection). Standardised gonopodial lengths (SGp) were plotted for each collection time at each study site. Individual animals were plotted to indicate where overlap of mature (presence of gonopodial terminal hook complex) and immature (no gonopodial terminal hook complex) groups of male animals took place.

There were significant interactions between site of collection and time of collection for the individual GL and population (sex ratio, proportion of mature males) traits measured making parametric analysis of the traits between study sites and collection times impossible. Since greatest potential for population level effects is at the time of breeding collection time was isolated using male GSI and relationships investigated between the above traits and measured sediment and water contamination data (Chapter 4) and the EROD biomarker of exposure to AhR ligands (Chapter 5). These analyses were conducted with a low sample size (n= 5 or 6 depending on the endpoint) and the results are discussed in this context. Relationships were investigated using simple and multiple regression techniques.

7.4 Results

SOP wetlands varied in the degree of diel temperature variation but Winter cooling commenced at the same time for each study site. The highest diel temperature range in SOP was in Boundary Ck and the lowest was in Lake Belvedere. Maximum temperatures for each SOP wetland occurred in February just prior to the commencement of Winter cooling. The highest daily temperatures were recorded at Boundary Ck (33°C) while other SOP wetlands never reached 30°C (Figure 7-1). Generally, the daily maximum water temperatures were within one or two degrees across the SOP wetlands and Macquarie Uni (Figure 7-2). Water at Upper Colo was consistently 2 to 3^{o}C cooler than at the other study sites (Figure 7-2). In Winter the diel temperature range was much narrower (maximum 2°C at Macquarie Uni) and 15°C cooler than maximum Summer temperatures (Figure 7-2).

Standard length-weight relationships indicate differences between the study sites. Fish from Boundary Ck were generally smaller and lighter than those at other study sites with few fish exceeding 25mm length. Upper Colo and EWQCP had few fish greater than 30 mm length and 500 mg while the remaining three study sites (Narrawang 22, Lake Belvedere and Macquarie Uni) had an abundance of larger, heavier fish (Figure 7-3). At Narrawang 22, Macquarie Uni and Upper Colo there were certain outliers in this distribution, which represent relatively very heavy fish (>1 g) of moderate size (<25 mm). These were heavily pregnant females and suggest some degree of precociousness in these individuals.

Comparisons of SMA regressions of log-log plots of standard length against wet weight showed significant ($p = 0.001$) differences in slopes. This analysis separated the overall population condition of populations at the study sites into three homogeneous subsets; (ordered by increasing population condition) [EWQCP, Narrawang 22, Upper Colo] < [Boundary Ck, Lake Belvedere] < [Macquarie Uni] (Table 7-1).

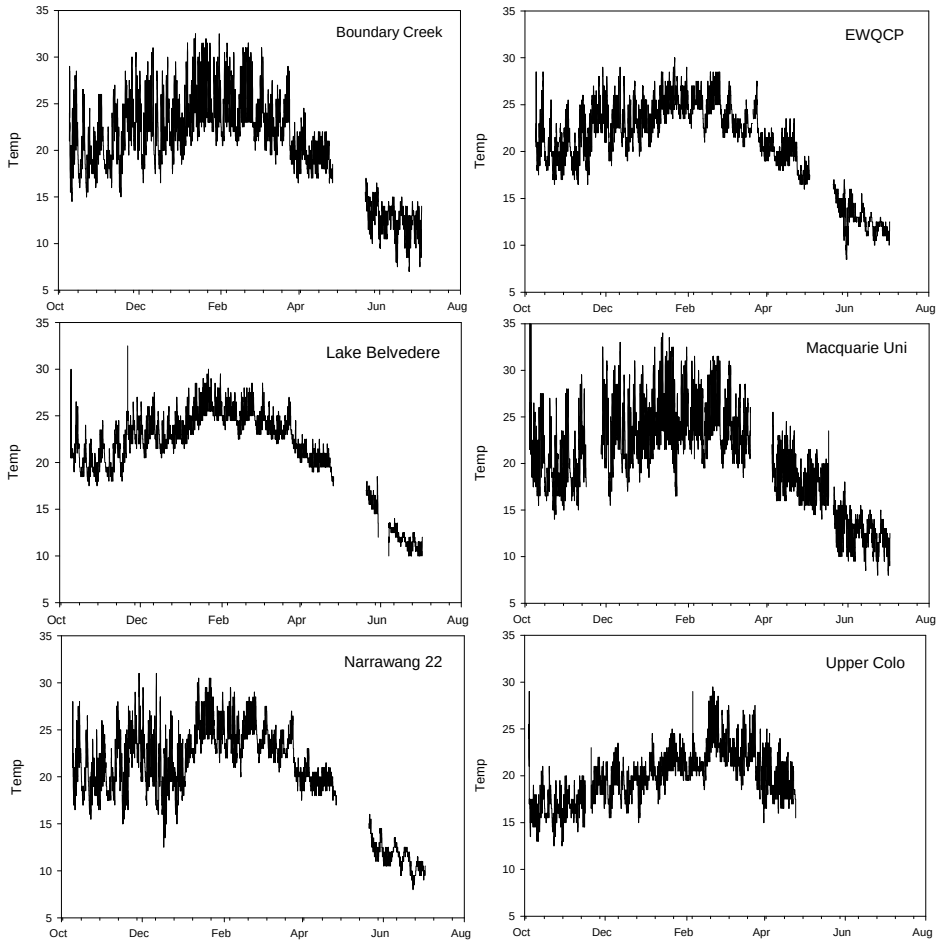


Figure 7-1 Summer and Winter daily and seasonal water temperature variation at SOP and reference wetlands.

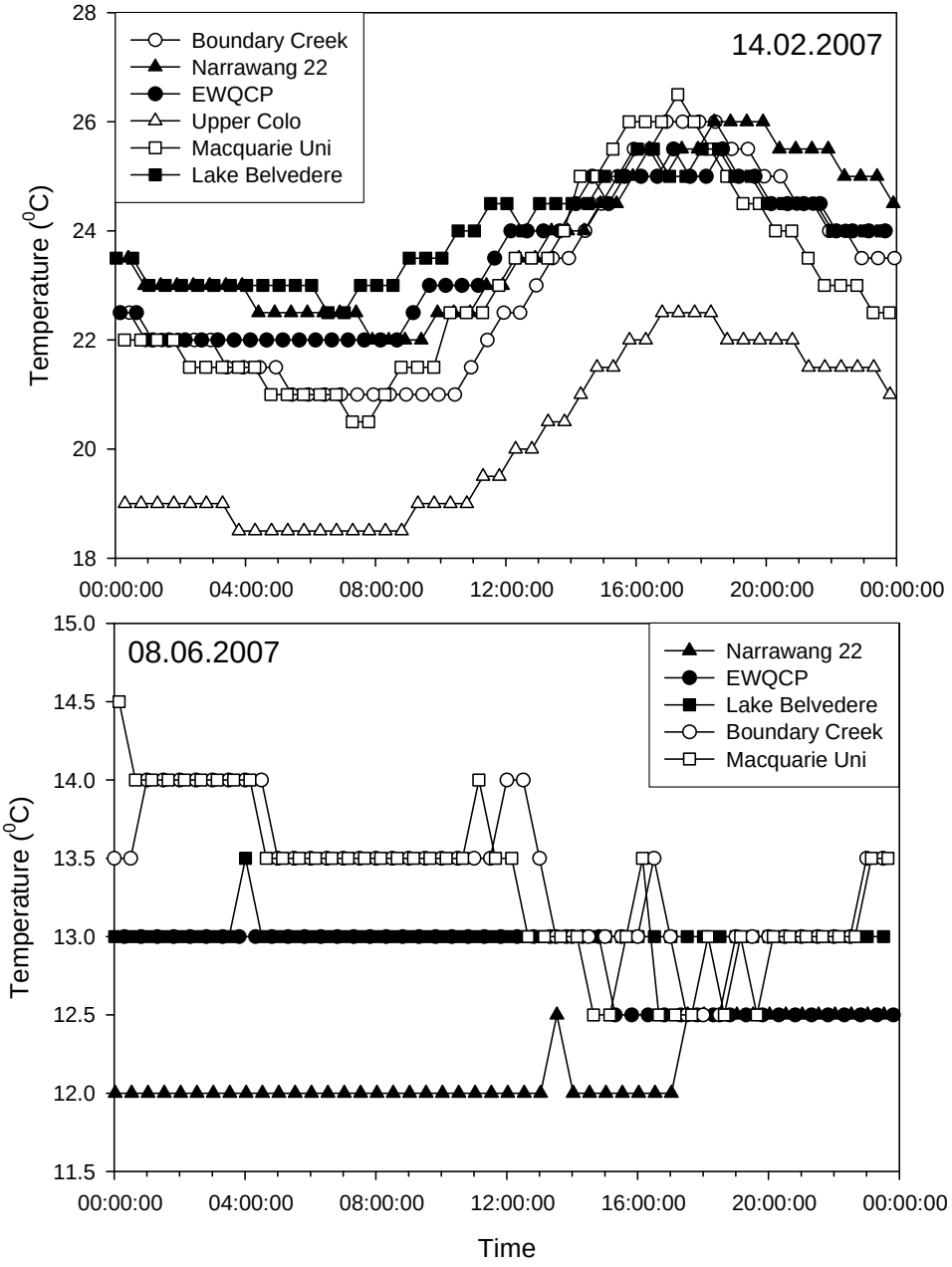


Figure 7-2 Diel variation during Summer (top) and Winter (bottom) at SOP and reference study sites. Note that there is no data for Upper Colo during Winter months due to missing logger.

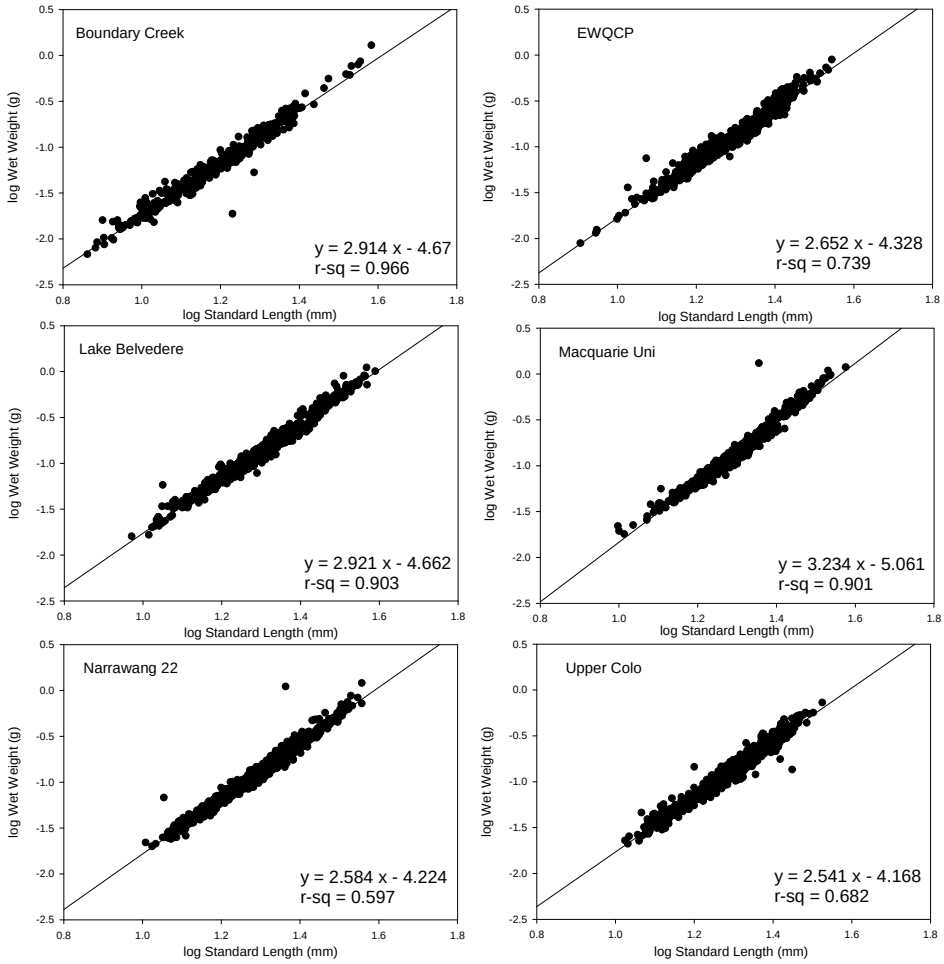


Figure 7-3 Log-log plots of standard length vs. wet weight of mosquitofish at SOP and reference sites. Linear regression equations and coefficients of variation are given for each regression.

Table 7-1 Matrix of p-values for pairwise comparisons of standardised major axis regression slopes of log-log plots of standard length vs. wet weight of mosquitofish from study SOP and reference sites using (S)MATR software (Falster et al. 2006). * = Significant difference (α = 0.05).

	Narrawang 22	EWQCP	Lake Belvedere	Boundary Ck	Upper Colo	Macquarie Uni
Narrawang 22	1.000	0.404	0.002*	0.001*	0.587	0.001*
EWQCP		1.000	0.001*	0.002*	0.098	0.001*
Lake Belvedere			1.000	0.874	0.001	0.001*
Boundary Ck				1.000	0.001	0.001*
Upper Colo					1.000	0.001*
Macquarie Uni						1.000

The proportion of male fish in the populations at Boundary Ck, Macquarie Uni, EWQCP and Lake Belvedere increased toward a maximum at the end of Winter and decreased abruptly at the beginning of Summer (Figure 7-4). At Upper Colo the inverse of this trend (a decrease though Winter and increase through Summer) was found, while at Narrawang 22 the proportion of males in the population was relatively constant throughout the study period (Figure 7-4). The proportion of males with gonopodial terminal hook complexes (sexually mature males) in 2006 was maximum in October 2006 at Boundary Ck (n=2), EWQCP, Lake Belvedere and Narrawang 22 and in November 2006 at the two reference sites, Upper Colo and Macquarie Uni. (Figure 7-5). Juvenile fish distinguishable as morphologically male (but without developed gonopodia) were first present in the first sampling period following this maximum for all study sites except Macquarie Uni where some were evident at the maximum sampling period (Figure 7-5). At the end of Winter (2006) there was a high proportion (≥20%) of males with developed gonopodia without gonopodial terminal hook complexes at Lake Belvedere, Macquarie Uni and Upper Colo while at other study sites the proportion of these animals was not high until November (EWQCP and Narrawang 22) or never high (Boundary Ck) (Figure 7-5).

