Polybrominated Diphenyl Ethers: Levels in Townsville sediments, Depuration and (Anti-) Estrogenic effects in Barramundi (*Lates calcarifer*)

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outline in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1101).

Elisabeth Knowles Muirhead **Date**

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Abstract

The purpose of this research was to study polybrominated diphenyl ethers (PBDEs) and the effect they have in North Queensland, Australia, specifically in reference to a commercially important fish species, barramundi (*Lates calcarifer*). This thesis is separated into four main sections: determination of PBDE levels in Ross Creek, Townsville, QLD; toxicokinetics of PBDE-47 in barramundi; optimization of an enzyme-linked immunosorbent assay (ELISA) for detection of vitellogenin (Vtg) in barramundi; and assessing the (anti-)estrogenic effect of PBDE-47 in barramundi.

Levels of two common PBDE congeners, PBDE-47 and PBDE-209 were measured in sediments at three sites along Ross Creek in Townsville, QLD. Levels were found to range from below detection (0.2 μg kg^{-1} dw) to 0.35±0.2 μg kg^{-1} (dw) for PBDE-47 and from below detection (0.2 μg kg^{-1} dw) to 0.85±0.07 μg kg^{-1} (dw) for PBDE-209.

Male juvenile barramundi were injected with either a low (1 mg kg^{-1} bw) or a high (10 mg kg^{-1} bw) dose of PBDE-47 and then sampled over the course of 14 days in order to determine the depuration rate of PBDE-47 in barramundi. PBDE-47 was found to depurate at a rate of 0.041-0.069 day^{-1}, a rate which falls well within the range of the literature for depuration of PBDE-47 in fish.

An optimal ELISA for the detection of Vtg production in barramundi was determined after comparing the component reagents of a pre-existing ELISA with component reagents developed during this study. Two commercially available Vtg standards, a lipophylised Rainbow Trout Vtg standard (RT Vtg standard) and a lipophylised Atlantic Salmon Vtg standard (Salmon Vtg standard) (both from Caymen Chemical Co), were compared to a purified barramundi Vtg fraction obtained after size exclusion chromatography of plasma from barramundi in which Vtg production was induced by repeated injection of large doses of 17β-estradiol (E2). In addition, a commercially available monoclonal mouse anti-striped bass Vtg primary antibody (ND-3G2, Biosense) was compared with two polyclonal sheep anti-barramundi Vtg antibodies (Sh-0404JCU and Sh-0404-SJCU) created by inoculating sheep with one of the size exclusion

chromatography purified Vtg fractions. The optimal ELISA was determined to be the pre-existing ELISA using ND-3G2 as the primary antibody and RT Vtg standard for quantification, although promising results obtained with the purified barramundi Vtg fractions, Sh-0404JCU and Sh-0404-SJCU suggest that further purification could lead to a better barramundi specific ELISA in the future.

Finally, male, juvenile barramundi were exposed to PBDE-47 in two separate experiments to study whether PBDE-47 has an estrogenic or anti-estrogenic effect, with Vtg production measured by ELISA as the endpoint for estrogenic behaviour.

In the first experiment barramundi were given either a low (1 mg kg^{-1} bw) or a high (10 mg kg^{-1} bw) dose of PBDE-47 by intraperitoneal (i.p.) injection, and then sampled over the course of 14 days to determine the time course induction of Vtg production. Vtg levels in samples were not quantifiable but the qualitative data allowed for assessment of trends and patterns. Two interesting conclusions were apparent from the data. The first is that male barramundi appear to produce Vtg without exposure to xeno-estrogens, a hypothesis that is supported by literature that has found low natural levels of E2 production in males of many fish species. The second is that the high dose of PBDE-47 suppressed Vtg production between days 7 and 14 with Vtg levels rising much slower in the high dosed fish than in either the control or low dosed fish.

In the second experiment barramundi were given either a single low (1 mg kg^{-1} bw) or a high (10 mg kg^{-1} bw) dose of PBDE-47 by i.p. injection then sampled 3 and 6 days after injection, or were given two low (1 mg kg^{-1} bw) or a high (10 mg kg^{-1} bw) doses of PBDE-47 by i.p. injection, with three days between injections, then sampled 3 and 6 days after the second injection. This was done to determine whether a repeated dose of PBDE-47 had more of an effect on Vtg production than a single dose. The Vtg levels in these samples was quantifiable and the results showed that a double injection of PBDE-47 significantly suppressed the production of Vtg ($P<0.0001$) at both a low and high dose. In addition, at 6 days post final injection there was a small, but significant difference ($P=0.0355$) between the fish that received a single low dose and a single high dose, confirming that a single high dose of PBDE-47 can suppress Vtg production as well.

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Glossary of Abbreviations

Abbreviation	Definition
1° Ab	Primary antibody
2° Ab	Secondary antibody
ABS	Acrylonitrile Butadiene Styrene
BFR	Brominated Flame Retardant
CF	Column Fraction
DMSO	Dimethyl Sulfoxide
E2	17β-Estradiol
EDC	Endocrine Disrupting Chemical
ELISA	Enzyme-linked Immunosorbent Assay
F_{OC}	Fraction by weight of Organic Carbon
GC-MS	Gas Chromatography – Mass Spectroscopy
H+L-HRP	Horseradish Peroxidase conjugate
i.p.	Intraperitoneal
K_D	Equilibrium Constant
K_{OW}	Octanol-Water Partition Coefficient
ND-3G2	Monoclonal Mouse Anti-Striped Bass Vtg antibody (Biosense)
OPD	O-Phenylenediamine Dihydrochloride
PBDE	Polybrominated Diphenyl Ether
PBDE-47	2, 2', 4, 4'-tetrabromodiphenyl ether
PBDE-99	2, 2’, 4, 4’, 5-pentabromodiphenyl ether
PBDE-153	2, 2’, 4, 4’, 5, 5’-hexabromodiphenyl ether
PBDE-154	2, 2’, 4, 4’, 5, 6’-hexabromodiphenyl ether
PBDE-166	2, 3, 4, 4’, 5, 6-hexabromodiphenyl ether
PBDE-183	2, 2’, 3, 4, 4’, 5’, 6-heptabromodiphenyl ether
PBDE-190	2, 3, 3’, 4, 4’, 5, 6-heptabromodiphenyl ether
PBDE-205	2, 3, 3’, 4, 4’, 5, 5’, 6-octabromodiphenyl ether
PBDE-209	Decabromodiphenyl ether
PCB	Polychlorinated Biphenyl
PCB-103	2, 2’, 4, 5’, 6-pentachlorobiphenyl
PMSF	Phenylmethylsulfonyl Fluoride
POP	Persistent Organic Pollutant
RT Vtg standard	Lipophylised Rainbow Trout Vtg standard (Caymen Chemical Co)
Salmon Vtg standard	Lipophylised Atlantic Salmon Vtg standard (Caymen Chemical Co)
Sh-0404JCU	Unscreened Polyclonal Sheep Anti-Barramundi Vtg antibody
Sh-0404-SJCU	Screened Polyclonal Sheep Anti-Barramundi Vtg antibody
Vtg	Vitellogenin

1. Introduction

1.1 Polybrominated Diphenyl Ether (PBDE): General Background

Most commercial products, including electronics, textiles and plastics contain flame retardant chemicals to protect consumers from accidental fires (de Wit, 2002; Watanabe and Sakai, 2003). Arguably the most important group of flame retardants are the brominated flame retardants (BFRs) which have been used for many years to reduce fire risks (Watanabe and Sakai, 2003; de Wit, 2002). Over the last two decades BFRs have been attracting attention and creating concern regarding environmental pollution and human exposure (Ikonomou et al, 2002; McDonald, 2002; Alaee et al, 2003; de Wit et al, 2006; Law et al, 2006). Recent studies highlighting a marked increase in BFR levels in human milk worldwide (Meironyté et al, 1999; Ohta et al, 2002; Fängström et al, 2004; Chao et al, 2007) as well as continued research showing that some BFRs have serious health effects (see, e.g. the review by Bergman and Ulrika, 2001) have increased this concern exponentially.

Polybrominated diphenyl ethers (PBDE) are one type of BFR widely used around the world. They can be found in a variety of products, such as clothing, furniture, electronics, vehicles and computers (Johnson and Olson, 2001). They are used with a wide range of polymers, from high-impact polystyrene to polyurethanes, and PBDE content can range from 5 to ~20% of the polymer (Arias, 2001).

Diphenyl ether is brominated in the presence of a Freidel-Craft catalyst in a solvent such as dibromomethane to create PBDE. Because diphenyl ether has 10 hydrogen atoms, any or all of which can be exchanged with a bromine, there are 209 possible PBDE congeners, although in most commercial mixtures relatively few congeners predominate. PBDE congeners are numbered according to bromination pattern using the same system as that used to number polychlorinated biphenyl (PCB) congeners. Figure 1.1 shows the chemical structure of PBDE-47, 2, 2', 4, 4'-tetrabromodiphenyl ether.

There are three main commercial products in which PBDEs have been used: Deca-BDE which consists mainly of PBDE-209 (97-98%); Octa-BDE which is made up of mainly PBDE-183, with a few unidentified octa- and nona-BDEs; and Penta-BDE which is 41-42% tetra-BDEs (mainly PBDE-47), 44-45% penta-BDEs (PBDE-99 and -100) and 6-7% hexa-BDEs (PBDE-153 and -154) (Alaee et al, 2003). There are two main types of BFR compounds, reactive and additive. Reactive BFR compounds are bound to the polymer of which they are a part by covalent bonds, while additive BFR compounds are mixed with the polymers during their production and are not chemically bonded to the polymer itself (de Wit, 2002). PBDEs are additive flame retardants which increase the likelihood of the chemical leaching out of the product (Alaee et al 2003).

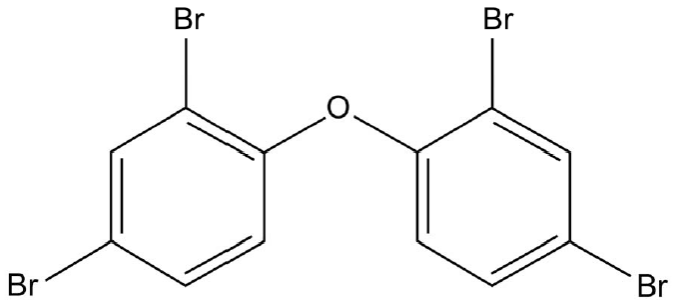

Figure 1.1 Chemical structure of 2, 2', 4, 4'-tetrabromodiphenyl ether (PBDE-47)

Deca-BDE is a general purpose flame retardant and can be used in almost any type of polymer. It is the most highly brominated class of PBDE with 83% bromine by weight. When combined with antimony oxide it can be used in high-temperature processing to make polymers such as high-impact polystyrene which is commonly used in TV and computer monitors (Alaee et al, 2003). Octa-BDE, which is 79% bromine by weight, is mainly used in Acrylonitrile Butadiene Styrene (ABS) resins, which can be used for injection molding and extrusion applications including automotive, appliances and packaging (Alaee et al, 2003). The final class of commercial PBDE is Penta-BDE, which is 70% bromine by weight and most commonly used in polyurethane foam and textiles.

PBDEs are structurally and behaviorally similar to polychlorinated biphenyls (PCBs), in that they are highly lipophilic and hydrophobic as well as thermally stable (Alcock et al, 2003). There is growing evidence to suggest that PBDEs are a widespread environmental pollutant (de Wit et al, 2006; Law et al, 2006) that are known to bioaccumulate in food chains much like PCBs (Boon et al, 2002; Tomy et al, 2004). However, unlike PCBs and other "classical" persistent organic pollutants (POPs), which have seen a marked decrease in global levels over the last two decades as restrictions have removed them from the global market, global levels of PBDEs have risen over the last twenty years according to several studies (Rayne et al, 2003; Ikonomou et al, 2002; Papke et al, 2000; Meironyte et al, 1999; Easton et al, 2002; Johnson and Olson, 2001; McDonald, 2002; Ohta et al, 2002; Zennegg et al, 2003). This is most likely due to a lack of global restrictions on their production and use until recently, as well as the disposal of products treated with PBDEs (Martin et al, 2004). In addition, although many countries have no primary production capabilities or direct import of PBDE commercial mixtures, contamination is occurring through indirect imports in the form of electronics, furniture, and textiles treated with PBDEs in their country of manufacture (Harrad and Porter, 2007).

1.2 PBDE in the global environment

The global use of brominated flame retardants grew over 100% in the decade between 1990 and 2000 (Alaee et al, 2003) while global demand for PBDE was ~67,000 metric tons in 1999, up from ~40,000 metric tons in 1992 (Zhu and Hites, 2003). Of the ~67,000 metric tons, 54,800 were Deca-BDE, 3825 were Octa-BDE, and 8500 were Penta-BDE (Alaee et al, 2003). In Australia it was estimated that ~210 metric tons of PBDE commercial products (penta-, octa- and deca-BDE) were imported in 2003-2004 (NICNAS, 2005). The last decade has seen regulations and restrictions placed on the lower brominated PBDEs, with Penta-BDE voluntarily withdrawn from the Japanese market (Watanabe and Sakai, 2003) and both Octa-BDE and Penta-BDE banned in Europe (Alaee et al, 2003). While in the period between 1999 and 2004 saw a decrease in penta- and octa-BDE use in Australia (before production of penta- and octa-BDE fully ceased worldwide in 2005) the use of deca-BDE, which is not restricted, increased slightly, from 177 to 180 metric tons, an increase of ~1% (NICNAS, 2005).

Due to the lipophilicity of PBDEs, in the aquatic environment fish and marine mammals typically have the highest levels of PBDEs (Ikonomou et al, 2002). Biomonitoring studies in salmon have identified relatively consistent PBDE congener profiles with up to 43 individual congeners identified, although PBDE-47 is typically the most abundant congener in biota (Hites et al, 2004). This pattern seems consistent in other studies, with the predominant congener most often being PBDE-47, not only in fish (Zennegg et al, 2003; Johnson and Olson, 2001), but also in marine mammals (Ikonomou et al, 2002) and humans (Ohta et al, 2002; Harrad and Porter, 2007; Toms et al, 2007). In sediments, on the other hand, the predominant congener tends to be PBDE-209, the main congener in the commonly used Deca-BDE commercial mixture (Law et al, 2006; Toms et al, 2008). The congener profile of sediment samples is reflective of the global use patterns of the different commercial mixtures, and one possible explanation of the predominance of PBDE-47 in biota is that it is the result of biotransformation of the chemical after ingestion (Tomy et al, 2004; Stapleton et al, 2004a; Stapleton et al, 2004b). Table 1.1 gives an overview of the PBDE levels reported globally for water, sediments, sewage sludge, select commercial fish species and humans in the last decade.

Table 1.1 Concentrations of PBDEs reported globally

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Water					
Dissolved	North America – Lake Michigan	2004	47, 66, 99, 100, 153, 154	18±1.8 pg L^{-1}	Streets et al (2006)
	North America - San Francisco Estuary	2002	17, 28, 33, 47, 6, 82, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203 – 209	3-513 pg L^{-1}	Oros et al (2005)
Particulate	North America – Lake Michigan	2004	47, 66, 99, 100, 153, 154	3.1±0.29 pg L^{-1}	Streets et al (2006)
Sediment					
	Australia - Queensland	2002-2003 & 2005	47, 99, 100, 153, 154, 183, 209	ND[1]-4.42 µg kg^{-1} dw^{2}	Toms et al (2008)
	Australia - NSW	2002-2003 & 2005	47, 99, 100, 153, 154, 183, 209	ND-36.4 µg kg^{-1} dw	Toms et al (2008)

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Sediment					
	Australia - South Australia	2002-2003 & 2005	47, 99, 100, 153, 154, 183, 209	ND-1.88 μg kg^{-1} dw	Toms et al (2008)
	Australia - ACT	2002-2003 & 2005	47, 99, 100, 153, 154, 183, 209	0.093-7.73 μg kg^{-1} dw	Toms et al (2008)
	Australia - Tasmania	2002-2003 & 2005	47, 99, 100, 153, 154, 183, 209	ND-0.53 μg kg^{-1} dw	Toms et al (2008)
	North America - San Francisco Estuary	2002	17, 28, 33, 47, 6, 82, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203 - 209	ND-212 μg kg^{-1} dw	Oros et al (2005)
	North America - Lake Michigan	2002	28, 47, 66, 85, 99, 100, 153, 154, 183, 209	66.1 μg kg^{-1} dw (mean)	Song et al (2005)
	North America - Lake Huron	2002	28, 47, 66, 85, 99, 100, 153, 154, 183, 209	30.3 μg kg^{-1} dw (mean)	Song et al (2005)
	North America - Niagara River	2006	28, 47, 66, 85, 99, 100, 153, 154, 183	ND-148 μg kg^{-1} dw	Samara et al (2006)
	Europe - Cinca River, NE Spain	2004	47, 100, 118, 153, 154, 183, 209	2.5-42 μg kg^{-1} dw	Eljarrat et al (2004)
	Europe - River Danube, Austria	2003	17 congeners including: 47, 99, 181, 183	0.3-10.4 μg kg^{-1} dw	Moche and Stephan (2003)
	Europe - River Viskan, Sweden	1998	209	68-390 μg kg^{-1} dw	Sellstrom et al (1998)
	Europe - Cork Harbour, Ireland	2004	28, 47, 66, 71, 75, 77, 85, 99, 100, 119, 138, 153, 154, 183, 190, 209	1.8-1.9 μg kg^{-1} dw	Kilemade et al (2004)
	Europe - Lake Mjosa, Norway	2004	28, 47, 49, 99, 100, 138, 153, 154, 183, 209	0.6-27 μg kg^{-1} dw	Schlabach et al (2004)

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Sediment					
	Arctic - Several Freshwater sites in N. Russia	2000-2001	47, 99	0.004-0.027 μg kg^{-1} dw	AMAP (2004)
	Arctic - Kola, Russia	1998	47, 99, 100, 153	0.1-241 μg kg^{-1} dw	Chernyak et al (2003)
Sewage Sludge					
	North America - US	2002	47, 99, 100, 209	3010 μg kg^{-1} dw	Hale et al (2002)
	Europe - Sweden	2000	47, 99, 100, 153, 154, 209	18-260 μg kg^{-1} dw	Swedish EPA study cited in Law et al (2006)
	Europe - Denmark	2003	17, 28, 47, 49, 66, 85, 99, 100, 153, 154, 183, 209	238±23 μg kg^{-1} dw	Christensen et al (2003)
	Europe - Spain	2002	7, 15, 17, 28, 47, 49, 66, 85, 99, 100, 119, 153, 154, 209	844-18100 μg kg^{-1} dw	Fabrellas et al (2004)
Fish and Molluscs					
Lake Trout	North America – Lake Michigan	2000-2002	47, 66, 77, 100, 153, 154	120-350 μg kg^{-1} (mean)	Streets et al (2006)
Salmon	North America - Pacific Coast	2000	39 congeners including: 15, 28, 47, 49, 66, 99, 100, 153, 154	0.2 μg kg^{-1} ww[3] (mean)	Easton et al (2002)
Salmon (farmed)	North America - Pacific Coast	2002	39 congeners including: 15, 28, 47, 49, 66, 99, 100, 153, 154	2.7 μg kg^{-1} ww (mean)	Easton et al (2002)
Rainbow Trout	North America - Spokane River, WA	1999	47, 99, 100, 153, 154	119-174 μg kg^{-1} ww	Johnson and Olson (2001)

