# DEVELOPMENT AND VALIDATION OF BIOMARKERS

# IN A FINFISH SPECIES FROM SOUTHERN

# AUSTRALIAN CONTAMINATED WATERS

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# Abstract

In ecotoxicology, biomarkers are measurable biological responses to xenobiotics which are present in the environment and in organisms. Such biological responses are limited to sub-organismic levels including organ or tissue level, cellular or sub-cellular level and molecular level. These lower levels of biological responses precede the higher level of biological organization changes such as population, community and ecosystem. As such, the most important function of biomarkers is to provide early warning signals of significant biological effects. Appropriate organisms should be employed to detect such biomarker signals. In an aquatic ecosystem, bottom dwelling fish are commonly used for biomarker studies.

Southern sand flathead (*Platycephalus bassensis*), is an endemic finfish species widely distributed along the southern Australian coastal waters. This species has been used as an indicator species since 1970s as it is demersal, sedentary, carnivorous and abundant, all favourable traits for environmental ecotoxicology research. However little is known about the effects of anthropogenic pollutants on this species so far and very few biomarker-related studies have been conducted on this ecologically important species.

The aim of this thesis was to develop and validate the molecular and histological biomarkers in sand flathead from southern Australian contaminated waters. The development of biomarkers was conducted in sand flathead from a historical metal polluted estuary (Derwent estuary, Tasmania). In terms of validation, the findings from the Derwent estuary were further examined in sand flathead from a more complex marine environment (Port Phillip Bay, Victoria). This research had four research chapters to address this question. 1) Assess the relationships between thyroid-related genes transcripts and trace metals in sand flathead from the Derwent estuary. 2)

Investigate gill histology and hepatic expression of metal homeostasis-related genes in sand flathead from the Derwent estuary. 3) Assess the liver histological biomarkers and usefulness of Diablo/SMAC homolog and GRP78 gene as biomarkers in sand flathead from the Derwent estuary. 4) Examine the applicability of all the candidate biomarker genes and liver histology in sand flathead from Port Phillip Bay.

The data from field studies suggested that trace metals such as mercury and arsenic are associated with the transcripts of thyroid hormone related genes and environmental metal exposure influenced metals homeostasis related genes on transcriptional level, Apoptotic genes, Diablo/SMAC1 and Diablo/SMAC2 responded differently to environmental metal exposure and the ER stress marker gene GRP78 could be induced by metal stress. The results of gill histology indicated the both metal pollution and pathogen infection could contribute to gill lesions. A negative correlation observed between mercury residues and parasites number suggested pollutants may affect the abundance of gill parasites. The results for liver histology suggested chronic environmental metal exposures may contribute to the increased prevalence of inflammation and bile duct fibrosis.

Port Phillip Bay is subject to high level of organic pollutants and heavy metal mainly due to anthropogenic activities and compared to the Derwent estuary is a more complex marine environment. Results for Port Phillip Bay suggested thyroid hormone receptor genes are the major target for pollutants; Corio Bay was the most affected sampling site as some genes such as transthyretin and ferroportin 1 transcripts were only regulated in fish from this area; Diablo/SMAC1 is a potential novel biomarker gene in female fish due to its high sensitivity response to environmental pollutant exposure. Combining the gene expression results from the Derwent estuary; these data implied different pollutants impact different genes of these pathways.

In terms of assessment of effects of pollution, pathway based molecular biomarker genes could provide a more comprehensive and integrated picture than the traditional single biomarker gene. In terms of histological biomarkers, a higher prevalence of granuloma was observed in the fish with high level of total arsenic, suggesting environmental arsenic exposure may contribute to the formation of this lesion.

In conclusion, this research focused on the development and validation of histological and molecular biomarkers in sand flathead. The gene transcripts involved in four different biological activities were newly identified. Two major organs, gill and liver, were used for histological assessment. The associations between the gene transcripts, pollution and parasites were observed in this work. Several gene transcripts, liver histological condition and absence of gill parasites potentially serve as biomarkers for future environmental monitoring. The results in this thesis have made a substantial contribution to understanding of interactions between pollutants and fish.

# Acknowledgements

# Chapter 1 : General Introduction

## 1.1 Pollutants in marine environment

Based on the nutritional requirement of organisms, heavy metals can be classified into biological essential metals (like zinc, copper and iron) and non-essential metals (like cadmium, mercury and lead). All can be toxic for organisms when accumulations exceed certain concentrations (Rainbow, 1995). Within an ecosystem, heavy metals can originate from both natural sources and anthropogenic sources. Currently the input of heavy metals mainly from anthropogenic activities, such as mineral mining, manufacturing and energy development, far exceed those from nature (Duruibe *et al.*, 2007). Therefore, heavy metals have become one of more serious contaminants in ecosystems as they are toxic, persistent, bio-accumulative and non-biodegradable (Uysal *et al.*, 2008). Heavy metal pollution in the marine environment is a serious global problem and is a growing threat to marine ecosystems. Estuaries are important sites for many forms of aquatic organisms and also play a role as final sinks of many pollutants that mainly arise from anthropogenic activities (Pérez-Carrera *et al.*, 2007). Thus, aquatic organisms are vulnerable to heavy metal contamination. Many studies have reported that environmental heavy metal exposure could pose adverse effects to aquatic organisms and the communities (Bryan, 1971; Dauvin, 2008; Jezierska *et al.*, 2009; Pinto *et al.*, 2003; Warnick and Bell, 1969).

Besides heavy metal, persistent organic pollutants (POPs) such as polychlorinated hydrocarbons (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides are also predominant pollutants in marine environments (Corsolini *et al.*, 2002; Jensen *et al.*, 1969; Pino *et al.*, 2000; Wenning and Martello, 2013). These compounds are widely used in agricultural and industrial activities (Jones and de Voogt, 1999). The POPs enter the marine environment through domestic and industrial waste water discharges, agricultural runoff, oil spills and atmospheric deposition (El-Shahawi *et al.*, 2010). As a result, these pollutants are passed up via the food

chain and ultimately pose adverse effects to marine organisms such as fish (Kelly *et al.*, 2007). There is increasing evidence that these pollutants could alter the fish reproductive, endocrine and immune system (Dunier and Siwicki, 1993; Kime, 1995; Kime, 2012).

## 1.2 Pollutants in Derwent estuary and Port Phillip Bay

It is well documented that marine environments that are close to developed and industrialized cities are heavily impacted by anthropogenic activities (Jones *et al.*, 2003). The Derwent estuary has been recognized as one of the most heavy metal polluted estuary in Australia (Bloom and Ayling, 1977). It is located in south eastern Tasmania and is surrounded by the capital city of Hobart (Fig 1.1). This estuary has significant ecological and recreational values in Tasmania (Green and Coughanowr, 2003). It is not only an important ecological niche for various aquatic plants and animals, but also supports recreational activities such as fishing, boating, diving and swimming (Green and Coughanowr, 2003). Nevertheless, the biota of this estuary is severely polluted by heavy metals (Green and Coughanowr, 2003). For example, the mercury level in the muscle of southern sand flathead (*Platycephalus bassensis*), black bream (*Acanthopagrus butcheri*) and sea-run trout (*Salmo trutta*) exceeded the maximum permitted level of Food Standards Australia and New Zealand (FSANZ) (Verdouw, 2008). The predominant metals such as mercury (Hg) and arsenic (As) are mainly from historical industrial effluent discharge (Bloom and Ayling, 1977). A heavy metal monitoring program is conducted annually in fish from the Derwent estuary (Green and Coughanowr, 2003). However, these trace metal data are unable to reflect the biological significance of pollution. This is because body burdens do not provide any information regarding effects of pollutants on the health of aquatic organisms (van der Oost *et*

*al.,* 2003). Therefore, more studies are needed to assess the impact of heavy metals on aquatic organisms in the Derwent estuary.
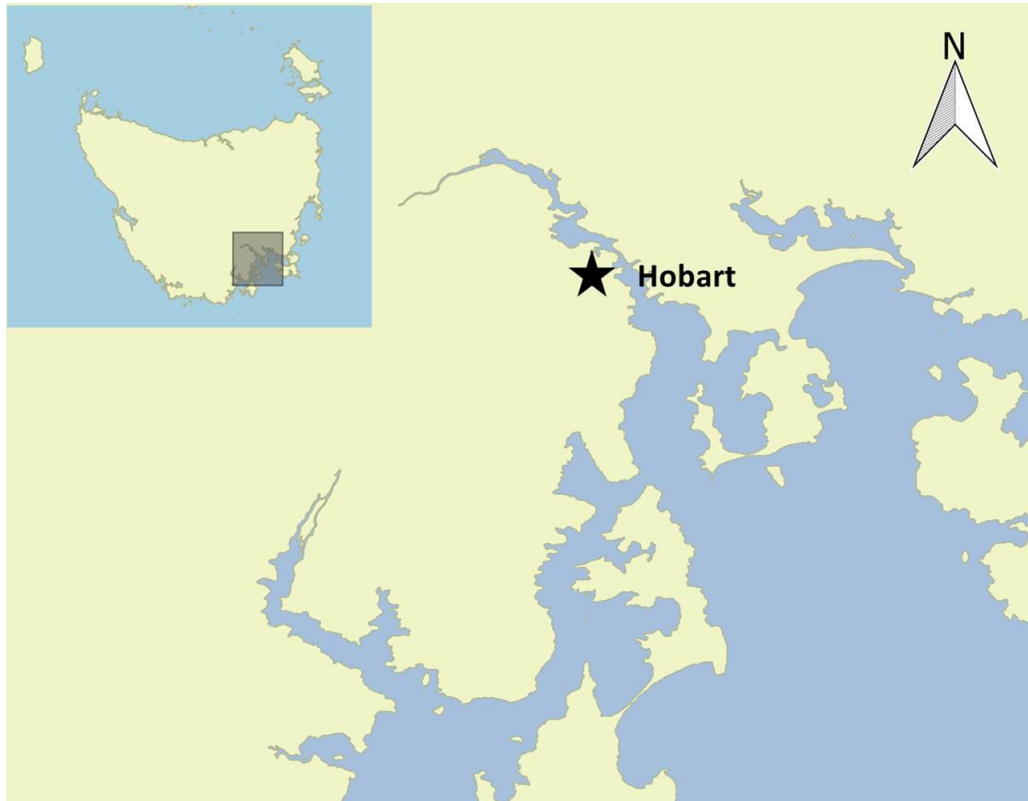


**Figure 1.1 Map of Derwent estuary, Tasmania**

Port Phillip Bay is a landlocked bay in Victoria, Australia (Fig 1.2). It has a large catchment (9800 km$^2$). Melbourne, the second biggest city of Australia with an approximate population of 4 million, is on the northern edge of Port Phillip Bay. The city of Geelong (population 200,000) is located on the west of Port Phillip Bay. Besides the densely populated surrounding areas, Port Phillip Bay also supports extensive port facilities and industries. It was reported that the Port

Phillip Bay sediment, water and biota are severely contaminated with heavy metals such as mercury, cadmium and organic pollutants including PCBs and PAHs (Phillips *et al.*, 1992). The pollutant level of Port Phillip Bay has become a public concern. Previously, studies in Port Phillip Bay have been focused on monitoring the level of these pollutants in species such as oyster (*Ostrea angasi*), mussel (*Mytilus edulis*) and sand flathead (*Platycephalus bassensis*) (Fabris *et al.*, 1992; Nicholson *et al.*, 1994; Phillips, 1976). Only a few studies were conducted for evaluating the effects of pollutants on fish more than a decade ago (Gagnon and Holdway, 2002; Holdway *et al.*, 1994). Therefore, more studies are needed to fully understand the biological response of fish to the environmental pollutants exposure in Port Phillip Bay.



**Figure 1.2 Map of Port Phillip Bay, Victoria.**

## 1.3 Biomarkers and indicator species

Pollutants that enter the aquatic ecosystem can cause multiple levels of biological responses that range from molecular to the whole ecosystem (Fig 1.3) (Bayne, 1985; Di Giulio and Hinton, 2008; van der Oost *et al.*, 2003). The environmental pollutants initiate these biological responses through altering molecular components and organelles in cells. Therefore, the biological responses which occur at molecular or cellular levels are highly sensitive to pollutants exposure, but may not have obvious ecological relevance (Di Giulio and Hinton, 2008). Compared to lower level biological responses, the population or community level responses have a higher degree of ecological relevance but less degree of response sensitivity (Di Giulio and Hinton, 2008) (Fig 1.3). In the past decades, much effort has been focused on developing methods that are able to detect these lower level biological responses within marine ecosystems (Lam and Gray, 2003). As such lower level biological responses are also referred to as biomarker signals or biomarkers (Fig 1.3) (Lam and Gray, 2003). Basically biomarkers are defined as measurements indicating sub-organismic level changes due to the presence of environmental pollutants (van der Oost *et al.*, 2003). These sub-organismic levels changes are often restricted to molecular, subcellular or cellular and tissue or organ changes that are determined within an organism (Lam and Gray, 2003). It is generally believed that these biomarker signals precede the higher level of biological responses such as population and community changes (Lam and Gray, 2003). Therefore, it is important to detect biomarker signals before more serious adverse effects occur on the higher levels that are difficult to be reversed through remedial actions and risk reduction (van der Oost *et al.*, 2003).
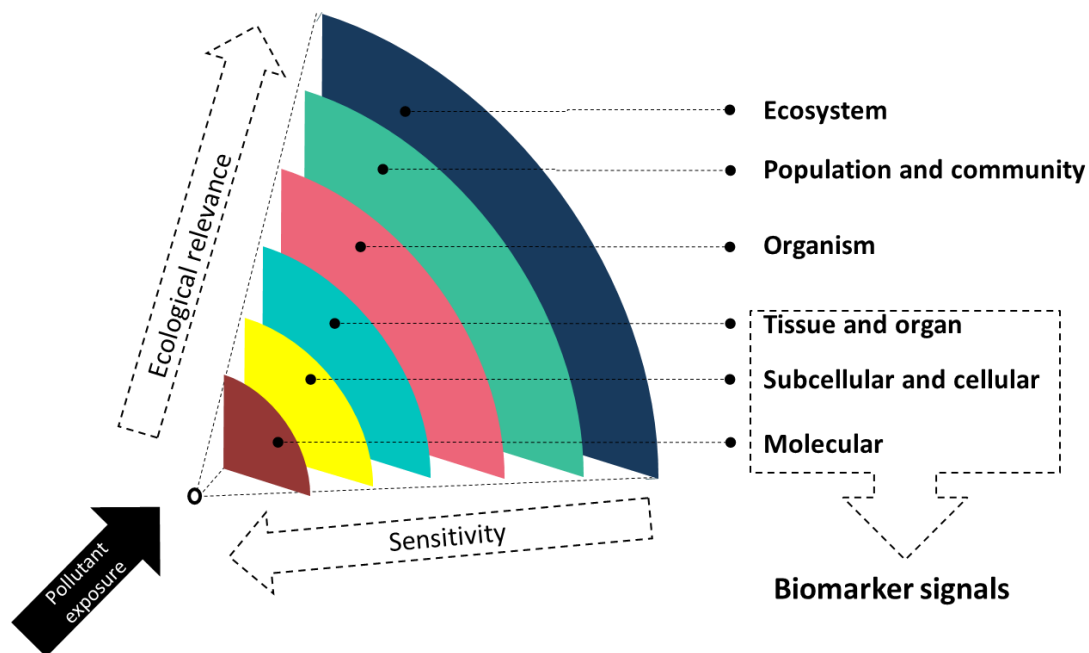
**Figure 1.3 Biological response sensitivity and ecological relevance to pollutants exposure within an ecosystem.** Biomarker signals are mainly focused on sub-organismic levels including molecular, subcellular and cellular, tissue and organ level. Modified from (Bayne, 1985; Di Giulio and Hinton, 2008; van der Oost *et al.*, 2003)

In the past decades, substantial efforts have been made to develop and validate the biomarkers. Some classic biomarkers have gained international recognition in both laboratory field studies such as cytochrome P4501A (EROD), vitellogenin (VTG), metallothionein (MT), liver histopathology and externally visible fish disease (Amiard-Triquet *et al.*, 2012). These biomarkers have been successfully deployed by regulatory bodies in environmental monitoring programs which were mainly carried out in Europe and North America (Amiard-Triquet *et al.*, 2012). For instance, biomarkers such as MT, EROD and histology in liver of fish have been recognized by OSPAR Commission (Oslo/Paris convention for the Protection of the Marine Environment of North-East Atlantic) (OSPAR Commission 2007). In the United States of America, the histological assessments of liver, gills and kidney of demersal fish species have been used to identify histological biomarkers in National Benthic Surveillance Project (NBSP)

(Myers *et al.*, 1993). The results have shown the significant associations between prevalence of certain liver lesions and pollutants level of sediment (Myers *et al.*, 1993). Another well-known example of using fish biomarkers in United States is Puget Sound Assessment and Monitoring Program, where the measurements of hepatic CYP1A, VTG and liver histopathology in English sole (*Parophrys vetulus*) were used to establish the linkage between the pollutants and biomarker signals (Myers *et al.*, 1998; Amiard-Triquet *et al.*, 2012). The application of biomarkers by regulatory agencies in these environmental monitoring programs has demonstrated that biomarkers are useful tools for environmental protection.

Indicator species or sentinel species are often used for monitoring the effects of pollution. An indicator species can be defined as an organism that is able to provide information regarding environmental conditions of its habitat (van der Oost *et al.*, 2003). In marine pollution studies commonly used indicator species are bivalves, gastropods and fish (Phillips, 1977). Several fish species are of particular interest in environmental monitoring programs because they are abundant in the aquatic environment and play a significant ecological role in the food chain (Beyer, 1996). For instance, European flounder (*Platichthys flesus*) and common dab (*Limanda limanda*) have been successfully used for environmental monitoring in Europe (Bucke *et al.*, 1996) and English sole (*Pleuronectes vetulus*) and winter flounder (*Pleuronectes americanus*) have often been employed to assess the effects of marine pollution in North America (Feist *et al.*, 2004). These studies suggested an appropriate indicator species should be demersal and sedentary, exist in relative abundance and be susceptible to pollutants (Bucke *et al.*, 1996).

## 1.4 Southern sand flathead

In southern Australia, southern sand flathead (*Platycephalus bassensis*) (Fig 1.4) are often used as indicator species for monitoring pollution (Ayling *et al.*, 1975; Holdway *et al.*, 1994). This species is an endemic species extensively distributed along the coast of southern Australia (Gomon *et al.*, 1994). In addition, this species exhibits strong site fidelity, it is always in association with bottom sediments and accumulates pollutants from a diet consisting of benthic organisms (Parry and Scientific, 1995). Because of these traits, southern sand flathead has attracted considerable interest in environmental monitoring study.



**Figure 1.4 Southern sand flathead (*Platycephalus bassensis*) (http://dpipwe.tas.gov.au/)**

A number of studies have shown that southern sand flathead is a reliable indicator species for assessing the biological effects of pollutants in both laboratory and field studies (Brumley *et al.*, 1995; Gagnon and Holdway, 2002; Holdway *et al.*, 1994). For instance, It has been reported that ethoxyresorufin *O*-deethylase (EROD), uridine diphospho-glucuronosyltransferase (UDP-GT) activities were increased after PCB mixture Aroclor 1254 exposure under laboratory conditions, suggesting these biological responses are sensitive biomarkers and sand flathead is an appropriate indicator fish species (Brumley *et al.*, 1995). This species was also successfully employed as an indicator species in field studies. A three year study demonstrated that measurements of two hepatic enzymes (ECOD and EROD) and a serum enzyme (s-SDH) in

flathead from Port Phillip Bay could reflect the pollution level in general (Holdway *et al.*, 1994). These studies suggest sand flathead is a suitable indicator species for environmental monitoring.

## 1.5 Molecular and histological biomarkers

### 1. 5.1   Molecular biomarker

Gene expression analysis is commonly used for development of molecular biomarkers. In this project, the traditional molecular biomarker genes such as MT and CYP1A combined with pathway based biomarker candidate genes were cloned and tested in sand flathead from the Derwent estuary and Port Phillip Bay. These genes are mainly involved in: 1) thyroid hormone pathway; 2) iron homeostasis; 3) apoptosis and endoplasmic reticulum (ER) stress.