There were significant ($p<0.05$) interactions between site and time of collection for both the condition index (BCI) (Fulton's K) and the gonado-somatic index (GSI). The body condition index (BCI) (Fulton's K) in male fish had a distinct peak during March 2006 followed by a rapid decrease and stabilisation through Winter, Spring and Summer 2006 (Figure 7-6). Some inter-annual variation was apparent across the study period with this peak not repeated in March 2007 (Figure 7-6). There were two peaks in female body condition across the sampling periods, one in March 2006 followed by a larger peak in October 2006 (Figure 7-6). This second peak was consistent across the SOP wetlands. At Upper Colo this peak was not evident until November 2006, while at Macquarie Uni the increase was more gradual with the peak not apparent until December 2006 (Figure 7-6). At most study sites there was a gradual decrease in the gonado-somatic index (GSI) of mature male fish between mid-Summer and mid-Winter with minimum GSI in June or August 2006 (Figure 7-7). This was followed by a sharp increase between August and October 2006 reaching maximum values in November 2006 for all SOP wetlands (Figure 7-7). The

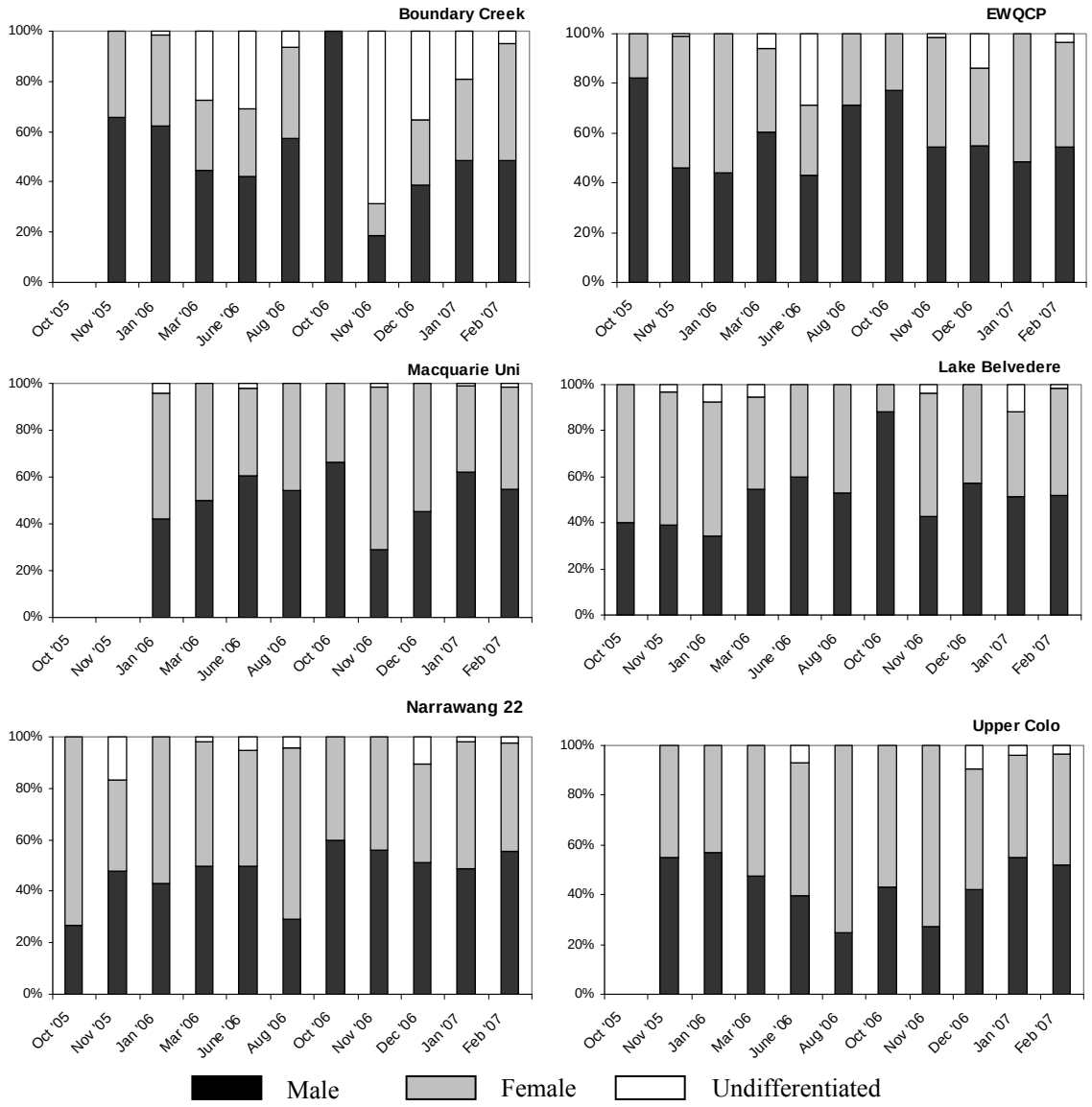


Figure 7-4 Proportion of males, females and undifferentiated individuals in mosquitofish populations at SOP and reference sites.

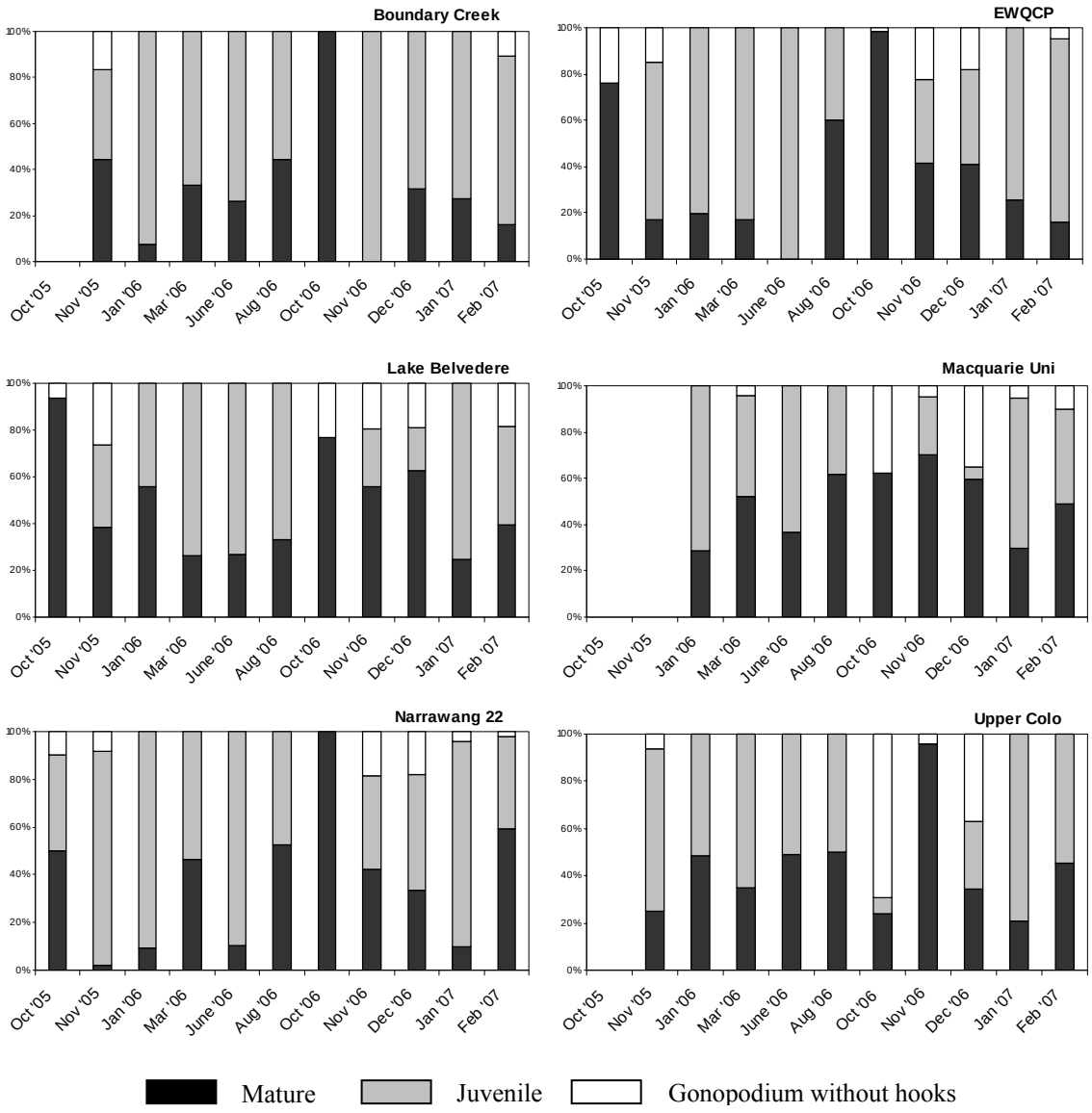


Figure 7-5 Proportion of mature, juvenile and individuals with a gonopodium but without gonopodial terminal hook complexes in male populations of mosquitofish in SOP and reference sites.

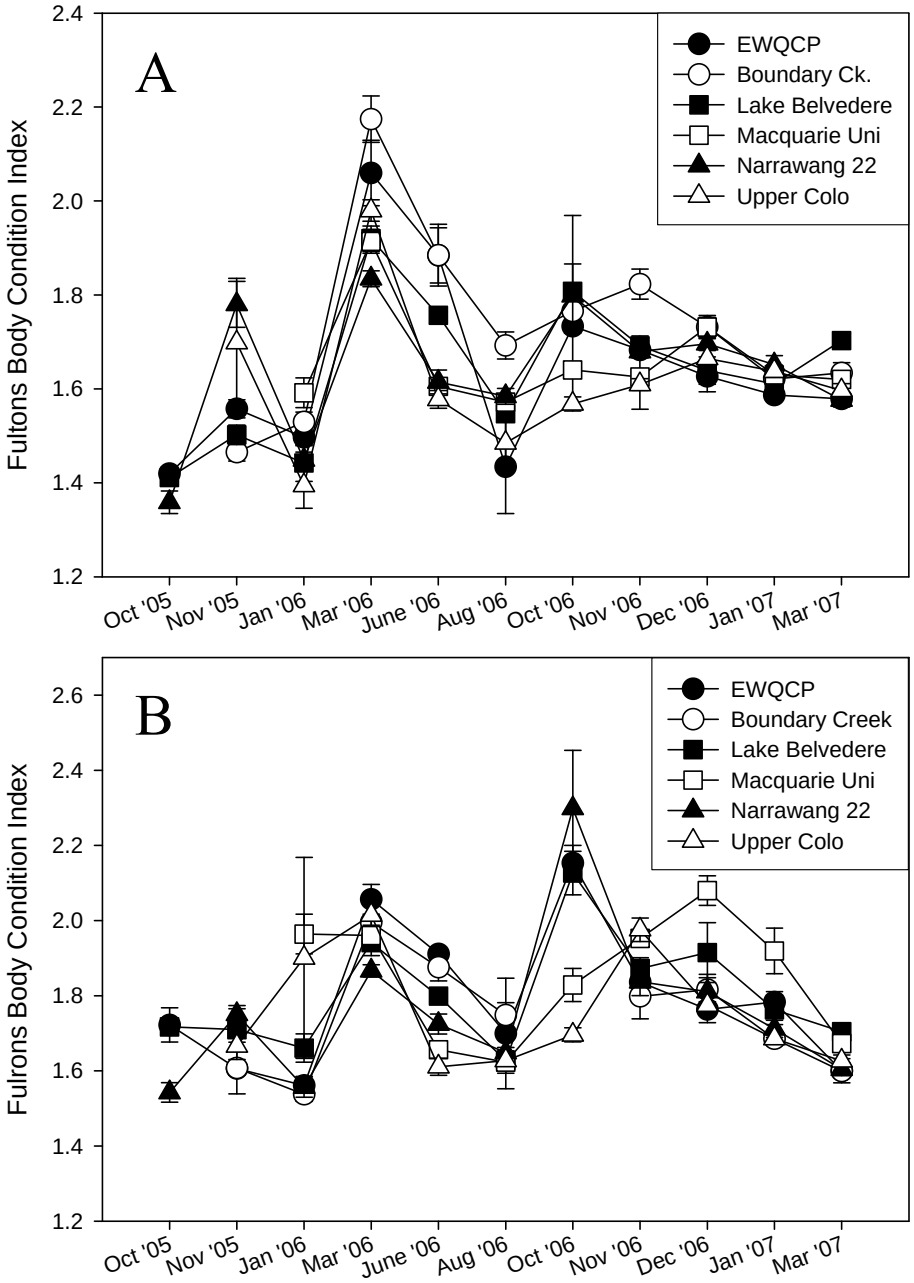


Figure 7-6 Mean (± standard error) of Fulton's K, body condition index (BCI) of A. male, and B. female mosquitofish from SOP and reference sites.