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Fish and Molluscs					
Oysters	North America - San Francisco Estuary	2002	17, 28, 33, 47, 6, 82, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203 - 209	9-64 μg kg^{-1} dw	Oros et al (2005)
Mussels	North America - San Francisco Estuary	2002	17, 28, 33, 47, 6, 82, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203 - 209	13-47 μg kg^{-1} dw	Oros et al (2005)
Clams	North America - San Francisco Estuary	2002	17, 28, 33, 47, 6, 82, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203 - 209	85-106 μg kg^{-1} dw	Oros et al (2005)
Brown Trout	Europe - Lakes in SE Norway	2003	28, 47, 99, 100, 153, 154, 183	3.6-18 μg kg^{-1} ww	Mariussen et al (2003)
Brown Trout	Europe - Lake Mjosa, Norway	2003	28, 47, 99, 100, 153, 154, 183	353 μg kg^{-1} ww (mean)	Mariussen et al (2003)
Brown Trout	Europe - Skerne and Tees Rivers, NE England	2003	28, 47, 99, 100, 153, 154	4.9-197 μg kg^{-1} ww	Allchin and Morris (2003)
Barbel	Europe - Cinca River, NE Spain	2004	40 congeners including: 47, 153, 154, 183	1.3-298 μg kg^{-1} ww	Eljarrat et al (2004)
Herring	Europe - North Sea	2004	17, 28, 47, 66, 77, 99, 100, 153, 154, 183, 209	14 μg kg^{-1} lipid (mean)	Paepke and Herrmann (2004)
Plaice	Europe - NE Atlantic	2004	17, 28, 47, 66, 77, 99, 100, 153, 154, 183, 209	6.7 μg kg^{-1} lipid (mean)	Paepke and Herrmann (2004)
Trout	Europe - NE Atlantic	2004	17, 28, 47, 66, 77, 99, 100, 153, 154, 183, 209	9.7 μg kg^{-1} lipid (mean)	Paepke and Herrmann (2004)
Halibut	Europe - NE Atlantic	2004	17, 28, 47, 66, 77, 99, 100, 153, 154, 183, 209	0.4 μg kg^{-1} lipid (mean)	Paepke and Herrmann (2004)

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Fish and Molluscs					
Blue Mussels	Europe - Norway	2003	28, 47, 99, 100, 153, 154, 183	0.1-0.3 μg kg^{-1} ww	Bethune et al (2004)
Crab	Europe - Norway	2003	28, 47, 99, 100, 153, 154, 183	0.6-7.0 μg kg^{-1} ww (shell meat)	Bethune et al (2004)
Whitefish	Europe - Switzerland	2003	28, 47, 99, 100, 153, 154, 183	2.0-7.4 μg kg^{-1} ww	Zennegg et al (2003)
Rainbow Trout (farmed)	Europe - Switzerland	2003	28, 47, 99, 100, 153, 154, 183	0.7-1.3 μg kg^{-1} ww	Zennegg et al (2003)
Unknown Fish Species	Asia - Taiwan Rivers and Estuaries	2007	28, 47, 99, 100, 153, 154, 183	2.9-1240 μg kg^{-1} lipid	Peng et al (2007)
Young Yellowtail	Asia - Japan	2002	28, 47, 99, 100, 153, 154	1.6-1.7 μg kg^{-1} ww	Ohta et al (2002)
Mackerel	Asia - Japan	2002	28, 47, 99, 100, 153, 154	1.4-1.6 μg kg^{-1} ww	Ohta et al (2002)
Yellowtail	Asia - Japan	2002	28, 47, 99, 100, 153, 154	1.0-1.3 μg kg^{-1} ww	Ohta et al (2002)
Humans					
Milk	North America - N. Quebec, Canada	2000	47	6.2 μg kg^{-1}	Pereg et al (2003)
Milk	Europe - Faroe Islands, Denmark	1999	47, 99, 100, 153	7.2 μg kg^{-1}	Fangstrom et al (2004)
Plasma	Europe - Russia	2001	Congeners not listed	0.1-0.9 μg kg^{-1}	AMAP (2004)
Milk	Europe - Russia	2001	Congeners not listed	0.1-0.3 μg kg^{-1}	AMAP (2004)
Milk	Asia - Japan	2002	28, 47, 99, 100, 153, 154	0.7-2.8 μg kg^{-1} lipid	Ohta et al (2002)

Environmental media	Region	Year	PBDE congeners analyzed	ΣPBDE	Reference
Humans					
Milk	Asia - Taiwan	2007	17, 28, 47, 66, 85, 99, 100, 138, 153, 154, 183, 209	3.9 μg kg^{-1} lipid (mean)	Chao et al (2007)
Blood Serum	New Zealand	2001	47, 99, 100, 153, 154, 183	7.2 μg kg^{-1} lipid (mean)	Harrad and Porter (2007)
Milk	Australia	2003	17, 28, 33, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, 166, 183	6.1-18.7 μg kg^{-1} lipid	Toms et al (2007)

[1]Not Detected, sample levels below the limit of detection for the analytical method used.
[2]dry weight
[3]wet weight

Concentration of PBDEs in environmental samples worldwide range from very low levels dissolved in water (18±1.8 pg L^{-1}, Streets et al, 2006) to very high levels in sewage sludge in the US (544 μg kg^{-1}, Hale et al, 2002) and Europe (844-18100 μg kg^{-1} dw, Fabrellas et al, 2004). In biota levels range from 0.2 μg kg^{-1} in Salmon off the Pacific Coast of North America (Easton et al, 2002) and 0.1-0.3 μg kg^{-1} (ww) in Blue Mussels from Norway (Bethune et al, 2004) all the way up to 350 μg kg^{-1} in Lake Trout from the Great Lakes in North America (Streets et al, 2006), 353 μg kg^{-1} (ww) in Brown Trout from Lake Mjosa in Norway (Mariussen et al, 2003) and 2.9-1240 μg kg^{-1} (lipid) in various fish species from Taiwan Rivers and Estuaries (Peng et al, 2007).

Sediment levels in Australia are fairly consistent with levels in the rest of the world, with fairly low levels at sites in Queensland, South Australia, the ACT and Tasmania (not detected (ND)-7.73 μg kg^{-1} dw, Toms et al, 2008) comparable to levels in sites such as the River Danube in Austria (0.3-10.4 μg kg^{-1} dw, Moche and Stephen, 2003) and Cork Harbour in Ireland (1.8-1.9 μg kg^{-1} dw, Kilemade et al, 2004) and higher PBDE levels at sites in the populous and industrialized areas in New South Wales and Victoria (ND-60.9 μg kg^{-1} dw, Toms et al, 2008) comparable to sites in North America, such as Lake Huron (30.3 μg kg^{-1} dw, Song et al, 2005) and Lake Michigan (66.1 μg kg^{-1} dw, Song et al, 2005), and sites in Europe, such as Cinca River

in NE Spain (2.5-42 μg kg^{-1} dw, Eljarrat et al, 2004) or Lake Mjosa in Norway (0.6-27 μg kg^{-1} dw, Schlabach et al, 2004).

There is no literature which describes any active attempt to clean up or reduce current levels of PBDE in the environment. However, as was stated earlier, rising levels of PBDE caught the attention of governments around the world, leading to the restriction of their production and use. The lower brominated commercial mixtures, penta- and octa-BDE, were the first to be regulated, due to the high level of uptake efficiency and accumulation of the lower brominated PBDE congeners (de Wit, 2002; Alaee et al, 2003). Penta- and octa-BDE production ceased worldwide in 2005 (NICNAS, 2005). While this action removed an obvious source of emission of PBDEs into the environment, in the form of effluents from the factories which produced them, there is no guarantee this will lead to an immediate or lasting reduction of PBDE levels worldwide.

The reason for this is two-fold. The first is that there are still large quantities of penta- and octa-BDE extant in products which were produced 10-20 years ago when all three commercial PBDE products were in frequent use. Prevedouros et al (2004) derived an inventory for the use and emissions of the penta-mixture of PBDE in Europe between 1970 and 2000. Their study suggests that the majority of the penta-BDE mixture is in polyurethane foams, up to 30% of it in cars, another 10% in furniture foam, and the rest in textiles, building materials, packaging and solid applications. As these products are discarded the additive PBDE will leach out into the environment. Watanabe and Sakai (2003) suggest possible sources of emissions into the environment ranging from municipal, hospital or hazardous waste incinerators to facilities recycling plastics and metals from electronic devices to final disposal sites and accidental fires.

In addition to the possibility of emissions from discarded penta- and octa-BDE containing products there is also the continued production and use of deca-BDE mixtures. A major argument made by manufacturers of deca-BDE products is that deca-BDE is too large a molecule to be highly bioavailable to organisms and would therefore not accumulate in biota like the lower brominated PBDE congeners and commercial mixtures did. However, laboratory experiments have shown that PBDE-209, the main congener in deca-BDE products, can be debrominated in the gastrointestinal tract of carp (*Cyprinus carpio*) after dietary exposure in the form of spiked

food pellets (Stapleton et al, 2004a). This study showed that, although PBDE-209 itself did not accumulate in carp tissue during the 60 days of exposure, at least seven penta- to octa-brominated congeners were formed. In another study looking at debromination of PBDE-99 and -183 in carp, Stapleton et al (2004b) they found significant debromination of the two congeners resulting PBDE-99 converting to PBDE-47 and PBDE-183 converting to PBDE-154. In addition, studies in eels (Allchin and Morris, 2003), large-scale suckers (*Catostomus macrocheilus*) (Johnson and Olson, 2001) and lake trout (*Salvelinus namaycush*) (Tomy et al, 2004) all found debromination of PBDE congeners after dietary exposure as well. This suggests that so long as deca-BDE commercial mixtures remain in production and worldwide use levels of PBDE occurrence in biota will continue to rise.

1.3 PBDE and endocrine disruption

As PBDE levels have risen worldwide, interest in the potential toxic side-effects of exposure to the chemical increases as well. Within the last decade numerous studies have been published exploring these possible negative side-effects which include neurodevelopmental defects (Kuriyama et al, 2005; Viberg et al, 2003; Branchi et al, 2003), thyroid hormone disruption and cancer (McDonald, 2002). It is recognized and well documented that PBDEs are endocrine disrupting chemicals which affect thyroid hormone homeostasis and metabolism (Labadie and Budzinski, 2006; Branchi et al, 2003; Legler and Brouwer, 2003; Jenssen et al, 2004; Ellis-Hutchings et al, 2006; Hall et al, 2003). One area where numerous studies have been done with little consensus being reached is in the area of estrogenic effects. Although there seems to be agreement that PBDEs do interact with the reproductive endocrine system, there is still some question as to what effect PBDEs have on it. The literature involved is difficult to review and compare because the research methods used in the various studies are not always clearly comparable to one another. Methodology includes both *in vivo* and *in vitro* experiments in animals ranging from mice to mussels to human cell lines. Narrowing the criteria to studies in aquatic organisms which measure vitellogenin (Vtg) production as an endpoint of estrogenic activity leaves three studies which illustrate well the continued uncertainty about the estrogenic or anti-estrogenic behavior of PBDEs.

In the first, an *in vitro* study published in 2005, Nakari and Pessala measured vitellogenin (Vtg) secreted from freshly separated hepatocytes of juvenile rainbow trout (*Oncorhyncus mykiss*) using an enzyme-linked immunosorbent assay (ELISA). They saw a clear dose-response curve in the presence of three different PBDE congeners (PBDE-47, -99 and -205), with the higher brominated congeners causing higher levels of induction and lower concentrations. PBDE-205 and PBDE-99 induced maximum Vtg production at concentrations of about 10 $\mu g\ L^{-1}$, while PBDE-47 needed a concentration of approximately 50 $\mu g\ L^{-1}$ for maximum induction. In addition, while PBDE-47 and PBDE-99 induced maximum Vtg production levels of approximately 800 and 1,100 ng mL^{-1}, respectively, the maximum Vtg concentration in the presence of PBDE-205 was about four times higher at almost 4,000 ng mL^{-1}.

On the other hand Boon et al (2002) looked at Vtg production *in vivo* in Atlantic salmon (*Salmo salar*) after oral dosing with two commercial PBDE mixtures (Penta- and Octa-BDE). In this case Vtg expression was measured by quantifying mRNA levels in the liver by hybridization to rainbow trout Vtg cDNA probes (clone AA-4). Groups of fish were dosed twice (oral intake at days 1 and 4) with 10 and 50 mg kg^{-1} body weight of both the penta- and octa-BDE commercial mixtures and sampled at days 7 and 14. While both mixtures were readily taken up into the different tissues that were analyzed, the slot blot for Vtg mRNA expression shows no observable Vtg expression except for E_2 positive control groups.

Finally, Aarab et al (2006) exposed both male and female mussels (*Mytilus edilus*) to 1 $\mu g\ L^{-1}$ of tetra-BDE for three weeks in a continuous flow-through system. Levels if Vtg-like proteins were measured using indirect method alkali-labile phosphate assay. This study found that exposure *in vivo* to tetra-BDE actually decreased Vtg levels. While Vtg-like protein concentration in control males and females was approximately 15 and 17 $\mu g\ g^{-1}$, respectively, in mussels exposed to tetra-BDE levels in both males and females fell to approximately 3 $\mu g\ g^{-1}$.

In the end, as these three studies show, the research is currently too varied and contradictory to conclusively state whether PBDEs act estrogenically or anti-estrogenically in fish. Further research is still needed before this debate can really be settled.

1.4 Enzyme-linked immunosorbent assay (ELISA): Measuring vitellogenin production as an endpoint of estrogenic potency

Vitellogenin (Vtg) is a large, complex phospholipoglycoprotein dimer normally produced in the livers of maturing female oviparous vertebrates in response to estrogen secreted by the ovaries (Booth and Skene, 2006). The molecular weight of a whole Vtg molecule ranges between 300 – 600 kDa, with the number and molecular weight of subunits varying among different species (Specker and Sullivan, 1994). It is an egg-yolk precursor protein that is transported from the liver by way of the bloodstream to the ovaries where it is incorporated into developing oocytes (Wallace, 1985).

The hepatic estrogen receptors that are responsible for inducing Vtg production are present in males even though estrogen production is normally nominal (Booth and Skene, 2006). Therefore, Vtg production can be induced in male fish in the presence of exogeneous estrogen (Hotta et al, 2003) highlighting Vtg as a useful biomarker for assessing estrogenic effects of suspected endocrine disrupting chemicals in male fish.

There are several enzyme-linked immunosorbent assays (ELISAs) for detecting and measuring Vtg. There are three main methods which form the basis of all ELISAs: a) the direct ELISA, b) the indirect ELISA and c) the sandwich ELISA (Crowther, 2001). The most common assay is an indirect ELISA (Specker and Sullivan, 1994). The main advantages of this ELISA are ease of development and validation, low equipment requirements, and high sensitivity and specificity (Specker and Sullivan, 1994; Bon et al, 1997).

The development of an ELISA for a specific species is based on obtaining an antibody against Vtg from that species. This is achieved by first obtaining purified Vtg from a target species, such as rainbow trout, and then injecting that Vtg into a second species, such as sheep, in order to produce an antibody response (Bon et al, 1997; Schafhauser-Smith and Benfey, 2002). In an indirect ELISA this primary antibody binds to Vtg from the first species, and then a secondary antibody specific to the species in which the primary antibody was produced binds to the primary antibody. The secondary antibody has an enzyme conjugate, for example, horseradish

peroxidase, which in the presence of an appropriate developing solution, o-Phenylenediamine dihydrochloride, develops a color which can be measured on a spectrophotometer (Crowther, 2001). This colorimetric method has two distinct advantages: results can be seen by eye allowing for immediate assessment of whether the assay has worked and quantification can be easily achieved by running Vtg standards of known concentration along with the unknown samples (Crowther, 2001).

1.5 This project

This thesis consists of 6 chapters. Chapter 1 and 6 are general introduction and conclusions with recommendations for future work, respectively, and Chapters 2-5 are:

Chapter 2: Determination of PBDE-47 levels in sediments from Ross Creek, Townsville, QLD

Sediment samples from Ross Creek in Townsville, QLD were analyzed to determine current levels of contamination of two PBDE congeners, PBDE-47 and PBDE-209. PBDE was extracted from the samples by soxhlet extraction and the extract underwent a further clean-up using a silica gel clean-up method. The samples were then analyzed by gas chromatography – mass spectroscopy (GC-MS) to determine the concentration of PBDE present in the samples.

Chapter 3: Toxicokinetics of PBDE-47 in barramundi (*Lates* calcarifer)

Barramundi (*Lates calcarifer*), a commercially valuable Australian fish species was exposed to PBDE-47, the most common PBDE congener found in biota, to ascertain the rate at which PBDE-47 depurates in barramundi. The fish were given an i.p. injection of saline (control), 1mg kg^{-1} (bw) of PBDE-47 (low dose) or 10 mg kg^{-1} (bw) of PBDE-47 (high dose), then sampled over the course of fourteen days. PBDE-47 was extracted from the samples by hexane extraction and the extract was analyzed by GC-MS to determine concentrations of PBDE-47 present at each time point. From this data the depuration rate of PBDE-47 in barramundi was determined.

Chapter 4: Development and optimization of an enzyme-linked immunosorbent assay (ELISA) for detection of Vtg production in barramundi (*Lates calcarifer*)

The work in chapter 4 included preliminary work towards creating a barramundi specific ELISA, including the creation of a primary polyclonal anti-barramundi Vtg antibody in sheep, but ultimately culminated in the optimization of a previously developed barramundi ELISA. In the initial stages Vtg production in barramundi was induced with an injection of 17β-estradiol (E2), then the Vtg was purified using size exclusion column chromatography. The purified Vtg was then injected into sheep to induce production of a polyclonal antibody against barramundi Vtg. The purified Vtg and the sheep polyclonal antibody were then compared to an existing ELISA for detecting barramundi Vtg which uses a rainbow trout Vtg standard (Caymen Chemical Co) and a mouse anti-striped bass Vtg monoclonal antibody (Biosense). This comparison was done to determine the optimal working ELISA for detecting Vtg production in barramundi.

Chapter 5: Estrogenicity of PBDE-47 in barramundi (*Lates calcarifer*)

The estrogenic or anti-estrogenic effect of PBDE-47 on barramundi was assessed using Vtg production as the measurable endpoint of estrogenic activity. Two experimental designs were used for this research, the first being a time course experiment designed to determine the induction of Vtg production over time after a single injection of either a low (1 mg kg^{-1} bw) or a high (10 mg kg^{-1} bw) dose of PBDE-47. The second experiment was designed to determine whether a repeated injection of PBDE-47 had a significantly different effect on Vtg production than a single injection of PBDE-47. The optimal ELISA for detecting Vtg production in barramundi, as determined in chapter 4, was used to measure Vtg production in response to exposure to PBDE-47.

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2. Determination of PBDE-47 levels in sediments from Ross Creek, Townsville, QLD

2.1 Introduction

As was discussed in Chapter 1, PBDEs are a group of commonly used flame retardants in many polymers, with global use increasing steadily over the last few decades. Research has been done worldwide over the last two decades to determine current PBDE contamination levels in the environment (Table 1.1).

Due to the hydrophobic nature of PBDEs (according to Braekevelt et al (2003) the octanol-water partition coefficients (K_{OW}) for PBDEs range from $10^{5.74\pm0.22}$ to $10^{8.27\pm0.26}$) in the aquatic environment, PBDE contamination tends to be concentrated in sediment samples rather than within the water column itself (Oros et al, 2005). A recent study published by the Australian Government Department of the Environment and Heritage looked at PBDE levels in sediments from around Australia (Toms et al, 2006). Prior to the publication of this research there was no data available on PBDE levels in aquatic environments in Australia. In Queensland the samples obtained by Toms et al (2006) were all taken from the southeast corner of the state, concentrated around the city of Brisbane, QLD. In this chapter data are presented regarding PBDE levels in sediment collected in Ross Creek which drains an urbanized catchment within the city of Townsville, a city of ~153,000 (Year Book of Australia, 2008) located 1,300 km north of Brisbane on the eastern coast of Australia. The aim of the research in this chapter is to provide additional data on current levels of PBDE occurrence in Australia. The goal is to establish a baseline that can be used in future research to determine whether levels are increasing, decreasing or remaining steady.

Sediment samples were collected in duplicate from three sites along Ross Creek in Townsville. Ross Creek was chosen because of its proximity to downtown Townsville and its role as a drain for the catchment area. The sites are representative of the areas through which the creek flows: residential, industrial, and urban. The samples were extracted by Soxhlet, followed by a clean-

up process described by the US EPA, and were then analyzed by gas chromatography – mass spectroscopy to determine the current level of PBDE contamination in Townsville.