### (1) Thyroid hormone related genes

In the past decades, there has been increasing evidence suggesting that environmental pollutants including heavy metals and the persistent organic pollutants can disrupt the thyroid system of fish (Besselink *et al.*, 1996; Bleau *et al.*, 1996; Brown *et al.*, 2004; Fok *et al.*, 1990; Leatherland and Sonstegard, 1980; Nichols *et al.*, 1984; Stephens *et al.*, 1997). It is generally accepted that thyroid disrupting chemicals disrupt the thyroid hormone system through their effects on thyroid hormone transportation, metabolism and reception (Patrick, 2009). The thyroid system is one of the most important endocrine systems in vertebrates and regulates many bioactivities including metabolism, development and reproduction (Zoeller *et al*., 2007). As such, the disruption of thyroid system may cause significant impacts on the health of individual fish and on sustainability of fish population. This project focused on a number of genes that are involved in these major targets of thyroid hormone pathway (Fig 1. 5). These genes were transthyretin gene

(TTR), deiodinase genes (D1 and D2) and thyroid hormone receptor genes (TRα and TRβ). In fish, two types of thyroid hormone (T4 and T3) are synthesized in the thyroid follicles and then are released in the circulation. Transthyretin is one of the major transport proteins that carry thyroid hormone into target tissues (Power *et al.*, 2009). The majority of T4 is converted into T3, a more biologically active form of thyroid hormone, by D1 and D2 in the target tissues (Orozco and Valverde, 2005). T3 binds to thyroid hormone receptors (TRs) as a transcription factor to regulate gene expression (Yamano, 2005).
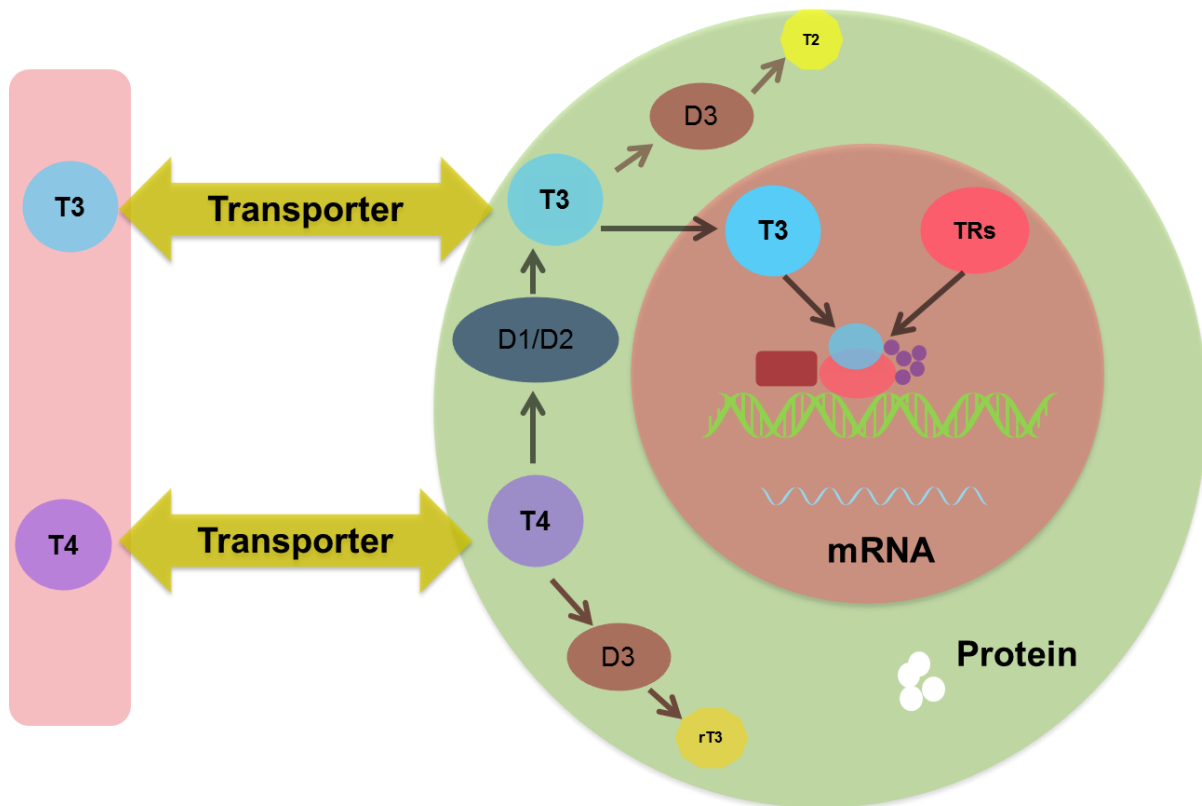


**Figure 1.5 Simplified thyroid hormones pathway.** Thyroid hormones (T4 and T3) are released into circulation, and then carried into target cells by transporter protein. T4 is converted into a more biological active form T3 by D1 (type I deiodinase) and D2 (type II deiodinase). T3 binds to thyroid hormone receptor (TRs) as a transcription factor regulated gene expression. (Modified from (Cohen-Lehman *et al.*, 2010)

**(2) Metal homeostasis related genes**

Iron (Fe) is a significant element that is involved many fundamental bioactivities including transportation of oxygen, energy metabolism and DNA replication (Sahu *et al.*, 2013). The function and homeostasis of Fe can be altered by heavy metals such as Hg (Taylor *et al.*, 1995). To investigate the effects of environmental heavy metals on fish liver iron homeostasis pathway, this project concentrated on four genes that are important for Fe homeostasis including MTF1 (metal regulatory transcription factor 1), ferroportin-1 (FPN1), transferrin (TF) and ferritin (Fig 1.6). Metal regulatory transcription factor 1 (MTF1) regulates the expression of genes including metallothioneins (MTs) and ferroportin-1 (FPN1). Ferroportin-1 (FPN1) is responsible for iron exporting and is significant for iron cellular homeostasis (Ward and Kaplan, 2012). Transferrin (TF) and ferritin are also involved in iron homeostasis in vertebrates (Ward and Kaplan, 2012). Transferrin is the major iron transport protein that carries iron to different locations, while ferritin plays an important role in iron storage.
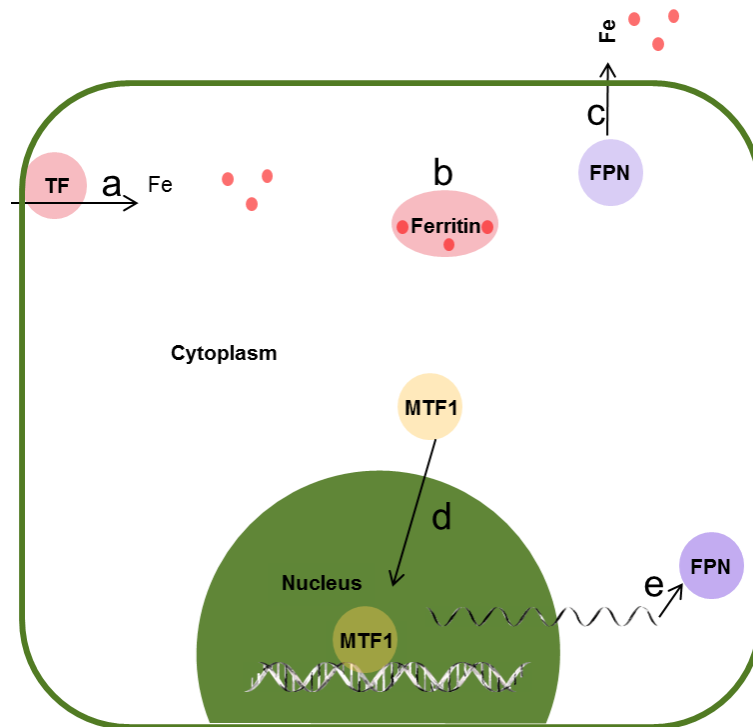
**Figure 1.6 Simplified scheme of iron metabolism.** (a) Fe is transferred by transferrin into target cells. (b) Excess Fe is stored in ferritin. (c) Fe is exported out of cell through FPN1. (d) MTF1 transfers into nucleus from cytoplasm. (e) MTF1 activates FPN mRNA synthesis. Modified from (Zhao *et al.*, 2014)

### (3) Diablo/SMAC homolog and GRP78

Diablo (Direct IAP binding protein with low P*I*)/SMAC (second mitochondria derived activator of caspase) is a mitochondrial released protein involved in apoptotic pathways (Burri *et al.*, 2005). A number of recent studies indicate that measurement of Diablo/SMAC gene mRNA is a promising biomarker for liver cellular damage in European flounder (Leaver *et al.*, 2010; Williams *et al.*, 2008; Zacchino *et al.*, 2012). However, the applicability of Diablo/SMAC homolog as biomarker in other fish species remains unknown.

GRP78 (glucose regulated protein 78) is a highly conserved endoplasmic reticulum (ER) molecular chaperone and plays important role in unfolded protein response during the ER stress

(Bertolotti *et al.*, 2000). Previous studies suggest that the transcripts of this gene could be induced under ER stress caused by metal exposure in both invertebrates and vertebrates (Ma *et al.*, 2014; Zhu *et al.*, 2013). These studies indicate this gene has the potential as a biomarker gene for environmental pollutants exposure. Therefore, this gene was selected and examined as a biomarker candidate gene in this project.

### 1. 5.2   Histological biomarker

Fish constantly exposed to environmental stressors usually exhibit high prevalence of certain types of histological changes and are more susceptible to diseases or pathogen infections (Bernet *et al.*, 1999). Fish gills and liver are two major target organs for histological assessment in order to detect a pollutant's effects (Bernet *et al.*, 1999). Gills are the main entry routes for pollutants in fish, while the liver is responsible for uptake, transformation and excretion of xenobiotics (Di Giulio and Hinton, 2008; Gernhöfer *et al.*, 2001). Numerous studies have demonstrated the usefulness of gills and liver histology as biomarkers in environmental monitoring programs (Bernet *et al.*, 1999). For instance, Stentiford et al 2003 successfully used flounder (*Platichthys flesus*) gills histology to an estuarine monitoring program. The presence of aneurysm in flounder gill has been reported with waterborne pollution (Mallatt, 1985; Stentiford *et al.*, 2003). Liver pathology of the European dab and flounder has been used in European monitoring programs (Feist *et al.*, 2004). An increased size or number of melano-macrophage centres (MMCs) in liver were often observed in flounder from contaminated water (Agius and Roberts, 2003; Pierce *et al.*, 1980). These pathologies are increasingly being applied as biomarkers of environmental stress. It is generally believed that these pathologies in marine flatfish are sensitive and reliable biomarkers that could be linked to marine environmental pollutants (Stentiford *et al.*, 2003).

These histological changes reflect alterations in tissue or organs and are more ecologically relevant biological responses (van der Oost *et al.*, 2003).

## 1.6 Thesis structure and research objectives

The general objective of this study was to assess potential molecular and histological biomarkers in southern sand flathead from the Derwent estuary and then further validate these findings in fish from, a more complex marine environment, Port Phillip Bay. To address this objective, there are four research chapters with specific aims as follows: (An overview of structure of these chapters is shown in Fig 1.7).
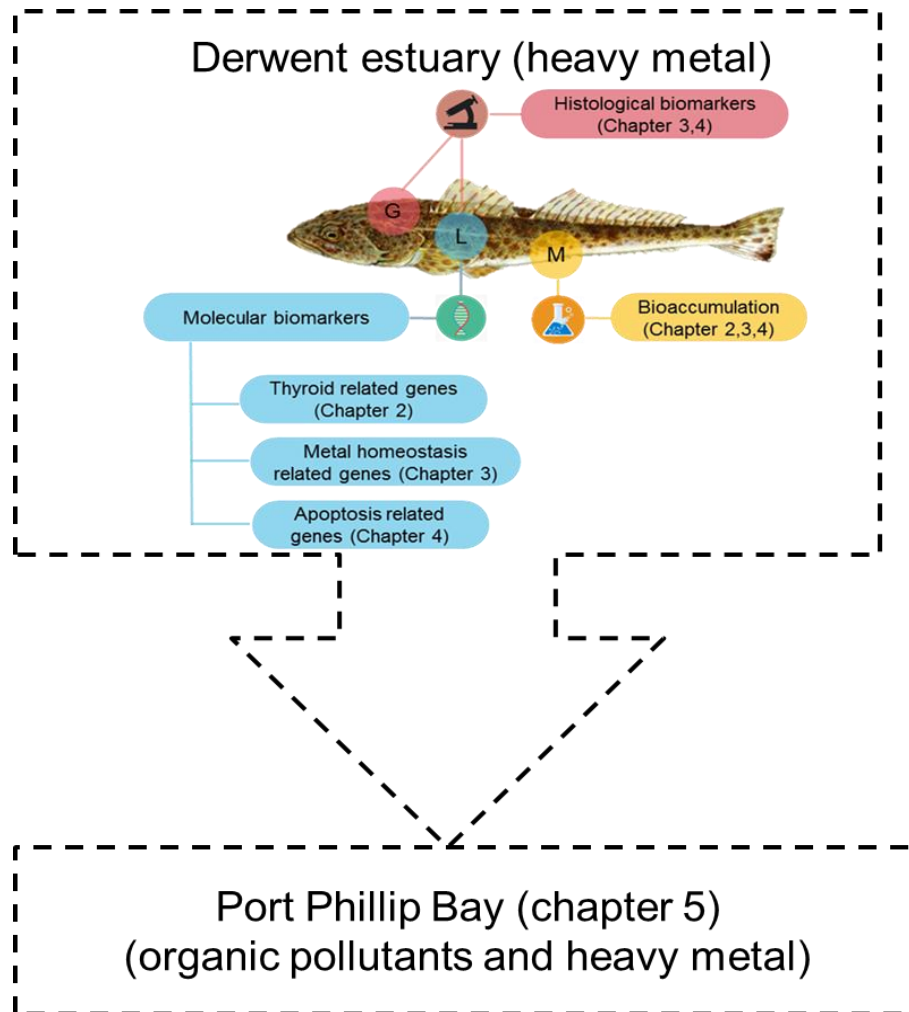
**Figure 1.7 Thesis structure.**

Chapter 2: Assess the relationships between As and Hg levels with thyroid related genes in sand flathead from the Derwent estuary, Tasmania, Australia.

- Analyse the hepatic relative gene expression level of thyroid related genes (D1, D2, TTR, TRα and TRβ) in fish from different sampling sites.

- Explore the relationships between the thyroid related genes transcripts and Hg and As residues in fish muscle.

Chapter 3: Assess gill histology and hepatic expression of metal homeostasis-related genes as potential biomarkers in sand flathead from the Derwent estuary, Tasmania, Australia.

- Evaluate the usefulness of metal homeostasis-related genes (MTF1, TF, FPN1, ferritin and MT) as biomarkers in sand flathead from the Derwent estuary.

- Assess gill histological changes in sand flathead from the Derwent estuary.

Chapter 4: Expression profiling of Diablo/SMAC gene homolog, GRP78 and liver histology of sand flathead from the Derwent estuary, Tasmania, Australia.

- Assess the applicability of Diablo/SMAC homolog genes and GRP78 gene in flathead as biomarker genes for environmental heavy metal exposure.

- Examine liver histological alterations in flathead from the Derwent estuary.

Chapter 5: Using hepatic gene expression and liver histological assessment to assess the effects of pollution in flathead from Port Phillip Bay, Victoria, Australia.

- Validate the usefulness of all the candidate biomarker genes including thyroid related genes (chapter 2), metal homeostasis related genes (chapter3), Diablo/SMAC homolog genes and GRP78 gene (chapter4) in sand flathead from Port Phillip Bay.

- Assess the liver histopathology in sampled fish from Port Phillip Bay.

- Make recommendation of using these findings in the future monitoring programs.

Chapter 2 : Thyroid hormone related gene transcription in southern sand flathead (*Platycephalus bassensis*) is associated with environmental mercury and arsenic exposure

## 2.1 Abstract

Arsenic (As) and mercury (Hg) are ubiquitous elements known to disrupt thyroid function in vertebrates. To explore the underlying mechanisms of Hg and As on the fish thyroid system, we investigated the associations between muscle concentrations of Hg and As with thyroid-related gene transcription in flathead (*Platycephalus bassensis*) from a contaminated estuary. We sampled fish at several sites to determine the hepatic expression of genes including deiodinases (D1 and D2), transthyretin (TTR), thyroid hormone receptors (TRα and TRβ) and related them to Hg and As levels in the same individuals. Negative correlations were observed between Hg levels and D2, TTR, TRα and TRβ, whereas positive associations were found between As concentrations and TTR and TRβ. These results suggest that Hg and As exposures from environmental pollution affect the regulation of genes important for normal thyroid function in fish. These thyroid-related genes could be used as biomarkers for monitoring environmental thyroid-hormone disrupting chemicals.

## 2.2 Introduction

Arsenic (As) and mercury (Hg) are environmental contaminants commonly associated with industrial pollution, which can be bioaccumulated and biomagnified and pose adverse effects on humans and wildlife (Abernathy *et al.*, 2003; Eisler, 2004; Mason *et al.*, 1996; Ward *et al.*, 2010). In recent years, there has been substantial evidence suggesting that both Hg and As can disrupt the thyroid system of vertebrates (Ciarrocca *et al.*, 2012; Iavicoli *et al.*, 2009; Tan *et al.*, 2009). The thyroid system is highly conserved throughout vertebrates and plays a crucial role in many bioactivities such as normal metabolism, reproduction and development (Power *et al.*, 2001; Zoeller *et al.*, 2007). Therefore, the impairment of the thyroid system by an exposure to Hg and

As may exert profound effects on the health of vertebrates. Previous studies reported Hg concentrations were positively associated with the circulating free thyroxine (T4)/ free triiodothyronine (T3) ratio in humans (Barregard *et al.*, 1994; Ellingsen *et al.*, 2000). In mammals, low level As exposure altered thyroid hormone receptor-mediated gene expression in pituitary cells (Davey *et al.*, 2008). Likewise, associations between Hg exposure and thyroid hormone concentrations have been observed in birds (Wada *et al.*, 2009) and exposure to As affected the thyroid hormone receptor dependent developmental processes of amphibians (Davey *et al.*, 2008). In fish, chronic As exposure decreased the plasma T4 concentration in coho salmon (*Oncorhynchus kisutch*) (Nichols *et al.*, 1984), while in juvenile rainbow trout (*Oncorhynchus mykiss*), both plasma T4 and T3 concentrations were increased when exposed to Hg (Bleau *et al.*, 1996). Although Hg and As have been proven to be associated with thyroid parameters in different vertebrate species, the relationships between Hg, As, and thyroid related genes are poorly understood. In particular, the synergistic effects of Hg and As on these genes are still unknown (Iavicoli *et al.*, 2009).

Fish can be used to provide valuable information on potential thyroid disrupting effects of environmental factors in fish or other animals that prey upon fish (Brown *et al.*, 2004; Leatherland, 2000). Although the mechanisms of chemical mediated disruption on the thyroid system are not fully understood, it is generally believed that these disruptors alter the thyroid function at different levels and can directly interfere with thyroid hormone synthesis, thyroid hormone metabolism, thyroid hormone transport and thyroid hormone receptors (Patrick, 2009). In most teleost fish, the synthesis of thyroid hormones occurs in the thyroid follicles, which are scattered diffusely along the afferent artery (Leatherland, 2005). As in other vertebrates, the basic subunits of the fish thyroid are follicles, which mainly secrete T4 into circulation (Brown *et*

*al.*, 2004). Transthyretin (TTR) is a liver secreted transporter protein, which carries T3 and T4 into different target tissues in fish (Power *et al.*, 2009). Thus, endocrine disrupting chemicals interfering with TTR could severely affect plasma thyroid hormone concentrations and the physiological status of organisms (Lans *et al.*, 1994). In teleost fish, the TTR gene has been cloned from few species (Yamauchi and Ishihara, 2009) and therefore the transcriptional regulation of TTR by thyroid endocrine disrupting chemicals is largely unknown. In the target tissues, T4 is converted into a more biologically active form of hormone T3 by deiodinases (D1 and D2) removing one of the iodide units of the outer ring of T4, which is a crucial step in regulating the peripheral thyroid status (Orozco and Valverde, 2005). It is believed that most T3 is not synthesized in the thyroid and that the primary control of T3 levels occurs in peripheral organs, particularly the liver. In target cells, the function of thyroid hormones is mediated by thyroid hormone receptors (TRs) implicating them as major targets of thyroid disruptors. T3 has ten times greater affinity for TRs than that of T4 and it binds to TRs as a transcription factor to regulate gene expression (Yamano, 2005).

The effects of Hg and As on the fish thyroid related genes have received little attention in both laboratory and field studies. In addition, the relationships between expression of thyroid associated genes and environmentally related concentrations of heavy metals are not well established. It is crucial to understand the interaction between pollutants and the thyroid system of fish, not only because of the important position of fish in the food chain but also because its thyroid system shares some basic properties with other wildlife and humans (Brown *et al.*, 2004). Therefore, investigations of chronic contaminant exposure on the thyroid system of indicator fish species would contribute to the development of molecular biomarkers to effectively monitor the aquatic system.

Southern sand flathead (*Platycephalus bassensis*) have been used as an indicator species to assess the health of contaminant-exposed fish populations (Yamano, 2005). Sand flathead are demersal, non-migratory and they accumulate heavy metals from their diet consisting of benthic organisms (Parry and Scientific, 1995). The Derwent estuary in Tasmania has been recognized as a river polluted with both Hg and As (Bloom and Ayling, 1977; Green and Coughanowr, 2003) and sand flathead in the Derwent estuary have been monitored for heavy metal residues since the 1970s (http://www.derwentestuary.org.au/). Metallurgical liquid effluent has been discharged into the estuary for decades (Bloom and Ayling, 1977; Langlois *et al.*, 1987). The bioaccumulations of heavy metals in fish and shellfish from the Derwent estuary are much higher than elsewhere in Tasmanian waters (Eustace, 1974; Ratkowsky *et al.*, 1975; Verdouw *et al.*, 2011).

The Derwent estuary is located in the southern part of Tasmania and is close to the capital city Hobart, which has a high level of industrial and urban activities, suggesting the accumulation of not only heavy metals but organic pollutants in flathead is high. Therefore, cytochrome P4501A (CYP1A) gene, a commonly used molecular marker for monitoring organic pollutants such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), furans and dioxins (Rees *et al.*, 2003), was employed as an additional marker. The metallothionein (MT) gene, the most commonly used molecular marker of metal pollution in fish, was also assessed in this study. However, previous studies have suggested that the use of the MT gene as a biomarker in fish may be inappropriate as it can be influenced by a variety of abiotic and biotic factors such as salinity, temperature, pH, fish species and tissues (Chen *et al.*, 2004; Cho *et al.*, 2008). Nonetheless, the use of the MT gene as a pollution biomarker has been widely adopted in a range of fish species even though most of the studies assessing the validity of MT as a biomarker were

conducted under laboratory conditions (Knapen *et al.*, 2007). The suitability of the MT gene as a molecular marker for metals exposure under field conditions in sand flathead is unknown.

In the present study, *P. bassensis* from the historically polluted Derwent estuary were used as to explore the relationships between As, Hg and thyroid-related gene expression levels. Thyroid related genes including, D1, D2, TTR, TRα, TRβ  and CYP1A were cloned and examined from the flathead liver. The hepatic mRNA levels of these genes were compared in flathead from different sites to investigate the effects of heavy metal exposure on thyroid related genes.


## 2.3 Materials and Methods

### 2.3.1   Sample collection

Juvenile *P. bassensis* were caught from Kingston Beach North (n = 8), Ralphs Bay (n = 8) and Cornelian Bay (n = 9) between October and November, 2012 (Fig 2.1). Only juvenile fish were used in gene expression analysis.  The gender was not identified due to these fish being sexually immature. All the fish used in this study were opportunistically sampled as part of a heavy metal monitoring project conducted by Nyrstar N. V Hobart (a mining and metals industry) under a permit issued under section 14 of the Tasmanian Governments *Living Marine Resources Management Act 1995* (2011-12 Annual Environment Review of Nyrstar N.V). Fish were captured from a small boat using a fishing rod with baited hook. Body weight (*W*) and standard length (*L*) were measured for each individual to calculate the condition factor (*K*) using the following formula: $K=100 \times W$ (kg) $\times L$ (cm)$^{-3}$. For As and Hg analysis, approximately 50 g of muscle was collected from individual fish and then put in a labeled plastic bag placed on ice for transport to the laboratory. All the samples were stored at -20 °C until Hg and As analyses. A sample of liver was collected from a small ventral incision in each fish and immediately stored in

a RNA preservation reagent (25 mM sodium citrate, 10 mM EDTA, 4 M ammonium sulphate, pH 5.2). The samples were held on ice until transport to the laboratory and stored at -80 °C until further processing.
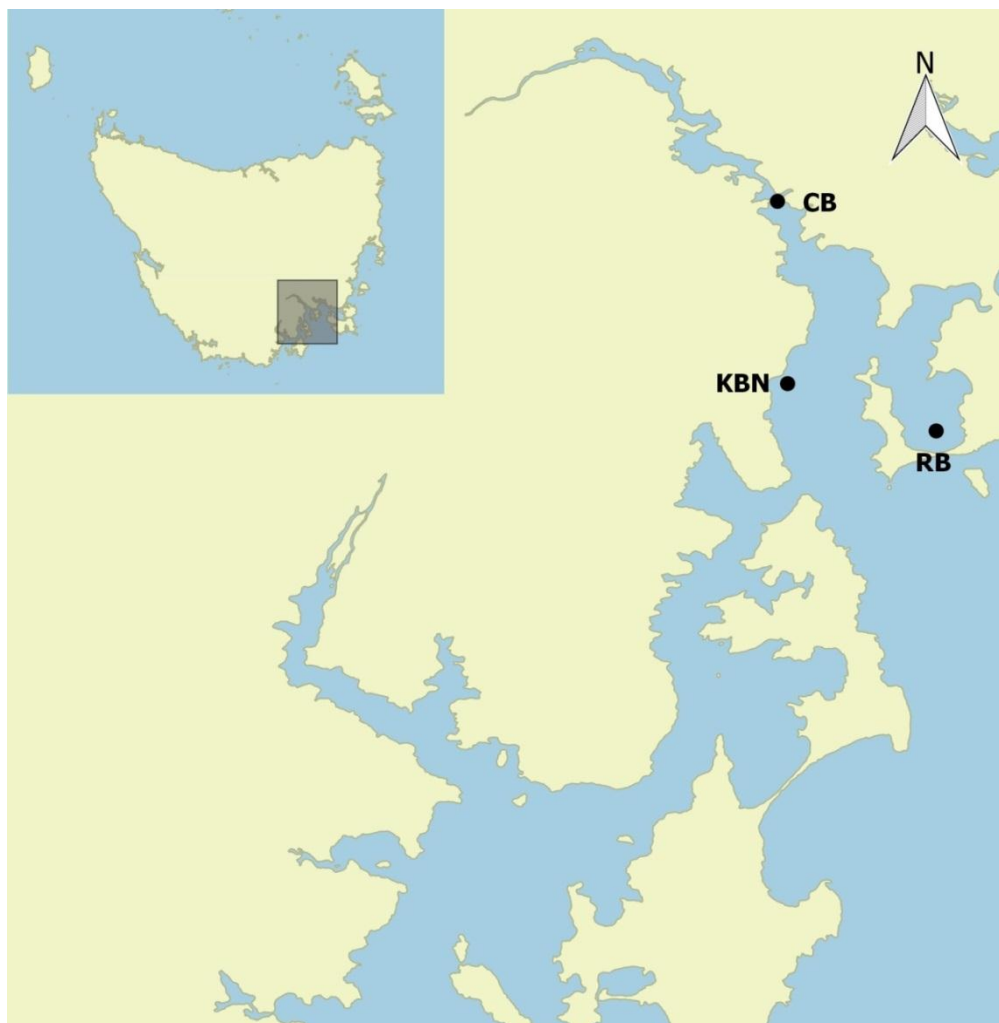


**Figure 2.1 Map of Derwent estuary sampling sites**: Cornelian Bay (CB), Kingston Beach North (KBN) and Ralphs Bay (RB)

### 2.3.2 Metals determination

All samples were analysed by Analytical Services Tasmania that is accredited by the National Association of Testing Authorities (NATA). The determination of total Hg in each sample was used as a proxy of methylmercury (MeHg) due to the assumption that MeHg is the primary form

of Hg in fish muscle (Harris *et al.*, 2003). Samples were digested and analysed using cold vapour atomic fluorescence spectrometry conducted on an atomic fluorescence analyser as described by (Verdouw *et al.*, 2011). For the analysis of other metals including, As, Cd, Co, Cr, Cu, Fe, Zn, Se, Pb, Ni and Mn, approximately 12 g of skinless and boneless muscle samples from individuals were dried and ground into powder. 1 g of each sample was digested and analyzed using an inductively coupled plasma atomic emission spectrophotometer. All the data of residues were given as milligrams per wet kilogram of sample with a minimum detection level of 0.02 mg/kg.