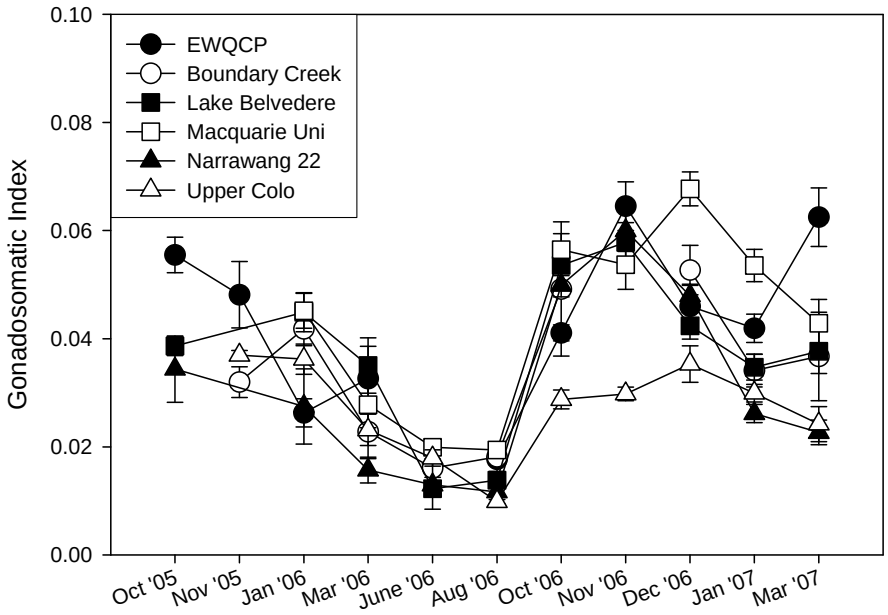


Figure 7-7 Mean (± standard error) of the gonado-somatic index (GSI) for mature (gonopodial terminal hook complex present) male mosquitofish from SOP and reference sites.

GSI of males from Upper Colo increased much less sharply than at other study sites in Spring 2006 and did not reach a maximum until December 2006. Males from Macquarie Uni did not reach maximum GSI until December 2006 (Figure 7-7). The GSI of males from EWQCP showed a secondary peak in March of both 2006 and 2007 (Figure 7-7).

The distribution of standardised gonopodial lengths was bimodal at most study sites over most of the sampling times indicating that fish either had a developed gonopodium or did not have one (Figure 7-8). At EWQCP (November 2005, November 2006, December 2006), Lake Belvedere (March 2007), Macquarie Uni (January 2007) and Narrawang 22 (November 2006, January 2007) there were sampling periods where this bimodality was less pronounced and there were fish with short gonopodia (Figure 7-8). To varying degrees the modal separation was between sexually mature males (those with gonopodial terminal hook complexes) and males without gonopodial terminal hook complexes.

The onset of the breeding period was isolated as October in 2006 (abrupt increase in male GSI (Figure 7-7). In October 2006 there was no relationship between the exposure of the fish to AhR ligands (EROD activity (Chapter 5)) and male GSI or between exposure to AhR ligands and the BCI (Figure 7-9). There was no relationship between male GSI and BCI (Figure 7-9). Multiple regression including sediment and water contamination data from these study sites (Chapter 4) indicated no relationship between overall population condition and wetland contamination and no relationship between the proportion of male fish in the population and wetland contamination (Table 7-2). There was a significant ($p<0.05$) negative relationship between the concentration of ΣDDT in the sediment and the proportion of sexually males in the population ($r^2 = 0.886$) (Table 7-2).

7.5 Discussion

Mosquitofish are an invasive species in Australia and feed opportunistically (potentially out-competing native species for limited food resources). The presence of an established mosquitofish population may, therefore, indicate a system with reduced native fish community structure. Hence, a "healthy" mosquitofish population in the current study does

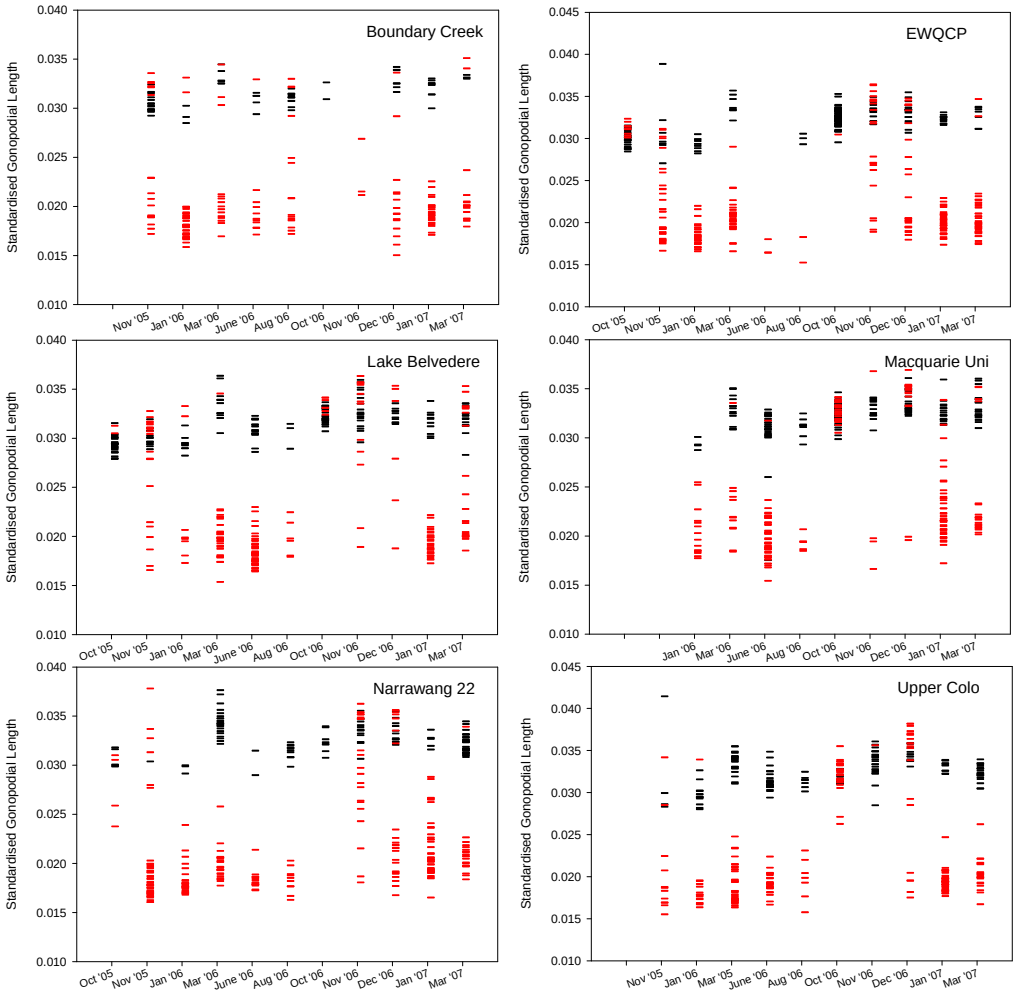


Figure 7-8 Gonopodial lengths of sexually mature (gonopodial terminal hook complex present) – black markers, and sexually immature (gonopodial terminal hook complex absent) mosquitofish from SOP and reference sites. Red = immature, Black = mature.

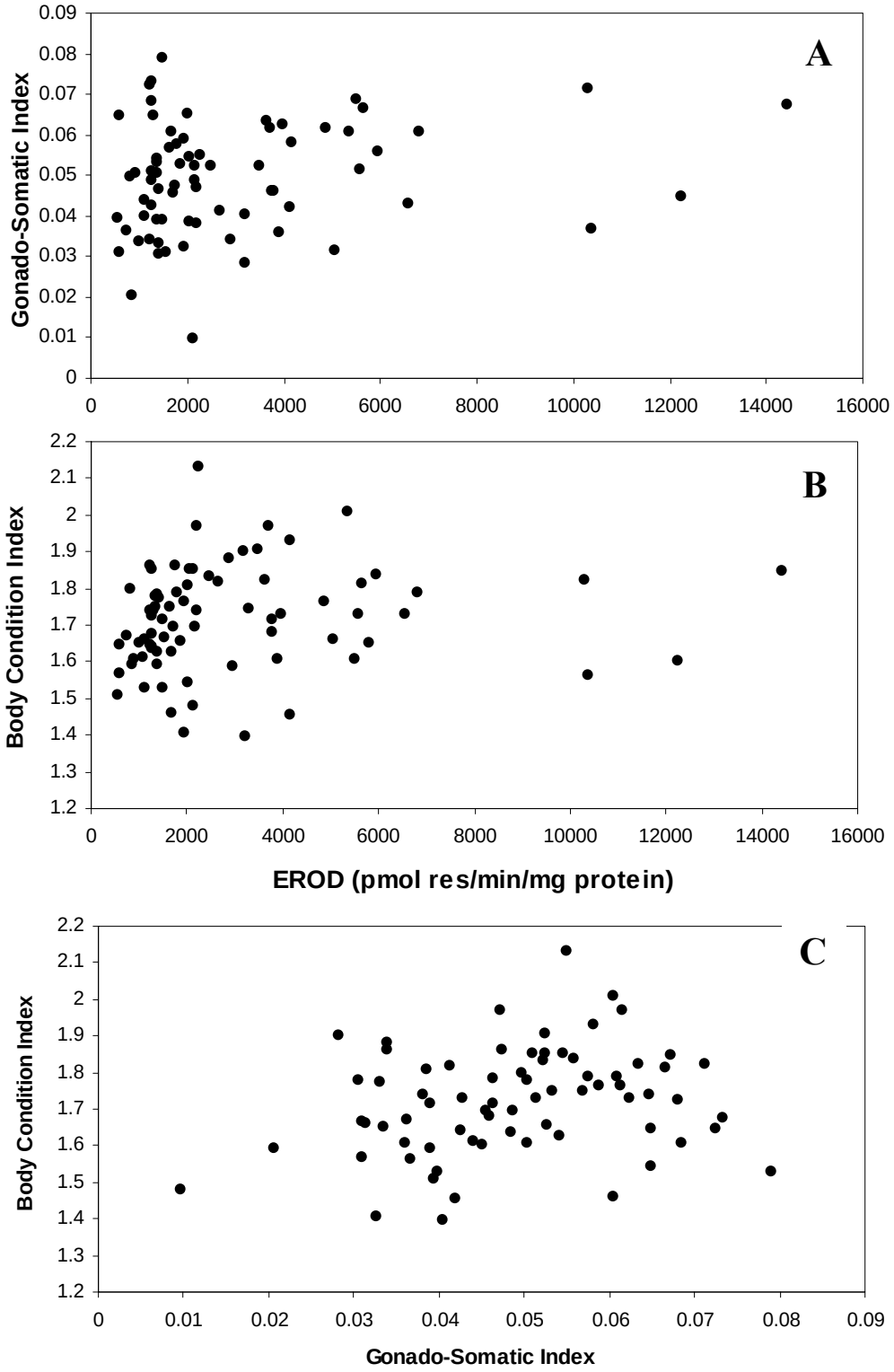


Figure 7-9 Scatter plots of hepatic CYP1A activity (EROD) against A. gonado-somatic index (GSI) and B. body condition index and C. GSI against BCI of mature (gonopodial terminal hook complex) of male mosquitofish from SOP and reference sites.

Table 7-2 Results of multiple regressions between population parameters measured in populations of mosquitofish in SOP and reference sites and contamination data. Contamination data included were sediment TCDDeq, aqueous phase TCDDeq, sediment concentrations of ΣDDT, ΣPCB, ΣPAH, and Σmetals and pore water Σmetal concentration.

	F	Sig. Level	Predictors	Correlation Type	r^2
Population condition[a]	No significant predictors				
Proportion of males[b]	No significant predictors				
Proportion of mature males[c]	24.32	0.39	ΣDDT	-	0.886

[a] Population condition taken from log-log standard length/wet weight regression. [b] Proportion of male fish in whole population. [c] Proportion of male fish which were sexually mature (had gonopodial terminal hook complexes).

not represent a "healthy" ecosystem. The species was selected as it is far more widely available across the study sites than any native species. The results are intended as an assessment of the potential for contamination at the site to affect established fish populations.

There were differences between the study sites in both biotic and abiotic factors. *Gambusia* spp. life-history traits are highly influenced by temperature (Dreze et al. 1998, Koya and Kamiya 2000). In the current study, both daily and seasonal water temperature varied between study sites. Over a small spatial scale (within SOP) seasonal temperature variation was similar. However, diel variation was different between study sites. An interesting comparison can be made between fish populations inhabiting Narrawang 22 and Macquarie Uni. These two wetlands are similar in size, with similar predation risk (both are wading and waterbird habitat, but are not habitat for any other carnivorous fish taxa), and have high levels of primary production and rarely receive flushing inputs. There was a difference between the two study sites in the level of riparian and littoral vegetation. At Narrawang 22 large rushes line the wetland's edge, providing shade and keeping the water temperature up to 2^{o}C lower (at daily Summer maximum). Since temperature has a substantial effect on fish developmental rate the shading effect could result in the differences described in length-weight relationships. The overall fish population condition at these study sites was significantly ($p<0.05$) different, with mosquitofish from Narrawang 22 being lighter for their size than those at Macquarie Uni. However, higher levels of predation have been shown to reduce fish size at birth and maturity and growth rates in guppies (Endler 1995) and the presence of submerged and littoral vegetation can provide efficient refugia from predation (Rozas and Odum 1988); therefore, the reverse trend (higher population condition at Narrawang 22) could be expected.

Impacts of biotic and abiotic stressors on fish populations may cause positive or negative changes in body condition (Munkittrick and Dixon 1989). It is, therefore, difficult to attribute differences in fish body condition to a general toxic effect caused by exposure to contamination. The presence of smaller, less heavy fish at certain wetlands in the current study (particularly Boundary Ck) could be viewed as a general toxic effect. Exposure to

metals (Schlueter et al. 1997, Canli and Atli 2003) and organic pollutants (Jee et al. 2004, Couillard et al. 2005) can result in reduced fish size. Chemical analysis suggests that Boundary Ck has the highest contaminant load (TCDDeq, ΣPCB, ΣDDT, sediment metals) of the wetlands included in this study, but contaminant load was not related to the measured variation in BCI during the breeding period. Mosquitofish from Boundary Ck also had the highest *in vivo* EROD activity, a biomarker indicating exposure to organic contaminants (Chapter 5). However, the other wetland with reduced numbers of large fish was Upper Colo, which, aside from having a relatively pristine catchment, had very low EROD activity (Chapter 5) and organic contaminant concentration (TCDDeq) (Chapter 4). Further, the lack of relationship between EROD activity and BCI at the crucial breeding period suggests no general toxic effect of AhR ligands. It is, therefore, not an insignificant matter to ascribe differences in body condition to any particular biotic or abiotic factor. More likely, differences are the result of complex interactions of biotic and abiotic factors and can be seen as inter-site variation over this medium spatial scale.