2.2 Materials and methods

2.2.1 Sampling

Duplicate samples were taken at each of the three sites marked by letters in Figure 2.1. These sites were chosen as representative of the different areas through which Ross Creek flows. Ross Creek is saline throughout its length, so all three sites would be considered estuarine, although sites B and C see greater variations in salinity than does site A (da Silva et al, 2005). Site A is located in the middle of the urban downtown area of Townsville. Site B is located near the rail yards and immediately downstream from the entry point of a drainage canal which travels from two man-made lakes west of the creek along a major roadway. Site C is located in the middle of a residential area of Townsville.

Samples were collected from the surface (top 1-5 cm) of fine-grained sediments in Ross Creek. The samples were collected using a hexane-washed aluminium spatula into hexane-washed glass containers. They were transferred to the laboratory within one hour of collection and stored at 4°C until being dried and prepared for analysis (see below).

Figure 2.1 Map of Townsville with sample sites labeled. Sample sites are: Ross Creek at Rooney's Bridge (A), Ross Creek at the Civic Theatre (B), and Upper Ross Creek (C). Vertical arrow points to entrance of drainage canals from The Lakes.

2.2.2 Extraction

Sediment samples were air dried and ground in a ring mill. Approximately 5.0 g (accurately weighed) was extracted with 50 mL of pesticide grade n-hexane (Envisol purity, Reidel-de Haen) in a soxhlet extractor for 16 hours. As an internal standard, PCB 103 (10 μL of 8 $\mu g.mL^{-1}$) was added to the extract and carried through the subsequent cleanup steps.

The extract underwent a silica gel cleanup step using silica gel, acidic silica gel, basic silica gel and anhydrous sodium sulphate using the procedure described in Section 13.3 of USEPA method 1614 (USEPA, 2007) . After clean up, the hexane extract was reduced in volume to 0.5 mL for analysis by quadrupole GC-MS (Varian 1200).

2.2.3 Gas chromatography – Mass spectroscopy (GC-MS)

PBDE-47 and PBDE-209 in all samples was quantified using a Varian 4000 GC-MS with a Varian 8400 AutoSampler and Varian 1177 Split/Splitless Injector. A 25m, 0.1μm film

thickness DB-5 column was used in the GC, and 2.0µL of sample was injected at an injection temperature of 250°C, with splitless injections and helium as the carrier gas in constant flow mode. The column temperature program used an initial column temperature of 80°C for 1.5 minutes, after which the temperature was increased at 12°C min^{-1} to a temperature of 250°C, followed by an increase of 25°C min^{-1} to a temperature of 300°C which was held for 5.3 minutes. The MS was in EI ionization mode, with helium damping gas at 2.5mL min^{-1}. The precursor ion used was at 486 m/z, with a product ion range (m/z) 320-335. For PBDE-47 the quantitation ions used were at 324, 326 and 328 m/z. For PBDE-209 the quantitation ions used were at 797 and 801 m/z.

2.3 Results

Table 2.1 shows PBDE-47 and PBDE-209 levels in Townsville, as determined by analysis by GC-MS. The highest overall PBDE levels were found at site B, while the next highest levels were downstream at site A. Site C samples both came in below the detection limit of the analysis, which was determined to be 0.2µg.

Table 2.1 Concentration of PBDE-47 and PBDE-209 in sediment samples from three sites along Ross Creek, Townsville, QLD.

	PBDE-47			PBDE-209			
Sample Site	Range	Mean	Standard Deviation	Range	Mean	Standard Deviation	Ratio PBDE-47:PBDE-209
A	ND - 0.5 µg kg^{-1} dw	0.15 µg kg^{-1} dw[b]	0.07	0.4 - 0.5 µg kg^{-1} dw	0.45 µg kg^{-1} dw	0.07	1:3
B	0.2 - 0.5 µg kg^{-1} dw	0.35 µg kg^{-1} dw	0.2	0.8 - 0.9 µg kg^{-1} dw	0.85 µg kg^{-1} dw	0.07	3.5:8.5
C	ND[a]	ND	0	ND	ND	0	ND

[a]Non-detectable levels (<0.2 µg kg^{-1} dw).
[b]Where one sample was below detection limits a value of ½ the detection limit (0.2 µg kg^{-1} dw) was used to determine the mean concentration of PBDE-209.

2.4 Discussion

In this study the levels of two PBDE congeners (PBDE-47 and -209) in sediment samples from Ross Creek in Townsville were determined. These two congeners were chosen because in the literature PBDE-209, as the main congener of the most common commercial PBDE product (deca-BDE), is consistently the predominant PBDE congener found in sediment samples (Law et al, 2006). PBDE-47 is typically the most abundant congener in biota (Hites et al, 2004) although, being the most common congener in penta-BDE commercial PBDE mixtures, PBDE-47 is usually present at detectable levels in sediments as well (Toms et al, 2006). The K_{OW} of PBDE-47 is within the range of optimum bioaccumulation potential (Fisk et al, 1998) while PBDE-209 may be too nonpolar or too large (Braekevelt et al, 2003). Therefore, one possible explanation put forward for the predominance of PBDE-47 in biota, as opposed to PBDE-209, is that PBDE-209 undergoes biotransformation by debromination after ingestion leaving PBDE-47 to bioaccumulate in the lipids of biota (Stapleton et al, 2004; Tomy et al, 2004). These two congeners were chosen for this research based upon their recurrence within the literature, and to confirm what has been seen by other studies, that PBDE-209 is present in higher levels in sediment samples than PBDE-47.

Water samples were taken at these sites, however PBDE concentrations were universally undetectable. Using the octanol-water partition coefficiencts for PBDE-209 and PBDE-47 determined by Braekevelt et al (2003) to determine the likely water concentration of these two congeners given the sediment concentrations found in Townsville sediments may help to explain why PBDE is undetected in water samples from these sites. If PBDE concentrations in the water are in equilibrium with the sediment at these sites then the equilibrium constant (K_D) can be estimated using the following equation from Karickhoff et al (1979):

Equation 2.1 $K_D = 0.6 \times f_{OC} \times K_{OW}$

where f_{OC} is the fraction by weight of organic carbon present in the sediment (Australian and New Zealand Environment and Conservation Council, 2000) and K_{OW} is the octanol-water partition coefficient of PBDE (Braekevelt et al, 2003). The f_{OC} in Townsville sediments is ~1%,

while the K_{OW} of PBDE-209 is 10^{10} (Braekevelt et al, 2003). The K_D of PBDE-209 was found to be $6x10^7$ L kg^{-1}. K_D is the ratio of the PBDE concentration in the sediment to the PBDE concentration in the water, as shown in the following equation:

Equation 2.2 $$K_D = \frac{[\text{sediment (mol kg}^{-1})]}{[\text{water (mol L}^{-1})]}$$

In this study a maximum sediment concentration of 0.9 μg kg^{-1} was found at one site, or ~1nmol kg^{-1}. Therefore, in equilibrium, the PBDE-209 concentration in the water at this site would be $1.67x10^{-17}$ mol L^{-1}, which is far below the detection limits available with GC-MS and which may account for the lack of detectable PBDE-209 in the water samples. Performing the same calculations for PBDE-47, using a K_{OW} of $10^{6.81}$ (Braekevelt et al, 2003) gives a K_D of $3.87x10^4$ L kg^{-1} and a water concentration of $2.58x10^{-14}$ mol L^{-1}, which is again far below the detection limits available with GC-MS.

In the sediment samples PBDE-47 and PBDE-209 were present at two of the three sites sampled, A and B. The concentrations determined for PBDE-47 were 0.35±0.2 μg kg^{-1} (dw) at site B and 0.15±0.07 μg kg^{-1} (dw) at site A (Figure 2.2). The concentration of PBDE-209 at these two sites was 0.85±0.07 μg kg^{-1} (dw) at site B and 0.45±0.07 μg kg^{-1} (dw) at site A. Ross Creek is saline throughout its length, up to and above site B, therefore both sites A and B can be classed as estuarine environments, which throughout the literature have been shown to have higher PBDE levels than freshwater sites (Oros et al, 2005; Song et al, 2005; Toms et al, 2006). What is harder to explain is why site B, which is further upstream, and sees greater variation in salinity than site A (da Silva et al, 2005), has noticeably higher levels of PBDE contamination. One possible explanation for these levels is that just upstream of site B a drainage canal enters Ross Creek which comes from two man-made lakes west of the creek (see Figure 2.1). The lakes are situated in the middle of a retail district and the canal runs along a major Townsville roadway, both of which could contribute to high contamination levels of the canal itself.

Comparing the PBDE concentration measurements found in Townsville to the values found by Toms et al (2006) in southeast Queensland, the PBDE levels from this research fall well within

the range seen by that group of researchers. The Brisbane River (in the city and Indooroopilly) had a PBDE-47 concentration of 0.23 μg kg^{-1} (dw), while it's PBDE-209 concentration was 0.88 μg kg^{-1} (dw) (Toms et al, 2006) a ratio of ~1:4, which correlates closely to the levels found in Ross Creek sediment samples at sites A and B. In addition, Toms et al (2006) compared PBDE concentrations (ΣPBDE concentrations for all congeners analyzed) based on land-use type as well as locations. They found that urban centers, the class which best defines Townsville, had a mean ΣPBDE concentration of 0.88 μg kg^{-1} (dw), with a median of 0.53 μg kg^{-1} (dw) (Toms et al, 2006).

It must be kept in mind that this study only looked at 2 PBDE congeners, yet compared with values worldwide the PBDE concentrations found in Townsville are relatively low. In Europe levels range from a range of 1.8-1.9 μg kg^{-1} (dw) in Cork Harbour, Ireland (Kilemade et al, 2004), to a range of 68-390 μg kg^{-1} (dw) in Sweden's River Viskan (Sellstrom et al, 1998). North America has levels ranging from non-detectable to 212 μg kg^{-1} (dw) in San Francisco Bay Estuary (Oros et al, 2005), to non-detectable to 148 μg kg^{-1} (dw) in Niagara River (Samara et al, 2006). There is no literature for either Africa or South America, but work has been done in the Arctic, with mostly low levels such as 0.004-0.027 μg kg^{-1} (dw) found in sediments from several freshwater sites in northern Russia (AMAP, 2004). However, sites on the Kola Peninsula of Russia, an area located within the Arctic circle that has seen military production and industrial mining had PBDE concentrations up to 241 μg kg^{-1} (dw) (Chernyak et al, 2003). The levels found in this study of sediment samples from Ross Creek seem to fall on the lower end of the spectrum when compared to more highly industrialized areas of Europe and North America (Table 1.1).

Production of penta-BDE commercial mixtures, of which PBDE-47 was a major component, was ceased in 2005, and even before that from 1994 to 2004 use of penta-BDE in Australia was falling (NICNAS, 2005). Conversely, over the same period the use of deca-BDE, which is not currently restricted, increased slightly, from 177 to 180 metric tons, an increase of ~1% (NICNAS, 2005). Therefore, it would be expected that future measurements will see levels of PBDE-47 in sediment samples fall while levels of PBDE-209 in the sediments rise. However, PBDE is an additive flame retardant, meaning it is not chemically bound to the products in which

it is used (Alaee et al, 2003). So, as products that were manufactured approximately 10-20 years ago, when penta-BDE commercial mixtures were still in frequent use, are discarded and destroyed it is reasonable to assume that levels of PBDE-47 will continue to rise. The only way to avoid this outcome would be to develop a method for disposing of products treated with PBDE in a way that would prevent the leaching of the chemical into the environment. Finding an efficient and cost-effective method to extract PBDE from discarded materials would be one way of doing this, but there is no literature at the moment to suggest anyone is looking into this option. Therefore it seems likely that future measurement will see levels continue to rise in the near future, followed by a gradual tapering off to a plateau as products containing PBDE-47 are discarded and replaced with products containing an alternative flame retardant.

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3. Toxicokinetics of PBDE-47 in Barramundi (*Lates calcarifer*)

3.1 Introduction

PBDE use worldwide has risen steadily in the last few decades and Australia has not been immune to this increase in PBDE levels, with ~210 tons of PBDE products imported in 2003-2004 (NICNAS, 2005). While overall PBDE levels in aquatic sediment in Australia are currently fairly low (0.72 μg kg^{-1} (dw) in freshwater systems, 8.09 μg kg^{-1} (dw) in estuarine systems) compared to levels in other countries (Table 1.1), there are areas, such as downstream of sewage treatment plants, where levels are quite high already (up to 60.9 μg kg^{-1} (dw)) (Toms et al, 2008). Global levels of PBDEs have risen over the last twenty years (Rayne et al, 2003; Ikonomou et al, 2002; Papke et al, 2000; Ohta et al, 2002; Zennegg et al, 2003) and though the last decade has seen some regulation of the lower brominated PBDE mixtures (Watanabe and Sakai, 2003; Alaee et al, 2003) levels can be expected to continue to rise with the continued use of Deca-BDE commercial mixtures and the disposal of products treated with all three major PBDE mixtures (Martin et al, 2004).

While Deca-BDE is the most commonly used class of commercial PBDE, and the most common congener found in sediments, soils and water is PBDE-209 (Alaee et al, 2003; de Wit et al, 2006; Law et al, 2006), most studies of animals and humans have found PBDE-47 to predominate in biological samples (Chao et al, 2007; de Wit et al, 2006; Harrad and Porter, 2007; Law et al, 2006; Ohta et al, 2002), possibly as a result of debromination of the more highly brominated PBDE-209 in the gastrointestinal tracts of the animal (Stapleton et al, 2004b; Tomy et al, 2004). The potential toxic side effects of PBDE have been shown to include neurodevelopment deficiencies (Kuriyama et al, 2005; Viberg et al, 2003; Branchi et al, 2003), thyroid hormone disruption (Ellis-Hutchings et al, 2006; Hall et al, 2003; Morgado et al, 2007) and cancer (McDonald, 2002). Studies looking into the possible negative side effects of PBDE exposure in animals have found that the lower brominated congeners are more potent than the higher brominated congeners, especially with regards to thyroid hormone disruption (McDonald, 2002). So, although only ~13% of the total commercial PBDE demand worldwide contains PBDE-47, it is important that the ability of organisms to metabolize and clear this congener be studied.

In addition, while PBDE exposure in humans could come through many routes, with PBDEs being detected in many areas from the atmosphere to common house dust (Wurl et al, 2006; Stapleton et al, 2005), studies have shown a definite correlation between consumption of contaminated fish and elevated levels of PBDEs in humans (Ohta et al, 2002; Ikonomou et al, 2002; Zennegg et al, 2003; Schecter et al, 2004; Tittlemier et al, 2004). Studying how PBDE-47 is cleared by an important commercial fish species along with work to determine current environmental levels would allow for the estimation of contamination levels in humans consuming fish on a regular basis.

Barramundi is a commercially important fish species throughout Australia and Southeast Asia. Barramundi production within Australia has been increasing over the past 15 years (Boonyaratpalin and Williams, 2002). In northeastern Australia the wild fishery for barramundi is a major industry, grossing more than 6 million dollars annually (Katersky and Carter, 2007). The importance of barramundi to the Australia fisheries and aquaculture industry is increasing steadily, and this is a good opportunity to determine how barramundi handle exposure to PBDE. Therefore, the aim of this chapter is to determine the depuration rate of PBDE-47, the PBDE congener most commonly found in biota (Hites et al, 2004), in barramundi. This could be considered preliminary toxicokinetic work which would be useful in combination with future work to determine how environmental levels of PBDE might affect humans with a diet heavy in fish, or in combination with work looking at the potential toxic effects of PBDEs in barramundi to determine at what point environmental levels sill become too high for the fish to remove PBDE faster than it is being exposed to it.

In the work described in this chapter male juvenile barramundi were injected with a low (1 mg kg^{-1} bw) dose of PBDE-47 suspended in DMSO. A random sample of fish from each treatment group was then sacrificed at regular intervals and tissue samples were analyzed by GC-MS to determine the amount of PBDE-47 present at the time of sampling.

3.2 Materials and Methods

3.2.1 Fish Treatment

Male juvenile barramundi (average weight = 10g) were obtained from stocks bred and grown at the Marine & Aquaculture Research Facilities Unit (M.A.R.F.U.) at James Cook University in Townsville. The fish were maintained in 150 L recirculating systems in a temperature controlled environment. The water temperature was maintained at 27 ± 1°C, with a photoperiod of 16:8 (light:dark). Fish were fed ~3% body weight daily with dry feed pellets obtained through the MARFU facility. Prior to the experiment fish were separated into four tanks (n=25) and allowed to acclimate for two days. This experiment and the time course work described in chapter 5 were performed using the same fish. More fish were bled for that experiment than sacrificed for this one, therefore the n above represents more fish than will be used for this experiment.

3.2.2 Experimental Protocol

For the experiment fish were anaesthetized in 0.05% 2-phenoxyethanol (Sigma Aldrich) and weighed, then one group was injected with 2mL kg^{-1} of acidified saline as a control while the other three groups were injected with 2mL kg^{-1} of 0.5mg mL^{-1} PBDE-47 in DMSO suspension. All injections were done by i.p. injection. Three control and four PBDE-47 dosed fish, randomly sampled from the three PBDE-47 dosed groups, were terminated at 0, 1, 2, 3, 4, 5, 7 and 10 days post- injection. On day 14 post-injection two control fish and three PBDE-47 dosed fish were sampled. The fish were terminated by over-anaesthetization with 2-phenoxyethanol and whole body tissue samples were frozen at -20°C until analysis.

3.2.3 PBDE Extraction

Fish samples were removed from the freezer immediately before extraction. Each sample was homogenized using a hand blender and approximately 1mL g^{-1} of double distilled water (ddH_2O). 100µL of 8µg mL^{-1} PCB-103 (internal standard) was added to the homogenate, then the sample was mixed on a Ratek RM4 rotary mixer at 38 rpm for 5 minutes. 10mL of HPLC

grade hexane was then added and the sample was mixed at 38 rpm for a further 30 minutes. The sample was then centrifuged at 4.4 krpm for 5 minutes to separate the hexane layer, which was collected and kept for GC-MS-MS analysis.

3.2.4 GC-MS

PBDE-47 in all samples was quantified using a Varian 4000 GC-MS with a Varian 8400 AutoSampler and Varian 1177 Split/Splitless Injector. A 25m, 0.1μm film thickness DB-5 column was used in the GC, and 2.0μL of sample was injected at an injection temperature of 250°C, with split injections and helium as the carrier gas in constant flow mode. The column temperature program used an initial column temperature of 80°C for 1.5 minutes, after which the temperature was increased at 12°C min^{-1} to a temperature of 250°C, followed by an increase of 25°C min^{-1} to a temperature of 300°C which was held for 5.3 minutes. The MS was in EI ionization mode, with helium damping gas at 2.5mL min^{-1}. The precursor ion used was at 486 m/z, with a product ion range (m/z) 320-335. The quantitation ions used were at m/z 324, 326 and 328.

3.2.5 Data Analysis

PBDE-47 standards (0.5, 1, and 2μg mL^{-1}) with a PCB-103 internal standard (0.8μg mL^{-1}) were run in triplicate. Equation 3.1 shows the relationship between the peak areas and concentration of the PBDE-47, PCB-103 and the response factor, F. F was calculated by plotting (Area Count PBDE-47/[PBDE-47]) vs. (Area Count PCB-103/[PCB-103]) for the set of standards, and was then used in equation 3.1 to calculate [PBDE-47] in unknown samples.

Equation 3.1 $$\frac{\text{(Area Count PBDE-47)}}{\text{[PBDE-47]}} = F\,\frac{\text{(Area Count PCB-103)}}{\text{[PCB-103]}}$$

3.2.6 Statistical Analysis

A two-tailed paired t-test with a 95% confidence interval was done to determine whether there was a statistically significant difference between the control fish and the PBDE-47 dosed fish.

3.3 Results

The response factor, F, from equation 3.1 was found to be 0.0705.