### 2.3.3    RT-qPCR analysis

Total RNA was isolated from the liver of *P. bassensis* using TRI Reagent (Molecular Research Centre, OH, USA) and treated with DNAse (Turbo DNase, Ambion, TX, USA) to remove any contaminating genomic DNA. RNA quality was assessed by electrophoresis in a 1.0% agarose gel. The RNA concentrations were determined using an Invitrogen Qubit fluorometer and Quant-iT RNA assay kit (Invitrogen, VIC, Australia). Total RNA (1 μg) was used for the first-strand cDNA synthesis using BioScript reverse transcriptase (Bioline, NSW, Australia) with Oligo (dT) 18 priming as per manufacturers instuctions.

Degenerate oligonucleotide primers (Macrogen, South Korea) were designed from the conserved regions of five teleost sequences retrieved from Genbank (Table 2.1) and used to obtain nine target partial genes by PCR amplification of *P. bassensi* hepatic cDNA; deiodinase type I (D1), deiodinase type II (D2), transthyretin (TTR) thyroid hormone receptor α (TRα), hormone receptor β (TRβ), cytochrome P4501A (CYP1A), metallothionein (MT), β-actin (β−actin) and elongation factor 1-alpha (EF1α). PCR amplification of each gene was conducted in a volume of 20 μl containing 2 × PCR mixtures (Bioline, NSW, Australia), 0.5-0.75 μM of each primer and

approximate 50 ng template cDNA. Amplification was carried out on an Eppendorf thermal cycler using the following PCR profile: 2 min at 94 °C, then 32 cycles of 94 °C for 30 s, 50-52 °C for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were separated via 1.5% agarose gel and purified from gel by using a gel extraction kit (Qiagen, NSW, Australia). Purified PCR products were Sanger sequenced directly in the forward direction (Macrogen). Homologs of these genes were then analysed and confirmed with published sequences by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the gene sequences deposited in Genbank (for accession numbers see Table 2.1)

**Table 2.1 Primers used for cDNA sequence and quantification of the mRNA expression by Real-time PCR**

| Gene (accession no.) | Oligo name | Primer DNA Sequence (5'→3') |
|---|---|---|
| cDNA sequence | | |
| D1 | D1F | ACBATGACCCAGAAYC |
| | D1R | TCCACMACCACWGGRCACAGGGG |
| D2 | D2F | GGYTTCTTYTCSAACTG |
| | D2R | GGCTKATRAAGGGGGGTCA |
| TTR | TTRF | TCCTGGTCCTCGAAGACCTCCA |
| | TTRR | TCAAACTCMCGATACACKCC |
| TRα | TRαF | ATGTGCCGAAGAGAAAGA |
| | TRαR | TCCTGGTCCTCGAAGACCTCCA |
| TRβ | TRβF | GTGTGYGGGGACAAAGC |
| | TRβR | AGCAGCTTDGGGCCAGAA |
| MT | MTF | ATGGAYCCTTGYGMMTGC |
| | MTR | TCACTGACAGCAGSTBGTGTC |
| CYP1A | CYP1AF | ATYGATCACTGYGARGAC |
| | CYP1AR | TTGTGYTTCATKGTGAGRCC |
| Real-time RT-PCR | | |
| D1 (KP893709) | qD1F | ACAGATCCTGGTTCAGAA |
| | qD1R | ATACTTCACGGCAGACAT |
| D2 (KP893710) | qD2F | GCACTCAACTCCAAAGTAG |
| | qD2R | ACCAGGTGACACATTAGT |
| TTR (KP893711) | qTTRF | AGGTCCATAATCTCATCAC |
| | qTTRR | CTCATCTTCCCAGTTAGC |
| TRα (KP893714) | qTRαF | CCATCCAGAAGAACCTCCA |
| | qTRαR | GTTGCGGGTGATCTTGTC |
| TRβ (KP893715) | qTRβF | TAAGCCTGAGGATATTGG |
| | qTRβR | TTTGTAAACTGACTGAAGG |
| CYP1A ( KP893713) | qCYP1AF | ATGACAAGGACAACATTC |
| | qCYP1AR | ATCTGACATCTGGACATT |
| MT (KP893712) | qMTF | ATCCGGCTGCACCAAATG |
| | qMTR | GTTTACTGACAGCAGGTGGT |
| β-actin (KR076429) | qβ-actinF | ACCTCACAGACTACCTCAT |
| | qβ-actinR | TTGATGTCACGCACGATT |
| EF1α ( KP893716) | qEF1αF | TTGGAGTCAACAAGATGG |
| | qEF1αR | GATGTAGGTGCTCACTTC |

The deiodinases (D1 and D2), transthyretin (TTR), thyroid hormone receptors (TRα and TRβ), metallothionein (MT), and cytochrome P4501A (CYP1A) mRNA expression were determined by quantitative real-time RT-PCR using gene-specific primers (Table 2.1). Real-time PCR was performed on an iQ5 Real-time PCR Detection System (Bio-Rad, NSW, Australia) in a volume of 10 μl containing 0.1 μM of each primer, 5 μl 2 × SensiMixPlus SYBR & Fluorescein PCR master mix (Bioline, NSW, Australia), and 1 μl of cDNA. The PCR cycling consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s with signal collection in each cycle. To assess the specificity of the PCR amplification, a melting curve was obtained at the end of the cycling and a single peak for each target gene was observed. The data analysis of the qPCR results was performed using qBasePlus Biogazelle software (Hellemans *et al.*, 2007). The target genes expression in different samples was normalized to β-actin and EF1α quantities. The geometric mean was used to normalize relative quantity (MNRQ) and was calculated for each gene of grouped replicates from the site. The expression level of all the genes was relative to that of expression in fish from CB.

### 2.3.4 Statistical analysis

Statistical analyses of spatial differences in Hg and As concentrations and hepatic gene expression were performed using one-way analysis of variance (ANOVA) followed by the Tukey HSD test to determine differences among the sites. To investigate the relationship between gene expression and heavy metal levels, correlation analyses were performed on As, Hg and hepatic genes expression from all fish. The Pearson's correlation coefficient was used to assess the relationships between parameters (heavy metal levels and relative genes expression after all samples pooled together). All statistical analyses were carried out using GraphPad Prism version 6.0 (GraphPad software Inc) with statistical significance at $P < 0.05$.

## 2.4 Results

### 2.4.1 Heavy metals concentrations

Four trace metals (Hg, Zn, Fe and As) were detected in the muscle of sampled flathead. There were significant spatial differences for mean As and Hg residues in flathead muscle from Cornelian Bay, Kingston Beach North and Ralphs Bay showed significant spatial differences (Fig 2.2). The Hg concentrations in flathead from Cornelian Bay (0.48 mg/kg) and Kingston Beach North (0.24 mg/kg) were significantly lower than in Ralphs Bay (0.91 mg/kg) ($P < 0.0001$, Fig 2.2B), whereas As residues in flathead from Kingston Beach North (10.2 mg/kg) were significantly higher than those of Cornelian Bay (3.0 mg/kg) and Ralphs Bay (3.39 mg/kg) ($P < 0.0001$, Fig 2.2A). No significant differences were found for the concentrations of Zn and Fe. Other metals were below the minimum detection level (0.02mg/kg).
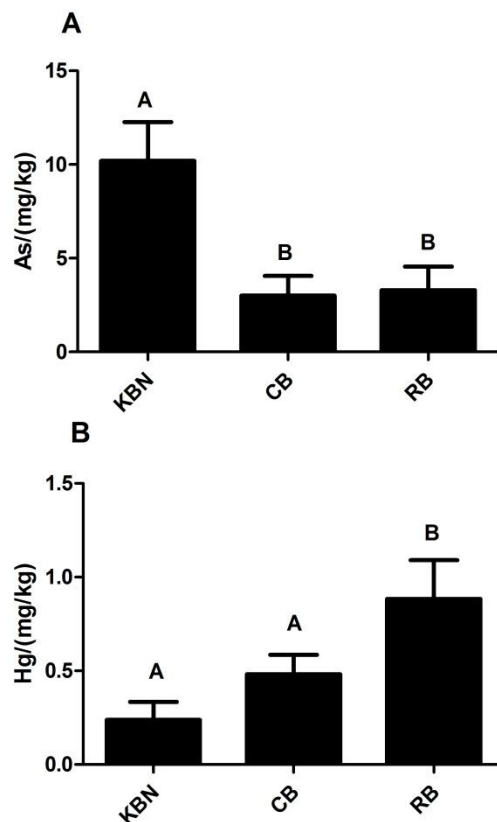
**Figure 2.2** The mean ± S.E arsenic (A) and mercury (B) concentrations in flathead muscle sampled at Kingston Beach North (KBN, n=8), Cornelian Bay (CB, n=9) and Ralphs Bay (RB, n=8). Vertical bars represent the means. Different letters indicated significant differences between sites.

### 2.4.2 Hepatic genes expression

Hepatic expression levels of D2 mRNA were significantly down-regulated in flathead caught at Ralphs Bay compared to those from Cornelian Bay (CB) ($P < 0.05$, Fig 2.3B), but not significantly different from fish at Kingston Beach North. There was no significant difference among these three sites for the expression levels of D1 (Fig 2.3A). For the transcripts of TTR, the highest expression level was observed in fish from Kingston Beach North (3.62-fold that of Cornelian Bay, $P < 0.0001$, Fig 2.3C). Similarly, TRβ mRNA levels were significantly higher in flathead from Kingston Beach North than those from Cornelian Bay and Ralphs Bay ($P < 0.0001$,

Fig 2.3E). In contrast, TRα hepatic mRNA levels were not significantly different in flathead sampled at different sites (Fig 2.3D). There were no significant differences in hepatic mRNA levels of CYP1A in flathead among all research sites (Fig 2.3F). MT mRNA expression was significantly higher in flathead from the Kingston Beach North (KBN) than in the fish from Cornelian Bay (CB) and Ralphs Bay (RB) ($P < 0.05$) (Fig 2.3G).
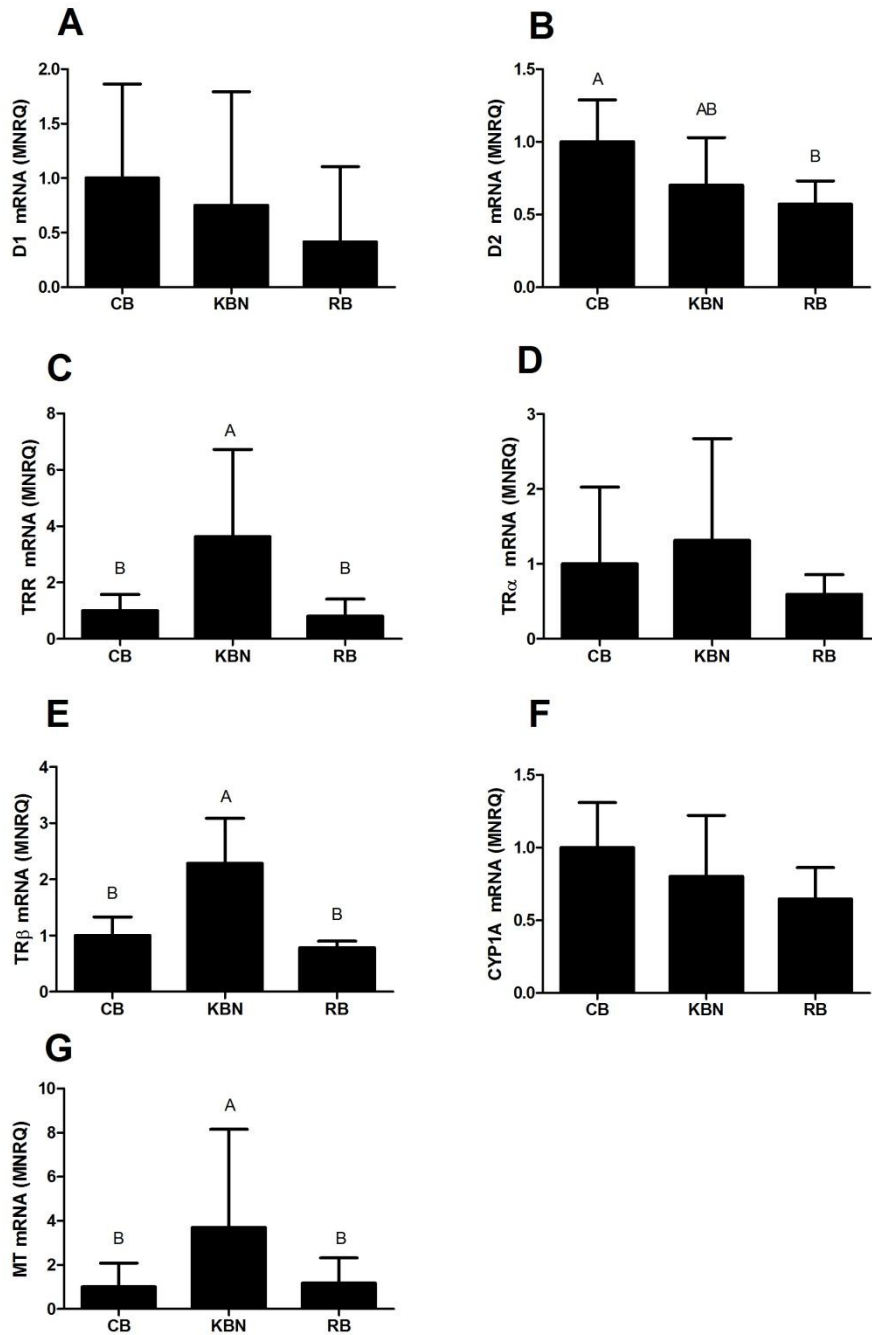
**Figure 2.3** Hepatic mRNA levels of D1, D2, TTR, TRα, TRβ, CYP1A and MT, relative to two reference genes (β-actin and EF1α) were analysed by qPCR in flathead captured at three sites of the Derwent estuary. The values are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval. Different letters indicated significant differences between sites.

**2.4.3    Relationships between gene transcripts, morphometric measurements, mercury and arsenic levels**

There were statistically significant positive correlations between As levels and expression of TTR (r = 0.6584, *P* = 0.0001, Fig 2.4A), expression of TRβ  (r = 0.6455, *P* = 0.0002, Fig 2.4B) and expression of MT (r = 0.4834, *P* = 0.0062, Fig 2.4G). Significant negative correlations were observed between the levels of Hg and the mRNA expression of D2 (r = -0.4021, *P* = 0.0232, Fig 2.4C), TTR (r = -0.4953, *P* = 0.0050, Fig 2.4D)*,* TRα ( r = -0.3663, *P* = 0.0329, Fig 2.4E), TRβ ( r = -0.6241, *P* = 0.0003, Fig 2.4F) and MT (r = -0.4101, *P* = 0.0187, Fig 2.4H). No correlation was observed between the morphometric measurements (fork length, weight and condition factor), gene transcripts, and the level of As or Hg (Fig 2.5).
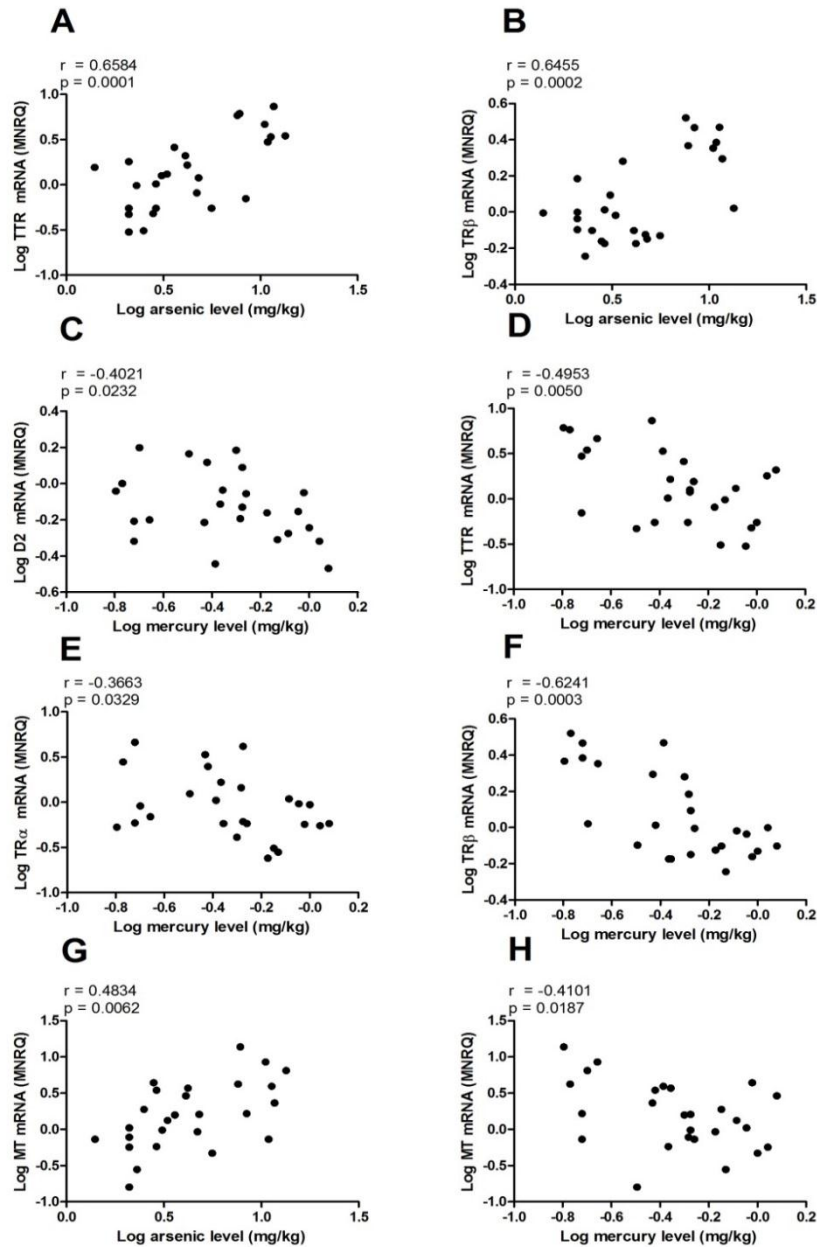
**Figure 2.4 Correlations between gene expression and heavy metal concentration in sampled fish.** The gene expression of TTR (A) ($r = 0.6584$, $P = 0.0001$), TRβ (B) ($r = 0.6455$, $P = 0.0002$) and MT (G) ($r = 0.4834$, $P = 0.0062$) were positively correlated with arsenic concentration. The significantly negative correlations were found between D2(C) ($r = -0.4021$, $P = 0.0232$), TTR (D) ($r = -0.4953$, $P = 0.0329$), TRα (E) ($r = -0.3663$, $P = 0.0329$), TRβ (F) ($r = -0.6241$, $P = 0.0003$) and MT (H) ($r = -0.4101$, $P = 0.0187$) gene expressional levels and mercury concentrations.
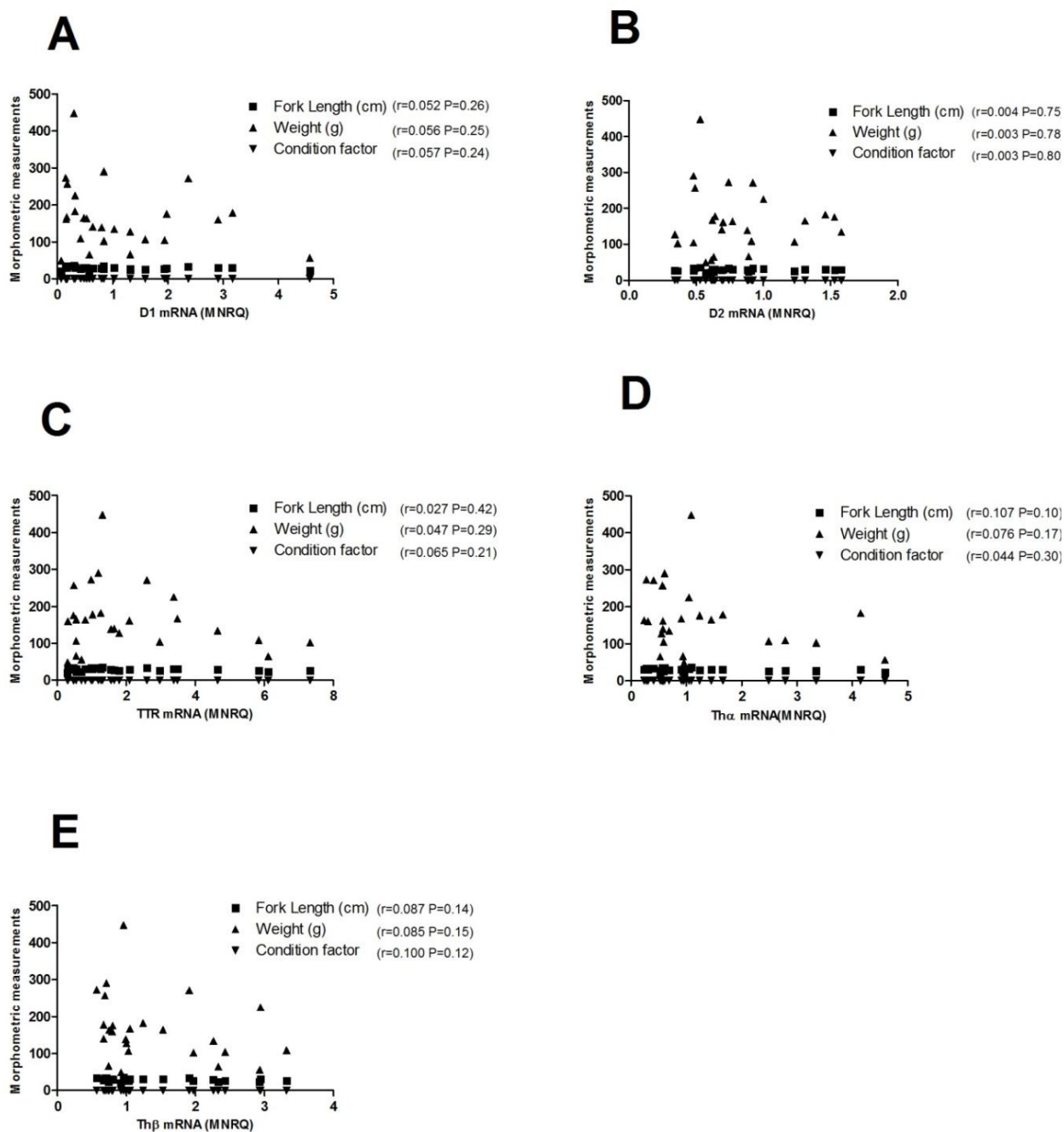
**Figure 2.5 Correlations between morphometric measurements, gene transcripts and heavy metal concentrations in sampled fish.**

## 2.5 Discussion

D2 mRNA in flathead livers was significantly lower in Ralphs Bay, where flathead had the highest Hg concentrations. In addition, a significantly negative correlation was observed between D2 transcripts and Hg levels for fish from all sites. It has been proposed that the synthesis and activity of 5'-deiodinases are generally inhibited by Hg exposure (Barregard *et al.*, 1994; Mori *et al.*, 2006). In previous studies, Hg exposure was associated with the decreased T4 concentrations of snakehead fish (*Channa punctatus*) (Bhattacharya *et al.*, 1989) and decreased both T3 and T4 in plasma of catfish (*Clarias batrachus*) (Kirubagaran and Joy, 1994). In birds, a recent study of the tree swallow (*Tachycineta bicolor*) inhabiting an area near a Hg polluted river found that both plasma T3 and T4 were suppressed compared to those in reference sites, suggesting Hg may exert inhibitory effects on 5'-deiodinases (Wada *et al.*, 2009). Deiodinases are selenoproteins, and selenium (Se) is the essential element required by deiodinases (Khan and Wang, 2009; Orozco and Valverde, 2005). Methylmercury (MeHg), the predominant form of Hg in the fish body, could impact on the availability of Se (Raymond and Ralston, 2004). In zebrafish (*Danio rerio*), selenium protein genes could be down-regulated by elevated MeHg level, suggesting MeHg regulates the selenium protein transcripts may be affected through low Se availability (Penglase *et al.*, 2014). More recently, it has been confirmed that low levels of Se were associated with elevated MeHg bioaccumulation in flathead from the Derwent estuary (Jones *et al.*, 2014). Hence, it is reasonable to hypothesise that the interaction between MeHg and Se may result in decreasing the bioavailability of Se thus contributing to the down-regulation of the deiodinase gene. The D1 gene showed a similar spatial expression pattern to D2 but there was no significant effect of site and it was not significantly correlated with Hg levels when all the samples were pooled together. It has been suggested that the teleost D1 is resistant to 5'-

deiodinase inhibitors (Orozco and Valverde, 2005). A field study on walleye (*Sander vitreus*) has shown that the D1 gene was not as sensitive as the D2 gene as a molecular marker for environmental pollutants (Picard-Aitken *et al.*, 2007). The data in our study suggest D2 gene was sensitive to Hg exposure.