Analysis of these geographically isolated populations of mosquitofish indicates variation over both small (within Sydney Olympic Park) and medium (compared with reference sites) spatial scales. In terms of overall population condition, while Macquarie Uni (a remote site) was significantly different to all other study sites and had greater weight for similar length fish than those at all other study sites, the other remote site Upper Colo was grouped with wetlands having the lowest slope suggesting no general geographic cline. Fish from wetlands separated by only small spatial variation also showed different growth characteristics (EWQCP ≠ Lake Belvedere, two wetlands separated by only 1.5 km), yet two other wetlands (Lake Belvedere and Boundary Ck) sharing the same catchment formed a separate intermediate group. So while spatial variation may not play a part in determining differences in overall population condition, catchment characteristics, and therefore contaminant inputs, may. Differences in overall population condition could not, however, be explained by sediment and/or water organic contamination data once more indicating no attributable general toxic effect.

There were significant outliers in the length-weight relationships from the study sites. These fish were small females, which were heavily pregnant suggesting some degree of female precociousness, a phenomenon reported upon exposure to environmental estrogen mimics (Jobling and Tyler 2003). These fish were only present at Upper Colo, Narrawang 22 and Macquarie Uni but there was no evidence of estrogen receptor ligands or estrogenic effects on male fish at these wetlands (Chapter 3). It is unlikely, given the rarity of these fish in the study, that they represent any overall trend toward precociousness at these wetlands but are simply early developing individuals.

Skewing of sex ratio is a known effect of some organic contaminants (Nimrod and Benson 1998, Bayley et al. 2002) and is likely to have population level effects. While there were differences in the sex ratio of mosquitofish populations both between study sites and across time reported here, there is no overall pattern suggesting that this represents anything other than natural variation. At the crucial breeding time, there was no variation in sex ratio that could be explained by contamination further indicating natural variation. Doyle and Lim (2002) have reported a failure of mosquitofish to develop gonopodial terminal hook complexes (i.e., attain sexual maturity) on exposure to 17β-estradiol concentrations greater than 20 ng/L. In the current study there was variation between the study sites in the proportion of males which were mature, through time. During the crucial breeding period variation in the proportion of mature males was explained by the concentration of sediment bound ΣDDT. ΣDDT includes estrogenic *o,p'*-DDT and the potent anti-androgen *p,p'*-DDE which can cause both skewing of sex ratio and delayed development in male fish (Kelce et al. 1995, Mills et al. 2001, Bayley et al. 2002, Papoulias et al. 2003) A low proportion of mature males during the breeding period has a high potential of detrimental impacts at the population level. This finding indicates the importance for monitoring urban wetlands for the presence of DDT and its metabolites. However, it must be noted that the 17β-estradiol concentration above which male mosquitofish fail to develop hooks is 20 ng/L. The estrogenic potency of DDT congeners is far lower than 17β-estradiol and concentrations of ΣDDT measured in this study is lower than 20 ng/L. This result must therefore be considered somewhat tentative and requires confirmation via laboratory investigation.

In sexually mature fish lower male GSI has been associated with decreased reproductive output (Kang et al. 2002). In the current study there was no correlation between EROD activity and GSI during the breeding period indicating that exposure to POPs does not impair seasonal increases in gonad size.

The bimodal pattern in gonopodial lengths in male mosquitofish at the study sites suggest that gonopodia develop rapidly. The substantial overlap between individuals with and without gonopodial terminal hook complex at some study sites may suggest that these develop more slowly once the gonopodium has attained its full length at these wetlands. The major period for gonopodial development was between October and December in 2006. The wetlands with the lowest levels of organic contamination (Upper Colo and Narrawang 22) showed no overlap in the distribution of gonopodial lengths of those with and without gonopodial terminal hook complexes in Autumn 2006, and Upper Colo (but not Narrawang 22) followed a similar pattern in Summer 2006. High levels of natural estrogens (Rawson et al. 2006), anthropogenic estrogen mimics (Dreze et al. 2000, Angus et al. 2005) and sewage effluent (Batty and Lim 1999) have been shown to decrease the development of the gonopodium. Under laboratory conditions the failure to develop a gonopodial terminal hook complex has been reported upon exposure to 17β-estradiol (Doyle and Lim 2002). While similar effects are not reported for the environmental contaminants identified in the current study the possibility remains that the delay in the development of a gonopodial terminal hook complex reported here may be further evidence of the presence of reproductive endocrine disrupting compounds.

7.6 Conclusions

This study has described the differences and similarities between isolated populations of mosquitofish over spatial and temporal scales. There was a great deal variation between study sites over small (within SOP) and medium (compared to reference sites) scales in many of the population variables described here. This indicates the importance of fully characterising reference sites (even if located close to the impacted sites) in studies using mosquitofish. Further, there was a degree of inter-annual variation, which must be taken

into account when designing studies using this species. The study incorporated sites with a known contamination profile and spanned the range of seasons. Attributing differences between mosquitofish populations to any individual pollutant or overall site contamination was not trivial. Only differences in the proportion of males which were sexually mature (had gonopodial terminal hooks) were explained by contamination data (ΣDDT). However, given the small sample size and the relatively low affinity of DDT and DDE for endogeneous steroid receptors this result is considered tentative. There was some evidence of the effects of reproductive endocrine disruption, which requires further investigation. Measures not included here including fecundity, size at birth and age at maturity may provide useful information in future studies examining the population effects of contamination at remediated sites.

8. Weight-of-Evidence Site Assessment

8.1 Introduction

The USEPA framework for ecological risk assessment (ERA) describes three main phases for the conduct of an assessment; problem formulation (identifying the potential stressors and receptors), analysis (characterising both the exposure of the receptor to the stressor and the effects of that exposure) and risk characterisation (an integration of the analysis into an estimation of risk) (Suter 2006). Potential stressors include contamination (e.g., chemical, thermal) to which the receptor (an organism within the environment which may be subjected to the stressor) may be exposed. Responses are quantifiable endpoints, which may constitute exposure and/or impairment and include sub-cellular, cellular, organ, whole animal, population and community level responses. The previous chapters of this thesis have completed the initial two phases of this framework (problem formulation and analysis) and this chapter presents a characterisation of the ecological risk associated with the Sydney Olympic Park (SOP) wetlands studied.

The weight-of-evidence (WOE) approach is a method reaching conclusions based on synthesising the information from multiple lines of evidence (LOE) (Burton et al. 2002b). Some methods are purely qualitative while others integrate data in a more quantitative form. Burton et al. (2002a) set out the key elements of a weight of evidence integration of multiple LOE, but the individual constituents of these elements will, based on the purpose of the integration, necessarily vary between studies (Burton et al. 2002a). The output of most WOE approaches is the WOE matrix, in which the results of all LOE of interest are presented. The method of input may be binomial ("+" or "-") (Chapman 1996, Grapentine et al. 2002), scores (e.g., 1 – 5) (e.g., Bailey et al. 1995, Reynoldson et al. 2002), relative ranks (Wiegers et al. 1998), indices (e.g., invertebrate taxon richness, sediment quality index, ecotoxicological rating (ETR)) (e.g., Soucek et al. 2000, Cherry et al. 2001), a number representing the degree of impairment relative to a reference value (Reynoldson et al. 2000) or combinations of the above. Individual LOE may be weighted based factors such as strength of association and data quality (Menzie et al. 1996, Johnston et al. 2002).

Based on the WOE matrix the risk associated with each site based on all lines of evidence can be evaluated.

In general, the design of the WOE framework and lines of evidence used in its construction are based around the study rationale and the needs of the end-user (e.g., environmental manager or regulatory body) and are, therefore, unique to each study. Where ecological and/or human health hazard and risk are to be evaluated quantitatively some estimate of uncertainty is incorporated in the final result and may cover such aspects as the variance associated with the response and the inherent assumptions associated with the choice of model (Burton et al. 2002a). Where a quantitative estimation of risk is not the key concern of a study (e.g., where sites are to be evaluated qualitatively against some reference condition) an evaluation of uncertainty may include the appropriateness of selected reference conditions and the error associated with the final conclusions.

While WOE approaches are commonly used in contaminated site risk assessment to define remediation goals (Suter 2006), post remediation site assessment using this approach is not well-represented in the literature. This is presumably due to the fact that such studies predominantly form parts of unpublished reports to regulators and industry.

The results presented and discussed previously in this thesis are different LOE used to assess the presence, potential and actual effect of contamination at the different wetlands studied. As such they lend themselves to synthesis using the WOE approach. The aim of this chapter was to synthesise the different LOE previously discussed into a WOE approach in order to discuss the relative ecological risk associated with each wetland. By including reference sites with an expected gradient of impairment the ranking system provides information regarding the efficacy of the remediation program at SOP. It is anticipated that the WOE will identify wetlands of high priority in future ecological monitoring and remediation. An additional aim was to evaluate the appropriateness of the selected LOE for future use in the assessment of sites affected by persistent organic pollutants (POPs).

8.2 Materials and Methods

8.2.1 Sites

Eleven study sites were chosen for inclusion in the site assessment process. Three were off-site reference sites and eight were within Sydney Olympic Park (SOP). The reference sites included one known "dirty" reference site (Homebush Bay), one representing a standard urban impacted reference site (Macquarie Uni) and one "pristine" reference site (Upper Colo). Inclusion of study sites were chosen based on the breadth of data collected across the study.

8.2.2 Selection of Lines of Evidence

A total of nine LOE across different levels of organisation was included in the WOE based on their relevance to the initial aims of the study. Not all LOE previously presented in the thesis were included. Concentrations of ΣPAH, ΣPCB and ΣDDT were included to indicate the potential for POP-related effects in the wetlands studied. Metals were not included as they were not the primary focus of the study. Receptor level *in vitro* responses (estrogen receptor and aryl hydrocarbon receptor ligand binding (E_2eq and TCDDeq)) were included as they provide an integration of potential biological response to different classes of chemicals (some of which were not measured). The hepatic EROD activity of mosquitofish, *Gambusia holbrooki*, was included as a biomarker of exposure to AhR ligands – the principal contaminants of concern. The overall condition of the mosquitofish populations was included as an estimate of general toxic effect and the macroinvertebrate taxon richness was included to suggest a community level response to between-wetland differences in POP contamination. The invertebrate taxon richness LOE was removed from Upper Colo as this study site was very different to all others in terms sediment size composition (a known driver of invertebrate taxon richness) (Chapter 6). Macroinvertebrate abundance was not included as, at certain sites, it was influenced by extrememly high numbers of tolerant taxa (e.g., nematodes at Boundary Creek), which suggests that its utility as an endpoint describing relative ecological health at the study sites is limited. biased Endpoints which did not vary significantly between study sites or which were considered of low ecological importance (aqueous phase E_2eq, mosquitofish gonopodial

characteristics, TCDDeq after agitation and physico-chemical parameters in sediments) were not included in the analysis.

8.2.3 Scoring and Weighting of LOE

The LOE scoring and weighting system employed in the current study is adapted from the semi-quantitative ecotoxicologic rating (ETR) system developed by Soucek et al. (2000) to rank sites affected by acid mine drainage (AMD) in which each LOE was scaled to a proportion of the highest recorded value and averaged within sites to give a percentile score. Cherry et al. (2001) improved on this method by adding a weighting to each LOE based on its ecological sensitivity to the contamination type investigated (AMD). In the current study, values for each LOE at each study site were converted to a percentage of the maximum observed value. Individual LOE were weighted based on relevance to ecological impairment using best professional judgment (Chapman et al. 2002). Chemical concentration data were weighted at 60%, receptor-based assays at 80% and the EROD biomarker of exposure to POPs at 90%. Invertebrate taxon richness and the overall mosquitofish population condition was weighted lower (50 and 20%, respectively) despite their higher degrees of ecological relevance as it was difficult to determine cause and effect based on the current study. Weighted LOE were averaged for each study site. The calculation for the score for each LOE at each study site (S_{LOE}) is presented in Equation 8-1 below.

8.2.4 WOE Matrix and Site Ranking

Weighted LOE scores were built into a WOE matrix and were averaged within individual sites (i.e., each site received an individual score). The calculation of site scores was conducted according to Equation 8-1, where S_{LOE} is the individual score for each LOE at each site, n is the number of LOE used for each site, w_i is the weighting applied to the i-th LOE, r_i is the raw value for the i-th LOE and r_{max} is the maximum observed value across all sites for the i-th LOE.

$$\text{Site Score} = \frac{\sum S_{LOE}}{n} = \frac{\sum_{i=m}^{n} w_i \left[\frac{r_i}{r_{\max}} \times 100 \right]}{n} \qquad \text{(Equation 8-1)}$$

Missing data are explained in previous chapters. Briefly, mosquitofish related endpoints are only available for those study sites with permanent populations of the species. Data for sediment related endpoints (chemistry and *in vitro* tests) are available only where sediment could be collected. In particular, Lake Belvedere sediment consisted of large pebbles within the zone of collection (accessible from the bank of the Lake). The sites were ranked such that higher (towards 1) ranking indicated a wetland with a combination of desirable characteristics.

8.2.5 Uncertainty in Ranking

Standard error about the mean score for each study site was calculated and presented graphically to indicate the uncertainty associated with the final ranking of the study sites. While this method of uncertainty incorporates the value of having increased LOE it does not take into account their weighting and, therefore, their ecological relevance.

8.3 Results and Discussion

This site assessment was designed to identify wetlands, which may require further monitoring/investigation. It is a qualitative ranking of the Sydney Olympic Park (SOP) wetlands to identify the success in returning them to a quality expected of an urban wetland in Sydney. This is accomplished by using the three reference sites, which display a gradient of impairment. The addition of quantitative data and the weighting system is purely for the development of the ranking. Average scores are not representative of the degree of impairment and were, therefore, not examined statistically.

The reference sites chosen for this site assessment represented a range of current and previous landuse. Homebush Bay is a well known, well characterised contaminated site, Macquarie Uni is located in an urban environment and subject to the impacts associated with this (hard surface runoff, aerial deposition etc.) and Upper Colo is a pristine wetland with a catchment made up almost entirely of National Park. These three reference sites cover the breadth of scores and ranks calculated using the approach described above (Table 8-1). In addition, the final score from the WOE approach reflected the expected level of

Table 8-1 The weight of evidence matrix using multiple lines of evidence (LOE) for the eleven sites studied. Ranks are based on the average score for each study site. LOE scores for each study site were calculated and weighted as described in sections 8.1.2.2 and 8.1.2.3 to yield a site score. The site ranking was based on the site score (i.e., the lower the site score the higher its position the ranking). Shaded cells represent no data available.