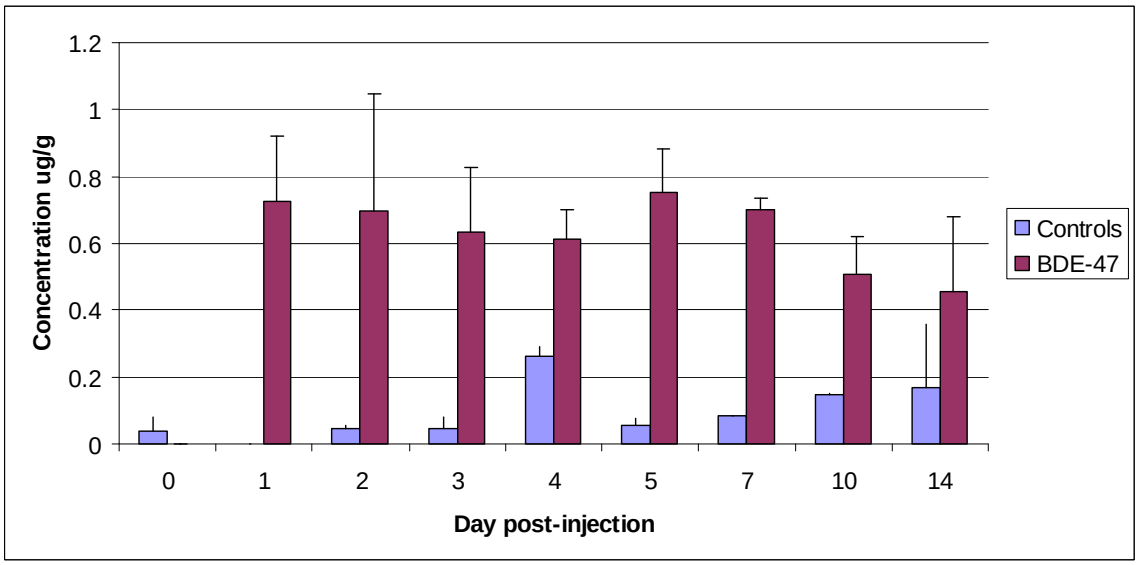

Figure 3.1 PBDE-47 concentration in barramundi dosed with 1mg kg^{-1} PBDE-47. Each bar represents mean $\pm$ sd (n=3).

Figure 3.1 shows the mean concentration of PBDE-47 in control (blue bars) and PBDE-47 dosed (maroon bars) barramundi in µg g^{-1} (ww). Unexpectedly, the control fish, those dosed with acidified saline, registered noticeable levels of PBDE-47. However, PBDE-47 dosed fish had significantly higher levels of PBDE-47 than did those dosed with acidified saline (P=0.0002). Fish were injected with 1µg g^{-1} of PBDE-47, and after 24 hours an average of 0.73±0.195µg g^{-1} was detected, indicating an uptake rate of approximately 73% of the dose. PBDE-47 levels then remained relatively consistent through the first 5 days post-injection before beginning to decrease. The depuration rate from day 5 on was determined assuming first-order kinetics (Muirhead et al, 2006), by determining the slope of a plot of Ln([PBDE-47]) vs. time. The depuration rate was found to be 0.069±0.03 day^{-1} (the uncertainty is the standard error of the

slope). If the depuration rate is calculated from day 1, a depuration rate of 0.041±0.02 day^{-1} is calculated. By day 14 PBDE-47 levels had decreased to an average of 0.46±0.225μg g^{-1}.

3.4 Discussion

The purpose of this study was to determine the depuration rate of PBDE-47 from barramundi (*Lates calcarifer*) after intra-peritoneal injection. Two depuration rates were calculated, the first for depuration starting at day 1 of 0.041±0.02 day^{-1}, the second if depuration begins after a lag of 5 days of 0.069±0.0.03 day^{-1}. The data appears to remain steady for the first five days after injection, supporting the determination of the depuration rate from day 5 to day 14. However, no literature example of this delayed depuration of PBDE can be found, and studies that have been done on depuration of PBDE in fish give depuration rates beginning immediately (Ciparis and Hale, 2005; Gustafsson et al, 1999; Stapleton et al, 2004b; Tomy et al, 2004) or within 24 hours of dosing (Muirhead et al, 2006).

Stapleton *et al* (2004b) and Tomy *et al* (2004) both studied the rate of depuration of multiple PBDE congeners in the common carp and juvenile lake trout, respectively. They found that PBDE-47 depurated at rates of 0.011±0.008 day^{-1} (Stapleton et al, 2004b) and 0.018±0.004 day^{-1} (Tomy et al, 2004), which are slightly slower than either rate found in this study of barramundi. However, Muirhead et al (2006) reported a depuration rate for PBDE-47 of 0.06±0.01 day^{-1} in Japanese Medaka (Muirhead et al, 2006) which is much closer to the rate found in this study. In addition, Ciparis and Hale (2005) and Gustafsson et al (1999) studied the rate of depuration of multiple PBDE congeners in two sedentary marine organisms, the oligochaete, *Lumbriculus variegatus* (Ciparis and Hale, 2005), and the blue mussel, *Mytilus edulis* (Gustafsson et al, 1999). Ciparis and Hale (2005) determined that *Lumbriculus variegatus* clears PBDE-47 at a rate of 0.031±0.006 day^{-1}, while Gustafsson et al (1999) found a depuration rate of 0.090±0.017 day^{-1}. The depuration rates determined in this study, therefore, fall right into the middle of the range of depuration rates for PBDE-47 found in the literature.

Recent studies have led to the recognition that consumption of contaminated fish is the predominant route of exposure to PBDEs in humans (Ohta et al, 2002; Schecter et al, 2004;

Tittlemier et al, 2004). Lower brominated PBDEs have shown very high uptake efficiency, such as 90% uptake of PBDE-47 in the gastrointestinal tract of pike during one dietary exposure study (Burreau et al, 1997), while the higher brominated PBDEs, such as PBDE-209, show a remarkable persistence, with one study in rats finding that the portion of a dose that reached the adipose tissue remained unchanged over 90 days of recovery (Norris et al, 1975). In addition there are multiple studies which find that the higher brominated PBDEs can undergo debromination in the gastrointestinal tract of fish, leaving behind lower brominated congeners (Allchin and Morris, 2003; Stapleton et al, 2004a; Stapleton et al, 2004b; Tomy et al, 2004). This makes studying the depuration rate of these lower brominated congeners important to understanding the possible effect on human consumption of continued rising levels of PBDE occurrence in commercial fish species.

In comparison with concentrations measured in marine organisms in the environment, summarized in Table 1.1, it is clear that a dose of 1 $\mu g\ g^{-1}$ or 1 $mg\ kg^{-1}$ is a fairly high dose compared to what is being seen in fish samples around the world at this time, although there are a few significant exceptions. In North America fish and mollusc samples have been found to contain ΣPBDE concentrations ranging from a low of 0.2 $\mu g\ kg^{-1}$ in Salmon off the Pacific coast (Easton et al, 2002) to 350 $\mu g\ kg^{-1}$ in Lake Trout in Lake Michigan (Streets et al, 2006). In Europe levels range from 0.1 $\mu g\ kg^{-1}$ in Blue Mussels from Norway (Bethune et al, 2004) to 298 $\mu g\ kg^{-1}$ in Barbel from the Cinca River in Spain (Eljarrat et al, 2004) and 353 $\mu g\ kg^{-1}$ in Brown Trout in Lake Mjosa in Norway (Mariussen et al, 2003). Asia sees the greatest variation of all from 1 $\mu g\ kg^{-1}$ in Yellowtail from Japan (Ohta et al, 2002) to 1240 $\mu g\ kg^{-1}$ in unidentified fish species in Taiwan's rivers and estuaries (Peng et al, 2007). At this point in time, humans are not being exposed to very high levels of PBDE contamination in the fish they consume. However, the continued use of deca-BDE commercial products means levels will most likely continue to rise, making the study of depuration rates in commercial fish species important to predicting the potential impact of these rising PBDE levels on the human diet.

Finally, as it was noted in the results the acidified saline dosed control fish contained unexpectedly high levels of PBDE-47. There are a couple possible explanations for this, the first being lab contamination. While steps were taken to ensure no cross-contamination of samples, it

is always a possibility and it cannot be conclusively ruled out here. The second possible explanation is that these fish had already come in contact with PBDE-47 in the environment. As was seen in the previous chapter, PBDE-47 is present in the local environment. While these fish were hatchery born and raised that is no guarantee that they haven't been exposed to PBDEs. Easton et al (2002) examined contaminant loadings in farmed salmon compared with wild salmon and found farmed salmon to have much higher levels of contamination due to high levels of contaminant loading in commercial feed. It would be worthwhile to look at both local biota, to determine current levels of PBDE accumulation, and the M.A.R.F.U. facilities and feed to determine whether the barramundi in this study faced contamination from a secondary source.

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4. Optimization of an enzyme-linked immunosorbent assay (ELISA) for detection of vitellogenin (Vtg) in Barramundi (*Lates calcarifer*)

4.1 Introduction

As PBDE levels rise worldwide interest in the potential for toxic side effects has increased as well. Within the last decade research has found that the side effects of PBDE include neurodevelopmental defects (Kuriyama et al, 2005; Viberg et al, 2003; Branchi et al, 2003), thyroid hormone disruption and cancer (McDonald, 2002). In addition, it is well documented that PBDEs are endocrine disrupting chemicals which affect thyroid hormone homeostasis and metabolism (Labadie and Budzinski, 2006; Branchi et al, 2003; Legler and Brouwer, 2003; Jenssen et al, 2004; Ellis-Hutchings et al, 2006; Hall et al, 2003).

One area where numerous studies have been done without reaching a consensus as to the effect of PBDE is that of estrogenic effects. While there is agreement that PBDEs affect the reproductive endocrine system there is little agreement on what these effects are exactly. Some studies have found that PBDE exposure mimics exposure to estrogen (Meerts et al, 2001; Nakari and Pessala, 2005; Stoker et al, 2005), while other studies have found PBDE exposure to have an anti-estrogenic effect (Aarab et al, 2006; Labadie and Budzinski, 2006).

One method for measuring estrogenic activity is to measure vitellogenin (Vtg) production. The use of enzyme-linked immunosorbent assays (ELISAs) in the measurement of Vtg production as an endpoint for assessing the estrogenic effects of suspected endocrine disrupting chemicals in male fish is discussed in Chapter 1 (section 1.4). Initially the goal of the work described in this chapter was to create and optimize a new indirect ELISA to detect Vtg in barramundi. The first step in developing an indirect ELISA was to inject male barramundi with 17β-estradiol (E2) to induce Vtg production, confirm production using size exclusion chromatography, attempt purification and to inject the final product into sheep to produce a working primary polyclonal antibody. Full details are provided in sections 4.2.1 and 4.2.2.

Project time constraints made the goal of fully developing an ELISA to detect Vtg induction in barramundi impractical. Fortunately, Susan Codi King at the Australian Institute of Marine Science (AIMS) in Townsville, Queensland, in collaboration with colleagues from the Royal Melbourne Institute of Technology (RMIT) in Melbourne and Biosense Laboratories in Norway were in the final stages of developing an indirect ELISA for detection and assessment of Vtg in barramundi and black bream (Codi King et al, 2008). This work included extensive cross-reactivity testing of blood plasma from E2 exposed barramundi against a wide range of mono- and polyclonal anti-Vtg antibodies available from Biosense Laboratories. They found that the mouse anti-striped bass Vtg monoclonal primary antibody, ND-3G2 (ND-3G2) (Biosense Laboratories, Norway), had the highest binding affinity in titration curves for plasma, as well as optimum binding over corresponding controls in ELISA results for barramundi. In addition, ND-3G2 binds with both intact and degraded Vtg (Codi King et al, 2008). A collaboration was established and samples from this research were used confirm the viability of their ELISA.

The ultimate goal of this portion of research was to create the best possible ELISA for the detection of Vtg in barramundi. This involved a comparison between the sheep anti-barramundi polyclonal primary antibody (Sh-0404JCU) produced in this study with the commercially produced monoclonal primary antibody (ND-3G2), created by Biosense and already in use by Codi King et al (2008). The focus was to determine the efficiency and accuracy of each antibody at detecting Vtg in male barramundi plasma samples. Then, after determining the most efficient primary antibody for use in the ELISA, two commercial Vtg standards (Rainbow Trout and Atlantic Salmon) (Caymen Chemical Co) and the purified Vtg produced in this study were compared in order to determine the best standard to quantitatively measure Vtg levels in male barramundi plasma samples.

4.2 Materials and Methods

4.2.1 Vtg induction and purification

4.2.1.1 Fish handling

Male, juvenile barramundi (weight range 262-654 g, mean weight 377 ± 104 g) were obtained from stocks bred and grown at the Marine & Aquaculture Research Facilities Unit (M.A.R.F.U.) at James Cook University in Townsville. Fish were maintained in 3,000 L recirculating systems at a temperature of ~25 ± 1°C, with a naturally occurring photoperiod of ~12 hr light: 12 hr dark. Fish were fed ~3% body weight daily with dry feed pellets.

For exposure experiments, fish were anaesthetized in 0.05% 2-phenyoxyethanol (Sigma Aldrich) and weighed before injection. Injections were done by i.p. injection and blood was collected from the caudal vein before being placed in a centrifuge tube and spun at 13,000 rpm for 5 min. Plasma samples were frozen at -20°C for later analysis.

4.2.1.2 E2-dosed barramundi for Vtg Induction

In order to induce Vtg production six male barramundi were dosed by i.p. injection with 5 mg kg^{-1} body weight, of 17β-estradiol (E2) suspended in acidified saline. Another six male barramundi were dosed by i.p. injection with acidified saline as controls. All fish were injected on three occasions, with a week between each injection. Blood samples were taken at the first injection (Day 0), the third injection (Day 14) and a week after the final injection (Day 21). The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the plasma from the final bleed (400 μg mL^{-1}) to inhibit enzyme activity which might break down the protein in the sample (Sun et al, 2003).

4.2.1.3 Vtg Purification

Vtg was purified using size-exclusion chromatography. A Sephacryl S-300 column (Pharmacia), loaded with Sepharose 4B was used to purify Vtg from the plasma samples obtained at the final bleed in section 4.2.1.1. Initially, blue dextran (2000 kDa) was run through the column to determine the void volume (66 mL). Next, a set of molecular weight markers were run to establish a standard calibration curve for the column. The molecular weight markers were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa) (Sigma).

1 mL saline (C) and E2 dosed fish plasma samples were mixed 1:1 with 50 mM Tris-HCl, pH 7.5 sample buffer. Samples were loaded onto the column (2 mL maximum loading capacity), then eluted with 50 mM Tris-HCl, 200 mM NaCl, pH 7.5 run buffer. Column fractions (CF) were collected (1 mL aliquots) and absorbance was monitored on a Beckman spectrophotometer at 280 nm for the fractions between 60 mL and 100 mL (Fractions 1-41). A control plasma sample was run followed by an E2-dosed plasma sample to confirm the Vtg peak was only present in E2-dosed fish (Figure 4.1).

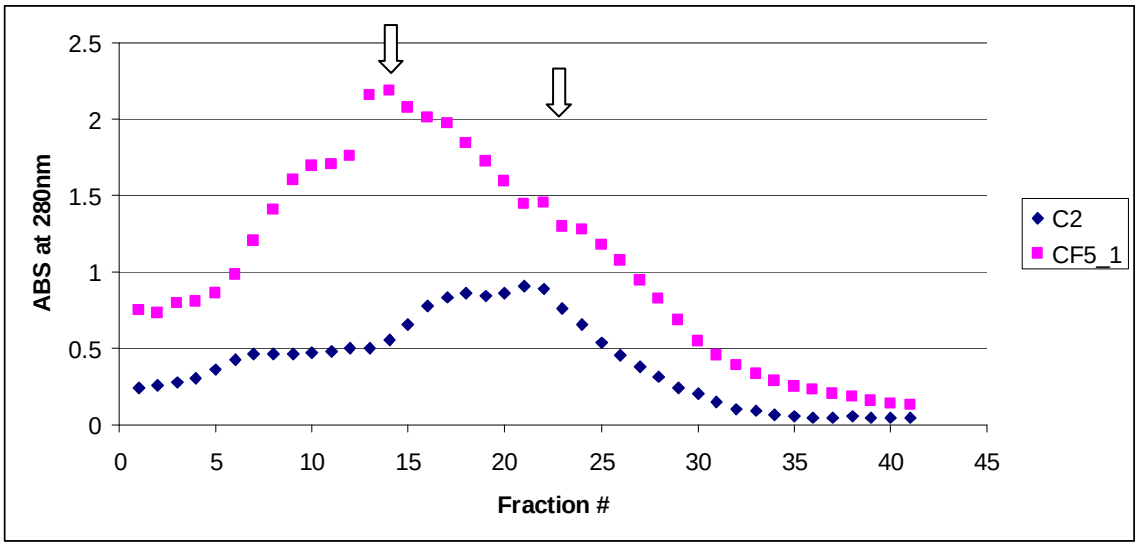

Figure 4.1 Purification of barramundi Vtg by size-exclusion chromatography. CF_C2 from control plasma sample C2 compared to CF_E5 from E2_5 plasma sample. C2 plasma sample was run on the column immediately before plasma sample E2_5. Arrow points to putative Vtg peak.

The column fractions will henceforth be referred to as CF followed by E (for E2 dosed fish) or C (for saline control fish) and fish sample # (1-6) (ex. Fish E2_1 becomes CF_E1).

Figure 4.2 shows the absorbance of the saline and E2-dosed barramundi column fractions at 280 nm, with each graph (a-e) showing the elution profile of one saline (C) and one E2 (E) dosed fish for direct comparison. A peak between fractions 13-17 was the appropriate molecular weight to be Vtg, 540 kDa (Sun et al, 2003). In Figure 4.2 b-e) this peak is evident, along with a smaller peak between fractions 20-26. No peak is evident in the saline (C) (Figure 4.2a) at the molecular weight ranged for Vtg, but a smaller peak centered around fraction 23 could suggest a slightly smaller protein produced naturally in barramundi (~300 kDa), although it's also possible that the peak represents contamination of the plasma samples or the column.

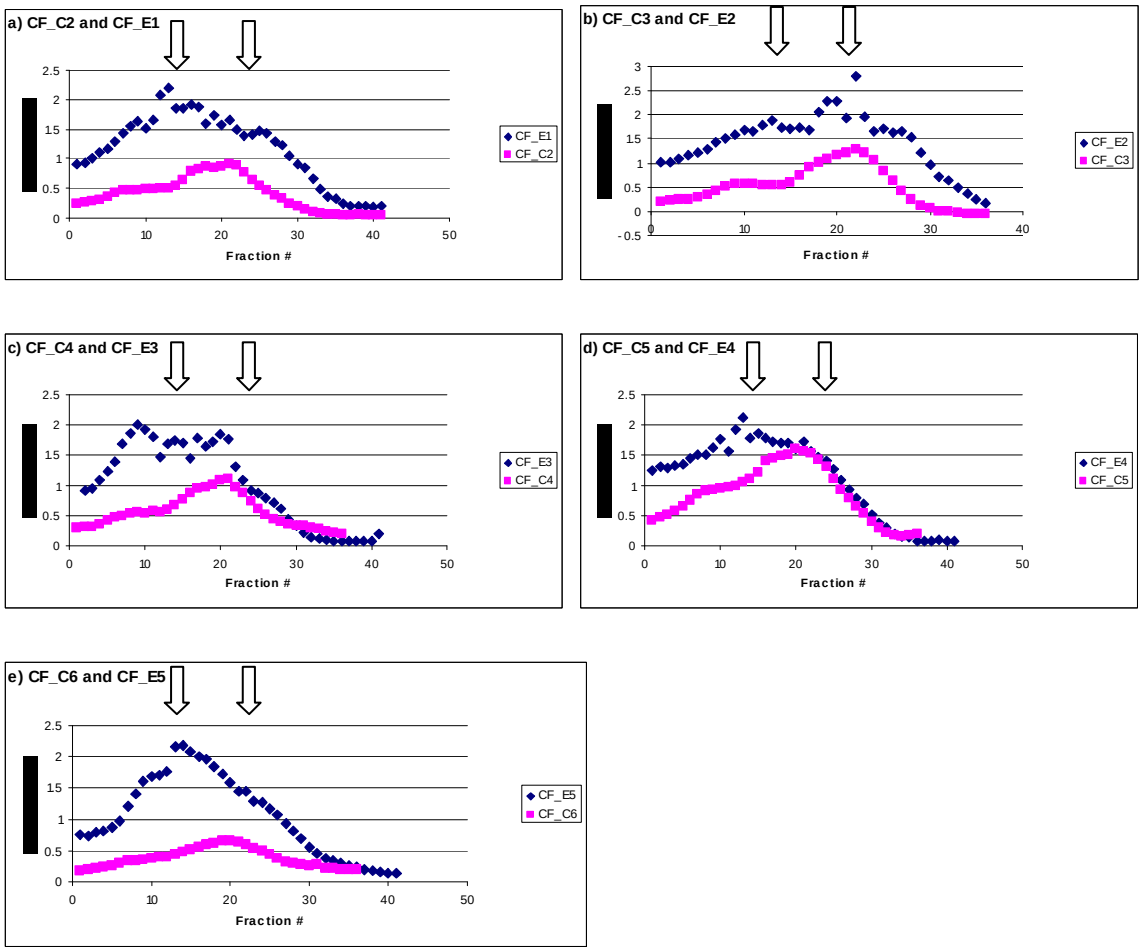

Figure 4.2 Purification of barramundi Vtg by size-exclusion chromatography. Each graph presents the elution profile of one E2 (E) dosed fish and one saline (C) dosed fish for comparison. Arrows point to putative Vtg peak (left arrow) and secondary serum protein peak (right arrow).