In this study, TTR gene expression levels were negatively correlated with Hg residues in flathead. In the rat, it has been reported that hepatic mRNA levels of TTR were significantly decreased due to selenium deficiency (Kendall and Christensen, 1997). As stated above, the accumulation of MeHg could cause Se deficiency which results in suppression of the synthesis of deiodinases. Thus, one possible explanation for the negative correlation between TTR mRNA levels and Hg residues is that Se deficiency in the fish liver may result in a decrease of TTR transcripts. However, further investigations are needed to confirm this and explain the detailed mechanisms.

The expression levels of two TRs genes, TRα and TRβ, were both negatively correlated with Hg concentrations in the flathead. Previously, it has been demonstrated that the TRs genes could be regulated by thyroid hormone levels. For instance, a study in frog (*Xenopus laevis*) reported that expression levels of TRα and TRβ were up-regulated by T3 at 2-fold and 20-fold, respectively (Yaoita and Brown, 1990). Similar results were observed in zebrafish (*Danio rerio*) and striped parrotfish (*Scarus iseri*), TRα and TRβ transcripts were increased by T3 (Johnson and Lema, 2011; Liu *et al.*, 2000). In this context, it can be hypothesized that the down-regulations of these two TRs might be due to the decrease of T3 in the fish livers. Consistent with this, in flathead, the expression levels of D2, the predominant 5'-deiodinase in the fish liver that is responsible for the synthesis of T3, was also negatively correlated with Hg concentrations. However, an additional endpoint, such as measurement of T3 levels in fish liver is required to support this hypothesis.

The mRNA levels of TTR in fish liver were increased in Kingston Beach North and positively correlated with As concentrations in fish body. The precise action of As as a toxin is not known, but it is believed to be a result of As binding to cellular proteins (Yan *et al.*, 2009). Proteins such as tubulin, PARP-1, thioredoxin reductase and estrogen receptor-alpha could be the As binding targets (Kitchin and Wallace, 2008). In other vertebrates, several environmental pollutants, such as bisphenol A (BPA), nonylphenol and polychlorinated biphenyls (PCBs), could bind to TTR and disrupt the thyroid hormones homeostasis (Simon *et al.*, 2011; Yamauchi *et al.*, 2003). Based on this observation, it could be assumed that As may bind to TTR and up-regulation of TTR mRNA levels could be a requirement of keeping hormone homeostasis in the fish liver.

Similarly, the thyroid hormone receptor gene TRβ was up-regulated in fish from Kingston Beach North as compared to flathead from Cornelian Bay and its transcripts were positively correlated with As concentrations. It has been reported that As has strong disruptive effects on thyroid hormones by altering gene regulation through the thyroid hormone receptors (Davey *et al.*, 2008; Freitas *et al.*, 2011), but the precise mechanism of As interfering with thyroid hormone receptors is still unclear. In the present study, the high expression of TRβ could contribute to maintaining the normal biological processes when TRs were impacted by As.

No significant differences were found for TRα mRNA expression among sampling sites and there was no correlation with As concentrations. Previous study reported that mRNA of TRα was not changed in the Chinese rare minnow (*Gobiocypris rarus*) liver but decreased in fish brain when exposed to amitrole (Li *et al.*, 2009). A similar phenomenon was observed in the frog (*Xenopus laevis*) when TRα was down-regulated in the brain but there was no effect on the transcription of this gene in the tail after MMI treatment (Zhang *et al.*, 2006). These results

indicated that TRα expression was organ or tissue-specific (Li *et al.*, 2009). The observation in the present study indicates TRα in flathead liver is not sensitive to As exposure. Therefore, these results may imply that thyroid hormone transporter proteins and their receptors could be the target sites for fish exposed to As and that both TTR and TRβ could be regulated by high levels of As in fish.

The highest expression level of MT in flathead was at Kingston Beach North and its transcript was positively correlated with the concentrations of As in flathead muscles. The toxicity of As has been reported in relation to the generation of reactive oxygen species (ROS) (Kondoh *et al.*, 2001; Thornalley and Vasak, 1985). Therefore, high levels of ROS could result in lipid peroxidation and cellular damage. In previous studies, it has been proposed that MTs acting as scavengers of ROS could be induced by cumulative ROS. However, a study on the effects of As compounds on hepatic MT expression in catfish liver, showed the induction of hepatic MT by As was not through an oxidative stress mechanism (Schlenk *et al.*, 1997). Additionally, a recent study in mammals suggested that the metal-activated transcription factor 1 (MTF1) was involved in As induction of metallothioneins (Shen *et al.*, 2013). It has been demonstrated that As covalently bound to the C-terminal cysteine cluster of MTF1, which results in inducing the binding of MTF1 to the metal response elements of metallothioneins (He and Ma, 2009). In the present study, the up-regulation of MT was present in flathead from Kingston Beach North where the fish had the highest concentrations of As, suggesting MT in flathead could be induced by As exposure. The positive correlation between As and MT transcripts strongly suggests that MT plays a critical role in protecting the liver cells from arsenic-mediated toxicity. These lines of evidence indicate MT could be used as a molecular marker to monitor the As exposure in the Derwent estuary.

The failure of MT mRNA expression to reflect Hg bioaccumulation in flathead is consistent with previous studies. For example, none of the investigated organs displayed significant correlations between total Hg and MT levels for two fish species, *Dicentrarchus labrax* and *Liza aurata* (Mieiro *et al.*, 2011). Although several investigations have associated an increased MT expression with Hg exposure (Schlenk *et al.*, 1995), the use of fish MTs gene as biomarkers of exposure to Hg is problematic and has been questioned (Mieiro *et al.*, 2011). It is well documented that metallic Hg (II) could induce MT in the injected mouse, while MeHg and Hg (0) are inert in inducing MT (Yasutake and Nakamura, 2011). MeHg is considered to be the primary form of Hg in fish muscle (Harris *et al.*, 2003). In our study, MT expression was not regulated at Ralphs Bay where the fish had the highest levels of Hg accumulation, suggesting the mRNA expression of MT is not sensitive for MeHg accumulation in flathead. Interestingly, a significant negative correlation was observed between the MT mRNA expression levels and the Hg concentrations in flathead. These results suggest that MT mRNA expression is not suitable for monitoring Hg levels in flathead.

In the present study, there were no significant differences among the relative expressions of CYP1A at different sites. The hepatic mRNA expression of the CYP1A gene in fish has been used to reflect the degree of organic pollution, such as PCBs, PAHs and PBDEs (An *et al.*, 2011; Ibhazehiebo *et al.*, 2011; Lehigh Shirey *et al.*, 2006). The expression profile of CYP1A in the present study supports the previous report of the Derwent Estuary Program (DEP) that organic pollutants were at low levels in the sediments of the Derwent estuary (http://www.derwentestuary.org.au/).

## 2.6 Conclusion

Flathead from sites contaminated with As and Hg showed changes in hepatic thyroid hormone-related gene expression. The transcription of TTR and TRβ genes were positively correlated with As concentration, suggesting they were sensitive to As exposure, while D2, TTR, TRα and TRβ gene expression were suppressed with increased Hg levels. The associations between the As, Hg and thyroid hormone related gene transcription in sand flathead were significant considering that the thyroid hormone system is conserved in vertebrates, implying that other fish species inhabiting in this area may also be impacted by As and Hg. Further studies of the thyroid hormone levels in fish blood, and histological assessment of fish thyroid follicles are required to adequately address the biological impact of the environmental Hg and As exposure.

Chapter 3 **:** Gill histology and hepatic expression of metal homeostasis-

related genes in an indicator finfish species from a metal contaminated

estuary

## 3. 1  Abstract

The biological responses of an indicator finfish species, southern sand flathead (*Platycephalus bassensis*), from a historical metal polluted estuary were assessed, using a suite of studies including gill histopathology, hepatic gene expression and trace metals analysis. Fish were sampled from five polluted sites and a reference site. Zinc (Zn), Iron (Fe), Mercury (Hg) and Arsenic (As) were detected in all the fish muscle samples. All these trace metals showed significant spatial difference in the fish from different sampling sites. Four types of gill lesions such as hyperplasia and lamellar fusion, epitheliocystis, telangiectasis, and deformed filament were observed in fish gills. The gill histopathological results indicated both metal contamination and pathogen infection could contribute to these lesions. Metals bioaccumulation appeared to decrease the infection of monogenean parasites and impact the parasites species diversity. The hepatic gene expression analyses were conducted on fish from two polluted sites (highest Hg and As bioaccumulation level) and the reference site. The metal homeostasis-related genes were metal-regulatory transcription factor 1 (MTF1), transferrin (TF), ferriportin1 (FPN1), ferritin and metallothionein (MT). MTF1, TF, and FPN1 were significantly up-regulated in the liver of fish inhabiting the two polluted sites.  Strong correlations between transcriptional expression of TF and MTF1, FPN1 and MTF1 and FPN1 and TF were also observed in the present study. In this study hepatic gene expression analyses and gill histology were integrated to evaluate the biological effects of environmental metal exposure from molecular to organ level. The findings in this study could be used in the future environmental monitoring.

## 3. 2   Introduction

Heavy metals are dominant pollutants in estuarine environments, mainly arising from anthropogenic sources including industrial wastewater, agricultural runoff, domestic sewage, vehicle emissions and urban runoff (Hu *et al.*, 2013; Ip *et al.*, 2007; Matthiessen and Law, 2002). Bottom-dwelling fish are commonly used as indicator species to monitor heavy metal levels in marine environments (Yilmaz *et al.*, 2007).  However, the determination of pollution level in an aquatic organism itself is unable to reflect the biological effects of the pollutants (van der Oost *et al.*, 2003). In the past decades, considerable efforts have been focused on the evaluation of the causal relationships between heavy metals exposure and the potential adverse effects in indicator fish species (Damek-Poprawa and Sawicka-Kapusta, 2003; Ribeiro *et al.*, 2005). In the field, the complexity of pollutants can cause a wide range of biological responses in aquatic organisms. Accordingly, it has been advocated that multiple levels of biological responses should be assessed at same time (Nogueira *et al.*, 2010). Histopathology and gene expression analyses have been commonly applied in assessing the effects of pollutants in aquatic organisms at the organ and molecular level, respectively (Levine and Oris, 1999; Mellanen *et al.*, 1999; Quiros *et al.*, 2007; Stentiford *et al.*, 2003).

Histological alterations in the gills of fish are increasingly being used to detect early warning signals of pollutants in bio-monitoring programs (Pandey *et al.*, 2008; Pereira *et al.*, 2013; Poleksic *et al.*, 2010). Fish gills are the organ most impacted by waterborne pollutants (Mallatt, 1985). Consequently gill functions, such as gas exchange, osmoregulation and acid-base balance can be severely affected by pollutants (Alazemi *et al.*, 1996). Many gill conditions have been reported to be associated with contaminants in both laboratory and field studies.  For instance, the severity of the epithelial hyperplasia increased in a dose dependent of manner after exposed

to copper sulfate in the gills of carp (*Cyprinus caripo*) under laboratory conditions (Karan *et al.*, 1998). In a field study, a higher prevalence of telangiectasis (aneurysms) was observed in the gills of flounder (*Platichthys flesus*) captured from contaminated estuaries compared to flounder from a reference site (Stentiford *et al.*, 2003). In addition to histopathological alterations, previous studies have shown associations between pollutants and fish gill parasites (Lafferty, 1997; Sures, 2001; Williams and Mackenzie, 2003). For example, monogeneans have been employed as an environmental indicator in many studies (Sures, 2001). Moreover, monogenean flukes are the most common parasite in the gills of southern sand flathead (*Platycephalus bassensis*) used in this study (Nowak *et al.*, 2004). As such, fish gill histology combined with parasitism was used in the present study to evaluate the impact of environmental heavy metal exposure.

Hepatic gene expression analyses of heavy metal related genes can also be used to provide valuable information before more serious effects of heavy metal pollution occur to the whole aquatic ecosystem. The fish liver is the major organ responsible for uptake, biotransformation and excretion of pollutants (Gernhöfer *et al.*, 2001). It has been commonly used for investigation of the interaction between pollutants and their related genes in the aquatic toxicological studies (Asker *et al.*, 2013; Leaver *et al.*, 2010). Previous studies have demonstrated that heavy metal exposure may interfere with the metabolism of essential metals such as iron (Elsenhans *et al.*, 1991; Moulis, 2010). Iron is an important cofactor for many fundamental biological processes including transportation of oxygen, energy metabolism and DNA replication (Sahu *et al.*, 2013). In the present study, we have focused on the effects of environmental heavy metal on four iron homeostasis-related genes namely, MTF1 (metal regulatory transcription factor 1), ferroportin-1 (FPN1), transferrin (TF) and ferritin. Metal regulatory transcription factor 1 (MTF1) regulates

the expression of genes which are involved in metal-detoxification, homeostasis of essential metals and against oxidative stress, including metallothioneins (MTs) and ferroportin-1 (FPN1) (Andrews, 2001; Troadec *et al.*, 2010). Ferroprotin (FPN) is the only iron exporter in vertebrates and it plays vital role in keeping cellular iron homeostasis (Troadec *et al.*, 2010). It has been reported the FPN1 gene also can be induced by other transition metals and the expression of FPN1 gene under the metal stress is regarded as a protective mechanism against metal toxicity (Ward and Kaplan, 2012). In vertebrates, cellular iron homeostasis is also managed by transferrin (TF) and ferritin (Ward and Kaplan, 2012). Transferrin is a mainly liver synthesized iron transport protein that carries iron to target locations such as the site of absorption, storage and utilization (Zakin, 1992). The major function of ferritin is iron storage that can be classified into three types including, intracellular iron needs, intracellular protection from iron overload and other specific cells such as macrophages (Theil, 1987). In addition to the iron homeostasis related genes, metallothionein (MT), a classic molecular biomarker gene, was used as an additional biomarker. In this study, these five candidate genes known to be related with metal stress were examined in the liver of southern sand flathead from a polluted estuary to determine the usefulness of these genes as potential molecular biomarkers.

Southern sand flathead, a ubiquitous, abundant and non-migratory demersal finfish species, is an endemic fish species which widely distributed along the southern Australian coast. It has been recognized as a significant indicator species for monitoring the heavy metals in estuaries (Ayling *et al.*, 1975; Fabris *et al.*, 1992). Although previous studies have shown that flathead can accumulate relatively high levels of heavy metals in muscle (Verdouw *et al.*, 2011), no investigations has been conducted to develop related biomarkers on this key indicator species.

Hence, studies on the effects of chronic heavy metals exposure in sand flathead under field condition are needed for monitoring the marine environment of Australia.

The aim of present study was to evaluate the effects of environmental metal exposure in southern sand flathead from a historical metal polluted estuary. Hepatic expression of metal-hemostasis related genes, gill histology, gill parasitological analysis and metal determination were applied to detect earlier biomarker signals.

## 3. 3    Materials and Methods

### 3.3.1    Sample collection

All the fish used in this study were opportunistically sampled as part of a routine heavy metal monitoring program carried out by Nyrstar (Hobart) under a permit issued under section 14 of *Living Marine Resources Management Act 1995* (2011-12 Annual Environment Review of Nyrstar N.V). Sand flathead were sampled at a number of sites along the Derwent estuary including Cornelian Bay (CB) (n = 10), Newtown Bay (NTB) (n = 10), Kingston Beach North (KBN) (n=10), Sandy Bay Beach (SBB) (n = 10) and Ralphs Bay (RB) (n = 15) (Fig 3.1). Mickey's Bay (MB) (n = 19), a relative less polluted site, was selected as the reference site. All the samples were collected between September and November, 2013. Morphometric measurements including body weight (*W*) and standard length (*L*) were measured for each individual fish. For metals determination, approximately 50 g of muscle sample was collected from each fish and put in a labeled plastic bag which was placed on ice until transport to the lab. All samples were stored at -20 °C until metals analyses were conducted.
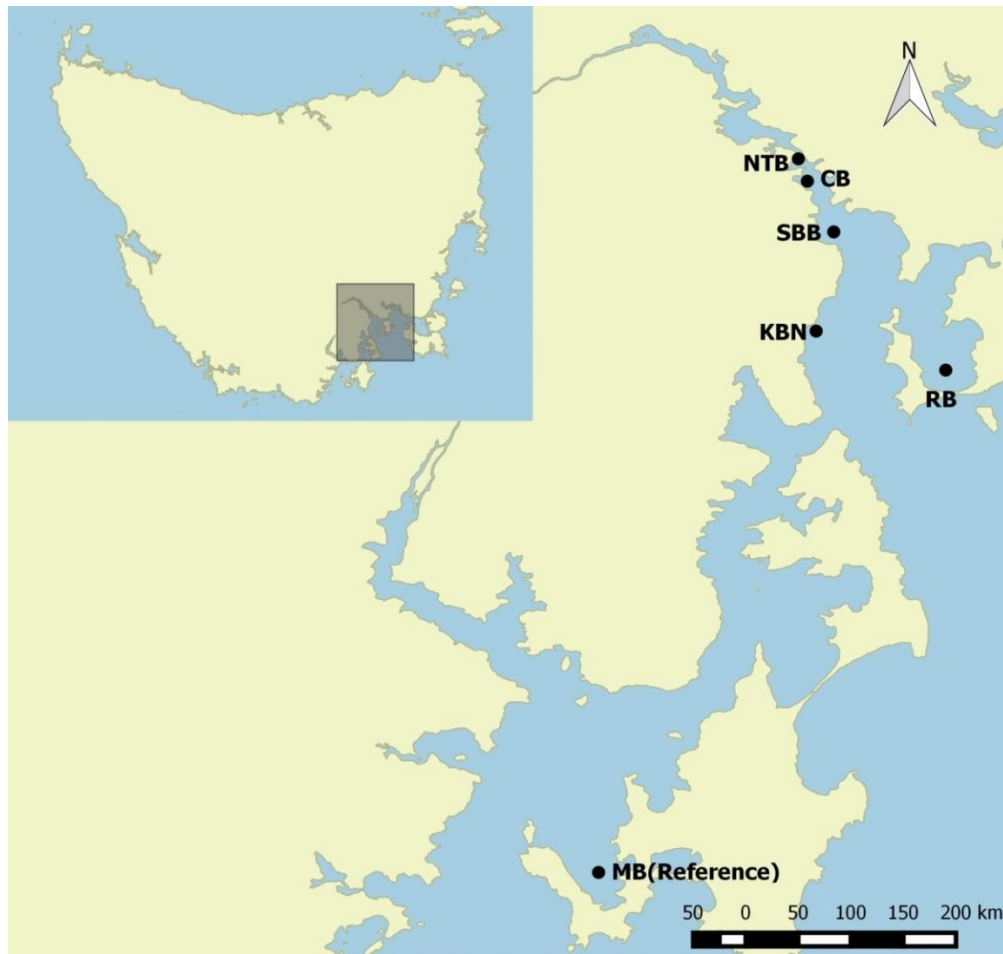
**Figure 3.1 Map of Derwent estuary sampling sites.** CB: Cornelian Bay, NTB: Newtown Bay, KBN: Kingston Beach North, SBB: Sandy Bay Beach, RB: Ralphs Bay and the reference site MB: Mickey's Bay.

For gene expression analyses, a small piece of liver (20 mg) was collected from individual fish and promptly stored in the labeled tube with ice-cold RNA preservation reagent (25 mM sodium citrate, 10 mM EDTA, 10 M ammonium sulphate, pH 5.2). The samples were held on ice until transport to laboratory and then placed at -20 °C to await further processing.

For histopathological examinations, gills from each individual fish were removed and immersed in seawater Davidson's fixative. Fixation lasted for 24 h, and then all the samples were transferred to 70 % ethanol for storage.

### 3.3.2 Metals determination

All samples were analyzed by Analytical Services Tasmania that is accredited by National Association of Testing Authorities (NATA). The determination of total Hg in sample was used as a proxy of methylmercury (MeHg) based on the assumption that MeHg is the primary form of Hg in fish muscle (Harris *et al.*, 2003). Samples were digested and analysed by using cold vapour atomic fluorescence spectrometry conducted on an atomic fluorescence analyser as described by (Verdouw *et al.*, 2011). For the analysis of other metals including, As, Cd, Co, Cr, Cu, Fe, Zn, Se, Pb, Ni and Mn, approximately 12 g of skinless and boneless muscle tissues from individuals were dried and ground into powder. 1 g of each sample was digested and analyzed using an inductively coupled plasma atomic emission spectrophotometer. All the data of residues were given as milligrams per wet kilogram of sample with a minimum detection level of 0.02 mg/kg.