		Line of Evidence (LOE) Score									Site Score	No. LOE used	Site Rank
		Sed. E_2EQ^A	Sed. $TCDD_{EQ}^B$	Aq. $TCDD_{EQ}^B$	ΣPCB	ΣPAH	ΣDDT	CYP1A Activity[C]	Macro. Invert. Rich.[D]	Fish Popltn. Cond.[E]			
Wetlands in Sydney Olympic Park	Boundary Ck	80	4.9	80	23	14	25	91	50	18	43	9	10
	Lake Belvedere			22				64		18	35	3	9
	Bicentennial Pk	28	6.9	30	7	22	10		39		20	7	5
	Wharf Pond	17	1.3		7	11	14		30		13	6	3
	Narrawang 22		0.8	29	5	1	14	1	19	16	11	8	2
	EWQCP	19	4.5	29	10	60	16	26	32	16	24	9	6
	SWQCP		5.1	55	11	5	54		42		29	6	8
	Nth Water Feat.[F]	17	4.1	26	7	12	12		29		15	7	4
Reference Sites	Homebush Bay	69	80.0		60	54	60				65	5	11
	Macquarie Uni		6.5	52	7	2	18	50	41	20	25	8	7
	Upper Colo		0.5	10				11		15	9	4	1

[a] 17β-estradiol equivalency in sediment as measured using a radioligand competitive binding assay. [b] 2,3,7,8-TCDD equivalency as measured by the H4IIE cell line bioassay. [c] hepatic CYP1A activity measured as EROD activity in sexually mature male mosquitofish. [d] benthic macroinvertebrate taxon richness. [e] overall population condition of mosquitofish inhabiting the wetlands studied was measured as the slope of the linear regression between log standard length and log wet weight. [f] Northern Water Feature.

impact at these sites. This points toward the appropriateness of the LOE chosen for the weight-of-evidence approach.

The ranks for all SOP wetlands fell between the ranks of the pristine reference site (Upper Colo) and the highly impacted reference site (Homebush Bay). Three wetlands (SWQCP, Lake Belvedere and Boundary Ck) were ranked higher than the urban impacted reference site. Boundary Ck is designed as a sink for urban catchment contamination and was, therefore, expected to have a high degree of impairment. The results for SWQCP and Lake Belvedere indicate that these wetlands require ongoing monitoring. Both are habitat for significant bird populations and SWQCP is primary frog habitat. The possibility of bioaccumulation and biomagnification from these sites is high and monitoring should include endpoints associated with chronic exposure to organic contaminants.

All other wetlands had ranks below that of the urban impacted reference site. These are remnant (Wharf Pond, Bicentennial Park) and created (Narrawang 22, Northern Water Feature, EWQCP) wetlands and appear to be impacted to varying degrees by urban contaminant inputs. Whether these input routes are via surface run-off, aerial deposition or some other means requires investigation, if these contaminant pathways are to be closed.

The aim of the current assessment was to qualitatively rank the study sites in an urban context, so absolute evaluation of uncertainty is not critical. An estimate of uncertainty is provided as the standard error about the mean site score (Figure 8-1). This represents the uncertainty associated with the correct ranking of the study sites. While Homebush Bay is clearly ranked lowest, the other study sites form a gradient with significant uncertainty between adjacent ranks. Study sites separated by more than three ranks are more likely to be ranked apart.

In the current study, the number of LOE varied between study sites (see Table 8-1) for reasons outlined above. Burton et al. (2002a) suggest that at least three LOE are required for an assessment to be considered a WOE. In general, the uncertainty associated with assigning a rank to a study site (standard error about the mean site score) will increase with

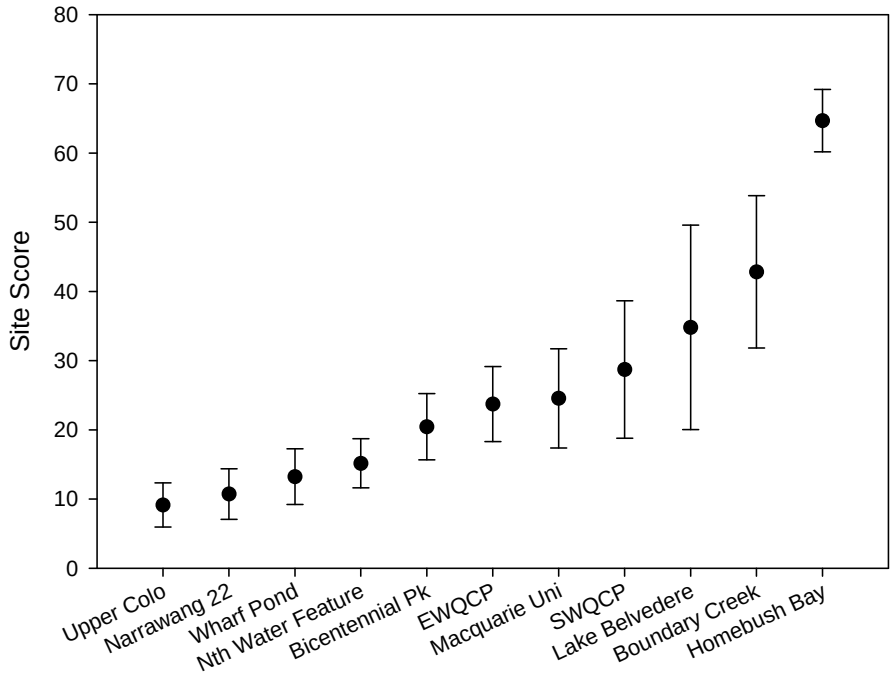


Figure 8-1 Mean (± standard error) of site scores for SOP and reference study sites as calculated from the weight-of-evidence data matrix (Table 8-1).

a decrease in the number of LOE used to calculate the site score. In the current study, only three LOE were able to be employed at Lake Belvedere (the minimum number) and this is reflected by the high standard error about the mean site score (Figure 8-1). The three LOE employed at this wetland, however, were of medium to high ecological relevance (reflected in the assigned weighting) so while this result could be considered of relatively low certainty based on the number of LOE this is counteracted by their ecological relevance. The WOE employed four to nine LOE for all other study sites and greater than six LOE for eight of the eleven study sites (Table 8-1). The certainty associated with the ranking of these sites is, therefore, higher.

The highest ranked SOP wetlands (i.e., with the lowest ecological risk) were Narrawang 22 and Wharf Pond, while the lowest ranked SOP wetland (i.e., with the highest ecological risk) was Boundary Ck. These wetlands represent logical best and worst case ecological risk scenarios in the range of impairment of SOP wetlands. Narrawang 22 and Wharf Pond are adjacent to the Newington Nature Reserve (Figure 2.1) and have catchments, which are in the precincts of the Park and do not include large areas of impervious surfaces. Boundary Ck is a (twice) remediated wetland subject to inputs from a highly urbanised catchment, and was designed to intercept contaminated sediment flowing into the Park. While the two water quality control ponds (EWQCP and SWQCP) were next highly ranked (separated only by a reference site) the major contributing endpoints to their scores were different. At EWQCP, ΣPAH, aqueous phase TCDDeq, and EROD activity influenced the site score while at SWQCP, aqueous phase TCDDeq, ΣDDT, benthic macroinvertebrate richness were important endpoints. This suggests that while the two catchments appear similar important differences remain to be investigated.

These results demonstrate the importance of including reference sites, which incorporate the range of responses expected (i.e., following an expected gradient of impairment), in correct interpretation of study site rankings. If only the pristine reference site was included in the analysis most wetlands would be identified as being impaired. Including the urban impacted site, however, suggests that the wetlands represent the level of impairment

expected for this stressor set. Including the non-remediated contaminated site, Homebush Bay, some degree of success in remediation can be implied.

The semi-quantitative WOE approach is a useful technique for the integration of multiple LOE across different levels of organisation in contaminated site assessment. While there is little evidence in the literature of this approach being used in post-remediation assessment there is no fundamental difference between pre- and post-remediation sites that would prevent its use. Given the breadth of response and ranking calculated for the references sites used in the study, the LOE and scoring methods employed are appropriate for ranking wetlands potentially affected by persistent organic pollutants.

8.4 Conclusions

The remediation and restoration project at Sydney Olympic Park has established a range of wetlands with purposes ranging from wildlife habitat to stormwater amelioration. In terms of ecological risk, these wetlands fall within the range expected of urban impacted wetlands and there is little evidence to suggest the presence of legacy contamination in their water or sediments. However, the studies making up this thesis have suggested that some sites in SOP are subject to current contaminant sources (particularly storm water runoff). In particular the site ranking presented here identifies SWQCP, Lake Belvedere and Boundary Ck as wetlands which should have high priority for inclusion in future ecological monitoring programs and Narrawang 22 as an appropriate reference site against which to measure impairment.

9. Concluding Remarks

9.1 Study Conclusions

This study aimed to investigate the presence and effects of organic contamination in wetlands in the remediated Sydney Olympic Park (SOP) site. Particular emphasis was placed on estrogenic compounds and persistent organic pollutants (POPs; PAHs, PCBs, OCPs, and PCDDs). The study used a suite of investigative tools including chemical analyses, *in vitro* bioassays (estrogen receptor radioligand competitive binding assay and H4IIE cell line bioassay), an *in vivo* biomarker of exposure (hepatic EROD activity), and population (fish) and community (invertebrate) level endpoints. The study also assessed the efficacy of the remediation program in attenuating these compounds and returning the wetlands on the site to a condition similar to that expected for an urban wetland in Sydney. In order to achieve this, a semi-quantitative weight-of-evidence approach was designed and the study sites ranked within the context of a gradient of contamination in remote reference sites.

There were measurable concentrations of estrogen receptor (ER) ligands (measured *in vitro* by a radioligand competitive binding assay) in the sediments of all the SOP wetlands studied, and in the dissolved fraction in some. While these did not translate to any effect on male secondary sexual characteristics in the mosquitofish, *Gambusia holbrooki* there is a possibility that effects could be seen in other taxa where the threshold of effect is lower. Further, this lack of observed impact of measured ER ligands may point to potential anti-estrogenic effects, which were not examined in this study.

Chemical analysis and *in vitro* bioassay (H4IIE) indicated measurable concentrations of POPs at most study sites. Even wetlands created during the remediation process (e.g., Narrawang 22) contained concentrations of sediment bound ΣPCBs, ΣPAHs and TCDDeq. The results suggest that the sources of contamination are related to the catchment of these wetlands rather than the presence of legacy material. Due to the relatively small catchment area of some of the wetlands aerial deposition of contaminants is likely to be a significant

source. The measured concentrations of ΣPAHs in the sediments of the water quality control pond, EWQCP, were exceptionally high (similar to those measured in the known contaminated sediments in the adjacent Homebush Bay). The source of ΣPAHs is likely to be the paved surfaces of the Park but requires further investigation.

Some individual SOP wetlands remain of concern for certain endpoints. Mosquitofish from Boundary Ck had significantly higher hepatic CYP1A activity than fish from the pristine reference site (used for basal activity estimation), suggesting exposure to AhR ligands. There is evidence that where the bioavailability of POPs to fish populations is of concern (i.e., where the presence of bird populations makes biomagnification a possibility) the disturbance of sediments should be avoided. Benthic macroinvertebrate taxon richness was very low across most of the SOP wetlands. While this is the expected response for urban impacts, the absence of pollution sensitive taxa at this low trophic level indicates that ecosystems at these wetlands may be somewhat impaired.

The weight-of-evidence approach used in this study ranked the SOP wetlands within the context of a reference gradient of suspected contaminant related impairment. The reference sites were ranked as expected based on prior knowledge and suspected contaminant influences. The chosen lines of evidence across multiple levels of organisation are, therefore, considered both appropriate and sufficient in the assessment of persistent organic pollutant (POP) impacted wetlands. The weight-of-evidence approach suggests that the SOP wetlands are, post-remediation, within the bounds expected for urban impacted sites in Sydney, in terms of the presence and potential receptor exposure to contaminants. The ranking indicates that Boundary Ck is the wetland of most concern within SOP. This wetland is subject to the highest off-site urban catchment influences and is designed to reduce pollutant loads into the Park by sedimentation. In this respect, the wetland does fulfill its role. However, it is also wading bird and waterbird habitat and must be monitored into the future with consideration of potential biomagnification and off-site migration of contaminants.

The remnant Wharf Pond and the created wetland, Narrawang 22, were ranked as the wetlands of least concern. These are situated in the Newington Nature Reserve and adjacent to it, respectively. These results are encouraging given their high ecological value as endangered frog and migratory bird habitat. Narrawang 22 is an appropriate reference wetland for inclusion in future monitoring of the SOP wetlands.

9.2 Future Research Directions

The results presented in this thesis imply that, while there is no necessity for immediate remediation of any of the wetlands studied within SOP, there are instances where further research is pertinent, particularly in the identification of contaminant source(s). In particular the creation of a mass balance of persistent organic pollutant flux between the catchment, aerial deposition, sediment, water column and biota at wetlands of high concern (e.g., EWQCP and Boundary Ck) would be valuable in determining the utility of any particular method of restricting or closing the current contamination pathways. This study has shown that persistent organic pollutants are common contaminants in the urban wetlands of Sydney, indicating need for a wide-scale examination of their sources and fate in the freshwater systems of the Sydney metropolitan area.

These results should form the basis of an ongoing monitoring program focusing on the bioavailability of persistent organic pollutants to the aquatic biota and resident water bird populations of the SOP wetlands. SOP presents a unique opportunity to examine the effects of a worlds-best-practice remediation project in fulfilling goals, not only in the short-term but into the future.