Fractions 13-17 from each E2-dosed fish were pooled and the two samples with two of the highest quality chromatographic peaks (CF_E1 and CF_E5) were chosen to be used in the antibody production step.

4.2.2 Polyclonal Antibody Production (sheep anti-barramundi)

4.2.2.1 Production of Polyclonal Antibody (sheep anti-barramundi)

Two male sheep (approx. 2 years old) from the flock held at the veterinary school at James Cook University were used for the antibody production. Each sheep was injected with the same E2 sample for each repeated exposure. The two sheep received a 1 mL injection (0.5 mL of E2 sample and 0.5 mL of Montanide (Tall-Bennett, Australia) as an adjuvant) four times, with two weeks between injections. Two weeks after the final injection, blood was collected and centrifuged at 13,000 rpm for 5 min. Plasma was separated into 1 mL aliquots and frozen until ELISA verification. These plasma samples contained the unscreened sheep anti-barramundi Vtg polyclonal primary antibody, which will be referred to as Sh-0404JCU throughout the remainder of the text.

4.2.2.1 Vtg antibody incubation

In order to remove antibodies which react to common plasma proteins, the sheep anti-barramundi Vtg polyclonal antibody was incubated with male barramundi saline (C) plasma. The antibody was incubated overnight at 4°C with male plasma (1:2 v/v), then centrifuged for 1 hour at 13,000 rpm. The supernatant was removed and stored at 4°C until use in the ELISA. The screened sheep anti-barramundi Vtg polyclonal primary antibody will be referred to as Sh-0404-SJCU throughout the remainder of the text.

4.2.3 ELISA validation with E2 plasma

4.2.3.1 ELISA plate set-up and protocol

The following is a brief description of the indirect ELISA protocol used in this study as detailed in Codi King et al (2008).

All ELISA reagents and solutions were made with milliQ water that had been autoclaved for 70 minutes at 120°C.

For the purpose of independently validating the ELISA, E2-dosed plasma samples obtained from the induction experiment (Section 4.2.1.1) were analyzed. E2-dosed fish plasma and saline (C) were thawed on ice, diluted 1:10 in coating buffer, then coated onto 96-well high-binding microplates ((Immulon 4 HBX, Thermo Scientific) and incubated overnight at 4^{o}C. The plates were then washed with PBS-Tween (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20) (Sigma-Aldrich) and free-binding sites blocked with 2% Bovine Serum Albumin (BSA) for 1 h (200 µL). Following another wash step (3X PBS-Tween), the plates were incubated with 100 µL of mouse anti-striped bass Vtg monoclonal primary antibody ND-3G2 (ND-3G2) (Biosense Laboratories, Norway), diluted 1:500 in 1% BSA in PBS and incubated for 1 h at 37^{o}C. Following a second wash step (3X PBS-Tween), the plates were incubated with 100 µL of goat anti-mouse IgG horseradish peroxidase conjugate (H+L-HRP) secondary antibody (anti-mouse 2° antibody) (BioRad) diluted at a 1:2000 in 1% BSA in PBS. The plate was covered and incubated for 1 h at room temperature. Following the final wash step (5X PBS-Tween), the plates were developed using 0.04% o-phenylenediamine dihydrochloride (OPD) in 0.012% H_2O_2 (Sigma-Aldrich) for between 5-30 minutes (depending on expected Vtg levels, lower levels getting a longer developing time). The samples were fixed by the addition of 4 N sulfuric acid (H_2SO_4, Merck). The absorbance of each plate was read at 492nm on a BioTek Powerwave plate reader (BioTek Instruments, USA). All procedures follow the standard protocol of Biosense Laboratories (http:// www.biosense.com/docs/Biomarker_ELISA_protocol.pdf).

A series of wells were incubated without samples and were used to determine non-specific binding (NSB). For the purposes of verifying the ELISA a qualitative approach was used, where absorbances could be used to compare relative Vtg levels in the samples, but no standards were used to determine exact concentrations.

4.2.4 Confirmation of E2-dosed Fish Plasma Using Western blot Techniques

4.2.4.1 Western blot set-up

Initial work indicated that the E2 plasma samples contained very high concentrations of Vtg. Three dilutions were done in order determine the dilution necessary to confirm the presence of Vtg without overloading the gel. Four plasma samples (E2_1; E2_2; E2_3 and E2_5) were diluted in sodium dodecyl sulfate (SDS) reducing buffer to dilution ratios of 1:6,000, 1:12,000 and 1:30,000.

4.2.4.2 Western blot protocol

Saline (C) and E2-dosed fish plasma samples were thawed on ice then, along with a combined protein standard ladder (Magic Marker (20-220 kDa) and SeeBlue (21-261 kDa), Invitrogen) were diluted in sodium dodecyl sulfate (SDS) reducing buffer to the dilution ratios described above. Samples and the standard were then heated for 5 minutes at 100ºC in a heating block to denature proteins.

A 15µL aliquot of sample or standard was loaded onto a 7.5% Tris HCL pre-cast polyacrylamide gels (Bio-Rad), in accordance with the method described by Laemmli (1970). The chamber was filled with running buffer and the gels were run at 200 V, 60 mA per gel (120 mA for two gels) at a constant voltage for 45 min. Once chromatographic elution was complete the gels were washed in transfer buffer to equilibrate. The gels were then transferred to 0.20 µm polyvinylidene difluoride (PVDF) membranes (Invitrogen) for Western blot analysis (Towbin et al, 1979) at 100 V, 400 mA, at constant voltage for 60 min.

The WesternBreeze® Chromogenic Immunodetection kit (Invitrogen) was used to detect Vtg antigens using ND-3G2 (Biosense) and an alkaline phosphatase-conjugated secondary antibody (Goat anti-mouse IgG, Invitrogen). A pre-stained molecular weight standard containing proteins from 20 to 261 kDa (Magic Marker (20-220 kDa) and SeeBlue (21-261 kDa), Invitrogen) was processed with all samples to allow for molecular weight estimates and determination of transfer efficiency.

4.2.5 Comparison of Primary Vtg Antibodies

In order to determine the most efficient and accurate of the available anti-Vtg primary antibodies, Sh-0404JCU, Sh-0404-SJCU, and ND-3G2 (Biosense) were compared.

The ELISA plate contained six sets of three samples (6 X 3) and a background reference and they included: one saline (C) barramundi, two E2-dosed barramundi (E2_1 and E2_5) and a set of non-specific binding (NSB) wells for background correction. Each of the six sample sets was treated with different antibody combinations, as shown in Table 4.1.

Table 4.1. Primary and secondary antibody matrix for comparison of primary Vtg antibodies.

	Sample set 1	Sample set 2	Sample set 3	Sample set 4	Sample set 5	Sample set 6
Primary antibody	Sh-0404JCU	ND-3G2	Sh-0404-SJCU	Sh-0404JCU	ND-3G2	Sh-0404-SJCU
Secondary antibody	Anti-sheep[a]	Anti-mouse[b]	Anti-sheep	Anti-mouse	Anti-sheep	Anti-mouse

[a]Refers to rabbit anti-sheep IgG horseradish peroxidase conjugate (H+L-HRP) secondary (2°) antibody (BioRad).
[b]Refers to goat anti-mouse IgG H+L-HRP 2° antibody (BioRad).

This matrix was designed in order to ensure there was no cross reactivity between primary and secondary antibodies from different animals (eg. ND-3G2 and anti-sheep 2º antibody), and to determine overall binding efficiency between the different polyclonal and monoclonal Vtg antibodies. The 1° and 2° antibodies were diluted 1:500 and 1:2000, respectively, which was the optimal dilution determined by Susan Codi King and her colleagues for this ELISA.

4.2.6 Comparison of Vtg Standards

4.2.6.1 Comparison of two commercial Vtg standards from Rainbow Trout and Atlantic Salmon

In order for an indirect ELISA to be used quantitatively, a Vtg standard curve must be evaluated to see if it produces a good linear regression which allows for concentrations of unknown Vtg samples to be calculated. There are two commercially available Vtg standards that have been recommended for use in the indirect ELISA, the lipophylised Vtg (Rainbow Trout) standard (RT Vtg standard) and lipophylised Vtg (Atlantic Salmon) standard (Salmon Vtg standard) (Caymen Chemical Co). Both these Vtg standards were analyzed to determine whether 1° and 2° antibodies already established for the indirect ELISA gave comparable results to the 1° and 2° antibodies developed in this study. Serial dilutions of both RT and Salmon Vtg standards were done to produce calibration curves ranging from 0 to 6 μg mL^{-1} and 0 to 4.5 μg mL^{-1}, respectively.

The two sets of standards were run with two different antibody combinations, as shown in Table 4.2.

Table 4.2. Primary and secondary antibody combinations for comparison of two commercially available Vtg standards.

	RT standards set 1	RT standards set 2	Salmon standards set 1	Salmon standards set 2
Primary antibody	Sh-0404JCU	ND-3G2	Sh-0404JCU	ND-3G2
Secondary antibody	Anti-sheep[a]	Anti-mouse[b]	Anti-sheep	Anti-mouse

[a]Refers to rabbit anti-sheep IgG horseradish peroxidase conjugate (H+L-HRP) secondary (2°) antibody (BioRad).
[b]Refers to goat anti-mouse IgG H+L-HRP 2° antibody (BioRad).

4.2.6.2 Comparison of column fraction (CF) with commercial Rainbow Trout Vtg standard

Theoretically the column fractions obtained in section 4.2.1.2 contain only Vtg, therefore using these samples (serially diluted) in a standard curve and assessing the linear regression would help

to determine if they would be a suitable alternate Vtg standard specific to barramundi (Sun et al, 2003). Protein concentrations of the column fractions were determined as previously stated and one column fraction (CF_E2) was compared to the RT Vtg standard. The working concentration range for the RT Vtg standard was 0 to 4.5 µg mL^{-1}. The column fraction (protein concentration = 5.31 mg mL^{-1}) was diluted 1:1000, then this dilution was used to obtain a working range of 0 to 5.31 µg mL^{-1}, which was similar to the range for the RT Vtg standard.

Two saline (C) samples, each diluted 1:10, and two E2-dosed plasma samples in a range of dilutions (1:10, 1:50, 1:100 and 1:200) were processed along with the standards. The plate was exposed to ND-3G2 followed by anti-mouse 2° antibody.

4.2.7 *Quantification of Vtg levels in column fraction sample*

Column fraction CF_E1 was serially diluted in a range from 1:7,500 to 1:40,000 and run, along with RT Vtg standards with a working concentration range from 0 to 4.5 µg mL^{-1} for quantification. The plate was processed with ND-3G2 1° antibody and anti-mouse 2° antibody.

4.3 Results

4.3.1 ELISA verification with E2 plasma

Figure 4.3 shows mean non-specific binding corrected absorbance for each saline (C) or E2 (E2) dosed fish at three sample points during the Vtg induction period. Fish E2_6 died between day 14 and day 21 post-injection, so there is no final sample data for that fish.

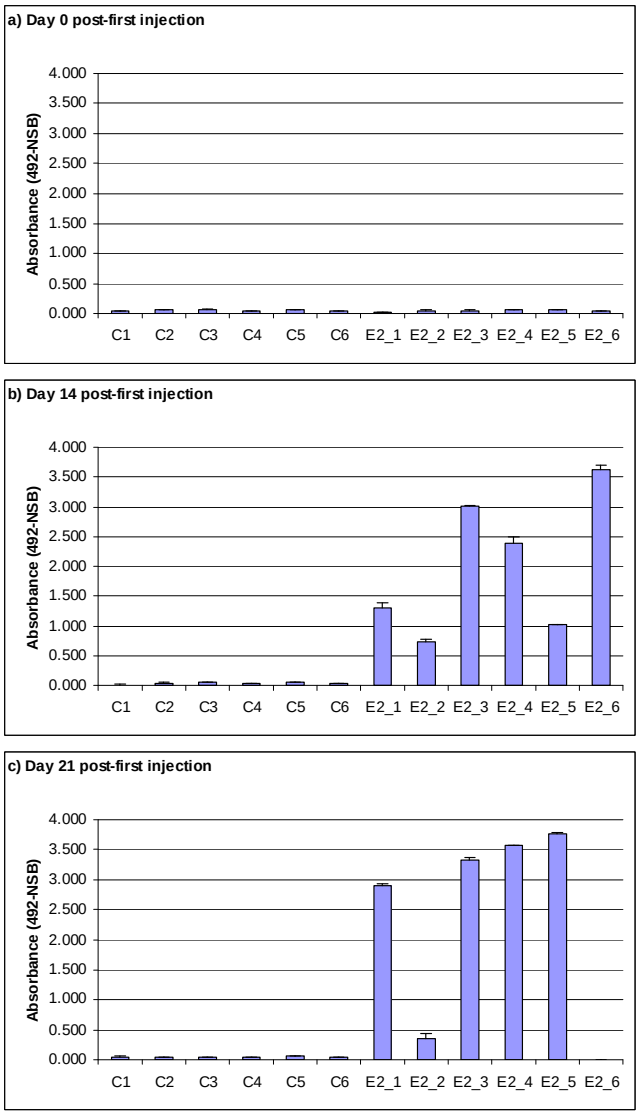

Figure 4.3 Mean absorbance (492-NSB) for the control (C) and E2–dosed fish plasma samples collected at 0 (a), 14 (b), and 21 (c) days post-injection. Each bar represents mean $\pm$ sd (n=3). On Day 21 post-first injection fish E2_6 was deceased.

On day 0 the mean absorbance in the E2 fish was 0.042±0.01, which was not significantly different from the mean absorbance for the control fish (P=0.3485). By day 14 the mean absorbance in the E2 fish had increased to 1.785±1.086, a ~40-fold increase from day 0. Between day 14 and day 21 the mean absorbance doubled, from 1.785±1.086 to 3.387±0.371.

4.3.2 Confirmation of E2 plasma using Western Blot Techniques

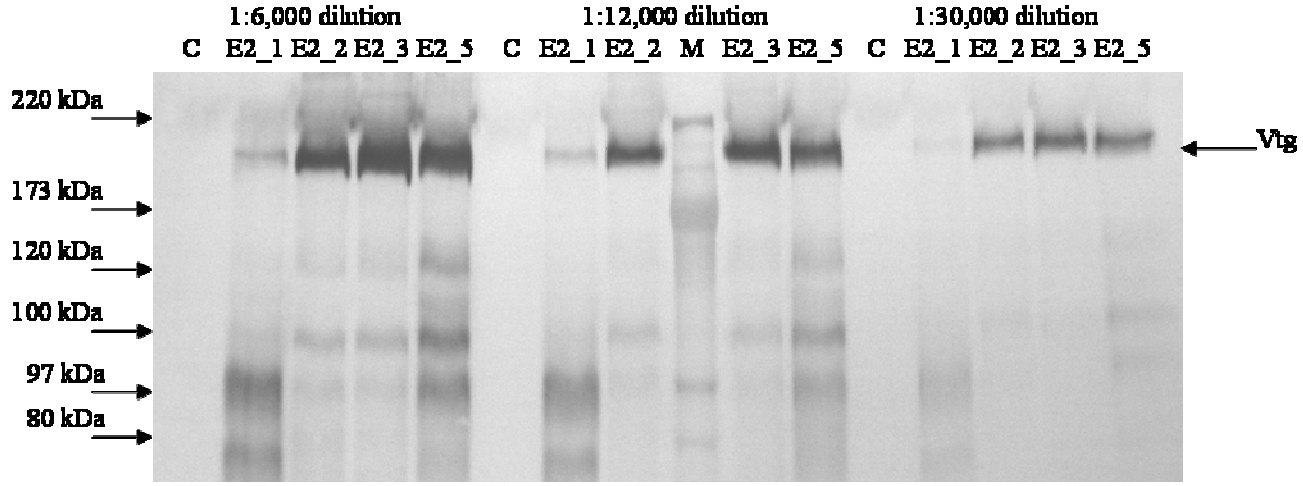

Figure 4.4 Western blot of E2 and saline (C) dosed fish plasma samples. The arrow to the right of the figure points to putative Vtg bands in the E2 plasma samples. Molecular weights of the protein standard ladder are labeled down the right side of the figure.

Figure 4.4 is a photograph of the western blot of the E2 dosed and saline (C) plasma samples. The control plasma sample (C) shows no evidence of Vtg, while the four E2 samples all show evidence of Vtg. E2_1 has the lowest concentration of Vtg in evidence while E2_2, E2_3 and E2_5 all present a thick Vtg, suggesting that even at a dilution of 1:6,000 the Vtg concentration in the samples is too high to present a clean band.

4.3.3 Comparison of Primary Antibodies

Table 4.3 is a summary of the absorbance corrected for non-specific binding (492-NSB) data from the ELISA plate run in the comparison of Sh-0404JCU, Sh-0404-SJCU and ND-3G2.

Table 4.3 Mean absorbance (492-NSB) for Control, E2_1 and E2_5 samples for each primary and secondary antibody combination.

1°Ab -->	Sh-0404JCU	ND-3G2	Sh-0404-SJCU	Sh-0404JCU	ND-3G2	Sh-0404-SJCU
2°Ab -->	Anti-sheep	Anti-mouse	Anti-sheep	Anti-mouse	Anti-sheep	Anti-mouse
Control	1.59	0.01	0.11	0.01	0.00	0.01
E2_1	2.24	3.64	1.57	0.01	0.02	0.01
E2_5	2.18	3.65	1.74	0.01	0.02	0.00

Comparing the 1° antibodies, ND-3G2 has the highest binding level as well as making the greatest distinction between the saline (C) and E2 dosed fish plasma, which is consistent with work already conducted. The mean absorbance (492-NSB) for the saline fish exposed to ND-3G2 and anti-mouse 2° antibody was 0.01±0.00 (sd), while the mean absorbance for E2_1 exposed to the same antibody combination was 3.64±0.04, and the mean absorbance for E2_5 was 3.65±0.05.

The next best antibody combination was Sh-0404-SJCU followed by anti-sheep 2° antibody. The mean absorbances for the C, E2_1 and E2_5 samples were 0.11±0.01, 1.57±0.03 and 1.74±0.03, respectively. However, the binding level of this combination was only ~50% that of ND-3G2 followed by anti-mouse 2° antibody.

The worst antibody combination in terms of distinguishing between the C and E2 samples was Sh-0404JCU followed by anti-sheep 2° antibody. The mean absorbances for the C, E2_1 and E2_5 samples were 1.59±0.03, 2.24±0.04 and 2.18±0.03, respectively.