### 3.3.3 Histology

The fixed second left gill arch was dehydrated in a progressive series of ethanol, cleared in xylene and finally embedded in paraffin (Tissue-Tek II tissue processor, Sakura, CA, USA). Sections (5µm) were cut on microtome (Microm HM340, Heidelberg, Germany), mounted on microscope slides and dried in an incubation oven at 37°C for 24 h. Sections were then stained with hematoxylin/eosin (H & E stain) and assessed microscopically using an Olympus BX40 microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

The prevalence of gill histological conditions from sampling sites was express as percentage of fish affected. Only well oriented filaments were used in the qualifications. For the quantification of monogenean gill flukes, the intensity and prevalence of parasite were quantified as described in (Rozsa *et al.*, 2000).

### 3.3.4   Gene expression analyses

Total RNA of each sample was isolated from the liver (around 5 mg) of *P. bassensis* using 400 µL ice-cold UREA buffer (4 M urea, 1 % SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7-8) with 20 units of proteinase K and disrupted tissue manually with a micro-pestle. Then the sample was incubated on ice until fully digested. 200 µL of 7.5 M ammonium acetate was added to samples and centrifuged at $16,100 \times g$ for 3 min at room temperature to remove detergent. Total nucleic acids were recovered by isopropanol precipitation at $16,000 \times g$ for 10 min at room temperature followed by once ethanol (70 %) wash of the nucleic acid pellet. All samples were treated with DNAse to remove any contaminating genomic DNA. RNA quality was assessed by electrophoresis on 1.0 % agarose gel. The RNA concentrations were determined using an Invitrogen Qubit fluorometer and Quant-iT RNA assays kit (Invitrogen, VIC, Australia). Total RNA (1 µg) was used for the first-strand cDNA synthesis by BioScript reverse transcriptase (Bioline, NSW, Australia) with Oligo (dT) 18 primer.

Five genes including metal regulatory transcription factor 1 (MTF1), Ferroportin-1 (FPN1), Transferrin (TF), Ferritin and 60S ribosomal protein L13a (RPL 13a, reference gene) were cloned by PCR amplification of flathead hepatic cDNA. Degenerate primers (Macrogene, NSW, Australia) were designed based on the conserved regions of other teleost sequences retrieved from Genbank (Table 3.1). The cloning of each gene was conducted in a volume of 20 μl consisted of 2 × PCR mixtures (Bioline, NSW, Australia), 0.5 - 0.75 μM of each primer and approximate 50 ng template cDNA. PCR was conducted on an Eppendorf thermal cycler using the following PCR profile: 2 min at 94 °C, then 35 cycles of 94 °C for 30 s, 52 - 55 °C for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were separated via 1.2 % agarose gel and purified from gel by using a gel extraction kit (Qiagen, NSW, Australia). Purified PCR products were sequenced directly in forward direction. Homologs of these genes were then analysed and confirmed with released sequences in Genbank by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the gene sequences deposited in Genbank.

24 fish were used for gene expression analysis. Gender of each individual fish was not identified during sampling. The liver samples were selected from KBN (n = 8, highest level of As), RB (n = 8, highest level of Hg) and MB (n = 8, reference site) (Table 3.2). MTF1, FPN1, TF, Ferritin and MT hepatic transcripts were measured by quantitative real-time RT-PCR using genes-specific primers (Table 3.1). Real-time PCR was performed on the iQ5 Real-time PCR Detection System (Bio-Rad, NSW, Australia) in a volume of 10 μl containing 400 nM of each primer, 5 μl 2 × SensiFast$^{TM}$ & SYBR mastermix (Bioline, NSW, Australia) and 2 μl of cDNA. Each individual sample was examined in duplicate and the PCR cycling procedure was initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 10 s with signal collection in each cycle. The melting curve was analyzed at the end of the

reaction to ensure the specificity of the PCR amplification. The data analysis of qPCR results was conducted using qBasePlus Biogazelle software (Hellemans *et al.*, 2007). All genes mRNA expression in different samples was normalized against the RPL13a quantities. The geometric mean was used to normalize relative quantity (MNRQ) and was calculated for each gene of grouped 8 biological replicates from the site. The expression level of all the genes was compared with that of expression in fish from MB.

**Table 3.1 Primers used for cDNA sequence and quantification of the mRNA expression by Real-time PCR**

| Gene | Oligo name | Primer DNA Sequence (5'→3') |
|---|---|---|
| cDNA sequence | | |
| MTF1 | MTF1F | ATGGAYCCTTGYGMMTGC |
| | MTF1R | TCACTGACAGCAGSTBGTGTC |
| FPN1 | FPN1F | GGTGACTGGGTDGACAA |
| | FPN1R | AAGGACCAAAGRCCRAHTCTAGC |
| TF | TFF | AAATGGTGTGCTGTGGGCC |
| | TFR | TGGRATCTCCTGGAGACAYTT |
| Ferritin | FerritinF | TCCCAGGTGMGACAGAACT |
| | FerritinR | CTCCTCATGTGACTGATG |
| RPL13a | RPL13F | GGMAACTTYTACCGYAACAA |
| | RPL13R | GTGATGGCCTGGTAYTTCCAGCC |
| Real-time RT-PCR | | |
| MTF1(KU313702) | qMTF1F | AGTGTGATGTGCAAGGCT |
| | qMTF1R | GATTCACAGTTGAATGTCTTGCCCGTG |
| FPN1 (KU313700) | qFPN1F | CAGAACAGTTGCGTCATC |
| | qFPN1R | GGTCAGAATCCATCCATTG |
| TF (KU313704) | qTFF | CCATTAGAGTGCCAGAAT |
| | qTFR | TCTCATCCATTGCTCATT |
| Ferritin (KU313699) | qFerritinF | TTGAGAAGAACGTGAACC |
| | qFerritinR | CCAGGAAATCGCACATAT |
| MT (KP893712) | qMTF | ATCCGGCTGCACCAAATG |
| | qMTR | GTTTACTGACAGCAGGTGGT |
| RPL13a (KU313703) | qRPL13F | ATCTTCTGGAGGACTGTCA |
| | qRPL13R | GAACACCTTCAGCCTCTC |

**Table 3.2 Morphometric measurements and metal levels of 24 flathead were used for gene expression analysis.** (KBN: Kingston Beach North, RB: Ralphs Bay, MB: Mickey's Bay). The metals levels are expressed as mg kg$^{-1}$ wet weight. All data are expressed as means ± SD. Different letters indicated significant differences between sites ($P < 0.05$).

| Location | n | Length(cm) | Weight(g) | Hg | As | Fe | Zn |
|---|---|---|---|---|---|---|---|
| KBN | 8 | 24 ± 2.4 | 129 ± 38.2 | 0.346 ± 0.082[a] | 9.06 ± 4.63[a] | 1.39 ± 0.30[a] | 5.13 ± 0.98[a] |
| RB | 8 | 26 ± 2.0 | 146 ± 35.6 | 0.781 ± 0.209[b] | 3.68 ± 1.20[b] | 1.65 ± 0.24[a] | 6.94 ± 2.58[a] |
| MB(reference) | 8 | 25 ± 1.0 | 137 ± 12.7 | 0.231 ± 0.069[a] | 3.83 ± 2.24[b] | 1.83 ± 0.35[a] | 5.23 ± 0.78[a] |

### 3.3.5    Statistical analyses

One-way ANOVA followed by a Tukey post hoc test was carried out to assess differences among means. For the prevalence and intensity of parasites, data analysis was performed on Quantitative Parasitology 3.0 (Rozsa *et al.*, 2000). The Pearson's correlation coefficient was used to assess the relationships between pollutants levels, gills lesions, parasites number and gene expression after all samples were pooled together. All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad software Inc). A probability (*P*) of < 0.05 was considered statistically significant. All results of heavy metal levels are presented as means ± S.E while the data for gene expression are shown as the geometric means of normalized relative quantity ± 95% confidence interval.

## 3. 4    Results

### 3.4.1    Element residues and the morphometric measurements

There were no significant differences in the length or weight of fish from different sampling sites (Table 3.3). Only three metals (Hg, Zn and Fe) and the metalloid As were detected in the muscle of sampled flathead. There were significant spatial differences for mean Hg ($F = 19.47$, *d.f.* 5, 68, $P < 0.0001$), As ($F = 5.833$, *d.f.* 5, 68, $P = 0.0002$), Fe ($F = 2.950$, *d.f.* 5, 68, $P = 0.0180$) and Zn ($F = 4.474$, *d.f.* 5, 68, $P = 0.0014$) residues in fish from different sampling locations (Table 3). The mean Hg levels of flathead from KBN (0.320 mg / kg) and MB (0.220 mg / kg) were significantly lower than those from RB (0.789 mg / kg), CB (0.668 mg / kg), NTB (0.614 mg / kg) and SBB (0.613 mg / kg). The mean As level of fish from KBN (8.30 mg / kg) was significantly higher than all other locations. The mean Fe residues of fish from SBB (2.38 mg /

kg) were significantly higher than other locations. For Zn level, the fish from RB (6.72 mg / kg) had a significantly higher mean value than fish from other sampling sites.

**Table 3.3 Morphometric measurements and metal levels of 74 flathead from 5 sampling sites in the Derwent estuary and a reference site** (**CB**: Cornelian Bay, **NTB**: Newtown Bay, **KBN**: Kingston Beach North, **SBB**: Sandy Bay Beach, **RB**: Ralphs Bay, **MB**: Mickey's Bay). The metals levels are expressed as mg/kg wet weight. All data are expressed as means ± S.E. Different letters indicated significant differences between sites ($P < 0.05$).

| Location | n | Length(cm) | Weight(g) | Hg | As | Fe | Zn |
|---|---|---|---|---|---|---|---|
| CB | 10 | 29 ± 2.8 | 167 ± 55.4 | 0.668 ± 0.164[a] | 2.93 ± 1.09[a] | 1.58 ± 0.39[a] | 6.67 ± 0.94[a] |
| NTB | 10 | 28 ± 3.0 | 165 ± 45.0 | 0.614 ± 0.145[a] | 4.63 ± 2.71[a] | 1.71 ± 0.34[a] | 5.33 ± 0.78[a] |
| KBN | 10 | 26 ± 3.0 | 119 ± 39.8 | 0.320 ± 0.092[b] | 8.30 ± 4.47[b] | 1.44 ± 0.31[a] | 5.13 ± 0.87[a] |
| SBB | 10 | 27 ± 4.0 | 142 ± 62.4 | 0.613 ± 0.257[a] | 3.64 ± 1.97[a] | 2.38 ± 1.35[b] | 5.49 ± 0.96[a] |
| RB | 15 | 29 ± 5.0 | 165 ± 89.8 | 0.789 ± 0.316[a] | 3.32 ± 1.29[a] | 1.70 ± 0.26[a] | 6.72 ± 2.03[b] |
| MB(reference) | 19 | 30 ± 3.5 | 177 ± 54.0 | 0.220 ± 0.074[b] | 4.38 ± 2.38[a] | 1.77 ± 0.46[a] | 5.39 ± 0.70[a] |

### 3.4.2    Hepatic genes expression and the relationships between the genes

The mRNA expression level of MTF1 was up-regulated in the liver of fish from KBN (8.1 fold) and RB (5 fold) compared to those from the reference site (MB), but there was no statistically significant difference between the fish from KBN and RB (Fig 3.2A). Expression of the TF gene was also significantly up-regulated in fish from KBN and RB with 16.4 fold and 4.2 fold respectively compared to the fish from reference site (Fig 3.2B). TF gene expression was also significantly higher in fish from KBN compared to RB. The mRNA expression level of FPN1 was significantly higher in the liver of flathead from KBN (5.6 fold) and RB (6.1 fold) compared to MB, however there was no significant difference between fish from these two polluted sites (Fig 3.2C). For the Ferritin and MT gene, no regulations were observed among all the sites (Fig 3.2D, 2E). Correlation analysis using all pooled data showed that there was strong positive relationship between MTF1 and TF (r = 0.7802, $P < 0.0001$, Fig 3.2F). Positive correlations were also observed between MTF1 and FPN1 transcripts (r = 0.7903, $P < 0.0001$, Fig 3.2G) and TF and FPN1 (r = 0.6554, $P = 0.0005$, Fig 3.2H). There was no significant correlation between the MTF1 and MT transcripts.
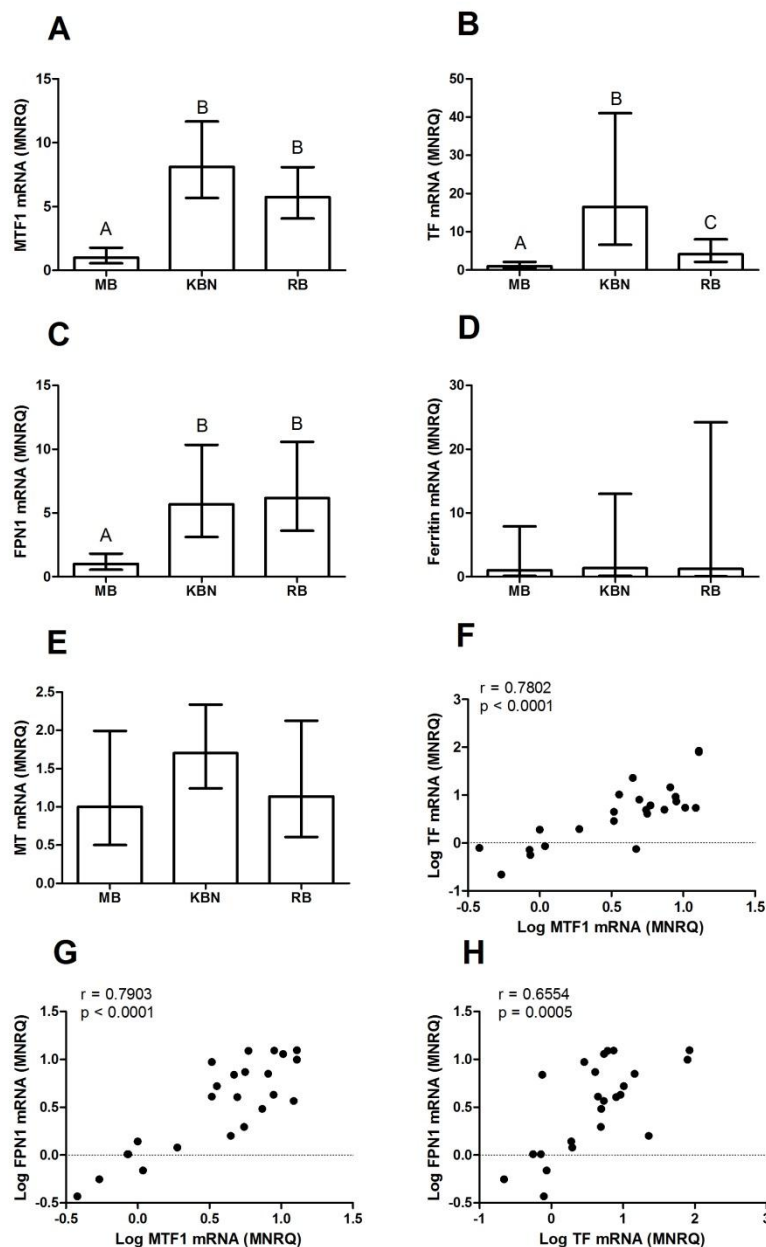
**Figure 3.2** Hepatic mRNA levels of MTF1 (A), TF (B), FPN1 (C), Ferritin (D) and MT (E) relative to reference gene (RPL13a) were analyzed by qPCR in flathead captured at two sites of Derwent estuary (KBN, Kingston beach and RB, Ralphs bay) and a reference site (MB, Mickeys bay). The values are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval. Different letters indicate significant differences between sites. The gene expression of TF (F) (r = 0.7802, *P* < 0.0001), FPN1 (G) (r = 0.7903, *P* < 0.0001) were positively correlated with MTF1 mRNA level. The gene expression of TF (H) was also positively correlated with FPN1 mRNA level (r = 0.6554, *P* = 0.0005).

### 3.4.3 Gill parasites, pathological conditions and relationships with element residues

Monogeneans from two groups, monopisthocotylean monogenean and polyopisthocotylean monogenean, were observed on the flathead gill section. For intensity and prevalence of monopisthocotylean monogenean numbers, there was no significant difference among flathead from different sites (Table 3.4). The polyopisthocotylean monogenean was only observed on the gills of 4 of the 19 flathead from reference site (MB). A weak yet significant negative correlation was observed between Hg level and the number of monopisthocotylean monogenean per section (r = -0.3094, $P$ = 0.0055) (Fig 3.3). There was no correlation between other metal levels and parasites.

**Table 3.4 The prevalence (%) and intensity of Monopisthocotylean monogenean in 74 flathead from 5 sampling sites in the Derwent estuary and a reference site** (**CB**: Cornelian Bay, **NTB**: Newtown Bay, **KBN**: Kingston Beach North, **SBB**: Sandy Bay Beach, **RB**: Ralphs Bay, **MB**: Mickey's Bay).

| Location | Total | Infected fish | Prevalence | Mean Intensity | Median Intensity |
|---|---|---|---|---|---|
| CB | 10 | 8 | 80 | 3.00 | 2.5 |
| NTB | 10 | 9 | 90 | 5.56 | 4.0 |
| KBN | 10 | 6 | 60 | 2.83 | 3.0 |
| SBB | 10 | 8 | 80 | 5.63 | 6.5 |
| RB | 15 | 9 | 60 | 2.22 | 2.0 |
| MB(reference) | 19 | 18 | 94.7 | 7.56 | 6.0 |
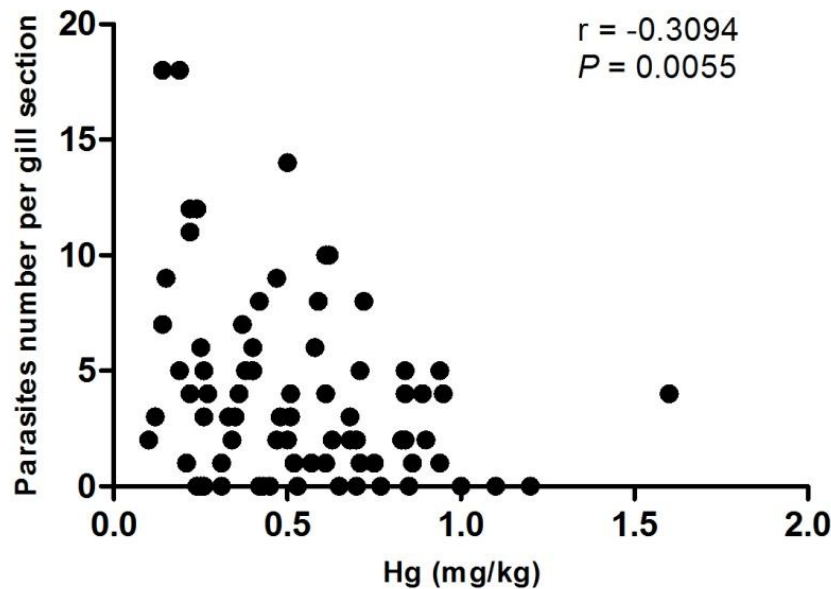
**Figure 3.3 Correlation between parasite number per gill section and Hg level in sampled fish.** The parasite number per gill section was negatively correlated with Hg level (r = -0.3094, $P$ = 0.0055).

Four different types of gill conditions were observed in fish gills (Fig 3.4). The most common condition, epithelial hyperplasia with lamellae fusion, was observed in all examined fish including those from the reference site (see Table 3.5).This type of lesion was frequently found in association with parasite infection. Epitheliocystis was found in 20% of fish from CB and 30% of fish from NTB, while it affected only 5.2% of fish from the reference site. The prevalence of telangiectasis was 60% in fish from CB, 10% in fish from NTB and 5.2% of fish from the reference site. Deformed filaments were only observed in fish from the reference site (15.8%).
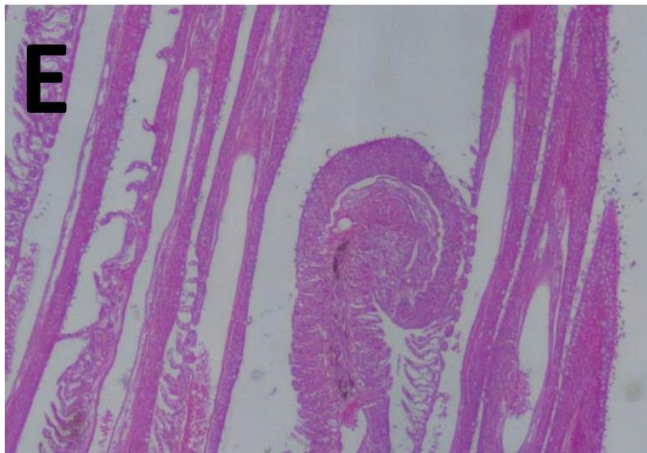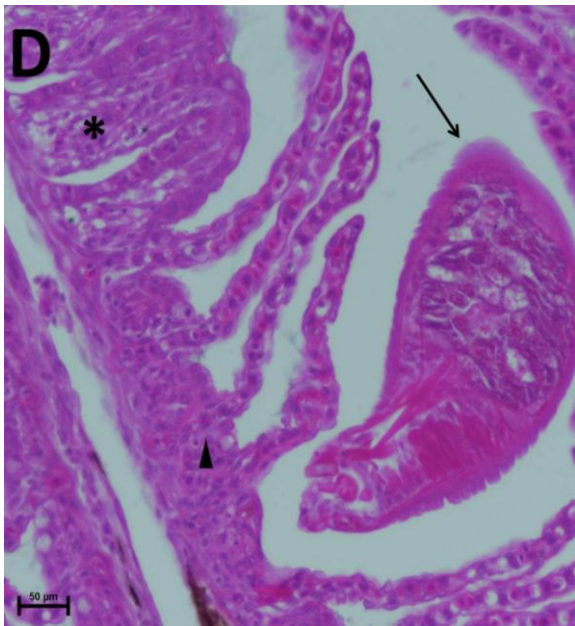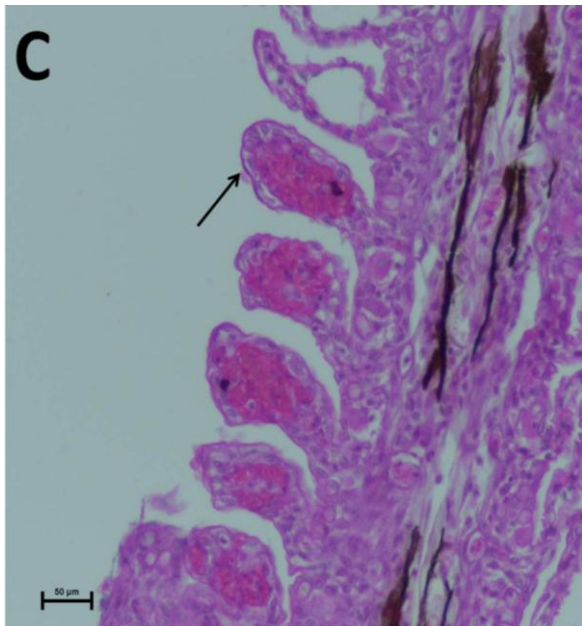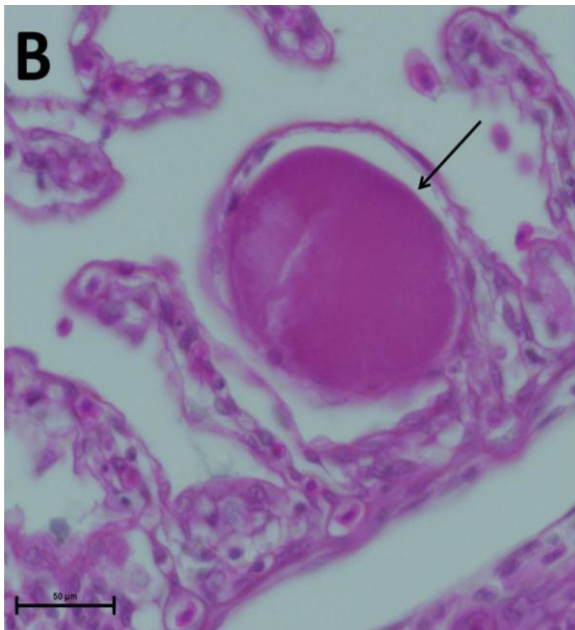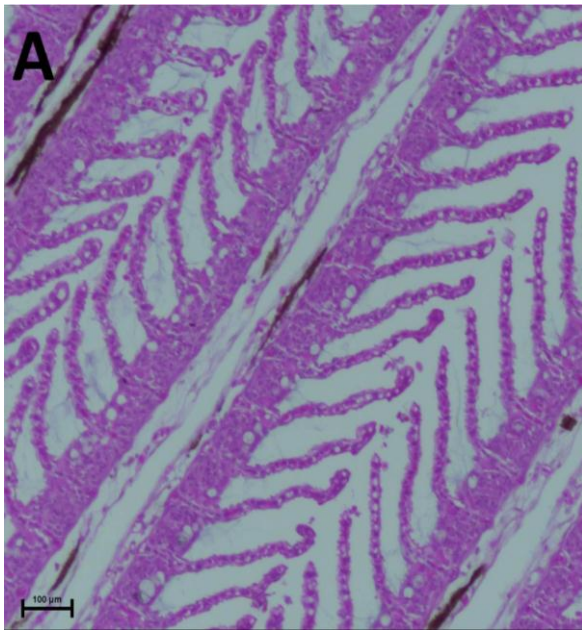
**Figure 3.1 Overview of sand flathead gill histology.** (A) Normal gill. (B) Arrow showing epitheliocystis. (C) Arrow showing telangiectasis. (D) Gill fluke (arrow) infection associated with epithelial hyperplasia (arrowhead) and complete fusion of secondary lamellae (*). (E) Deformed filament.