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Appendix A – Method: Estrogen Receptor Isolation

UTS SOP: EDC 1.1 – Estrogen Receptor Isolation

The following describes the standard method for isolating estrogen receptors (by differential centrifugation) from a sheep uterus. The uterus should be snap frozen (liquid nitrogen) as soon as possible after slaughter and the isolation procedure carried out within 2 months. Experience has shown that different uteri differ greatly in the number of high affinity receptors. The receptor characterisation protocol should be carried out prior to assay in the competitive binding assay

Note: As the estrogen receptor protein degrades quickly at room temperature all manipulations should be carried out in cold room where possible or in ice.

Equipment Required
Medium Velocity refrigerated centrifuge (1000g)
Ultra centrifuge (104,000g)
Ultra turax homogeniser
Grater
TEDG Buffer (Tris-Base, EDTA, DDT, Glycerol, MilliQ water)
2 d.p. balance
Clean rinsed centrifuge tubes
Microfuge tubes
Repeater Pippettor

Method

1. Turn on medium centrifuge and set to 4^{o}C to cool down
2. Place ultracentrifuge rotor in cold room to chill while prep.
3. Remove uterus from –80^{o}C freezer and weigh.
4. Place in Liq N_2
5. Grate uterus
    a) This is easiest if done in sections.
    b) Break up, if possible.
    c) Work on one section while the others are in Liq N_2. Once tissue becomes soft, place back in Liq N_2.
6. Weigh tissue as it is grated (4-5 batches) and place in beaker with ~100ml TEDG buffer.
7. When all is grated add TEDG to make up to ratio 50mg:1ml.
    a) As tubes hold 40ml – no more than ~1.75g / tube
    b) With 8 tubes = no more than 14g tissue
    c) If there is more tissue – can concentrate to no more than 30mg:1ml
8. Homogenise in beaker (3-4 passes cooling the probe after each pass)
    a) To cool probe – place in measuring cylinder in ice for 2 mins
9. Decant into polycarbonate tubes and balance tubes using 2 pan balance
10. Centrifuge at 1000g for 10 min @ 4^{o}C.

11. Rinse in TEDG buffer and chill Oakridge bottles.
12. Remove tubes from centrifuge and pour the supernatant into the Oakridge bottles. Fill tubes to shoulder only (~26ml)
13. Balance tubes using balance to 2 dec. places
14. Centrifuge at 104,000g at 4^{o}C for 1 hour (with T865 rotor in Biochem 104,000g = 32,000rpm)
15. Place microfuge tubes in rack and collect liq N_2 in thermos
16. Pour off supernatant into beaker.
17. Using repeater pipettor aliquot into tubes and place in liq N_2.
18. Place in –80 freezer
19. Tubes contain low-salt unoccupied cytosolic receptors

Appendix B – Method: Estrogen Receptor Characterisation

UTS SOP: EDC 1.2 Estrogen Receptor Characterisation

This protocol describes the method for characterizing a preparation of mammalian estrogen receptors in terms of number and binding affinity. It should be conducted on each new receptor preparation prior to use in the estrogen receptor competitive binding assay.

Equipment Required
TEDG buffer
Radiolabelled (^{3}H) Estradiol (^{3}H-E_2)
pH meter
1M NaOH and 1M H_2SO_4 for adjusting pH
Scintillation counter, cocktail and vials
Refrigerated centrifuge
Dextran T70
Activated charcoal

Method

1. Prepare 1L TEDG buffer and adjust pH to 7.2 (note TEDG buffer must be discarded at the end of the day.
2. Dilute ^{3}H-E_2 to required concentration in absolute ethanol based on breakdown rate. Breakdown concentrations are kept with the tracer.
3. Prepare Standards (work in ice tray as much as possible)
    - Place rack in ice tray
    - Label small (15ml 12x75mm) borosilicate tubes according to prepared assay setup sheet (see Appendix B). Place tubes in rack.
    - Dilute E_2 stock solution to 2μM. Primary stock is 2mM in EtOH
    - Serially pipette volumes of E_2 in tubes to appropriate concentrations
    - Serially pipette volumes of hot E_2 in tubes to appropriate concentrations
    - Add buffer to ^{3}H-E_2 tubes, E_2 tubes and TB tubes as per Table A2.1
4. Prepare assay tubes
    - Dispense 100uL of relevant standards into each tube (duplicate each assay tube). See Table A2.1 for example of tube setup
    - Thaw out on receptor preparation on ice and add 50uL to appropriate assay tubes (Table A2.1)
    - Place tray with assay tubes in fridge in overnight (18 hours)
    - Place centrifuge buckets are in centrifuge and turn on and chilling to 4^{o}C.
5. Termination
    - Prepare 100ml TEDG buffer
    - Add 450uL buffer to 3 assay tubes as blanks
    - Weigh activated charcoal and Dextran T70
    - Add 0.5g charcoal and 0.05g Dextran to TEDG
    - Mix well (preferable with magnetic stirrer). Keep chilled on ice.

- Add 200uL of TEDG/DCC solution to each tube except TB tubes and buffer blanks
- Vortex each tube on hand vortex mixer and place in chilled centrifuge buckets
- Place in centrifuge to incubate for 12 minutes.
- Centrifuge at 3000rpm for 12 minutes.
- Remove from centrifuge and place in rack on ice
- Pipette supernatant (450uL) into appropriate labeled scintillation vials. Carefully avoid pipetting any of the charcoal pellet.
- Add 2.5ml scintillation cocktail and cap tubes

6. Scintillation Counting
    - Wipe outside of vials well and shake well
    - Place scintillation vials (in order) into counter racks
    - Place racks in counter with a blank rack after last rack as a stop "indicator"
    - Turn on computer and printer
    - Protocol is under user: Labmaster and protocol: AnneC
7. Data Analysis
    - Counter CPM data are entered into the "ER_characterisation.xls" spreadsheet which will calculate
        i. Specific activity (CPM per fmol) of the tracer (^{3}H-E_2).
        ii. Total Binding (TB) – amount of ^{3}H-E_2 remaining after charcoal washing in absence of E_2 competitor
        iii. Non Specific Binding (NSB) – amount of ^{3}H-E_2 remaining after charcoal washing in presence of excess E_2 competitor
        iv. Specific Binding (SB) – amount of binding to receptor sites (= total binding – non-specific binding
    - From these values the B_{max} (maximum binding capacity of the receptor preparation) and K_d (the receptor dissociation constant) are calculated in two ways (the non-linear regression is generally considered more accurate but the fit of the linear equation can be descriptive of the accuracy of the assay.
        i. By linear regression (SB/F = a(SB) + b) of SB/F vs. SB where F is "free radioligand" (= tracer concentration – TB). $B_{max} = (-b/a)$, $K_d = (-1/a)$
        ii. By non-linear regression of SB vs. [^{3}H-E_2]:
            $SB = (B_{max} * [^3H\text{-}E_2])/(K_d + [^3H\text{-}E_2])$

Table B-1

	E2*			Inert E2			Buffer	Cytosol ER prep		TEDG/ DCC	Final volume
Tube #	Initial [ ] nM	Vol uL	# Final [ ] nM	Initial [ ] nM	Vol uL	Final [ ] nM	Vol uL	Vol uL	total vol before addition of charcoal	Vol uL	uL
1	0.1	100	0.025				250	50	400	200	600
2	0.1	100	0.025				250	50	400	200	600
3	0.3	100	0.075				250	50	400	200	600
4	0.3	100	0.075				250	50	400	200	600
5	0.6	100	0.15				250	50	400	200	600
6	0.6	100	0.15				250	50	400	200	600
7	0.8	100	0.2				250	50	400	200	600
8	0.8	100	0.2				250	50	400	200	600
9	1	100	0.25				250	50	400	200	600
10	1	100	0.25				250	50	400	200	600
11	3	100	0.75				250	50	400	200	600
12	3	100	0.75				250	50	400	200	600
13	6	100	1.5				250	50	400	200	600
14	6	100	1.5				250	50	400	200	600
15	10	100	2.5				250	50	400	200	600
16	10	100	2.5				250	50	400	200	600
17	30	100	7.5				250	50	400	200	600
18	30	100	7.5				250	50	400	200	600
19	0.1	100	0.025	10	100	2.5	150	50	400	200	600
20	0.1	100	0.025	10	100	2.5	150	50	400	200	600
21	0.3	100	0.075	30	100	7.5	150	50	400	200	600
22	0.3	100	0.075	30	100	7.5	150	50	400	200	600
23	0.6	100	0.15	60	100	15	150	50	400	200	600
24	0.6	100	0.15	60	100	15	150	50	400	200	600
25	0.8	100	0.2	80	100	20	150	50	400	200	600
26	0.8	100	0.2	80	100	20	150	50	400	200	600
27	1	100	0.25	100	100	25	150	50	400	200	600
28	1	100	0.25	100	100	25	150	50	400	200	600
29	3	100	0.75	300	100	75	150	50	400	200	600
30	3	100	0.75	300	100	75	150	50	400	200	600
31	6	100	1.5	600	100	150	150	50	400	200	600
32	6	100	1.5	600	100	150	150	50	400	200	600
33	10	100	2.5	1000	100	250	150	50	400	200	600
34	10	100	2.5	1000	100	250	150	50	400	200	600
35	30	100	7.5	3000	100	750	150	50	400	200	600
36	30	100	7.5	3000	100	750	150	50	400	200	600
37	0.1	100	0.025	for determining total dpms			500				600
38	0.1	100	0.025	for determining total dpms			500				600
39	0.3	100	0.075	for determining total dpms			500				600
40	0.3	100	0.075	for determining total dpms			500				600
41	0.6	100	0.15	for determining total dpms			500				600
42	0.6	100	0.15	for determining total dpms			500				600
43	0.8	100	0.2	for determining total dpms			500				600
44	0.8	100	0.2	for determining total dpms			500				600
45	1	100	0.25	for determining total dpms			500				600
46	1	100	0.25	for determining total dpms			500				600
47	3	100	0.75	for determining total dpms			500				600
48	3	100	0.75	for determining total dpms			500				600
49	6	100	1.5	for determining total dpms			500				600
50	6	100	1.5	for determining total dpms			500				600
51	10	100	2.5	for determining total dpms			500				600
52	10	100	2.5	for determining total dpms			500				600
53	30	100	7.5	for determining total dpms			500				600
54	30	100	7.5	for determining total dpms			500				600
55	Buffer	Blank									
56	Buffer	Blank									

Appendix C – Method: Estrogen Receptor Competitive Binding Assay

UTS SOP EDC 1.3: Estrogen Receptor Binding Assay

This protocol describes the performance of the Estrogen Receptor Competitive Binding Assay to evaluate estradiol equivalence (E_2eq) in environmental samples. Extracted water and sediment samples stored in DMSO can be used in the assay. The concentration factor of the extraction process must be included in the final calculation for E_2eq in the original sample.

Equipment Required
TEDG Buffer
pH meter and adjustment solutions (1M NaOH and 1M H_2SO_4)
^{3}H-E_2 (stock solution concentration originally = 0.05mCi/ml)
Refrigerated centrifuge
Small borosilicate assay tubes
E_2 standard solution (2μM)
Scintillation counter, cocktail and vials
Dextran T70
Activated charcoal

Method

1. Dilute Tracer
    - Bring radiolabelled tracer to room temp (stored in freezer)
    - Dilution factor calculated based on decomposition rate of undiluted tracer
    - Dilute to 2nM in TEDG buffer
2. Prepare Standards and Samples
    - Label small (15ml 12x75mm) borosilicate tubes according to prepared assay setup sheet (see Appendix B)
    - Dilute E_2 stock (2mM in 100% EtOH) to 2μM
    - Dilute samples in TEDG buffer such that the highest concentration has a storage solvent (DMSO) concentration of not more than 0.1%
    - Serially pipette volumes of E_2 in tubes based on Appendix C.
    - Serially pipette volumes of ^{3}H-E_2 in tubes based on Appendix C
    - Add buffer to ^{3}H-E_2 tubes, E_2 tubes and TB tubes
    - Add diluted samples to appropriate assay tubes
    - Assay tubes with only ^{3}H-E_2 are included for evaluation of the total activity of the tracer.
2. Prepare assay tubes
    - Dispense 100uL of relevant standards into each tube (duplicate each assay tube).
    - Thaw out characterised ER prep on ice and add 50μL ER prep to appropriate assay tubes using repeater pipettor

- Place trays with tubes in refrigerator overnight (18 hours). This allows the ^{3}H-E_2 and the competitor (the E_2 standard or the sample) to compete for receptor binding sites and an equilibrium state to be reached).
- Place centrifuge buckets are in centrifuge and turn on and chilling to 4^{o}C

3. Termination
   - Prepare 100ml TEDG buffer as described above
   - Add 450uL buffer to 3 assay tubes as blanks
   - Add 0.5g activated charcoal and 0.05g Dextran to TEDG (TEDG/DCC)
   - Mix well and keep chilled on ice.
   - Add 200uL of TEDG/DCC solution to each tube except TB tubes and buffer blanks
   - Vortex each tube on hand vortex mixer and place in chilled centrifuge buckets
   - Place in centrifuge to incubate for 12 minutes.
   - Centrifuge at 3000rpm for 12 minutes.
   - Pipette supernatant (450uL) into appropriate labeled scintillation vials. Carefully avoid pipetting any of the charcoal pellet.
   - Add 2.5ml scintillation cocktail and cap tubes
4. Scintillation Counter
   - Wipe outside of vials well and shake well
   - Place scintillation vials (in order) into scintillation racks
   - Place racks in counter with a blank rack after last rack as a stop "indicator"
   - Turn on computer and printer
   - Protocol is under user: Labmaster and protocol: AnneC
   - Place floppy disk in drive and enter protocol details into counter. Press Start
5. Data Analysis
   - Enter CPM data into "ERBA_workbook_blank.xls" with sample names (This spreadsheet is arranged for the analysis of 5 dilutions of each sample).
   - Enter extraction and sample dilution data for calculation of Eeq in original sample and the detection limit of the assay
   - The spreadsheet will calculate the E_2 standard curve (4-parameter) giving a value for the concentration for 50% binding (EC50). a = curve minimum, b = curve maximum, x = EC50, d = curve slope at EC50.

$$y = \frac{a + (a - b)}{1 + 10^{((c - \log x) * d)}}$$

   - Sample data are fitted to 4-parameter curves giving an EC50 for each sample binding to estrogen receptors
   - Eeq = (sample EC50)/(standard curve EC50)