4.3.4 Comparison of Vtg Standards

4.3.4.1 Rainbow Trout vs. Atlantic Salmon standards

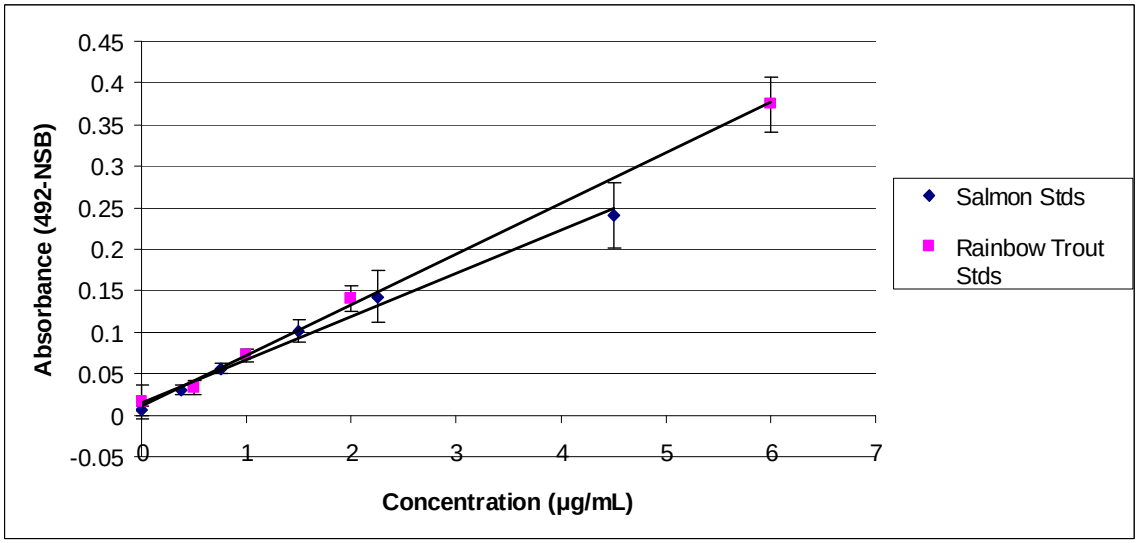

Figure 4.5 Comparison of standard curves produced with Salmon Vtg and RT Vtg standards run with ND-3G2 and anti-mouse 2° antibody. All values are means ±sd of triplicate analyses.

Figure 4.5 shows the calibration curves obtained when the two sets of standards were run with ND-3G2 and anti-mouse 2° antibody. The Salmon Vtg standards produced a curve described by the equation y=0.0521x+0.0148 with an R^2 of 0.9905. The RT Vtg standards produced a curve described by the equation y=0.0608x+0.0119 with an R^2 of 0.9981. While the standard curves were very similar in quality the RT Vtg standard curve provided a slightly better standard curve over a larger working range than the Salmon Vtg standards, so this standard was chosen for the quantitative ELISA.

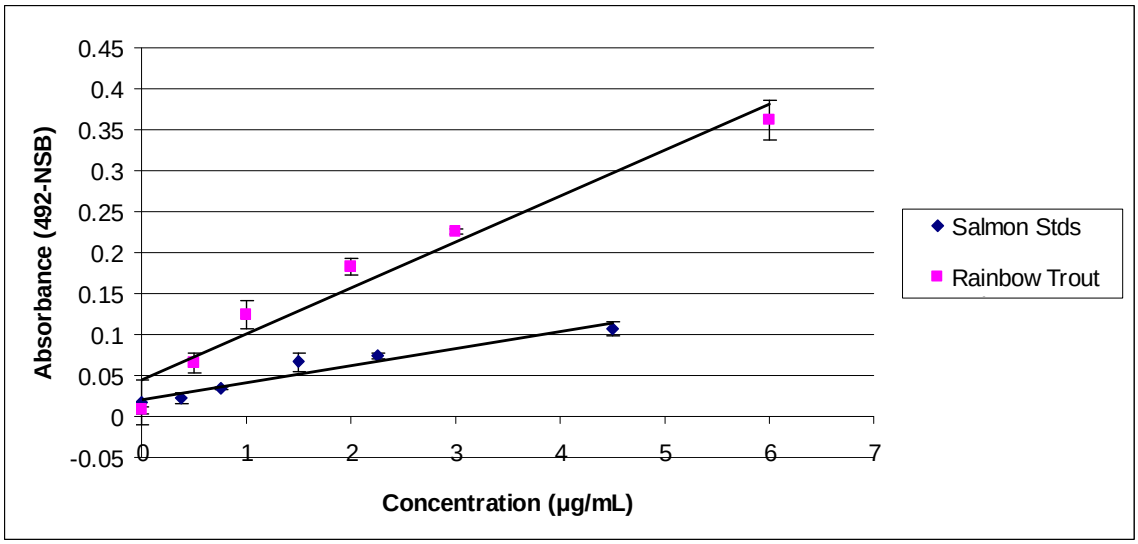

Figure 4.6 Comparison of standard curves produced with Salmon Vtg and RT Vtg standards run with Sh-0404JCU and anti-sheep 2° antibody. All values are means ± sd of triplicate analyses.

Figure 4.6 presents the standard curves obtained using Sh-0404JCU as the 1° antibody and anti-sheep 2° antibody. The Salmon Vtg standards produced a curve described by the equation y=0.0206x+0.0213 with an R^2 of 0.9378. The RT Vtg standards produced a curve described by the equation y=0.0559x+0.045 with an R^2 of 0.96. Here the RT Vtg standards provided a calibration curve with a slope similar the slope obtained using ND-3G2 and a higher R^2 value. Again, these results help confirm the use of the RT Vtg commercial standard for use in a quantitative ELISA in the absence of a barramundi specific Vtg standard as confirmed by Codi King et al (2008)

4.3.8.2 Rainbow Trout vs. Column Fractions

Figure 4.7 shows the calibration curves obtained when RT Vtg standard and column fraction CF_E2 were run with ND-3G2 and anti-mouse 2° antibody. The goal here is to determine whether the column fraction, which theoretically should contain only purified barramundi Vtg, can be used as a standard for the ELISA used in this work. The RT Vtg standards produced a curve described by the equation y=0.5016x+0.0548 with an R^2 of 0.9748. The column fraction CF_E2 dilutions produced a curve described by the equation y=1.0921x+0.7596 with an R^2 of 0.8012. While the RT standard gives a strong, linear standard curve with the expected doubling of absorbance as concentration doubles, the curve of the column fractions dilutions is not linear,

though a linear trend line can be fitted, and rises quite rapidly early on before reaching plateau above an absorbance reading of 3 at the higher end of the range. The maximum absorbance for the ELISA used here is ~3.5, suggesting that the Vtg concentration in CF_E2 column fraction is too high for the assay, making CF_E2 an impractical choice for a Vtg standard for this assay.

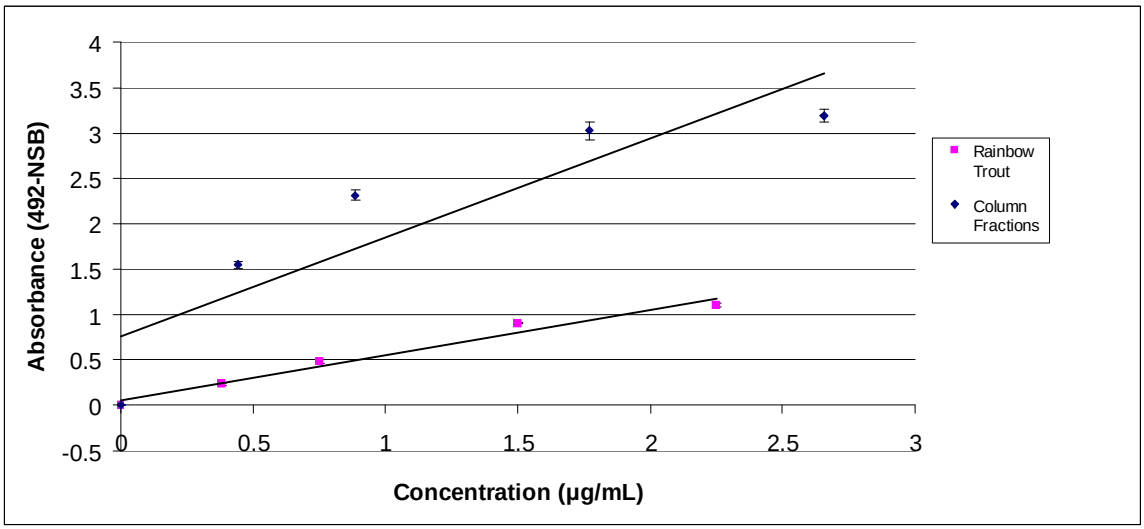

Figure 4.7 Comparison of standard curves produced with column fraction CF_E2 and RT Vtg standards run with ND-3G2 and anti-mouse 2° antibody. All values are means ± sd of triplicate analyses.

4.3.5 Quantification of Vtg levels in column fraction sample

Table 4.4 shows the Vtg concentration of six serial dilutions of the column fraction CF_E1 as determined by ELISA using ND-3G2 and anti-mouse 2° antibody. The results were quantified using RT Vtg standards and concentrations ranged from 20.92 to 33.40 mg mL^{-1}.

Table 4.4 Vtg concentration of CF_E1

Dilution Factor	Concentration (µg mL^{-1})	Final Concentration (corrected for DF) (mg mL^{-1})	Mean Concentration of CF_E1 (mg mL^{-1})	Std Dev
1:7,500	2.79	20.92	29.04	4.95
1:10,000	2.54	25.43		
1:12,000	2.45	29.42		
1:15,000	2.23	33.40		
1:20,000	1.63	32.54		
1:40,000	0.81	32.50		

4.4 Discussion

One of the goals of this research was to achieve the best ELISA possible for detecting Vtg production in barramundi. To this end Vtg production was induced in male, juvenile barramundi by repeatedly injecting them with large doses (5 mg kg^{-1} bw) of estradiol (E2). The large dose was to ensure E2 saturation and maximize Vtg production. While male fish have been shown to possess the hepatic estrogen receptors responsible for Vtg production (Booth and Skene, 2006; Schafhauser-Smith and Benfey, 2002) the E2 dose necessary to induce Vtg production can vary among species. The minimum threshold dose of E2 for induction of Vtg can range from 8 μg kg^{-1} bw in English sole (Lomax et al, 1998) up to ~100 μg kg^{-1} bw in greenback flounder (Sun et al, 2003). While these doses are quite low, for a rapid, strong Vtg response a dose between 2 mg kg^{-1} bw and 10 mg kg^{-1} bw is generally used (Bon et al, 1997; Korsgaard and Pedersen, 1998; Lomax et al, 1998; Vaccaro et al, 2005). Sun et al (2003) found that 5 mg kg^{-1} bw caused a significant response in greenback flounder within 48 hours post-injection while lower doses (0.5 and 1.0 mg kg^{-1} bw) needed 96 hours to see an increase in Vtg.

The expectation for the verification of the ELISA with E2 plasma samples is that Vtg concentration in the blood plasma of E2 dosed barramundi will continue to rise over the course of the induction period as E2 injections are repeated (Sun et al, 2003). Figure 4.3 clearly shows that this is what is happening and the qualitative ELISA is working well. The qualitative ELISA appears to be supported by the western blot of the E2 plasma samples (Figure 4.4) which all have a band present at ~150kDa, which corresponds to the literature as being the right size for a Vtg subunit (Sun et al, 2003; Codi King et al, 2008).

Vtg was then purified from the blood plasma of the E2 dosed male barramundi using size exclusion gel chromatography. The large Vtg peaks apparent in Figure 4.2 graphs b)-f) have a molecular weight of ~540kDa which is the right size for Vtg (Specker and Sullivan, 1994; Sun et al, 2003). There was a second apparent peak with a molecular weight of ~300kDa. This peak was present in both saline (C) and E2 dosed fish, suggesting a naturally occurring barramundi

protein, although it is possible it could be the effect of contamination of the plasma samples or column.

A polyclonal antibody (Sh-0404JCU) against barramundi Vtg was created by inoculating sheep with the Vtg column fractions from two E2-dosed fish. There are two classes of antibody used in ELISAs, polyclonal antibodies, like Sh-0404JCU created in this project, and monoclonal antibodies, such as the commercially available ND-3G2 (Biosense).

Polyclonal antibodies are present in the serum of an animal (in this case sheep) inoculated with an antigen (Crowther, 2001). These antibodies are usually heterogeneous as they are the product of multiple clones of cells responding to the injected antigen (Crowther, 2001). Also, the specificity of polyclonal antibodies is wholly dependent on the purity of the injected antigen, so cross-reactivity with other serum proteins is possible (Schafhauser-Smith and Benfey, 2002).

Monoclonal antibodies on the other hand, are derived from a single antibody producing cell-line (Crowther, 2001). The monoclonal antibody is, therefore, highly specified towards a defined epitope and demonstrates little or no cross-reactivity with other serum proteins (Crowther, 2001).

There are two methods for purifying polyclonal antibodies, in order to remove or reduce the incidence of cross-reactivity. The first is pre-incubation of the serum with male plasma (Bon et al, 1997; Lomax et al, 1998; Parks et al, 1999). The second is further purification by affinity chromatography (Korsgaard and Pedersen, 1998; Schafhauser-Smith and Benfey, 2002). In this study an aliquot of Sh-0404JCU was incubated overnight with plasma from a C male to create Sh-0404-SJCU.

These sheep primary antibodies, Sh-0404JCU and Sh-0404-SJCU, were then compared to an anti-striped bass Vtg antibody (ND-3G2) available commercially from Biosense (Norway) to determine the optimal 1° antibody for this ELISA. Studies have shown that the amino acid composition of Vtg in fish is highly conserved (Mananos et al, 1994; Heppell and Sullivan, 1999; Parks et al, 1999), which means that a Vtg antibody for one fish can be used for detection of Vtg in another fish. However, the molecular weight of Vtg varies between fish by up to

300kDa (range 300-600kDa) suggesting species specific differences in Vtg (Specker and Sullivan, 1994). In addition some species have been found to have multiple forms of Vtg (Kishida and Specker, 1993; Matsubara et al, 1999; Shimizu et al, 2002; Hiramatsu et al, 2002). Therefore, the most accurate ELISA is going to employ an antibody for Vtg from the specific species being studied.

There were two criteria for the optimal 1° antibody in this research. The first is a high binding affinity to the protein in question, Vtg. The second is a low cross-reactivity with other serum proteins in barramundi, which could lead to false positive results.

ND-3G2 fulfills both these criterion better than either Sh-0404JCU or Sh-0404-SJCU. ND-3G2 gives the highest readings for the E2 dosed fish (Table 4.3), which after 3 doses of 5 mg kg^{-1} should be close to saturation with Vtg. ND-3G2 also distinguishes quite strongly between the C and E2 dosed fish. While the E2 dosed fish are expected to have very high levels of Vtg (Sun et al, 2003), the C fish are expected to have very low or nonexistent Vtg levels (Korsgaard and Pedersen, 1998; Vizziano et al, 1996; Yamada et al, 1993). ND-3G2 shows exactly that.

Therefore, for the purposes of this project the commercial monoclonal anti-striped bass Vtg antibody, ND-3G2 (BioSense), was confirmed as the optimal 1° antibody. It had the highest binding affinity and optimum binding over corresponding controls of the commercially available mono- and polyclonal antibodies tested by Codi King et al (2008) and was superior to the polyclonal antibodies produced in this research. However, the high binding affinity of Sh-0404JCU, along with the low cross-reactivity of Sh-0404-SJCU (Table 4.3) suggests that further efforts, such as affinity chromatography to purify the antibody in Sh-0404JCU could lead to a better polyclonal anti-barramundi Vtg antibody in the future. In addition, ELISA optimization usually includes a step in which the optimal 1° and 2° antibody dilutions are determined. For the purposes of this research the dilutions determined by Codi King et al (2008) were used. These dilutions were 1:500 for Sh-0404JCU, Sh-0404-SJCU and ND-3G2 and 1:2000 for the anti-mouse 2° antibody and anti-sheep 2° antibody. Since dilution most directly affects binding affinity, it is possible that varying the dilution of Sh-0404-SJCU could lead to higher binding affinities for that 1° antibody with no further purification. It should be noted that multiple forms

of Vtg have been found in a wide range of fish species (Ding et al, 1989; Kishida and Specker, 1993; Chang et al, 1996; Hiramatsu et al, 2002). While the monoclonal primary antibody, ND-3G2, exhibited the highest binding affinity and optimum binding among commercially available antibodies, both mono- and polyclonal, finding a polyclonal primary antibody specific to barramundi would ensure that any multiple forms of Vtg produced by barramundi would be detected.

After an optimal primary antibody was established a Vtg standard was required in order to achieve a quantitative ELISA. Two commercially available standards, RT Vtg and Salmon Vtg standards, were run with both ND-3G2 followed by anti-mouse 2° antibody (Figure 4.5), and Sh-0404JCU followed by anti-sheep 2° antibody (Figure 4.6), then compared in order to determine which was most efficient and accurate for use in a barramundi Vtg ELISA. The optimal standard for this ELISA would give a curve in which as the Vtg concentration doubles the absorbance doubles, and the standards have a wide working range over which Vtg concentrations can be determined. While neither sets of standards gave the optimal curve, with both antibody combinations the RT Vtg standard gave a better curve (higher slope and R^2 value) over a greater range of standards compared to the curve obtained using the Salmon Vtg standard (Figures 4.5 and 4.6).

Since a species specific Vtg standard would be optimal for use in the barramundi ELISA the RT Vtg standard was also compared with a Vtg column fraction CF_E2 obtained earlier in this research (Figure 4.7). The CF_E2 standard curve achieves the doubling of absorbances with doubling of concentration that would be optimal in the lower end standards, but the higher end standards are at the saturation limit for this particular ELISA. Moreover, the CF_E2 standard curve exhibits a significant y-intercept, indicating that the standard curve wouldn't be useful for determining Vtg concentration in samples with low absorbances. The RT Vtg standard gives a shallower curve, but with a much better fit with a y-intercept that makes it possible to use in a much wider range of absorbances. One possible explanation for the CF_E2 standards not being as good as the RT Vtg standards is that the column fractions have not been purified enough (Schafhauser-Smith and Benfey, 2002). Further protein purification could result in a pure

barramundi Vtg which would be valuable as a standard for a quantitative ELISA, but at the moment the RT Vtg standard is the best Vtg standard available for this ELISA.

The quantitative results for the determination of Vtg concentration in the column fractions (Table 4.8) were similar to a wide range of Vtg plasma levels determined in fish such as 1.7 mg mL^{-1} in English sole (Lomax et al, 1998), 3 mg mL^{-1} in sea bass (Mananos et al, 1994), and as high as 60 mg mL^{-1} in rainbow trout (Bon et al, 1997).

In summary, the best indirect ELISA optimized for detecting Vtg induction in barramundi is the method already described in detail by Codi King et al (2008). The optimal 1°- 2° antibody combination was determined to be the commercially available ND-3G2 (Biosense) and anti-mouse 2° antibody (BioRad), while the optimal Vtg standard proved to be RT Vtg standard (Caymen Chemical Co). In addition, there were promising results with the polyclonal antibodies, Sh-0404JCU and Sh-0404-SJCU, and barramundi Vtg column fraction, CF_E2). All of this suggests that future refinement, through affinity chromatographic purification of the polyclonal antibody and through protein purification of the column fraction, could lead to a better barramundi specific ELISA.

4.5 References

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5. (Anti-)Estrogenicity of PBDE-47 in Barramundi (*Lates calcarifer*)

5.1 Introduction

Chapter 1 discussed the increasing PBDE levels worldwide and the potential toxic side effects of PBDE exposure. This included the contrasting results of research on whether PBDE acts estrogenically or anti-estrogenically in fish. Many species of fish are hermaphroditic, with the ability to change gender at certain points during their lifecycles, a process which is controlled by the reproductive endocrine system (reviewed by Devlin and Nagahama, 2002). Since exposure to xeno-estrogens could seriously affect the reproductive capabilities of hermaphroditic fish species it is important that PBDEs be studied further to determine what estrogenic or anti-estrogenic effects they have.