**Table 3.5 The prevalence (%) of gill conditions in 74 flathead from 5 sampling sites in the Derwent estuary and a reference site** (**CB**: Cornelian Bay, **NTB**: Newtown Bay, **KBN**: Kingston Beach North, **SBB**: Sandy Bay Beach, **RB**: Ralphs Bay, **MB**: Mickey's Bay). **EF**: Epithelial hyperplasia with lamellar fusion. **EP**: Epitheliocystis. **TE**: Telangiectasis. **DF**: Deformed filament.

| Location | n | EF | EP | TE | DF |
|----------|-----|-----|-----|-----|------|
| CB | 10 | 100 | 20 | 60 | 0 |
| NTB | 10 | 100 | 30 | 10 | 0 |
| KBN | 10 | 100 | 0 | 0 | 0 |
| SBB | 10 | 100 | 0 | 0 | 0 |
| RB | 15 | 100 | 0 | 0 | 0 |
| MB(reference) | 19 | 100 | 5.2 | 5.2 | 15.8 |

## 3. 5    Discussion

### 3.5.1  Hepatic gene expression analyses

The genes of metal regulatory transcription factor 1 (MTF1) and ferroportin1 (FPN1) were significantly up-regulated in the fish with high levels of Hg and As. A strong positive relationship was detected between these two genes transcripts when all samples were pooled together. Metal-activated transcription factor 1 (MTF1) is a coordinator for the transcriptional regulation of a set of genes that are involved in metal homeostasis, heavy metals detoxification and oxidative stresses (Andrews, 2001). The transcript of this gene was up-regulated from sites with fish that had the highest Hg and As levels (RB and KBN) compared to the reference site (MB), implying the crucial role of MTF1 in both Hg and As exposures in fish liver. These observations are consistent with previous studies that MTF1 could be induced by As and Hg in mammals (He and Ma, 2011; Liu *et al.*, 2001). The up-regulation of MTF1 would consequently trigger expression of other related genes against metal stress including FPN1 (Gunther *et al.*,

2012). FPN1 encoding the ferroportin 1 is the only iron exporter in vertebrates (Troadec *et al.*, 2010). The measurement of FPN1 mRNA has been reported as one of promising molecular biomarker amongst microarray analysis of As exposure in zebrafish and medaka fish (Xu *et al.*, 2013). FPN1 has been also proven to accept other transition metals as substrates, suggesting the importance of FPN1 in protecting cells from a wide range of metal toxicity (Troadec *et al.*, 2010; Yin *et al.*, 2010). Similarly, in present study, FPN1 was up-regulated in the fish with high levels of Hg and As, indicating FPN1 also plays an important role in fish liver cells against environmental Hg and As exposure. Moreover, it has been demonstrated that the induction of FPN1 mRNA occurs through the action of MTF1 (Gunther *et al.*, 2012; Troadec *et al.*, 2010). In our study, the strong positive correlation was observed between these two genes, suggesting the induction of FPN1 mRNA by As and Hg may be also mediated by MTF1. Whether the transcriptional levels of these two genes are paralleled with protein levels remains to be confirmed however the measurement of these two genes transcripts could possibly be useful molecular biomarkers for sand flathead.

The mRNA level of transferrin (TF) was up-regulated in the fish from KBN and RB compared to those from the reference site (MB). TF transcript level was higher in fish from KBN compared to those from RB. Moreover, strong positive correlations were observed between the transcripts of TF with MTF1 and FPN1. The up-regulation of TF gene in fish suggests TF gene is involved in mitigating with environmental As and Hg stress. The highest expression level of TF gene was in the fish with high As residues. In mammals, It has been previously reported that 18% of total plasma As binds to transferrin after injection of labelled As in rabbits (Shen *et al.*, 2013; Vahter and Marafante, 1983). Moreover, structure of transferrin is highly conserved throughout vertebrates (Ford, 2001). Therefore, It is reasonable to hypothesise that transferrin has high

affinity to As and the increase of TF mRNA is needed to maintain its basic function in fish liver. The up-regulation of TF transcripts was also observed in fish from RB with the highest level of Hg. The mechanism of Hg induced TF gene mRNA is not known due to the complexity of Hg metabolism pathways and the little available information regarding the interaction of Hg and TF gene. In vertebrates, transferrin and ferriportin-1 belong to the iron metabolism pathway. These two proteins play crucial roles in keeping the cellular iron homeostasis. The up-regulation of these two genes in the present study suggests environmental As and Hg exposures may interfere with the transportation and exportation of iron in fish liver. In addition, the strong positive correlation between MTF1 and TF genes mRNA has also implicated the connection between these two genes under the metal stress.

There was no significant difference for the mRNA of ferritin gene. A large individual variability was observed in the expression of this gene. In vertebrates, ferritin is assembled by twenty-four subunits (Arosio and Levi, 2002). In the present study, the ferritin gene of flathead encodes a middle chain subunit. In fish, some of the subunit genes have been demonstrated to be associated with metal stress. For instance, two ferritin heavy subunit genes (fth1 and frh3) are highly sensitive to As exposure in zebrafish (Xu *et al.*, 2013). However, for the ferritin middle subunit gene, only a few of genes in fish have been released in the public database. Moreover, the information regarding the interaction between this gene and environmental stressors is very limited. The large individual variability in the expression of this gene suggests it may be impacted by multiple environmental factors. Although we carefully controlled a number of variables in the wild fish samples, such as fish size differences and seasonal differences, considering the complexity of the field environment, the measurement of this ferritin gene mRNA may not suitable as a molecular biomarker.

For MT gene transcripts, there was no significant difference between fish from RB and KBN compared to fish from reference site MB. These data are generally consistent with our previous study on flathead from Derwent estuary that found hepatic MT gene expression may be not suitable as molecular biomarker to reflect the Hg bioaccumulation. This is also consistent with previous study of MT genes in carp (*Cyprinus carpio*) that has shown that MT genes in liver were unable to respond to environmental Hg exposure or injection (Navarro *et al.*, 2009). It also has been suggested MT genes in fish kidney were more sensitive than in fish liver (Gonzalez *et al.*, 2005; Navarro *et al.*, 2009). Fish from KBN the high As site did not exhibit up-regulated MT mRNA. This indicates that flathead were either not responding or were not exposed to sufficiently high concentrations of As to regulate the MT mRNA. It has been suggested that MTF1 was involved in As induction of MT in mammals (Shen *et al.*, 2013). However, there was no significant correlation between MTF1 and MT transcripts. This result was different with the up-regulation of MT gene in Chapter 2. The inconsistent results of MT gene are discussed in Chapter 6. Therefore, further studies are needed to confirm the usefulness of hepatic MT transcripts as molecular biomarker in flathead.

Gene expression analysis was conducted on fish that sex was not identified. The applicability of the potential biomarker genes including MTF1, FPN1 and TF should be further assessed in the mature fish including investigation of the effect of gender.

### 3.5.2 Parasites and gill histopathology

Monogeneans are the most common parasites on sand flathead gill (Nowak *et al.*, 2004). In this study, two monogenean groups, the polyopisthocotylean monogenean and monopisthocotylean monogenean, were observed on the fish gills. However, the polyopisthocotylean monogenean was absent in all the samples from the polluted sites, indicating that water contamination may

affect the diversity of the monogenean species. Apart from this, the intensity of monopisthocotylean monogeneans was higher in fish from the reference site than fish from other polluted sites, suggesting metals accumulation in fish body may also have the toxic effects on this parasite. This also has been confirmed by the negative correlation between the numbers of monopisthocotylean monogenean and Hg level of fish muscle in our study. In general, monogenean parasites are considered as the sensitive indicators for environmental changes (Galli *et al.*, 2001). In this study, monogeneans on flathead gills could be employed as a complementary indicator to the determination of metals level in fish body.

The most common histopathological changes observed in flathead gills were epithelial hyperplasia with secondary lamellae fusion. Prevalence of this condition was observed in all fish irrespective of sites. Previous studies have shown the casual relationships between epithelial hyperplasia with secondary lamellae fusion and pollutants (Al-Bairuty *et al.*, 2013; dos Santos *et al.*, 2012; Naidu *et al.*, 1983; Sola *et al.*, 1995). In addition to the pollutants, this type of gill condition has also been found in association with the presence of gill parasites. For example, a high infection prevalence of a copepod (*Lernaeocera branchialis*) was associated with epithelial hyperplasia of the secondary lamellae in flounder (*Platichthys flesus*) (Stentiford *et al.*, 2003). In flathead, this condition was also observed as a response to infestation of the monogenean in a previous study, suggesting parasites could be a contributing co-factor (Nowak *et al.*, 2004). In the present study, this histopathological alteration is likely to be the consequence of combined effects of pollutants exposure and parasites infection. Therefore, it is difficult to link epithelial hyperplasia and secondary lamellae fusion solely with the metals exposure and hence do not consider it a biomarker for sand flathead.

The higher prevalence of telangiectasis (aneurysm) was found in the fish from Cornelian Bay (CB). Telangiectasis on the fish gills is general believe as the result of disturbance of fish gill blood flow that caused by toxic substances in the ambient water (Mallatt, 1985). This lesion has been proposed as a useful toxic biomarker in a previous study (Stentiford *et al.*, 2003). The high prevalence of this lesion was observed in Nile tilapia (*Oreochromis niloticus*) from heavy metals polluted water (Abdel-Moneim *et al.*, 2012). Another field study with common sculpin (*Myoxocephalus scorpius*) also found a relationship between gill telangiectasis and Hg level (Sonne *et al.*, 2014). In the laboratory studies, telangiectasis in fish gills could be induced by Zn, Cu, As and Hg exposures (Ahmed *et al.*, 2013; dos Santos *et al.*, 2012; Giari *et al.*, 2008; Oliva *et al.*, 2009). Both the field and laboratory studies suggest this lesion could be related to the metals level of water. In the present study, however, telangiectasis was not observed in any fish from KBN or RB where fish had the highest level of As and Hg respectively. This observation suggests there is no direct link between telangiectasis and As or Hg bioaccumulation. The highest prevalence of telangiectasis was found in fish from CB. In fact, CB is the closest location to the industry area, in which the concentration of waterborne metals is higher than other selected sites (Bloom and Ayling, 1977). Based on these observations, it is reasonable to hypothesise that the occurrence of telangiectasis may be associated with waterborne pollutant exposure. Further studies are needed to build the linkage between this lesion in flathead and waterborne metals level of the Derwent estuary.

The fish from the reference site had the only occurrence of filament deformation observed. This lesion was characterized by distorted or twisted filament. Previous studies have shown that the presence of deformed gill filaments could be caused by a number of factors such as deficiency of nutrition or parasites infection (Fracalossi *et al.*, 1998; Naldoni *et al.*, 2014). In this study, the

high intensity of monogenean infection could be responsible for this type lesion as the fish from reference sites had the highest infection intensity.

Besides parasites and pollutants, epitheliocystis, an intracellular bacterial caused lesion was also observed in the fish gills. Epitheliocystis is normally considered as the benign condition that could infect various wild and farmed fish species in Tasmania (Nowak and Clark, 1999; Stride and Nowak, 2014). This condition has been reported in sand flathead from eastern Tasmanian water before and the prevalence of epitheliocystis in this study was generally consistent with the previous study (Stride and Nowak, 2014). The prevalence of epitheliocystis could be affected by many factors such as water temperature, fish size and parasites infections in the wild fish (Stride and Nowak, 2014). In this study, whether the metal contamination impact on the epitheliocystis infection remains unknown.

## 3. 6    Conclusion

This is the first comprehensive study to examine histological and molecular biomarkers on this indicator species from Derwent estuary. The abundance and diversity of gill parasites has previously been proven useful in reflecting the bioaccumulation of metals in fish and appeared to be consistent with the results of this study. The gill histological observations suggest gill pathology is a promising tool for monitoring pollutants but also highlights the complexity of the environment stressors, pathogens and fish interaction system. The histological alterations in the fish gill suggest both pollution and pathogens could be causative factors. The pollutant related histological changes observed in other studies indicate further research is needed to fully assess the relationships between gill conditions observed in this study with heavy metals found in the Derwent. For the gene expression analysis, measurement of metal-homeostasis related genes

(MTF1, FPN1 and TF) mRNA in the present study suggests the protective roles of these genes against the environmental Hg and As exposure. These biomarker candidate genes have potential to be applied in environmental monitoring research.

Chapter 4 **:** Hepatic expression of Diablo/SMAC, GRP78 genes and

liver histology of sand flathead (*Platycephalus bassensis*) from a metal

polluted estuary

## 4.1 Abstract

Trace metals determination, hepatic gene expression analysis and liver histological assessment were conducted in sand flathead (*Platycephalus bassensis*) from five polluted sites in the Derwent estuary and a reference site. Metals including zinc (Zn), iron (Fe), mercury (Hg) and arsenic (As) were detected in the fish muscle. There were significant spatial differences for Hg and As in flathead from the polluted sites and the reference site. The transcript levels of Diablo/SMAC homolog genes and GRP78 in the liver of flathead from different locations were analyzed using qPCR. There were significant spatial differences for all these tested genes, suggesting regulation of these genes are possibly associated with environmental contaminants. The mRNA of GRP78 was significantly increased in fish from four polluted sites compared to fish from reference site. The transcripts of Diablo/SMAC1 and Diablo/SMAC2 were up-regulated in fish from most of the polluted sites. Diablo/SMAC1 gene appeared to show a greater sensitivity than the Diablo/SMAC2 gene to environmental metal exposure. In addition, there was a significant positive correlation between the transcripts of these two Diablo/SMAC genes. For the liver histology, non-specific lesions including inflammation, granuloma, parasitic infections and bile duct fibrosis were observed in sampled fish. The prevalence of melano-macrophage centres (MMCs) was also recorded in the fish sample. There was significant difference in the prevalence of bile duct fibrosis and inflammation between the fish from polluted sites and the reference site, suggesting increased prevalence of these two lesions is associated with environmental pollutants exposure. This study suggests that measurement of Diablo/SMAC homolog and GRP78 transcripts and liver histological assessment are promising tools for assessing the effects of environmental metal exposure on fish.

## 4.2 Introduction

Heavy metals contaminations in estuaries have been a globally serious problem due to their bioaccumulation, persistence and deleterious effects on wildlife (Kennish, 1996; Wang *et al.*, 2013). Estuaries are important sites for the feeding, spawning, nursery and breeding of aquatic organisms (Boehlert and Mundy, 1988; Yamashita *et al.*, 2003). For decades, substantial efforts to investigate casual relationships between heavy metal exposure and their adverse effects in the aquatic ecosystem have been made (Baatrup, 1991; Bryan, 1971; Mclusky *et al.*, 1986; Phillips, 1977). Fish species are often employed in studies assessing the early stage of effects as they play significant ecological role in the food-webs, have high ecological relevance and are very abundant (Beyer, 1996). It is important to detect early biological responses before the more serious effects happen such as population or community changes. Such early biological responses are also referred as biomarkers, which are mainly focused on the sub-organism levels (van der Oost *et al.*, 2003). Histological examination, enzyme activity measurement and gene expression analysis are commonly used techniques in the development of biomarkers (McCarthy and Shugart, 1990).

Fish liver histological examination has been increasingly used as a tool in estuarine environmental monitoring (Stentiford *et al.*, 2003). Substantial evidence from both laboratory and mesocosm studies have demonstrated causal relationships between fish liver histological alterations and pollutants exposure (Arellano *et al.*, 1999; Figueiredo-Fernandes *et al.*, 2007; Malins *et al.*, 1985; van Dyk *et al.*, 2007). As such, the quantification of certain hepatic lesions could reflect the degree of pollution (Bernet *et al.*, 1999).

In addition to liver histological assessment, hepatic gene expression analysis in fish has also been demonstrated as a powerful and reliable endpoint in environmental monitoring (Cuklev *et al.*, 2012; Haasch *et al.*, 1993; Talbot *et al.*, 1976). The application of transcriptomic profiling techniques in sentential fish species provide comprehensive information of pollutant effects and identify novel potential molecular biomarkers. A recently study of hepatic transcriptomic analysis on European flounder (*Platichthys flesus*) exposed to pollutants found the Diablo/SMAC gene was one of most highly up-regulated in the cDNA microarray (Williams *et al.*, 2008). Additionally, long term mesocosm research has also shown this gene could be regulated by both chronic and acute exposure to multiple pollutants even in the absence of traditional exposure biomarker signals (Leaver *et al.*, 2010). A follow up study further confirmed these findings and proposed Diablo/SMAC as a novel molecular biomarker of pollutant exposure (Zacchino *et al.*, 2012). Diablo (direct IAP binding protein with low p*I*) or SMAC (second mitochondria derived activator of caspase) is a protein involved in mitochondrial apoptotic pathways (Burri *et al.*, 2005). It inhibits the actions of inhibitors of apoptosis proteins (IAP). IAPs are the key regulators in both apoptosis and inflammation (Silke and Meier, 2013). Therefore, changes of Diablo/SMAC transcripts could be a protective strategy for cells response to damage (Zacchino *et al.*, 2012). However, the Diablo/SMAC gene has only been characterized and examined on European flounder (*Platichthys flesus*) exposed to organic pollutants (Zacchino *et al.*, 2012). The usefulness of this gene homolog as a biomarker for heavy metals exposure is largely unknown.

Another molecular biomarker candidate gene is GRP78 (glucose regulated protein 78) gene. This gene is a multifunctional gene belongs to heat shock protein (HSP) gene family (Kennish, 1996). GRP78 is a highly conserved endoplasmic reticulum (ER) stress marker and plays an important

role in unfolded protein response during the ER stress (Bertolotti *et al.*, 2000). GRP78 mRNA is inducible by heavy metals in both invertebrates and vertebrates. For instance, the expression of GRP78 mRNA was increased in flatworm (*Dugesia japonica*) after exposure to Hg or Pb. In fish, it has been reported that hepatic GRP78 gene transcripts is significantly up-regulated after $Pb^{2+}$ stress (Zhu *et al.*, 2013). These studies suggest GRP78 gene has a highly conserved function in responding to ER stress caused by metal stress. However, whether this gene is inducible under environmental mixed metals exposure remains unknown. As such, the usefulness of Diablo/SMAC gene homolog and GRP78 gene as molecular biomarkers were assessed in sand flathead from the Derwent estuary.

The Derwent estuary has been considered as one of the most polluted estuaries in Australia (Verdouw, 2008). It is located in the south eastern area of Tasmania, surrounded by the capital city of Hobart. The estuary has been receiving industrial waste water for many years (Bloom and Ayling, 1977). As a result, biota of this area is heavily contaminated with metals (Bloom and Ayling, 1977; Green and Coughanowr, 1997; Verdouw, 2008). Metals bioaccumulation in sand flathead (*Platycephalus bassensis*), an important recreational fish species, has been examined annually since 1970s (Ayling *et al.*, 1975; Verdouw, 2008).

Sand flathead has been regarded as an ideal indicator species, because of its great advantages, e.g., it is a demersal and sedentary fish species, it has a relatively high trophic position in the food chain and they are very abundant along the southern Australian coasts (Holdway *et al.*, 1994; Walker, 1982). Due to these traits, this species has been used for biomonitoring of the coastal ecosystem of southern Australia in many environmental programs (Fabris *et al.*, 1992; Gagnon and Holdway, 2002; Holdway *et al.*, 1994; Langdon, 1986; Nicholson *et al.*, 1994).

The aims of the present study were to examine liver histological alterations in flathead from the Derwent estuary and to assess the applicability of Diablo/SMAC homolog gene and GRP78 gene in these fish as molecular biomarkers for environmental heavy metal exposure.

## 4.3 Materials and Methods

### 4.3.1 Sample collection

All fish used in this study were opportunistically sampled as part of a routine heavy metal monitoring program carried out by Nyrstar (Hobart) under a permit issued under section 14 of *Living Marine Resources Management Act 1995* (2011-12 Annual Environment Review of Nyrstar N.V). Sand flathead were sampled at a number of sites along the Derwent estuary including Cornelian Bay (CB, n = 10), Newtown Bay North (NBN, n=10), Kingston Beach North (n = 10), Sandy Bay Beach (n = 9) and Ralphs Bay (n = 16) (Fig 4.1). Mickey's Bay (MB, n = 13), a relatively less polluted site, was selected as the reference site. All the samples were collected between September and November, 2013. For metals determination, approximately 50 g of muscle sample was collected from each fish, put in a labeled plastic bag and placed on ice for transport to the laboratory. All samples were stored at -20 °C until processed for metals analyses.

For gene expression analyses, a small piece of liver (20 mg) was collected from individual fish and promptly stored in the labeled tube with ice-cold RNAlater. The samples were held on ice for transport to the laboratory and then placed at -20 °C until RNA extraction.