Table C-1

	Tube #	E2*	E2	Sample	TEDG	ER Prep	Inc Vol	DCC	Final Vol
BB	1				600		600		600
BB	2				600		600		600
TCR	3	100			500		600		600
TCR	4	100			500		600		600
TB	5	100			250	50	400	200	600
TB	6	100			250	50	400	200	600
E2 2000	7	100	100		150	50	400	200	600
E2 2000	8	100	100		150	50	400	200	600
E2 200	9	100	100		150	50	400	200	600
E2 200	10	100	100		150	50	400	200	600
E2 20	11	100	100		150	50	400	200	600
E2 20	12	100	100		150	50	400	200	600
E2 2	13	100	100		150	50	400	200	600
E2 2	14	100	100		150	50	400	200	600
E2 0.2	15	100	100		150	50	400	200	600
E2 0.2	16	100	100		150	50	400	200	600
E2 0.02	17	100	100		150	50	400	200	600
E2 0.02	18	100	100		150	50	400	200	600
E2 0.002	19	100	100		150	50	400	200	600
E2 0.002	20	100	100		150	50	400	200	600
S1 - 1	21	100		100	150	50	400	200	600
	22	100		100	150	50	400	200	600
S1 - 2	23	100		100	150	50	400	200	600
	24	100		100	150	50	400	200	600
S1 - 3	25	100		100	150	50	400	200	600
	26	100		100	150	50	400	200	600
S2 - 1	27	100		100	150	50	400	200	600
	28	100		100	150	50	400	200	600
S2 - 2	29	100		100	150	50	400	200	600
	30	100		100	150	50	400	200	600
S2 - 3	31	100		100	150	50	400	200	600
	32	100		100	150	50	400	200	600
S3 - 1	57	100		100	150	50	400	200	600
	58	100		100	150	50	400	200	600
S3 - 2	59	100		100	150	50	400	200	600
	60	100		100	150	50	400	200	600
S3 - 3	61	100		100	150	50	400	200	600
	62	100		100	150	50	400	200	600
↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
S10 - 1	69	100		100	150	50	400	200	600
	70	100		100	150	50	400	200	600
S10 - 2	71	100		100	150	50	400	200	600
	72	100		100	150	50	400	200	600
S10 - 3	73	100		100	150	50	400	200	600
	74	100		100	150	50	400	200	600

Appendix D – Method: Tissue Culture

UTS SOP: EDC – 3.1 – Maintenance of the H4iiE rat hepatoma cell line

This SOP describes the standard method for the initiation and maintenance of the H4iiE rat hepatoma cell line for use in the H4iiE assay for determination of TCDD (or other aryl hydrocarbon ligand) equivalence in environmental samples. This work should only be carried out by personnel who are trained in using aseptic technique. Unless specified all work should be carried out in a Type II Laminar Flow cabinet (UTS 04.06.14).

Required Reagents

Trypsin/EDTA 5% stock solution:(Invitrogen 15400-54 – 100ml) aliquot in 10ml falcon tubes and store at -20^{o}C.

Trypsin/EDTA 1% working solution: Thaw a 10ml aliquot of 5% Trypsin/EDTA in a water bath at 40^{o}C. Dilute 1:5 with sterile Phosphate Buffered Saline (PBS) in a 50ml falcon tube. Store at -20^{o}C after use.

Phosphate Buffered Saline (PBS): Dissolve 5 PBS tablets (Sigma-Aldrich P4417-100TAB) in 1L ultrapure H_20 in 1L Schott bottle. Sterilise in autoclave (120^{o}C for 20mins). Store at 4^{o}C until use.

Foetal Bovine Serum: (Source and Code): FBS varies from batch to batch and should be tested prior to ordering large quantities. New bottles should be heat-inactivated before addition to media. Thaw the bottle at 37^{o}C and place in a 60^{o}C water bath for 30mins swirling a few times to ensure even heating. After heating cool immediately (in fridge). Aliquot in 25 ml lots and refreeze.

L-Glutamine (200mM): (Gibco: 21051-024): Dissolve 1.46g ini 50 ml ultrapure H_2O, sterilise by passing through a 0.22μm syringe filter, and aliquoted to 10ml lots. Store at -18^{o}C until use

Gentamycin: (Gibco: 15710-064): Comes in batches of 10x5ml sterile bottles with relatively short shelf life.

HEPEs Buffer (2.5M): (Sigma: H6147-500G): Dissolve 29.79g in 50ml ultrapure H_2O and sterilise by passing through a 0.22μm syringe filter. Aliquote into 10ml lots in 15ml falcon tubes. Store at -18^{o}C until use

Dulbeccos Modified Eagles Media: Add 25ml Foetal Bovine Serum (FBS), 10 ml 200mM L-Glutamine, 1.25ml Gentamycin and 0.5ml 2.5M HEPEs to 500 ml DMEM (Gibco 11054-020). Store at 4^{o}C until use.

Method

N. B. The following methods must be followed in a TypeII Laminar Flow Cabinet

1. Cell Line Initiation

- Warm media to 37^{o}C in water bath
- Add 30ml to T75 tissue culture flask (Source and Code).
- Thaw cells by warming in hands
- Pipette all cells into flask
- Cap and place flask in incubator (37^{o}C, 5% CO_2)
- Leave to attach for 24 hours.
- Decant media into waste container (containing approx 50ml bleach)
- Add 25ml media to flask.
- Return to incubator.
- Incubate for 72hours (over the weekend)

Note: The replacement of media after 24hours is essential as it removes the DMSO (which inhibits cell growth) from the culture.

2. Cell Passaging

A passage is defined as the exposure of the cells to Trypsin. It is necessary to replace media and continue growth at optimal rates. The method below is for a 1:3 split of the cell culture. This is best for maintaining optimal growth rates. Cells are passaged on Monday, Wednesday and Friday. It may be necessary to lower the seeding density on Friday to account for an extra day's growth.

- Warm media, Trypsin/EDTA working solution and PBS (sterile) in 37^{o}C water bath for 30mins
- Examine the culture flask and continue if cells are >80%confluent. At confluency <80% cells can be passaged if necessary but seeding rates will need to be decreased at the workers discretion.
- Decant media into waste container. It is important to ensure that as much of the media is removed from the flask as it prevents Trypsin from working
- Wash the cell monolayer with approx 30ml PBS (sterile)
- Decant wash solution into waste container
- Add 2ml Trypsin/EDTA working solution to the flask and ensure that the whole monolayer is covered.
- Return the flask to the incubator for 8-10 mins.
- Mark 3 new T75 flasks with name, cell line, date and passage number
- Add 25mls media to each flask
- Remove flask from the incubator and visually inspect. Cells should be becoming loose on the surface of the flask and should come completely

loosen with gentle tapping. If not return to the incubator and check again after 2 mins.

- Add 10ml media to flask and pipette the cell suspension up and down a few times to separate the cells (clumps of cells are undesirable).
- Pipette up all 12mls of the cell suspension and add 4 mls to each of the new marked T75 flasks.
- Cap the flasks and return to the incubator.

Appendix E – Method: H4iiE Cell Counting

UTS SOP: EDC – 3.2 – Cell Counting for H4iiE Assay

This SOP describes a standard method for counting H4iiE cells. The H4iiE requires a reasonable accurate estimate of cell density for addition to assay plates. Note that absolute accuracy is not required as fluorescence readings are standardised for the amount of protein per cell as a surrogate for the cell density. As a rough guide 2xT75 flasks at >80% confluency will be sufficient to create 2xassay plates. This will vary greatly however with actual cell density.

Equipment Required

- Inverted Microscope
- 50ml Falcon Tubes
- 10ml Falcon Tubes
- Haemocytometer
- Pipettes – 1000ul, 100ul
- Sterile Pipette tips – Blue, Yellow
- Calculator
- Power pipettor
- 10ml. 25ml transfer pipettes

Reagents Required

- PBS (Sterile)
- DMEM
- Trypsin/EDTA working solution
- Trypan Blue

Method

1. Preparation of cells for counting.
    - Calculate the required number of culture flasks required for the number of samples and grow in accordance with UTS SOP: EDC – 3.1.
    - Detach cells using the Trypisn/EDTA as described in UTS SOP: EDC – 3.1
    - Once cells are detached add 5ml media to each flask to deactivate the trypsin/EDTA.
    - Pipette the suspension in each of the flasks up and down a few times to separate the cells and pipette them into a 50ml Falcon tube and cap.
    - Add 50uL of Trypan Blue solution to 3x10ml falcon tubes
    - Set up the haemocytometer with the supplied cover slip under the inverted microscope and focus on the grid ensuring that it is clean. If not clean with alcohol.
    - Add 50uL of cell suspension to one of the Trypan Blue tubes and mix well.
    - Take 50uL of this suspension and dilute it twice more using the remaining Trypan Blue tubes. The result is an 8 times dilution of the original suspension

- Carefully pipette a small amount of the final dilution under the cover slip of the hemocytometer so that the chamber is just filled.

2. Counting the cells
   - Inspect the stained suspension under the microscope. Cells should not be clumped and should have a clear "halo" around them. Cells that are stained dark blue or do not have a halo should not be counted.
   - Count the number of cells in each of the four corner sections of the chamber. Count cells touching the top and left edges of each square and not the bottom and right edges.
   - Record the value for each section.

3 Calculation of cell density and required dilution factor
Calculate the average of the four measures and calculate the density of the cells using the equation below:

$$\text{Density (cells.ml}^{-1}) = \text{Average Count} \times 8 \times 10000$$

Dilution Factor

Volume under corner square = 0.0001ml

e.g., Counts

1	23
2	26
3	18
4	28
Average =	23.75

$$\text{Density} = 23.75 \times 8 \times 10000 = 1.9 \times 10^{6} \text{ cells.ml}^{-1}$$

Appendix F – Method: H4iiE Assay

UTS SOP: EDC – 3.3 – H4iiE Assay

The H4iiE assay is used for the determination of 2,3,7,8-TCDD (or other aryl hydrocarbon ligand) equivalence in environmental samples. The assay should only be performed by those who have been specifically trained in its operation as it involves the use of a number of hazardous chemicals.

Equipment Required
Multi-Channel Pipettor
Pipettes 10ul, 100ul, 1000ul, 5ml
Sterile pipette tips: white (reach), blue, yellow
Non-sterile pipette tips: yellow, blue, 5ml white
70ml reservoirs: sterile (1), non-sterile (7)
Fluorescence plate reader: e.g., Fluostar Optima (Source and Code)
Pierce BCA Protein assay kit (Source and Code)
Sterile single wrapped 96 well plates (Source and Code)

Stock Reagents Required:

- **Resorufin Stock Solution (1mM):** Dissolve 0.47mg resorufin (source and code) in 2ml DMSO. Store in 100uL aliquots at -80^{o}C until use.

- **7-Ethoxyresorufin Stock Solution (7-ER, 0.5mM):** Dissolve 5mg 7-ethoxyresorufin (source and code) in 41ml 1:1 methanol:DMSO. Store in 400µl aliquots at -18^{o}C until use.

Working Solutions:

- **Phosphate Buffered Saline (PBS non-sterile, pH 7.5):** Dissolve 5 PBS tablets (Source and code) in 1L Ultrapure H_2O.Adjust to pH 7.5 and store at 4^{o}C until use.

- **HEPEs Buffer (0.1M, pH 7.8):** Dissolve 13.82g HEPES (Source and Code) in 1L Ultrapure water. Adjust to pH 7.8 and store at 4^{o}C until use.

- **Resorufin (0.01mM):** Thaw 100µL resorufin stock quickly under hot water. Dilute with 9.9ml HEPES Buffer (100x dilution).

- **7-Ethoxyresorufin:** Add 400µL of 7-ER stock to 100ml HEPES buffer (pH 7.8).

Method:

1. Day 1 – Cell Plating

- Count cells in accordance with UTS SOP: EDC 3-2.
- adjust to 2.5x10^{5} as follows:

Volume (V) of cell suspension required for 40ml at $2x10^5$:

$$V = \frac{(2\times10^5)\times 40}{\text{Cells.ml}^{-1}}$$

e.g., cells.ml^{-1}=$1.9x10^6$

V = 4.2 ml cell suspension required (added to 35.8 ml media)

- Make up as many of cell suspension at $2x10^5$cells/ml as needed (one 40ml tube = two assay plates
- Add contents of one tube to sterile reservoir and, using multi-channel pipettor add 200ul to each well of a sterile plate. Mark the plate with name, cell line, date and plate number.
- Return all plates to the incubator for 24hours at 37°C and 5% CO_2

2. Day 2 – Well Innoculation

- Dilute 2,3,7,8-TCDD standard in amber vials in DMSO in 2x series from stock () to 5 nM - 0.02 nM and return to freezer. These dilutions can be reused.
- Dilute samples to appropriate concentrations in DMSO in amber vials
    - For range-finding test dilute – 100%, 10%, 1%, 0.1%, 0.01%
    - For definitive test dilute in 2x series from maximum signal in range finding test. Top concentrations in many samples will be cytotoxic.
- Innoculate wells of prepared plates with 1µl of the appropriate sample dilution (Figure 1, Figure 2)
- Innoculate wells with 1µl of the appropriate standard dilution (Figure 1, Figure 2)
- Return all plates to the incubator for 24hours at 37°C and 5% CO_2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		S1 100%	S1 10%	S1 1%	S1 0.1%	S1 0.01%	S2 100%	S2 10%	S2 1%	S2 0.1%	S2 0.01%	
C		S1 100%	S1 10%	S1 1%	S1 0.1%	S1 0.01%	S2 100%	S2 10%	S2 1%	S2 0.1%	S2 0.01%	
D		S3 100%	S3 10%	S3 1%	S3 0.1%	S3 0.01%	S4 100%	S4 10%	S4 1%	S4 0.1%	S4 0.01%	
E		S3 100%	S3 10%	S3 1%	S3 0.1%	S3 0.01%	S4 100%	S4 10%	S4 1%	S4 0.1%	S4 0.01%	
F		STD 5NM	STD 2.5NM	STD 1.25NM	STD 0.63NM	STD 0.31NM	STD 0.16NM	STD 0.08NM	STD 0.04NM	STD 0.02NM	BLANK	
G		STD 5NM	STD 2.5NM	STD 1.25NM	STD 0.63NM	STD 0.31NM	STD 0.16NM	STD 0.08NM	STD 0.04NM	STD 0.02NM	BLANK	
H												

Figure F-1. Plate layout for range-finding test. Outside shaded wells are left blank as they are subject to evaporation.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		S1 1	S1 2	S1 3	S1 4	S1 5	S1 6	S1 7	S1 8	S1 9	S1 10	
C		S1 1	S1 2	S1 3	S1 4	S1 5	S1 6	S1 7	S1 8	S1 9	S1 10	
D		S2 1	S2 2	S2 3	S2 4	S2 5	S2 6	S2 7	S2 8	S2 9	S2 10	
E		S2 1	S2 2	S2 3	S2 4	S2 5	S2 6	S2 7	S2 8	S2 9	S2 10	
F		STD 5NM	STD 2.5NM	STD 1.25NM	STD 0.63NM	STD 0.31NM	STD 0.16NM	STD 0.08NM	STD 0.04NM	STD 0.02NM	BLANK	
G		STD 5NM	STD 2.5NM	STD 1.25NM	STD 0.63NM	STD 0.31NM	STD 0.16NM	STD 0.08NM	STD 0.04NM	STD 0.02NM	BLANK	
H												

Figure F-2. Plate layout for definitive test. Outside shaded wells are left blank as they are subject to evaporation.