Barramundi, *Lates calcarifer*, a protandrous hermaphrodite, with the smaller length classes being predominantly male and the percentage of females increasing as total length increases (Moore, 1979), is a good example of a fish species at risk from xeno-estrogens. Barramundi is a commercially important fish species throughout Australia and Southeast Asia. Barramundi production within Australia has been steadily increasing over the past 15 years (Boonyaratpalin and Williams, 2002) and aquaculture of barramundi has recently expanded to North America and Europe (Katersky and Carter, 2007). In the east part of northern Australia the wild fishery for barramundi is a major industry, grossing more than $6 million annually (Katersky and Carter, 2007). So as barramundi become increasingly important to the Australian fisheries market, this research provides a good opportunity to assess the estrogenic or anti-estrogenic effects of PBDEs in a valuable Australian fish species.

This research attempts to elucidate the estrogenic or anti-estrogenic effects of PBDE in barramundi, a commercially important fish species in Australia and southeast Asia, with vitellogenin (Vtg) production measured as an endpoint of estrogenic activity. Juvenile male barramundi were exposed to PBDE in experiments designed to study both dose response and time course induction of Vtg production. Vtg was measured using the ELISA method described previously in Chapter 4.

5.2 Materials and Methods

5.2.1 Fish Handling

Male juvenile barramundi were obtained from stocks bred and grown at the Marine & Aquaculture Research Facilities Unit (M.A.R.F.U.) at James Cook University in Townsville, Queensland, Australia. Fish for the dose response PBDE-47 experiment were maintained in 1,000 L recirculating systems at a temperature of ~25 ± 1°C, with a naturally occurring photoperiod of ~12 hr light: 12 hr dark, while fish for the time course PBDE-47 experiment were maintained in 150 L recirculating systems in a temperature controlled environment, with a water temperature of 27 ± 1°C and a photoperiod of 16 hr light: 8 hr dark. Fish were fed ~3% body weight daily with dry feed pellets.

For all exposure experiments, fish were anaesthetised in 0.05% 2-phenyoxyethanol (Sigma Aldrich) and weighed before injection. Injections were done by i.p. injection and blood was collected from the caudal vein before being placed in a centrifuge tube and spun at 13,000 rpm for 5 min. Plasma samples were frozen at -20°C for later analysis.

5.2.2 Time course of Vtg induction by PBDE-47: Low and High Dose

Prior to the low dose time course study, fish (~ 6 weeks old, 11.42±6.47g) were separated into four tanks (n=25 per tank) and allowed to recover for two days. After recovery, fish were anaesthetised and fish from the first tank were injected with acidified saline (control), while the fish in the three remaining tanks (tanks 2-4) were injected with PBDE-47 in dimethyl sulfoxide (DMSO) at a dose of 1 mg kg^{-1} body weight (low dose).

Prior to the high dose time course study, fish (~ 6 weeks old, 11.42±6.47g) were separated into four tanks (n=21 per tank) and allowed to recover for two days. After recovery, fish were anaesthetised and the fish from the first tank (tank 1) were injected with acidified saline (control), while the fish in the three remaining tanks (tanks 2-4)were injected with PBDE-47 in

DMSO at a dose of 10 mg kg^{-1} body weight (high dose). Due to a limited number of fish for this high dose exposure, time points 2 and 4 days were eliminated, and fewer fish were sampled in the final days. The sampling design for these studies is presented in Table 5.1.

Table 5.1 Sample design for Time Course experiments

	Low Dose[a]				High Dose[b]			
	Tank 1		Tanks 2-4		Tank 1		Tanks 2-4	
Treatment -->	Saline		PBDE-47		Saline		PBDE-47	
Sample Day	Total Fish	# Fish Sampled	Total Fish	# Fish Sampled	Total Fish	# Fish Sampled	Total Fish	# Fish Sampled
0	25	1	25	1[c]	21	4	21	0
1	24	3	24	3[d]	17	3	21	4[e]
2	21	3	21	3	14	0	17	0
3	18	3	18	3	14	3	17	4
4	15	3	15	3	11	0	13	0
5	12	3	12	3	11	3	13	4
7	9	3	9	3	8	3	9	4
10	6	3	6	3	5	3	5	3
14	3	3	3	3	2	2	2	2

[a] 1 mg kg^{-1}
[b] 10 mg kg^{-1}
[c] For a total of n=4 for time point 0
[d] For a total of n=9 for PBDE-47 dosed fish
[e] For a total of n=12 for PBDE-47 dosed fish

5.2.3 Dose Response of Vtg induction by PBDE-47: Single and Double Injections

Prior to the dose response study, fish (~ 3 months old; weight range 55-215g) were separated into five tanks (n=6 per tank) and allowed to recover for two days. After recovery, fish were anaesthetised and injected with a) a single dose of acidified saline (control), b) a single injection of PBDE-47 in DMSO at a dose of 1 mg kg^{-1} body weight, c) a single injection of PBDE-47 in DMSO at a dose of 10 mg kg^{-1} body weight, d) two injections of PBDE-47 in DMSO at a dose of 1 mg kg^{-1} body weight, with an interval of three days between injections or e) two injections of PBDE-47 in DMSO at a dose of 10 mg kg^{-1} body weight, with an interval of three days between injections. Blood samples were taken at 0, 3 and 6 days after the final injection

5.2.4 Analysis of samples

5.2.4.1 ELISA

The protocol for ELISAs has been previously described in section 4.2.3.1. Plasma samples were diluted in coating buffer at a ratio of 1:5, except for those instances where plasma volumes were low. For these samples a 1:10 dilution in coating buffer was done. Samples were analyzed with lipophylised Vtg (Rainbow Trout) standard (RT Vtg standard) (Caymen Chemical Co) run in a range from 0.375 to 4.5µg mL^{-1}. Standards were run on each ELISA plate and samples were analyzed using the corresponding standard curve. Samples and standards were all run in triplicate.

5.2.4.2 Protein Concentration

The juvenile barramundi used for the time course experiments provided enough plasma to analysis by ELISA only, so protein concentrations were not determined for those samples.

Protein concentration was determined for the barramundi from the dose-response experiment prior to Western Blotting using the Lowry protocol (Lowry, 1951).

5.2.4.3 Western Blot

Western blots were run using the protocol as previously described (Section 4.2.4.2). Onto each 7.5% Tris-HCL gel, sample was loaded corresponding to 10 µg of protein for each sample from the dose-response experiment. E2-dosed fish plasma was used as a positive control and was loaded at a dilution of 1:400. A final gel was run with the dose-response samples run with no dilution beyond a 1:2 dilution in the sample buffer, and an E2 positive control diluted to 1:500.

5.2.4.4 Statistical Analysis

For the Time course experiments a two-tailed paired t-test with 95% confidence interval was done to determine if there was a statistically significant difference between the data from the control and the PBDE dosed fish.

For the Dose Response experiments a two-way ANOVA was used to compare the effect of dose (high or low) against the effect of treatment (single or double injection) for the data set from each day post-final injection.

5.3 Results

5.3.1 Time Course of Vtg induction by PBDE-47

5.3.1.1 ELISA: Low Dose

Figure 5.1 presents the time course induction of Vtg by a low dose (1 mg kg^{-1} body weight) of PBDE-47. RT Vtg standards were analyzed with these samples. A barramundi specific Vtg standard does not exist, and the RT Vtg standard was considered the best available commercial standard. All barramundi Vtg levels fell below the lowest standard (0.375µg mL^{-1}), and therefore represent non-quantifiable concentrations of Vtg in barramundi exposed to a low dose of PBDE-47. Normally, in this instance the ELISA would be repeated using a lower standard range, however the fish used in the experiment were quite small and, unfortunately, the plasma samples were too limited to perform multiple analyses. Therefore, the data are presented in a qualitative manner (mean absorbance corrected for non-specific binding) to assess overall patterns in the data. The two-tailed paired t-test showed no statistically significant difference between the control and PBDE-47 dosed fish.

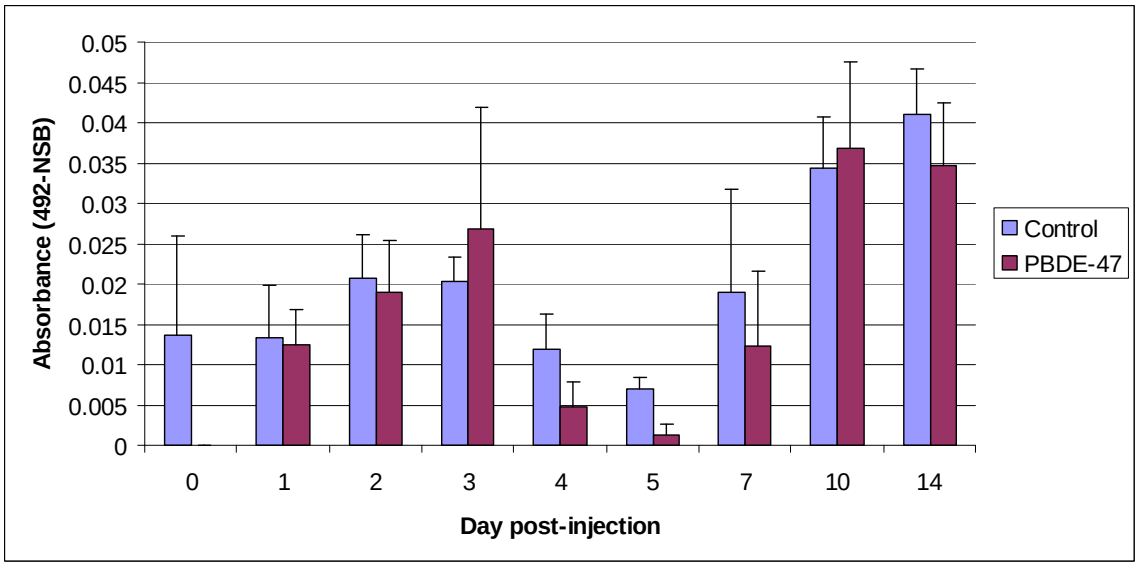

Figure 5.1 Time course induction of Vtg with a low (1 mg kg^{-1} bw) dose of PBDE-47. Each bar represents mean absorbance (492nm-NSB) + sd (n=3 for controls and n=4 for PBDE-47 dosed). For time 0 control data point, 5 samples were obtained using uninjected fish from both the low and high dose control tanks, hence the bar for standard deviation.

5.3.1.2 ELISA: High Dose

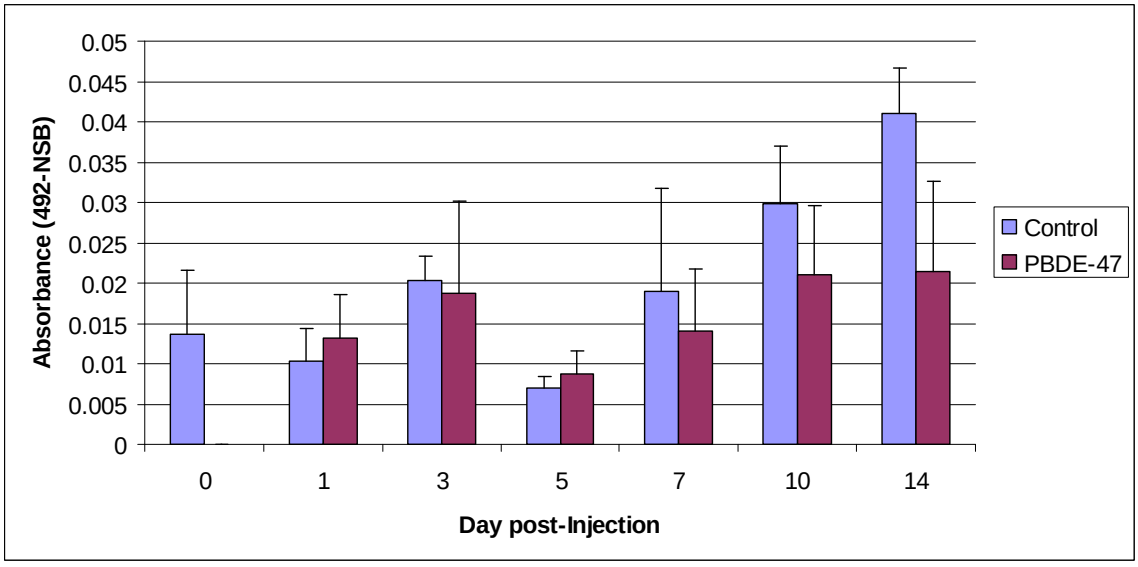

Figure 5.2 Time course induction of Vtg with a high (10 mg kg^{-1} bw) dose of PBDE-47. Each bar represents mean absorbance (492nm-NSB) + sd (n=3 for controls and n=4 for PBDE-47 dosed).

The graph in Figure 5.2 presents the time course induction of Vtg by a high dose (10 mg kg^{-1} body weight) of PBDE-47. The RT Vtg standards were analyzed with these samples as well, but again all barramundi Vtg levels fell below the lowest standard and the samples were not large enough for repeat analysis. Therefore, values are reported as mean absorbance corrected for non-specific binding instead of concentrations in µg mL^{-1} normalized to RT Vtg. The two-tailed paired t-test showed no statistically significant difference between the control and PBDE-47 dosed fish.

5.3.2 Dose Response of Vtg induction by PBDE-47: Single and double injections

5.3.2.1 ELISA

Figure 5.3 shows the dose response of Vtg production after a single or a double injection of either a high (10 mg kg^{-1} bw) or low (1 mg kg^{-1} bw) dose of PBDE-47 at three days post-final injection (a) and six days post-final injection (b). These samples were analyzed with RT Vtg standards and, in contrast to the time course data which was qualitative, the concentrations are shown in µg mL^{-1}. A two-way ANOVA of the data was conducted comparing the effect of dose (high or low) against the effect of treatment (single or double injection) for the data set from each day post-final injection. The two-way ANOVA results for the data set from day 3 post-final injection show that there is no significant interaction between dose and treatment ($P=0.5864$) and no significant difference between the data based on dose size ($P=0.5259$). There is, however, a very significant difference between the data for the single and double injected fish on day 3 post-final injection ($P<0.0001$). The two-way ANOVA results for the data set from day 6 post-final injection show that there is no significant interaction between dose and treatment ($P=0.2897$). There is a small, but significant difference in the data based on dose ($P=0.0335$) and a very significant difference between the data based on treatment on day 6 post-final injection ($P<0.0001$).

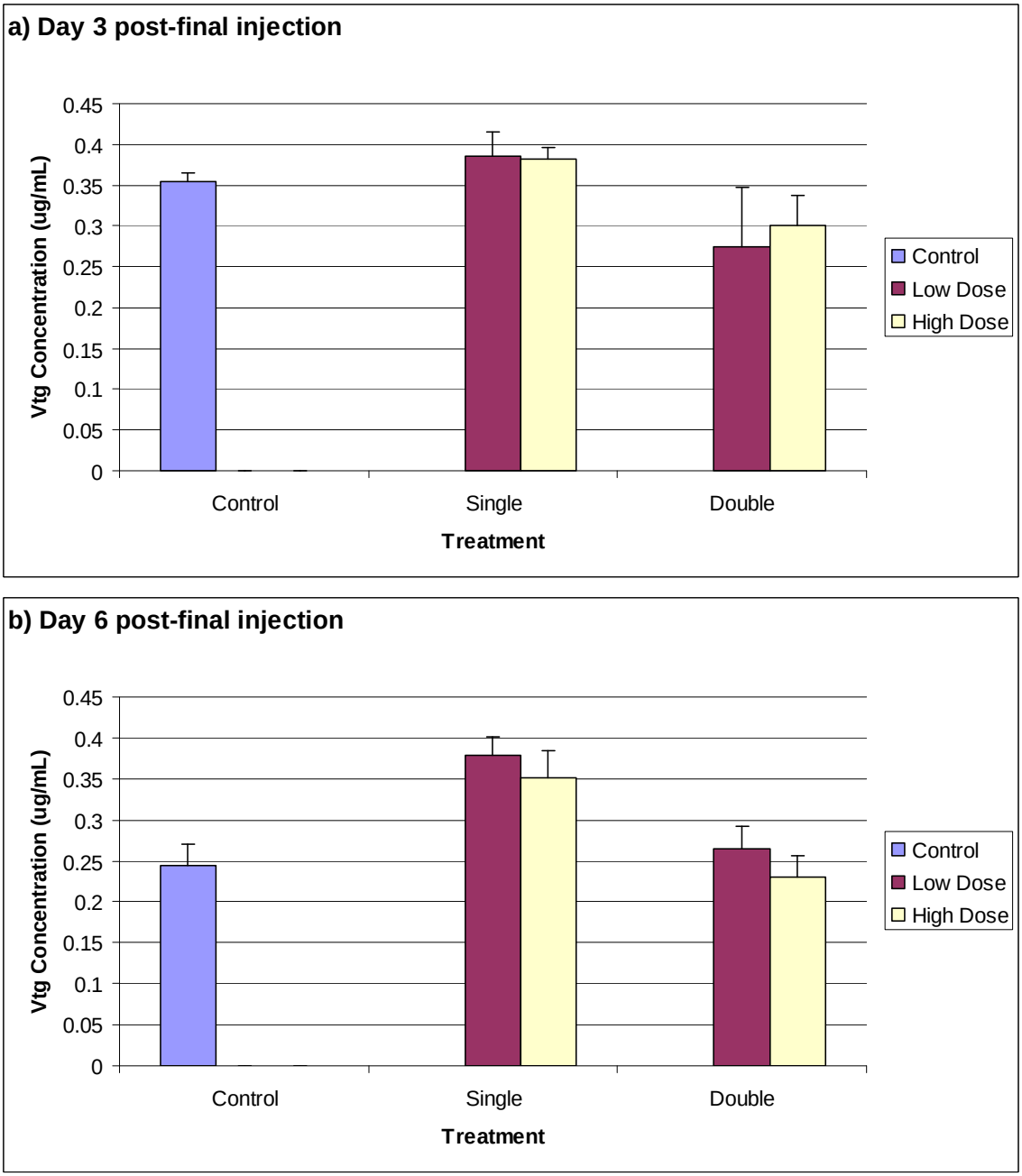

Figure 5.3 Dose response of Vtg production in response to a single or double injection of PBDE-47 compared based on day post-final injection. Each bar represents the mean absorbance (492nm-NSB) + sd (n=6). Graph a) shows the data 3 days after the final injection of saline (control) or PBDE-47 and graph b) shows the data from 6 days after the final injection.

5.3.2.2 Protein concentration and Western Blots

Protein concentration of the plasma samples from the dose response experiment was determined by Lowry assay and that protein concentration was used to determine the dilutions necessary to obtain 10 µg of protein (total protein, not Vtg) to load onto the gels for Western Blotting.

Figures 5.4 – 5.6 are images of the Western blot membranes of the fish plasma from the dose response experiment. While the E2 positive control saturated the column with Vtg, with a particularly dark band at ~180 kDa where the main Vtg subunit would be expected (Sun et al, 2003), the columns containing saline control and PBDE-47 dosed plasma samples were completely clean, with no colour development, indicating an absence of Vtg . This contrasts with the ELISA data from which quantifiable levels of Vtg in the dose response samples were obtained. This suggests that the western blot technique is less sensitive than the ELISA for detecting Vtg production in barramundi.