For histopathological assessment, liver samples from each fish were removed and directly immersed in 10% neutral buffered formalin (NBF). Fixation lasted for 24 h, and then all the samples were transferred to 70 % ethanol until further processing.
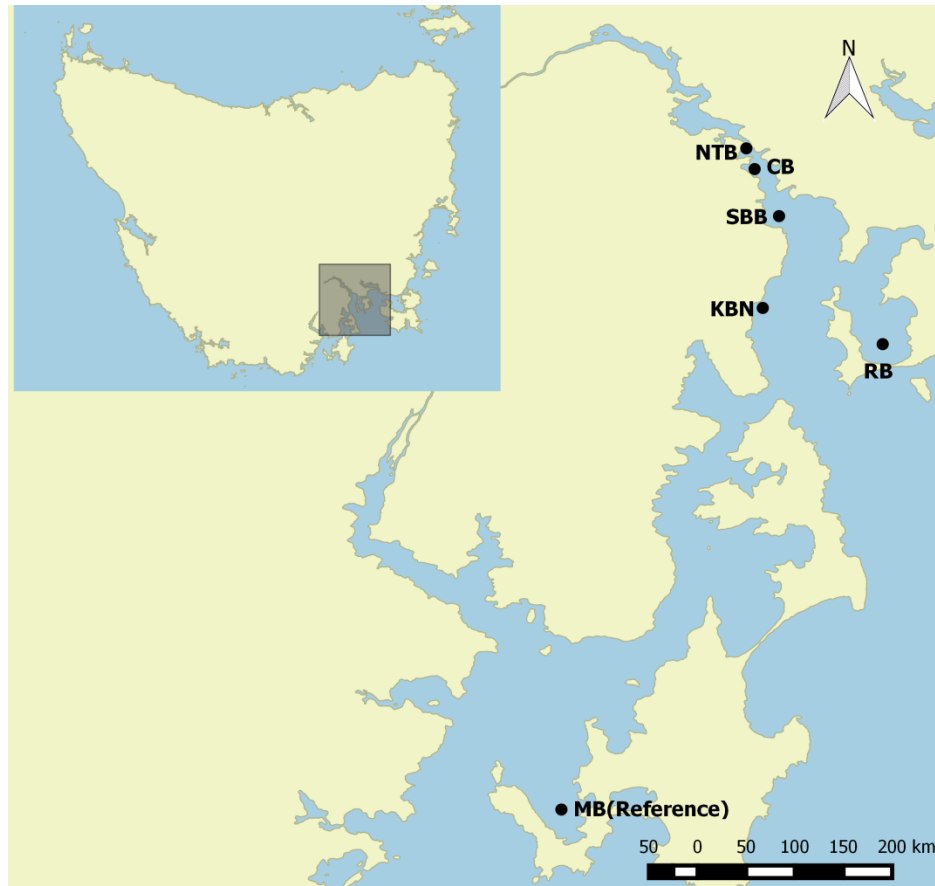


**Figure 4.1 Map of Derwent estuary sampling sites.** CB: Cornelian Bay, NTB: Newtown Bay, KBN: Kingston Beach North, SBB: Sandy Bay Beach, RB: Ralphs Bay, MB: Mickey's Bay

### 4.3.2 Metals determination

Muscle samples were analyzed by Analytical Services Tasmania that is accredited by National Association of Testing Authorities (NATA). The determination of total Hg in sample was used as a proxy of methymercury (MeHg) due to the assumption that MeHg is the primary form of Hg in fish muscle (Harris *et al.*, 2003). Samples were digested and analysed using cold vapour atomic fluorescence spectrometry conducted on an atomic fluorescence analyser as described by (Verdouw *et al.*, 2011). For the analysis of other metals including, arsenic, cadmium, cobalt, chromium, copper, iron, zinc, selenium, lead, nickel and manganese, approximately 12 g of skinless and boneless muscle tissues from individuals were dried and ground into powder. 1 g of each sample was digested and analyzed using an inductively coupled plasma atomic emission spectrophotometer. All the data of residues were given as milligrams per wet kilogram of sample with a minimum detection level of 0.02 mg/kg.

### 4.3.3 Histological assessment

The fixed livers were dehydrated in a progressive series of ethanol, cleared in xylene and finally embedded in paraffin. The livers were sectioned at 5 μm on microtome, mounted on microscope slides and dried in an incubation oven at 37 °C overnight. Sections were then stained with hematoxylin and eosin (H & E stain) and analysed microscopically.

### 4.3.4 Gene cloning and gene expression analyses

The GRP78 and Diablo/SMAC homolog partial genes were obtained by PCR amplification of flathead hepatic cDNA. Degenerate primers (Macrogene, South Korea) were designed based on the conserved regions of three teleost sequences retrieved from Genbank (Table 4.1). PCR amplification of each gene was conducted in a volume of 20 μl containing 2 × PCR mixtures (Bioline, NSW, Australia), 0.75 μM of each primer and approximate 50 ng template cDNA.

Amplification was conducted on an Eppendorf thermal cycler using the following PCR profile: 2 min at 94 °C, then 40 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were separated via 1.5 % agarose gel. The anticipated size PCR products were sequenced directly in forward direction (Macrogen Inc, Korea). Homologs of these genes were then analysed and confirmed with published sequences by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Table 4.1 Primers used for cDNA sequence, RACE reactions and quantification of the mRNA expression by Real-time PCR**

| Gene | Oligo name | Primer DNA Sequence (5'→3') |
|---|---|---|
| cDNA sequence | | |
| Diablo/SMAC1 | SMAC1F | ATGCARGYKGYTCGTCAGTG |
| | SMAC1R | TCAGTCYTCTCKTARATA |
| Diablo/SMAC2 | SMAC2F | GGCGGYGGRCTGTGYGCTGTMCCTTT |
| | SMAC2R | CGCTGGCCAATGATCRCCTGCCAGA |
| GRP78 | GRP78F | ACCAGGGWAACCGCATCAC |
| | GRP78R | GACTCCAWCCACTCRATCTTCTCCTC |
| Real-time RT-PCR | | |
| Diablo/SMAC1 (KU323388) | qSMAC1F | CAGAGGAGGATTCATATC |
| | qSMAC1R | AAGTTGACAGCATTTATC |
| Diablo/SMAC2 (KU323389) | qSMAC2F | TGTAGCGTCTTTAACTGT |
| | qSMAC2R | TGAACTGTCTGTAACCAA |
| GRP78 (KU313701) | qGRP78F | TGAAGAAGTCTGACATTG |
| | qGRP78R | GCCATTGAATAACTCCTT |
| RPL13α (KU313703) | qRPL13F | ATCTTCTGGAGGACTGTCA |
| | qRPL13R | GAACACCTTCAGCCTCTC |

8 fish were selected from each site for gene expression analysis and gender was not identified during the sampling. The total RNA of each sample (around 3-5 mg) was placed in a 400 µL ice-cold extraction buffer (4 M urea, 1 % SDS, 0.2 M NaCl, 1 mM sodium citrate pH 7-8) contains 20 units of proteinase K (Bioline) and disrupted tissue manually with a micro-pestle. The detergent, protein and cellular debris were removed by adding 200 µL of 7.5 M ammonium acetate and centrifugation at $16,100 \times$ g for 3 min at room temperature. The TNA (total nucleic acids) were then recovered by isopropanol precipitation at $16, 000 \times$ g for 10 min at room temperature followed by an ethanol (70 %) wash. The genomic DNA was digested by twice DNase and RNA quality was checked on a 1 % agarose gel. 1 µg of total RNA from each sample was used for cDNA synthesis by reverse transcriptase (Bioline, NSW, Australia) with Oligo (dT) 18 primer.

The real-time PCR was conducted on the iQ5 Real-time PCR Detection System (Bio-Rad, NSW, Australia) in a volume of 10 µL containing 0.4 µM of each primer, 5 µl 2 × SensiFast$^{TM}$ & SYBR mastermix (Bioline, NSW, Australia), and 2 µl of cDNA template. All the samples were assayed in duplicate and the PCR cycling procedure were initial activation of DNA polymerase at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 10 s. The melting curve analysis was performed at the end of the cycling protocol to ensure the amplification specificity.

### 4.3.5 Statistical analyses

Statistical analyses of spatial differences in Hg, As, Zn and Fe concentrations were performed using one-way analysis of variance (ANOVA) followed by the a Tukey HSD test to determine differences among the sites. For the hepatic genes expression, data analysis was conducted on qBase software (Hellemans *et al.*, 2007). All genes mRNA expression in different samples was

normalized against the RPL13a quantities. The geometric mean was used to normalize relative quantity (MNRQ) and was calculated for each gene of grouped 8 biological replicates from the site. The expression level of all the genes was compared with that of expression in fish from MB. One-way ANOVA followed by a Tukey post hoc test was carried out to assess differences between a pollute site and the reference site. The prevalence of liver histological conditions from sampling sites was expressed as percentage of fish affected. The difference in the prevalence of liver lesions and MMCs in fish from different sites were assessed using the Pearson Chi-square test (IBM SPSS statistics 23.0). The statistical analyses were carried out using GraphPad Prism version 6.0 (GraphPad software Inc, USA) and probabilities ($P$) of $< 0.05$ were considered statistically significant.

## 4.4 Results

### 4.4.1 Heavy metal levels in fish muscle

Zn, Fe, Hg and As were detected in the fish muscle from all sampling sites. The muscle metal concentrations of fish that used for gene expression analysis were shown in Fig 4.2. There were significant spatial differences for mean Hg ($F = 19.47$, $d.f.$ 5, 42, $P < 0.0001$) and As ($F = 5.833$, $d.f.$ 5, 42, $P = 0.0002$) residues of fish from different sampling sites. Fish from CB ($0.678 \pm 0.15$ mg/Kg), NTB ($0.585 \pm 0.10$ mg/Kg), SBB ($0.640 \pm 0.28$ mg/Kg) and RB ($0.768 \pm 0.21$ mg/Kg) showed higher concentrations of Hg residues than those from reference site (MB) ($0.217 \pm 0.07$ mg/Kg) (Fig 4.2A). No significant difference in Hg concentration was observed between fish from MB and KBN ($0.346 \pm 0.08$ mg/Kg). Fish from CB, SBB and RB had higher concentrations of Hg than fish from KBN. Hg levels were similar between fish from NTB and KBN. For As concentrations, fish from KBN ($8.738 \pm 4.07$ mg/Kg) had significantly higher As

than fish from all the sites except NTB (Fig 4.2B). No other differences were found for As level in fish among the sites including CB (2.938 ± 1.29 mg/Kg), NTB (5.163 ± 2.58 mg/Kg), SBB (3.838 ± 2.15 mg/Kg), RB (3.925 ± 1.43 mg/Kg), and MB (3.76 ± 2.14 mg/Kg). There was no significant difference in Zn (Fig 4.2C) and Fe (Fig 4.2D) residues in fish muscle among all the sites.
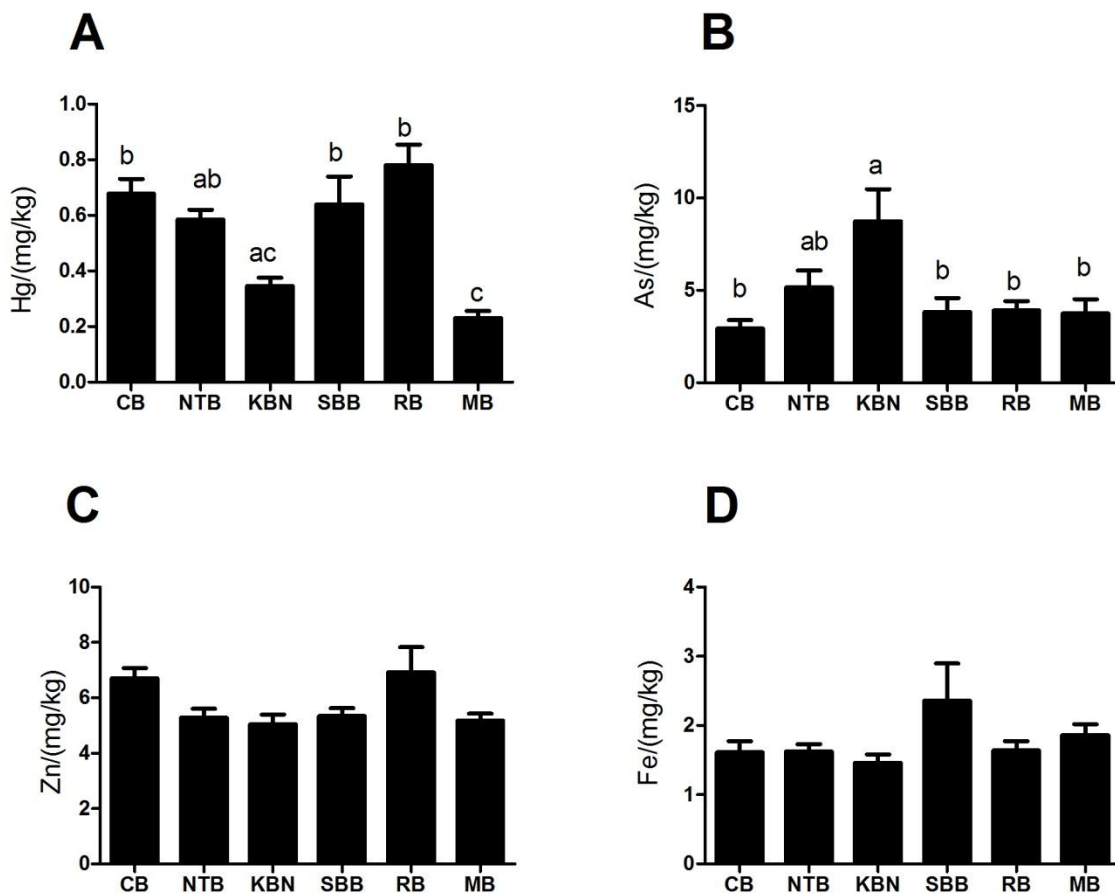


**Figure 4.2** The mean ± S.E (n = 8) mercury (A), arsenic (B),  zinc (C) and iron (D) concentrations in flathead muscle sampled at five sites in the Derwent estuary (CB: Cornelian Bay, NTB: Newtown Bay, KBN: Kingston Beach North, SBB: Sandy Bay Beach and RB: Ralphs Bay) and a reference site (MB: Mickey's Bay). Different letters indicate significant differences between sites.

### 4.4.2   Hepatic genes expression

Hepatic expression level of GRP78 mRNA was significantly up-regulated in fish from NTB (52 fold), SBB (36 fold), CB (26 fold) and KBN (20 fold) but not RB compared to fish from the reference site MB ($P < 0.05$, Fig 4.3A). The mRNA level of Diablo/SMAC1 was increased in fish from three polluted sites including KBN (8.0 - fold, $P < 0.0001$), CB (8.2 - fold, $P < 0.0001$) and NTB (6.1 - fold, $P < 0.0001$) compared to MB, while no up-regulation was observed in fish from RB and SBB (Fig 4.3B). The hepatic transcripts of Diablo/SMAC2 were also slightly increased in the fish from KBN (3.0 - fold, $P < 0.05$), NTB (2.9 - fold, $P < 0.05$), SBB (2.6 - fold, $P < 0.05$) and CB (2.4 - fold, $P < 0.05$) but not RB compared to the reference site (MB) (Fig 4.3C). A significant positive correlation was observed between the transcripts of Diablo/SMAC1 and Diablo/SMAC2 (r = 0.5735, $P < 0.0001$, Fig 4.3D).
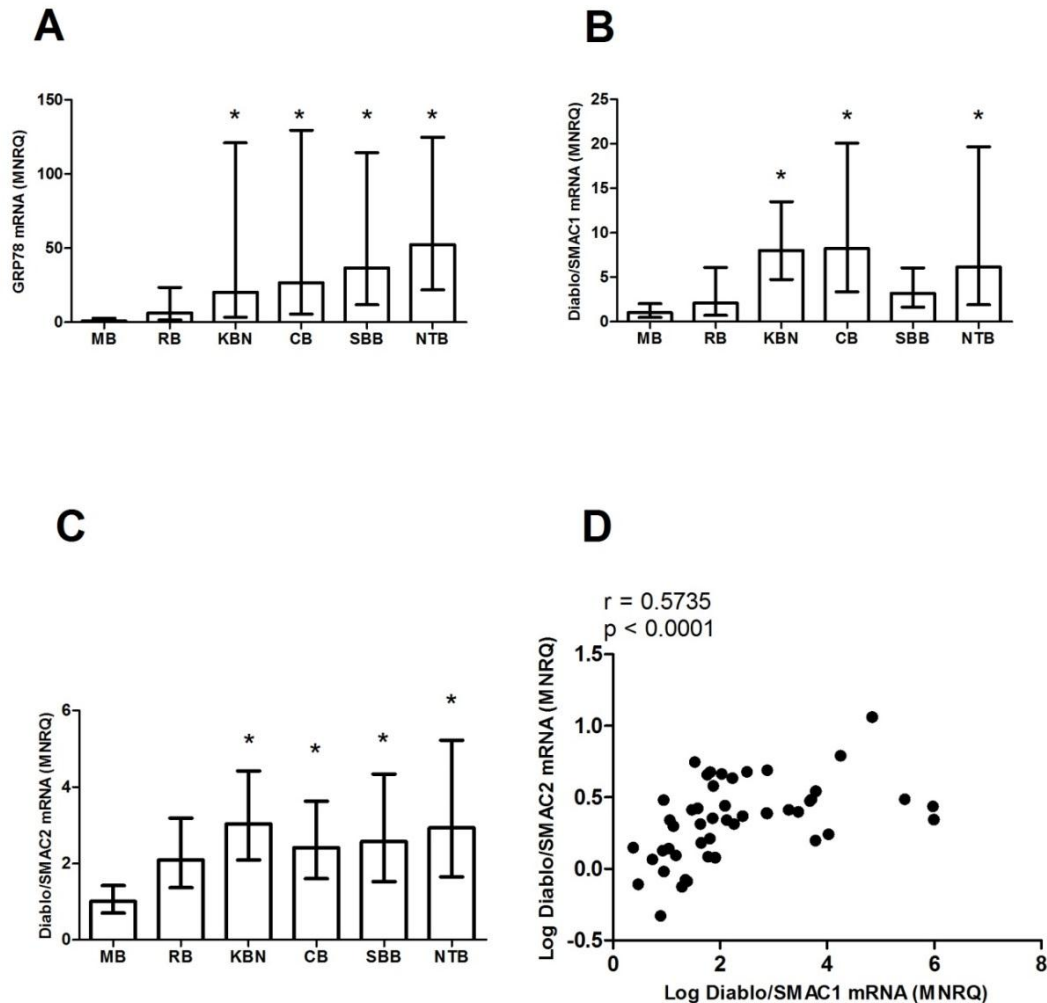
**Figure 4.3** Hepatic mRNA levels of GRP78 (A), Diablo/SMAC1 (B) and Diablo/SMAC2 (C) relative to reference gene RPL13) were analysed by qPCR in flathead captured at five sites in the Derwent estuary (CB: Cornelian Bay, NTB: Newtown Bay, KBN: Kingston Beach North, SBB: Sandy Bay Beach and RB: Ralphs Bay) and a reference site (MB: Mickey's Bay). The values are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval, a significant difference from reference site is indicated with an asterisk ($P < 0.05$). The positive correlation was observed between Diablo/SMAC1 and Diablo/SMAC2 (D) (r = 0.5735, $P < 0.0001$).

### 4.4.3 Liver histopathology

Four major types of histological conditions were found in all sampled fish including bile duct fibrosis, inflammation, granuloma and parasites (Fig 4.4). The prevalence of these liver histological alterations and melanomacrophage centres (MMCs) is shown in Table 4.2. The granulomas in flathead liver were formed around eosinophilic pigmented material encapsulated

with multiple layers of fibrous tissue (Fig 4.4A). No significant difference was found in the prevalence of granuloma among the different sites ($\chi^2$ = 5.829, $d.f.$ = 5, $P$ = 0.323).Granulomas affected40 % of fish from KBN, 25 % fish from RB, 22.2 % of fish from SBB, 20 % of fish from CB and NTB, and only 7.7 % of fish from reference site. Bile duct fibrosis was characterized by proliferation of connective tissue surrounding bile ducts (Fig 4.4B). There was significant difference in prevalence of this lesion among the sampling locations ($\chi^2$ = 15.555, $d.f.$ = 5, $P$ < 0.05). The bile duct fibrosis was found in the 78 % of fish from SBB, 50 % of fish from NTB and KBN, 37.5 % of fish from RB and only 15.4 % of fish from reference site, while this lesion was not observed in fish from CB. Inflammation was often observed around bile ducts, MMCs, granulomas and blood vessels (Fig 4.4C). There was significant difference in the prevalence of inflammation among the different sites ($\chi^2$ = 14.567, $d.f.$ = 5, $P$ < 0.05). The prevalence of inflammation was found to range from 40% to 89% in fish from polluted sites while it was only observed in 7.7 % of fish from reference site. MMCs were relatively common in flathead liver. They were characterized by clusters of golden or dark brown pigmented cells were occasionally surrounded with a thin layer of fibrous tissue and lymphocytes (Fig 4.4D). No significant difference in prevalence of this lesion was observed in fish from different sites ($\chi^2$ = 5.252, $d.f.$ = 5, $P$ = 0.386). The prevalence of MMCs was 90 % in fish from NTB and KBN, 89% in fish from SBB, 81.3% in fish from RB, 50% in fish from CB and 77% in fish from reference site. Digenean parasites were found in fish from four sites of Derwent estuary (CB, KBN, SBB, and RB), while no parasites was observed in fish from NTB and reference site (Fig 4.4E). No significant difference in the prevalence of parasites in fish liver ($\chi^2$ = 7.800, $d.f.$ = 5, $P$ = 0.168).

**Table 4.2 The prevalence (%) of liver histological conditions in flathead sampled at five locations in the Derwent estuary** (**CB**: Cornelian Bay, **NTB**: Newtown Bay, **KBN**: Kingston Beach North, **SBB**: Sandy Bay Beach, **RB**: Ralphs Bay) and a reference site, (**MB**: Mickey's Bay). **BF**: Bile duct fibrosis. **MMC**: Melano-macrophage centres.

| Location | n | BF | MMC | Inflammation | Granuloma | Parasites |
|---|---|---|---|---|---|---|
| CB | 10 | 0 | 50 | 40 | 20 | 10 |
| NTB | 10 | 50 | 90 | 50 | 20 | 0 |
| KBN | 10 | 50 | 90 | 40 | 40 | 10 |
| SBB | 9 | 78 | 89 | 89 | 22.2 | 30 |
| RB | 16 | 37.5 | 81.3 | 43.8 | 25 | 12.5 |
| MB (reference) | 13 | 15.4 | 77 | 7.7 | 7.7 | 0 |
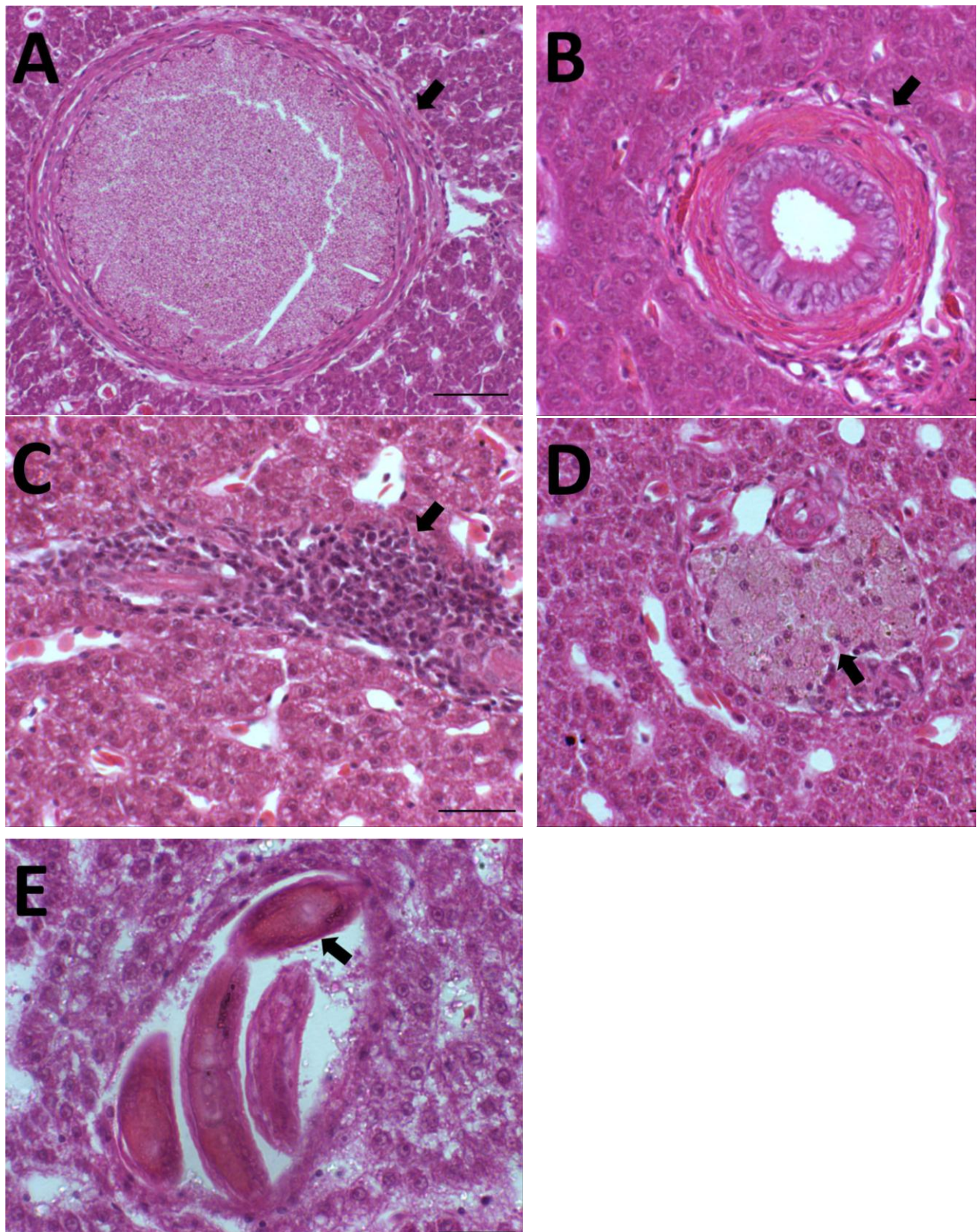
**Figure 4.1 Overview of sand flathead liver histology.** (A) Granuloma (arrow). (B) Bile duct (arrow). (C) Melanomacrophage centre (MMC) (arrow). (D) Inflammation (arrow). (E) Parasite Bar (A) = 50 µm. Bar (B, C, D, E) = 25 µm.