3. Day 3 – Plate Reading

- Switch plate reader on and set the incubation temperature to 37C
- Add 100ul HEPEs to each well of a black microwell plate. Serially dilute resorufin working solution to create a x2 series down the plate. Leave final column free of resorufin as blank.
- Read the fluorescence of the resorufin plate
- Remove the first plate from the CO_2 incubator
- Remove media from wells by gently tapping against wadded paper.
- Rinse the plate 3 times with PBS
- Remove as much PBS as possible from the wells and add 100ul 7-ER working solution to each assay well (A2-G11)
- Read plate immediately in the fluorescence plate reader (excitation = 540 nm, emission = 590 nm, time = 30 mins)
- Once the reader cycle is complete remove the liquid from wells by gently tapping against wadded paper
- Add 25µl of MilliQ water to each assay well (A2-G11) and place in freezer. This will lyse the cells and allow protein determination.
- Repeat steps 1 - 4 for each plate

Day 4 – Protein Assay (Pierce BCA Protein Kit)

- **Biconchoninic acid working solution**: 20:1 (Reagent A : Reagent B). Amount made up should be calculated on the plates to be assayed

- Remove all plates from the freezer and allow well contents to thaw at room temp
- Switch plate reader on and set and incubator temperature to 37C

- Prepare reagent working solution from reagents supplied with Pierce Macro BSA Protein Kit
- Prepare BSA protein standard x2 dilutions in a clear microwell plate in HEPEs buffer
- Allow standard plate to incubate at 37C for 30 mins
- Read absorbance (562 nm) of BSA standard plate
- Add 200µl of reagent working solution to each well of the first assay plate and allow to incubate for at 37C for 30 mins
- Read absorbance (562 nm) of assay plate
- Repeat steps 7-8 for each remaining plate

Appendix G – Method: Mosquitofish EROD activity assay

UTS SOP: EDC – 3.3 – Mosquitofish EROD activity assay

This protocol describes the method for determining the ethoxyresorufin-*O*-dethylase (EROD) activity liver tissue of the mosquitofish (*Gambusia holbrooki*). EROD activity is proportional to levels of cytochrome P450-1A (CYP1A) in the tissue and is a biomarker for exposure to aryl hydrocarbon receptor (AhR) ligands.

Equipment Required
Multi-Channel Pipettor
Pipettes 10ul, 100ul, 1000ul, 5ml
Sterile pipette tips: white (reach), blue, yellow
Non-sterile pipette tips: yellow, blue, 5ml white
70ml reservoirs: sterile (1), non-sterile (7)
Fluorescence plate reader: e.g., Fluostar Optima (Source and Code)
Pierce BCA Protein assay kit (Source and Code)
Sterile single wrapped 96 well plates (Source and Code)

Stock Reagents Required:

- **Phosphate Buffer Stock (pH 7.5 1L):** Add 11.60g K_2HPO_4, 4.54g KH_2PO_4, 372mg EDTA disodium salt, 200ml glycerol to 700ml in MilliQ Water and pH to 7.5. Make up to 1L with MilliQ water. Store in a Schott bottle at 4^0C until use.

- **Hepes Buffer (Reaction Buffer) (pH 7.8 1L):** Add 28.83g HEPEs and 1.0g $MgCl_2.6H_2O$ to 900ml MilliQ water and pH to 7.8. Make up to 1L using MilliQ. Store in a Schott bottle at 4^0C until use.

- **7-Ethoxyresorufin Stock (0.5mM):** Dissolve 2.412mg 7-ER in 20ml 1:1 MeOH:DMSO. Aliquot into 400ul lots in microfuge tubes and store at -20^{o}C in the dark

- **Resorufin Stock (1mM):** Dissolve 1.176mg resorufin in 5ml DMSO and aliquot into 100µL lots. Store at -80^0C.

Working Reagents (prepare on day of assay):

- **Phosphate Buffer Working Solution (100ml):** Sonicate 3.5mg PMSF in 1ml Phosphate Buffer Stock for 2 min in ultrasonic bath. Add 0.5ml PMSF solution to chilled beaker. Add 100ml Phosphate Buffer Stock. This solution must be discarded at the end of the day.

- **7-Ethoxyresorufin Working Solution (188uM):** Dilute 400µL of the 0.5mM stock with 663µL reaction buffer (pH 7.8). Add 200µL of diluted stock to 9.8ml reaction buffer. Remember that 7-ER is light sensitive so store in the dark and discard at the end of the day. The quantity of 7-ER needed will need to be calculated based on the number of samples to be used in the test

- **Resorufin Working Solution:** Thaw resorufin stock quickly under hot water. Add 900uL Phosphate Buffer working solution to the tube. Add contents of tube to a multi-pipette trough. Add 9ml Phosphate buffer working solution to the trough. If more resorufin (more samples) is needed repeat procedure.

- **NADPH solution – (247µM):** Dissolve 2.8mg NADPH in 12.5 ml reaction buffer.

Method

1. Resorufin Standard Curve
    - Add 100uL reaction Buffer to each well of columns 11 and 12 of a black NUNC 96 well plate using the multi-channel pipettor (This is the **ASSAY PLATE** – Figure 1)
    - Add 100uL of the resorufin Working Solution to A11 and A12
    - Create a doubling dilution down columns 11 and 12 leaving the H11 and H12 as blanks (remove 100ul from G11 and G12).
    - Add 100ul resorufin working solution to wells A3-A12 of a clear 96 well plate (This will be the **PROTEIN PLATE** – Figure 2)

2. Sample s9 Preparation
    - Remove samples in microfuge samples from the -80^{0}C freezer and thaw on ice
        - If using fish from multiple sites/ multiple collections, random number generation should be used to assign the samples to assay runs
    - Add 250µL phosphate buffer working solution to the microfuge tube
    - Using a 1ml syringe with a flat tip 18 gauge drawing up tip which has been rinsed in Phosphate buffer working solution repeatedly draw up the liquid and tissue until well homogenised (approx 1min).
    - Place back in ice.
    - When all samples have been homogenised centrifuge at 10,000g for 20min at 4^{0}C
    - Remove as much supernatant from each microfuge tube as possible (avoiding fatty layer) and place in another labeled, chilled 1.5ml tube on ice.

3. Plating out and measurement
    - Add 25µl of sample to a clear NUNC 96 well plate in quadruplicate as below

SAMPLES										STD CURVE (NM)	
1	2	3	4	5	6	7	8	9	10	5000	5000
1	2	3	4	5	6	7	8	9	10	2500	2500
1	2	3	4	5	6	7	8	9	10	1250	1250
1	2	3	4	5	6	7	8	9	10	625	625
11	12	13	14	15	16	17	18	19	20	313	313
11	12	13	14	15	16	17	18	19	20	156	156
11	12	13	14	15	16	17	18	19	20	78	78
11	12	13	14	15	16	17	18	19	20	0	0

Figure G-1 – Well layout of ASSAY PLATE

- Add 75µl Reaction buffer to each well.
- Add 80µl 7-ER working solution to each well
- Add 20µL NADPH solution to each well
- Read fluorescence (excitation: 512 nm emission: 596 nm) immediately. [Fluostar Optima plate reader (user: Chris Rawson; protocol: 'Mosquitofish EROD')]. Each plate takes 30mins to read.

4. Pierce Protein Assay

- Make up 20ml working solution (WR) for each 10 samples at ratio of 50:1 Reagents A:B (20ml Reagent A + 400uL Reagent B) in multi-pipette trough.
- Add 25ul of sample to each appropriate well as shown below. Each sample is in duplicate
- Serially dilute 'Pierce Protein' BSA standard in assay plate (shown in table 1)

STD1 µG/ML	STD2 µG/ML	SAMP	SAMP	SAMP	SAMP	SAMP	SAMP	SAMP	SAMP	SAMP	SAMP
1500	1500	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS	RES ABS
1000	1000										
750	750	1	2	3	4	5	6	7	8	9	10
500	500	11	12	13	14	15	16	17	18	19	20
250	250	21	22	23	24	25	26	27	28	29	30
125	125	31	32	33	34	35	36	37	38	39	40
25	25										
0	0	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK

Figure G-2 – Well layout of PROTEIN PLATE. Res Abs = Resorufin absorbance.

Table G-1 – Dilution calculations and volumes for protein standards

DILUTION CALCULATIONS: PROTEIN ASSAY STANDARDS							
ROW	FINAL CONC. (µG/ML)	DILUENT VOL. (µL)	VOL. BSA (µL)	BSA SOURCE (µG/ML)	SOURCE ROW	VOL. REMAIN (µL)	FINAL VOL. (µL)
A	1500	20	61	2000	STOCK	82	25
B	1000	28	57	1500	A	85	25
C	750	20	60	1000	B	80	25
D	500	28	55	750	C	83	25
E	250	58	58	500	D	115	25
F**	125	90	90	250	E	180	25
G	25	20	5	155	F	25	25
H	0	25	0	NA	NA	25	25

**150 µL MUST BE DISCARDED PRIOR ADDITION OF PROTEIN WR

- Add 200µl of the WR to each well.
- Incubate at 37^0C for 30mins
- Cool to room temp
- Measure absorbance at 562 nm. [Fluostar Optima plate reader (User: Chris Rawson; Protocol: Pierce Prot EROD)].
- Measure the resorufin absorbance of wells A3-A12 at 571nm [Fluostar Optima plate reader (user: Chris Rawson; protocol: 'Resorufin Abs')]. Each plate takes 2 mins to read.

5. Calculation of EROD activity (pmol res min^{-1} mg $protein^{-1}$)

- Open "Blank EROD Spreadsheet" and save under appropriate name immediately. Do not alter this spreadsheet unless necessary
- Load appropriate "RESORUFIN ABS" results in "Optima Evaluation Part". Copy resorufin absorbance to pink section (B35-B42) of "resorufin standard" sheet in EROD workbook. This gives the actual concentration of resorufin in the standard curve.
- Load appropriate "PIERCE PROT EROD" results in "Optima Evaluation Part". Copy absorbance results (whole plate) to blank plate in "Protein" sheet in EROD workbook. Run SOLVER (minimise D52 by changing B53-B55). This calculates the standard curve and the amount of protein in each well/sample.
- Load appropriate "MOSFISH EROD" results in "Optima Evaluation Part". Transpose-copy Raw Maximum values for A11-H12 to B11-J12 in "resorufin standard" sheet in EROD workbook. This creates a resorufin standard curve (FU against nmoles/well). Type the slope of this line to J34 to complete the equation for the standard curve.
- In "MOSFISH EROD" results in "Optima Evaluation Part" load "slope/min" results and copy A1-H10 into blank pink cells B3-K10 in sheet "Max V" in EROD spreadsheet.
- With Resorufin Standard Curve (FU to nmol) and protein content (mg protein) each well should give a measure for EROD activity (pmol res min^{-1} mg $protein^{-1}$). These

are then averaged and presented in the "Final" sheet in the EROD spreadsheet. Numbered samples can then be converted to relevant site and fish number labels.

- Note: in some cases not enough sample will available for quadruplicate evaluation (this is especially the case where livers have high fat content). In these cases the "Max V" sheet should be altered to reflect this.
- Similarly, wells with unusual values should be identified and removed from the final averaging of replicates.

Appendix H – Output Arising from this Project

Conference Presentations

1. Rawson C., Tremblay L., Lim R., Warne M., Chapman J., Laginestra E., 2005, Radioligand assay detection of EDCs in waters of Sydney Olympic Park. Australasian Society for Ecotoxicology, 25 – 28 September, 2005, Melbourne, Australia.
2. Rawson C., Tremblay L., Lim R., Warne M., Chapman J., Laginestra E., 2006, Hepatic CYP1A activity in fish of Sydney Olympic Park. Australasian Society for Ecotoxicology, 24-28 September, 2006, Perth, Australia.
3. Rawson C., Ying G-G., Warne M.,Tremblay L., Kookana R., Laginestra E., Chapman J., Lim R., 2007, Presence and potential exposure of fish populations to persistent organic pollutants at a remediated site in Sydney Australia, SETAC North America, 11 – 15 November, 2007, Milwaulkee, USA
4. Rawson C., Ying G-G., Warne M.,Tremblay L., Kookana R., Laginestra E., Chapman J., Lim R., 2007, Macroinvertebrate assemblages in sediments of a remediated site in Sydney, Australia, SETAC North America, 11 – 15 November, 2007, Milwaulkee, USA
5. Rawson C., Ying G-G., Warne M.,Tremblay L., Kookana R., Laginestra E., Chapman J., Lim R., 2008, An effects based assessment of the aquatic environment of the remediated Sydney Olympic Park site. SETAC World Congress, 3 -7 August 2008, Sydney Australia.