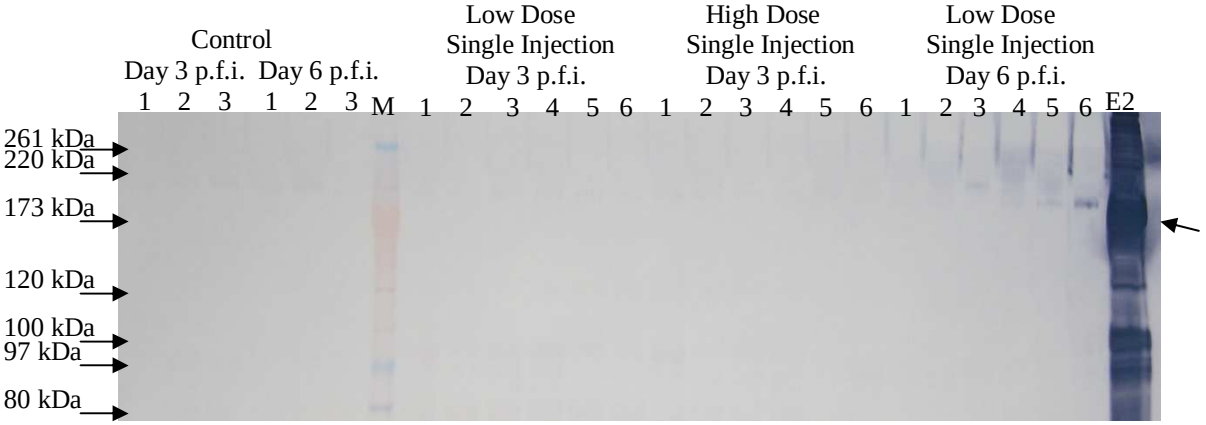

Figure 5.4 Western blot of dose response plasma samples. Putative Vtg band in E2 dosed positive control plasma sample (E2) is labeled with an arrow on the right hand side of the Figure. Standard protein ladder is labeled M and molecular weights are labeled along left hand side of the figure. P.f.i. is post-final injection

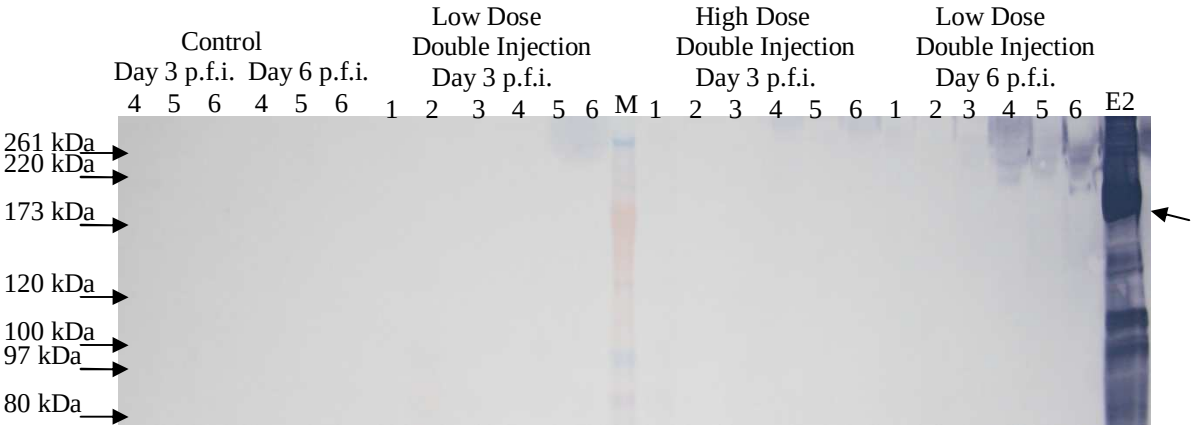

Figure 5.5 Western blot of dose response plasma samples. Putative Vtg band in E2 dosed positive control plasma sample (E2) is labeled with an arrow on the right hand side of the Figure. Standard protein ladder is labeled M and molecular weights are labeled along left hand side of the figure. P.f.i. is post-final injection.

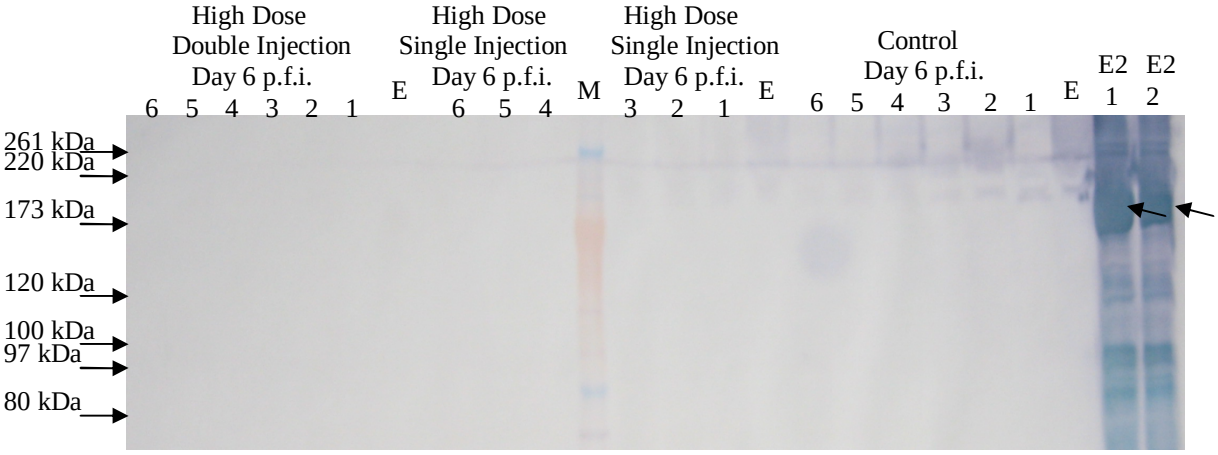

Figure 5.6 Western blot of dose response plasma samples. E2 1 is E2 dosed fish plasma diluted 1:100 and E2 2 is E2 dosed fish plasma diluted 1:200. Putative Vtg band in E2 dosed positive control plasma samples (E2) is labeled with an arrow. Standard protein ladder is labeled M and molecular weights are labeled along left hand side of the figure. E represents an empty lane and p.f.i. is post-final injection.

Figure 5.7 presents a final Western blot done on the dose response plasma samples. The plasma was not diluted at all beyond a 1:2 dilution in sample buffer before being loaded onto the gel. These undiluted samples were run to ensure that there was no dilution error causing low but detectable levels of Vtg to be missed in the other Western blots that were run.

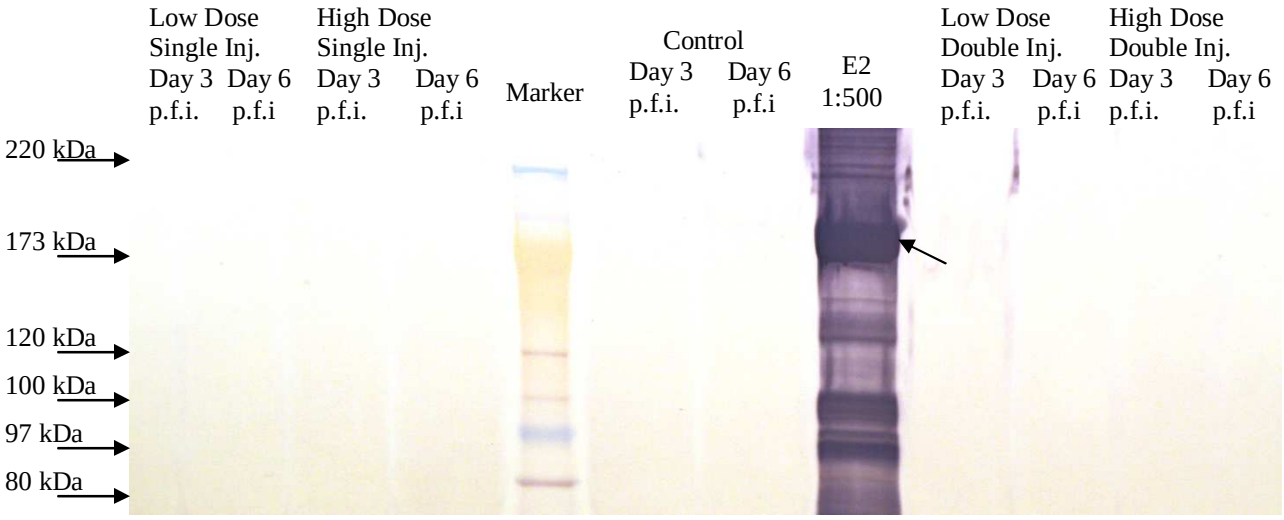

Figure 5.7 Western blot of selected dose response plasma samples run without dilution. Putative Vtg band in E2 dosed positive control plasma sample (E2) is labeled with an arrow. Standard protein ladder is labeled M and molecular weights are labeled along left hand side of the figure. P.f.i. is post-final injection.

5.4 Discussion

Published studies have been inconclusive when attempting to answer the question of what effect PBDE exposure has on the reproductive endocrine system, especially in the area of estrogen mimicry (Aarab et al, 2006; Boon et al, 2002; Labadie and Budzinski, 2006; Legler and Brouwer, 2003; Meerts et al, 2001; Nakari and Pessala, 2005; Stoker et al, 2005) The purpose of this research was to use the ELISA developed for barramundi and analyze samples taken from fish dosed with PBDE-47 to determine whether PBDE displays estrogenic or anti-estrogenic properties in barramundi, using Vtg production in juvenile males as an endpoint measurement of estrogenic activity.

Time course induction of Vtg production by PBDE-47

The time course experiments were designed to study the induction of Vtg over fourteen days after being dosed with either a low or high dose of PBDE-47. The data from the two time course experiments is reported as mean absorbance corrected for non-specific binding. Statistically there is no significant difference between the data from the control and PBDE-47 dosed fish.

The patterns and trends in this qualitative data illustrate the behavior of Vtg production in barramundi in response to injection with PBDE-47. Through the first seven days post injection Vtg production follows the same pattern in both the low and high dosed fish (Figures 5.1 and 5.2). Vtg production increases slightly in both the saline (C) and PBDE-47 injected fish in the first 3 days post injection, followed by a drop in production between days 5 and 7 in both C and PBDE-47 injected fish. It is reasonable to hypothesize that this initial increase and subsequent drop in Vtg production could be the result of a stress reaction to being handled and dosed by i.p. injection, as it is seen in both control and PBDE-47 dosed fish, although no other studies on the time course induction of Vtg by exposure to PBDE could be found to support or refute this hypothesis.

Vtg production appears to rebound between days 7 and 14 in the C and both sets of PBDE-47 dosed fish. Barramundi are protandrous hermaphrodites which begin their lives as males and subsequently change sex to female later in life (Moore, 1979). Therefore, male barramundi could be producing low levels of E2 naturally, a hypothesis which is supported by research which has found naturally occurring low levels of E2 in male salmonids (Sower et al, 1984; Yamada et al, 1993), goldfish (Trudeau et al, 1993), eels (Miura et al, 1999), Japanese hunchen (Amer et al, 2001), rainbow trout (Vizziano et al, 1996), and Atlantic croaker (Loomis and Thomas, 1999). The studies listed above all deal with older or larger fish than those used here. However, Amer et al (2001) studied prepubertal Japanese hunchen and Trudeau et al (1993) studied sexually regressed male goldfish in addition to sexually mature males, and both studies found E2 present in the absence of sexual maturity. In addition, Miura et al (1999) induced spermatogenesis in otherwise testicularly immature males and E2 was present before induction. While it can't be known if very young male barramundi like those studied here will have naturally occurring E2 present without further research, the existing literature does suggest that in other fish species E2 is present before sexual maturity. If this is the case, then low levels of Vtg may be being produced naturally in response to naturally occurring E2, and the rebounding Vtg production apparent in the time course experiments would be a return to normal baseline levels for male barramundi. While Vtg production in the low PBDE-47 dosed fish increases at the same rate as the C fish (Figure 5.1), Vtg production in the high PBDE-47 dosed fish increases somewhat slower (Figure 5.2), although statistically there is not a significant difference

between the control and PBDE-47 dosed fish. It is possible this could indicate suppression of Vtg production by the high dose of PBDE-47. This finding is supported by previous research which found PBDE to act anti-estrogenically in mussels (*Mytilus edulis*) (Aarab et al, 2006).

Dose response induction of Vtg by PBDE-47

In accordance with the time course data, which showed that a high dose of PBDE-47 suppressed Vtg production from days 7 to 14 after injection (Figure 5.2), statistical analysis by two-way ANOVA of the ELISA data from the fish which received a single or a double injection of PBDE-47, shows dose size has a small, but significant effect on day six post-final injection ($P=0.0355$). The difference in data based on dose size at day 6 post-final injection in the dose response experiment supports the conclusion that a higher dose of PBDE-47 is suppressing production of Vtg in barramundi, regardless of whether the dose was administered once or twice (Figure 5.3b).

The dose response experiments were designed to study whether repeating the injection of either a low or high dose of PBDE-47 had an effect on the induction of Vtg production in barramundi. Statistical analysis by two-way ANOVA shows treatment (single vs. double injection) has a very significant effect on the data on both day three and six post-final injection ($P<0.0001$). The time course data for both low and high dose showed a marked decrease in Vtg production between 5 and 7 days post-injection, independent of dose size, a trend which is repeated in the control fish between days 3 and 6 post-final injection in the dose response data. The first sample taken from the double injected fish falls 6 days after the first injection of PBDE-47. Therefore, it is difficult to determine whether the apparently greater suppression of Vtg production in the double injected fish is the result of suppression by PBDE-47 or the result of that apparently natural drop in Vtg production 5-7 days post-injection. A two-way ANOVA of the data from the double injected fish shows that there is a significant ($P=0.0022$) decrease in Vtg levels between day 3 post-final injection and day 6 post-final injection, which corresponds to day 9 post-first injection. In the time course work Vtg levels began to rebound between days 7 and 10 post-injection. The continuing decrease in Vtg levels six days after the second injection (nine days after the first injection) of PBDE-47 in the double injected fish would indicate continued suppression of Vtg levels after fish that received a single injection in the time course experiment began to see levels

rebound. While this conclusion would fit with the time course data and the hypothesis that PBDE-47 acts anti-estrogenically, it is difficult to say with certainty this is what is happening because of the unforeseen, apparently natural drop in Vtg levels 5-7 days after injection. Having a double injected control to compare the data with would help to clear up this uncertainty in future research, by providing data on whether a double injection of saline causes an increased period of suppression of Vtg production.

The Western blots shown in Figures 5.4 – 5.7 show no detectable levels of Vtg in the dose response samples. In contrast it will be recalled that for the dose-response results quantifiable levels of Vtg were detected using the ELISA (Figure 5.3). These results suggest that the western blot technique is less sensitive than the ELISA method for detecting Vtg production in barramundi, and therefore will not always be useful for confirming ELISA results.

In summary PBDE-47 appears to have an anti-estrogenic effect in barramundi, suppressing Vtg production in male barramundi when a high dose (10 mg kg^{-1} bw) is injected by i.p. injection, although this effect is not apparent when a low dose (1 mg kg^{-1} bw) is administered. In addition, the Vtg levels in the C fish suggest that male barramundi have a baseline level of Vtg production, possibly due to a natural production of E2, a phenomenon seen by other researchers in a variety of other fish species (Amer et al, 2001; Loomis and Thomas, 1999; Miura et al, 1999; Sower et al, 1984; Trudeau et al, 1993; Vizziano et al, 1996; Yamada et al, 1993). It would be worthwhile to take a look both at E2 and Vtg levels in male barramundi at different stages of life and seasonally to determine whether a baseline level of either does occur naturally. If this is the case in the future research concentrating on determining how the baseline levels of Vtg production in barramundi fluctuate, both seasonally and over the life-cycle of the species, could be used to help clarify the anti-estrogenic potency of PBDE-47.

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6. Conclusions and Recommendations for Future Work

6.1 Conclusions and Recommendations

This thesis encompasses multiple projects in multiple disciplines. It is comprises a pilot study investigating the current levels of PBDEs in the aquatic environment, combined with a study of the potential effects of PBDEs on a locally important fish species. The broad nature of this work has given rise to numerous questions which can only be resolved with further research. This chapter offers the key conclusions of the work and some recommendations on where the research could go from here.

The key outcomes of the research are:

a) PBDEs were detected in sediments at low levels. For the two congeners assessed (PBDE-47 and PBDE-209) levels were found to range from below detection (0.2 μg kg^{-1} dw) to 0.35±0.2 μg kg^{-1} (dw) for PBDE-47 and from below detection (0.2 μg kg^{-1} dw) to 0.85±0.07 μg kg^{-1} (dw) for PBDE-209. These levels are similar to levels found in other parts of Australia (Toms et al, 2008) and are a little lower than levels from similar sites around the world (Table 1.1).

b) PBDE-47 was found to depurate slowly, with a depuration rate constant between 0.41 and 0.69 day^{-1} which is consistent with similar studies (Gustafsson et al, 1999; Ciparis and Hale, 2005; Muirhead et al, 2006). In addition, PBDE-47 was detected in control fish at low levels, which was unexpected.

c) A viable and effective ELISA for the detection of Vtg in barramundi was produced, although unfortunately it was not possible to use the barramundi Vtg or the polyclonal sheep anti-barramundi Vtg antibody created during this research due to purification difficulties.

d) Finally, PBDE-47 was found to suppress the apparently naturally occurring production of Vtg in male barramundi.

Taken as a whole it can be seen that PBDEs exist in the Townsville environment and that therefore barramundi in a similar environment will be exposed to PBDEs. It has been shown that

PBDEs depurate relatively slowly and there is some evidence that PBDEs suppress Vtg production. Accordingly the following recommendations are made for future work targeting the key conclusions and outcomes of this research:

1) With the continued use of deca-BDE commercial mixtures worldwide and the discarding and destruction of products containing the now defunct penta- and octa-BDE commercial mixtures it is expected that levels of PBDE will continue to rise. Regular sampling along Ross Creek should only be the beginning point for future work. Townsville is the largest city in Northern Australia and is the site of an army and air force base, as well as a busy regional airport. A larger study which takes samples from a wider range of land use sites, such as along Ross Creek, at the armed forces bases, the airport, and the town landfill, would be a good start allowing for analysis of the samples based on land use as well as potentially revealing point sources for PBDE release into the local environment. This research would complement studies done in other parts of Australia and overseas.
2) Detection of PBDEs in control fish needs to be confirmed and if confirmed the source of PBDE contamination should be investigated. A future study sampling barramundi from the M.A.R.F.U. complex hatchery as well as sampling the feed and testing the equipment used there would determine whether the contamination seen in the control fish from this experiment is a result of prior exposure at some point or points during the rearing process.
3) While the ELISA used in this research using the mouse anti-striped bass Vtg primary antibody, ND-3G2, has proven to be both accurate and sensitive, an ELISA making use of a polyclonal antibody created to be specific to barramundi would detect all the forms of Vtg that barramundi produce. The column fractions used to inoculate the sheep and tested as a Vtg standard were not purified enough, but there was a strong Vtg signal present and future work to further separate and purify that Vtg would almost certainly result in a product which could be used as a barramundi specific Vtg standard in a quantitative ELISA. Using this purified Vtg to inoculate sheep would lead to a polyclonal primary antibody which is specific to barramundi.
4) Male barramundi in this study produced measurable levels of Vtg, without any external stimulation, suggesting Vtg is produced by male barramundi naturally, most likely due to low levels of naturally occurring E2. Many studies support the idea that male fish

produce E2 naturally (Amer et al, 2001; Loomis and Thomas, 1999; Miura et al, 1999; Sower et al, 1984; Trudeau et al, 1993; Vizziano et al, 1996; Yamada et al, 1993). While a few of these studies look at E2 production in the context of sexual maturation in males (Amer et al, 2001; Miura et al, 1999; Sower et al, 1984; Trudeau et al, 1993; Yamada et al, 1993) and one even looks at E2 expression over the course of a year (Amer et al, 2001) there is no study that looks at the fluctuation of E2 or Vtg over the whole lifecycle. Future research ought to include a lifecycle study of barramundi for E2 expression and Vtg production, from fingerlings through to sexual maturity.

5) Finally, this study suggests that PBDEs may suppress Vtg production in male barramundi. The levels of Vtg seen here were very low, making it hard to make a conclusive argument that this is the case. Repeating this study with sexually mature females, which would be expected to produce large concentrations of Vtg, would allow for the determination of the extent of Vtg suppression (if any) in the presence of PBDEs.

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