## 4.5 Discussion

### 4.5.1 Hepatic gene expression

A significant spatial difference of hepatic GRP78 mRNA expression in flathead was observed between the four polluted sites and the reference site. Previous studies with different organisms suggested that metals such as mercury, arsenic, lead and cadmium exposure can enhance the expression of GRP78 gene (Qian *et al.*, 2001; Shao *et al.*, 2014; Yang *et al.*, 2014; Zhu *et al.*, 2013). The up-regulation of GRP78 is considered to be a result of unfolded protein response during Endoplasmic reticulum (ER) stress (Lee, 2005). A number in vitro studies have reported that Hg and As exposure could induce the expression of GRP78. For instance, it has been demonstrated that GRP78 mRNA was up-regulated in myogenic cell lines from mouse after 9 hours of methylmercury exposure (Usuki *et al.*, 2008). Similarly, GRP78 mRNA was significantly increased in rat brain after methylmercury injection (Zhang *et al.*, 2013). It has been shown that GRP78 transcripts were dose-dependently induced in the SVEC4-10 cell line by As exposure (Weng *et al.*, 2014). These studies suggest that both As and Hg are ER stress inducers and could up-regulate GRP78 mRNA level. Not unexpectedly, these findings were consistent with our study, in which GRP78 mRNA levels were increased in fish with relatively high levels of Hg or As. Based on these results and previous studies on GRP78 gene, it reasonable to hypothesise that increasing GRP78 transcripts in flathead liver is a strategy for cells to against ER stress caused by Hg or As exposures. In the present study, the measurement of this gene mRNA has the potential to be a biomarker in flathead. However, dose-response relationships between the hepatic GRP78 mRNA level and Hg or As levels in fish needs to be confirmed.

Hepatic Diablo/SMAC1 and Diablo/SMAC2 mRNA levels were both significantly elevated in the fish from almost all of the polluted sites compared to those from reference site. The

Diablo/SMAC is a major apoptotic executioner that released from mitochondria and could activate caspase-9 by inhibiting its repressor (Du *et al.*, 2000). A mesocosm study in flounder (*Platichthys flesus*) showed that up-regulation of hepatic Diablo/SMAC homolog is associated with long-term polluted estuarine sediment exposure, suggesting the induction of its mRNA is an indication of cellular damage (Leaver *et al.*, 2010). A follow-up study further confirmed this finding where two isoforms of Diablo/SMAC gene were isolated from the same species and these two genes respond differently to pollutants (Zacchino *et al.*, 2012). In the present study, two isoforms of Diablo/SMAC gene were also identified in sand flathead. The up-regulation of these two genes in flathead from some of the polluted sites, suggests both of these isoforms could be associated with heavy metal exposure. The specific reason of increase Diablo/homolog transcripts are not known, but it is likely due to cellular damage which can be caused by high levels of environmental As and Hg exposure.

The significant positive correlation between the transcripts of Diablo/SMAC1 and Diablo/SMAC2 gene suggests these two genes have a similar expression pattern however the up-regulation of Diablo/SMAC1 transcripts was greater than Diablo/MSAC2. This indicates Diablo/SMAC1 gene is more sensitive than Diablo/SMAC2 gene response to environmental metal exposure. In contrast to our findings, a previous study reported that Diablo/SMAC2 gene is more sensitive than Diablo/SMAC1 gene in European flounder (*Platichthys flesus*) from distinct contaminated estuaries (Zacchino *et al.*, 2012). The specific reasons of these different observations are unknown, but it has been suggested that large inter-individual variability may confound the gene expression result of Diablo/SMAC1 in flounder (Zacchino *et al.*, 2012). Taken together all these observations indicate Diablo/SMAC genes are potential biomarkers and worthy for further study in environmental monitoring.

Gene expression data showed there were no significant differences for all candidate biomarker genes between the fish from reference site and fish from Ralphs Bay where the fish had the highest level of Hg. It has been proposed that environmental factors including diet, temperature and salinity could mask contaminants signals or confound the gene expression analysis (Thain *et al.*, 2008) . Previous study in this area reported that there are distinct physical differences between Ralphs Bay and other areas of Derwent estuary (Verdouw, 2008). For instance, Ralphs Bay has relatively shallow water and higher temperature and salinities compared to other regions of Derwent estuary (Jones *et al.*, 2003). These physical attributes may also result in different prey group of flathead inhabiting in Ralphs Bay (Verdouw, 2008). It is reasonable to hypothesise that the unique environmental differences of Ralphs Bay may impact the gene expression results. Therefore, further studies are needed to evaluate impacts of environmental variables on molecular biomarkers.

The influence of gender difference on the induction of these potential biomarker genes was not assessed due to the fish gender was not identified. The effects of sex on these gene responses to environmental pollutants are unknown. This should be further examined in the sexually mature fish.

### 4.5.2 Liver histopathology

There was no significant difference in prevalence of MMCs in fish liver with regard to different sites. MMCs are groups of melanin-containing cells that often found in the haemopoietic tissues of fish (Agius, 1980). The appearance of MMCs in flathead liver is brown or dark brown colour after H & E staining and they are occasionally capsuled with connect tissue or surrounded with lymphocytes. The one of the major roles of MMCs is accumulation of foreign materials such as products from detoxification (Agius and Roberts, 2003). The number, size and morphology of

MMCs could be influenced by various factors including the environmental stress (Agius and Roberts, 2003). Therefore, the measurement of the density or prevalence of MMCs has been proposed as a biomarker for contamination exposure (Fournie *et al.*, 2001; Wolke, 1992). It also has been reported that MMCs are associated with age changes (Agius, 1981). In addition, the parameters of MMCs could also be impacted by the metal content in the diet (Manera *et al.*, 2000). In the present study, our data suggest the prevalence of MMCs could not reflect the heavy metal pollutants, the size and density of MMCs should be measured when further validate the usefulness of MMCs as biomarker.

The prevalence of inflammation was higher in all polluted sites than those from reference site. In general, inflammation is considered to be a normal liver condition and it is commonly observed in wild fish (Fricke *et al.*, 2012). In the present study, inflammation occurred frequently in association with MMCs, granuloma, blood vessel and liver parasites, suggesting it can be caused by multiple factors. However, the prevalence of inflammation in fish from polluted sites was more commonly observed than in fish from the reference site, suggesting this pathology may be associated with environmental stressors.

The infection of digenea parasites was observed in fish from four polluted sites of the Derwent estuary while no parasites were found in fish from reference site. Digenea infections in wild fish are very often observed in digestive tracts such as liver and bile ducts (Bruno *et al.*, 2006). Liver tissue damage was frequently found in the surrounding area of parasites due to the parasites motion. In addition, these parasites may also cause other non-specific lesions such as inflammation and granuloma. The higher prevalence of parasite infection in fish from polluted water may due to the pollutants enhancing the susceptibility of fish to parasitic infections (Moller, 1987).

No significant difference in prevalence of granuloma was found in the liver samples of fish among all the sites. A granuloma is characterized by compact group of inflammatory cells capsuled with multiple layers of connective tissues. It is general believed that the major function of granuloma is to wall off irritants in liver such as various pathogens infection (Adams, 1976; Fricke *et al.*, 2012). The exact etiologies of this lesion are normally hard to identify. In the present study, it is hard to link this lesion with anthropogenic factors.

The prevalence of bile duct fibrosis (peribiliary fibrosis) was higher in fish from four polluted sites of the Derwent estuary compared to those from the reference site, and this lesion was not observed in fish from a polluted site. Previous studies showed the peribiliary fibrosis is often associated with fish age, liver trace metal level, or pathogen infection (Bunton *et al.*, 1987; Fricke *et al.*, 2012; Mikaelian *et al.*, 2002). This lesion was characterized by multiple layers of connective tissue around bile duct. The Peribiliary fibrosis is thought to be a healing condition in fish (Feist *et al.*, 2004). The data suggested environmental metal exposure may have contributed to the development of this lesion.

In the present study, the predominant liver lesions observed were non-specific hepatic alterations, which can be caused by multiple factors (Bucke and Feist, 1993). Therefore, it is difficult to establish the links between the heavy metals and these histological changes. It is worthy note that the prevalence of some lesions appeared to be considerably different between the fish from Derwent estuary and reference site in general. It has been suggested that the increased prevalence of non-specific lesions can serve as the generic stress indicator for the changes of aquatic ecosystem (Fricke *et al.*, 2012). As such, histological alterations in Derwent estuary sand flathead seem to be related with environmental stressors. However, further systemic, long-term

and large scale of monitoring studies on flathead is required to validate the usefulness of liver histology as biomarker.

## 4.6 Conclusion

In conclusion, a GRP78 cDNA and two Diablo/SMAC cDNAs were cloned from sand flathead liver. Hepatic relative gene expression of these genes was analysed in fish from different polluted sites and a reference site, suggesting the regulation of these genes is associated with environmental metal exposure and these genes are potential biomarkers for future environmental monitoring. For liver histological assessment, the absence of early non-neoplastic toxicopathic lesions, foci of cellular alteration (FCA) and neoplasms indicates the effect of environmental heavy metal on the organ level of sand flathead is not a significant concern. However, the relatively higher prevalence of some non-specific lesions in flathead from polluted sites suggests this species is sensitive to environmental stressors. Overall, the data suggest that southern sand flathead is a reliable indicator species, further systemic, long-term and large scale of monitoring studies on flathead is required to validate the usefulness of these biological responses as biomarker.

Chapter 5 : Using multi-biomarker approach to assess the effects of

pollution on sand flathead (*Platycephalus bassensis*) from Port Phillip

Bay, Victoria, Australia

## 5.1 Abstract

Hepatic gene expression and liver histology were assessed in sand flathead (*Platycephalus bassensis*) from a number of locations in Port Phillip Bay, Victoria, Australia. Four sets of gene including thyroid related genes (D1, D2, TTR, TR$\alpha$ and TR$\beta$), metal metabolism related genes (MT, MTF1, TF, Ferritin and FPN1), apoptosis related genes (Diablo/SMAC1, Diablo/SMAC2 and CYP1A) and endoplasmic reticulum stress biomarker gene (GRP78) were examined in female flathead using qRT-PCR. There was significant spatial difference for the relative expression of thyroid hormone receptor gene (TR$\alpha$ and TR$\beta$), transthyretin gene (TTR), ferrorportin-1 gene (FPN1), Diablo/SMAC1 and GRP78. TR$\beta$ and Diablo/SMAC1 gene expression was significantly up-regulated in fish from all polluted sites compared to those from the reference site. In this study Corio Bay was the most affected site. The transcripts of TR$\alpha$ and FPN1 were significantly increased in flathead from this area, while the hepatic mRNA of TTR and GRP78 were significantly decreased in those fish compared to fish from the reference site. In addition, positive correlations were observed between Diablo/SMAC1 and CYP1A, D2 and TR$\beta$, TR$\alpha$ and TR$\beta$. A negative correlation was observed between Diablo/SMAC1 and GRP78. Three major types of non-specific liver lesions and melano-macrophage centers (MMCs) were observed and recorded in the sampled fish. These lesions included bile duct fibrosis, inflammation and granuloma. The prevalence of granuloma in fish liver samples varied significantly with regard to sampling sites. There was no significant difference in prevalence of bile duct fibrosis, inflammation and MMCs in fish from different sites. This study showed that application of pathway based biomarker genes and histopathology could provide comprehensive and integrated information on the impact of environmental pollutants on fish.

## 5.2 Introduction

The assessment of anthropogenic impact on sentinel fish species could provide valuable information regarding the potential effects at the higher levels in aquatic ecosystem (van der Oost *et al.*, 2003). Biomarkers are usually employed to assess the effects of pollution on individual fish before the fish population is affected (Daniel *et al.*, 2008). Biomarkers have been defined as measurements indicating biochemical or cellular changes due to the presence of toxicants (van der Oost *et al.*, 2003). Biomarkers are strictly based on sub-organism biological response levels to pollutants exposure, including molecular level, subcellular or cellular level and organ or tissue level. These biological responses are sensitive, easily detected and they have been regarded as early warning signals of changes that may impact on the whole ecosystem (Lam, 2009). Gene expression analysis (molecular biomarkers) and histology (histological biomarkers) are commonly used to identify changes at molecular, cellular and organ levels in the sentinel species (Sanchez and Porcher, 2009). As such, a multi-biomarker approach is usually applied in assessing the impacts of complex mixture of pollutants in aquatic environment, as it can provide more comprehensive and integrative information.

Port Phillip Bay is a shallow (8 m in depth on average) and large (9800 $km^2$ catchment area) land-locked bay in the south coast of Victoria, Australia. The water exchange is very limited due to a narrow entrance (approximately 3 km) with Bass Strait (Fabris *et al.*, 1992; Harris and Scientific, 1996). The city of Melbourne (population 4 million) is on the northern edge the Port Phillip Bay and the city of Geelong (population 200, 000) is located on the west of Port Phillip Bay. In addition to these densely populated surrounding areas, there are also extensive port facilities, manufacturing industries and chemical industries (Gagnon and Holdway, 2002). As such, pollutants, including heavy metals, organochlorines and hydrocarbons, could easily

accumulate in water, sediments and biota of Port Phillip Bay from different sources. Port Phillip Bay supports a high level of commercial and recreational fishing activities (Holdway *et al.*, 1994). There has been growing public concern about the adverse effects of these pollutants to the biota of Port Phillip Bay (Phillips *et al.*, 1992).

Southern sand flathead (*Platycephalus bassensis*) has been successfully used to assess the adverse effects of contaminants in both laboratory and field studies (Brumley *et al.*, 1995; Gagnon and Holdway, 2002; Holdway *et al.*, 1994). Sand flathead is a bottom-dwelling, sedentary finfish; in a relatively high position in the food chain; easily caught and extensively distributed in Southern Australia (Ayling *et al.*, 1975) and as a result it appears to be an ideal sentinel species. A number of investigations have demonstrated that southern sand flathead is a reliable sentinel species for monitoring biological effects of pollutants in Port Phillip Bay (Gagnon and Holdway, 2002; Holdway *et al.*, 1994). For instance, a long term study has measured the activities of two hepatic enzymes (ECOD and EROD) and a serum enzyme (s-SDH) on the flathead from different sites of Port Phillip Bay, suggesting the enzyme induction generally happened in fish from the sites that were close to highly industrial and urbanized areas (Holdway *et al.*, 1994). Another follow-up study has also successfully applied these biochemical biomarkers (EROD and SDH) to assess the health status of flathead inhabiting Port Phillip Bay (Gagnon and Holdway, 2002). However, the information regarding the impacts of anthropogenic activities at organ levels or molecular levels of this sentinel species from Port Phillip Bay is scarce.

Some of the contaminants present in Port Phillip Bay such as PCB and PAHs have strong thyroid hormone disrupting effects in fish (Brown *et al.*, 2004). Thyroid hormone system, one of fundamental endocrine systems, is involved in many biological activities, such as growth,

reproduction and normal metabolism (Power *et al.*, 2001; Zoeller *et al.*, 2007). Therefore, the disrupting effects of thyroid hormone system could severely impact on the wellbeing of individual fish and even on the sustainability of fish population. In addition to the thyroid hormone system, a few recent studies suggested exposure to multiple pollutants such as PCB and PAHs could cause severe cellular damage on wild fish liver and measurement of Diablo/SMAC gene homologs, a pro-apoptotic gene, has been proposed as a novel molecular biomarker (Falciani *et al.*, 2008; Leaver *et al.*, 2010; Zacchino *et al.*, 2012). Apart from these organic pollutants, high levels of trace metals, such as Cd, Cu, Pb and Hg could be detected in the sediment, water and biota Port Phillip Bay (Fabris *et al.*, 1992; Phillips *et al.*, 1992). Previous studies have shown these heavy metals interfere with the homeostasis of essential metals, such as iron and zinc (Goyer, 1997; Martelli *et al.*, 2006). However, the interactions between the metal metabolism-related genes and the contaminants mixture in the wild fish are not clear. The exposure to some of these heavy metals could induce the endoplasmic reticulum (ER) stress in fish (Zhong *et al.*, 2014). The measurement of the molecular chaperone GRP78 gene has been using as a molecular biomarker of ER stress (Lee, 2005). This study concentrated on the genes that are involved in thyroid hormone system, apoptosis, metal metabolism and endoplasmic reticulum stress.

Besides the molecular biomarkers, histological biomarkers are robust tools to assess the effects of pollutants (Bernet *et al.*, 1999). The associations between certain types of lesions and chemical pollutants have been observed in field studies (Bernet *et al.*, 1999; Feist *et al.*, 2004). Fish liver, the main organ metabolising xenobiotics, has been extensively used for environmental monitoring (Brusle and Anadon, 1996; Fernandes *et al.*, 2008; Gul *et al.*, 2004). Fish liver lesions can be reliable and sensitive biomarkers of exposure to marine contaminants (Stentiford

*et al.*, 2009; Stentiford *et al.*, 2010). These histological biomarkers are more ecologically relevant compared to molecular biomarkers, while molecular biomarkers are more sensitive and specific (van der Oost *et al.*, 2003). Therefore, the combination of histological biomarkers and molecular biomarkers would provide a more comprehensive assessment regarding the impacts of pollutants on fish.

The aims of this study were to determine the hepatic relative gene expression levels of pathway based biomarkers that are involved in thyroid hormone system, metal metabolism, apoptosis and endoplasmic reticulum (ER) stress in southern sand flathead from a number of sites with different contaminants loads. Additionally, liver histopathology in these fish was assessed. Lastly, this study makes recommendations of using these multiple biological responses as biomarkers in the future monitoring programmes.

## 5.3 Materials and Methods

### 5.3.1 Sample collection

A total of 96 sand flathead (female = 72; male = 24) were sampled at six sites in Port Phillip Bay including Portsea (female = 16; male = 2), Clifton Springs (female = 7; male = 5), St Leonards (female = 9; male = 4), Corio Bay (female = 11; male = 6), Mordialloc (female = 12; male = 6) and Hobsons Bay (female = 17; male = 1). Portsea, a relatively less polluted site, was selected as the reference site. All the samples were collected during February 2015 (Fig 5.1).

**Figure 5.1 Map of Port Phillip Bay sampling sites.**

For gene expression analyses, a small piece of chopped liver was collected from individual fish and immediately fixed in RNAlater. The samples were incubated in RNAlater at 4 °C overnight and then placed at -20 °C until RNA extraction.

96 fish were used for histopathological assessment. A 1 cm$^3$ liver sample cube was removed from each fish and immediately fixed in 10 % neutral buffered formalin (NBF). After 48 h all the samples were transferred to 70 % ethanol until further processing.

### 5.3.2 Quantitative Real-time PCR

The liver samples for gene expression analysis were selected from Portsea, St Leonards, Corio Bay, Mordialloc and Hobsons Bay (Table 5.1). Samples from Clifton Springs were degraded due to storage in insufficient RNAater volumes and could not be used for gene expression analysis.

**Table 5.1 Morphometric measurements of 35 flathead were used for gene expression analysis.** All data are expressed as means ± SD.

| Location | n | Sex | Standard length(mm) | Weight(g) |
|---|---|---|---|---|
| Hobsons Bay | 7 | female | 231 ± 11.3 | 118 ± 23.0 |
| Mordialloc | 7 | female | 216 ± 9.3 | 104 ± 14.1 |
| Corio Bay | 7 | female | 226 ± 18.5 | 111 ± 30.4 |
| St Leonards | 7 | female | 208 ± 11.5 | 81 ± 15.2 |
| Portsea | 7 | female | 222 ± 6.0 | 96 ± 11.0 |

The total RNA of each sample (around 5 mg) was placed in a 500 µL pre-heated (60 °C) GITC buffer contains 20 mM DTT and disrupted tissue manually with a micro-pestle. The genomic DNA was sheared by vortex and total RNA was released by incubating at 60 °C for 5 min. All the samples were subjected to the silica-column RNA extraction kit (Qiagen, Australia). The genomic DNA was digested by DNase. RNA was purified with the RNA extraction kit (Qiagen, Australia) following manufacturer's instructions. RNA quality was checked on a 1 % agarose bleach gel. 1 µg of total RNA from each sample was used for cDNA synthesis by M - MuLV reverse transcriptase (NEB, Australia) with Oligo (dT) 18 primer and random hexamers.

For gene expression, four sets of genes were selected (Table 5.2). 1. The thyroid related genes, namely, deiodinase type I (D1), deiodinase type II (D2), transthyretin (TTR) thyroid hormone receptor α (TRα) and hormone receptor β (TRβ). 2. Metal metabolism related genes included metal regulatory transcription factor 1 (MTF1), ferroportin-1 (FPN1), Metallothionein (MT) and Transferrin (TF). 3. Cellular damage related genes: the Diablo/SMAC 1, Diablo/SMAC 2 and

cytochrome P4501A (CYP1A). 4. The endoplasmic reticulum (ER) stress biomarker gene GRP78. The real-time PCR was conducted on the iQ5 Real-time PCR Detection System (Bio-Rad, NSW, Australia) in a volume of 10 μL containing 0.4 μM of each primer, 5 μl 2 × SYBR mastermix (Bioline, NSW, Australia), and 2 μl of cDNA template. All the samples were assayed in duplicate and the PCR cycling procedure were initial activation of DNA polymerase at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 10 s. The melting curve analysis was performed at the end of the cycling protocol to ensure the amplification specificity. The data analysis of qPCR results was conducted using qBasePlus Biogazelle software (Hellemans *et al.*, 2007). All the target genes expression in different samples was normalized against the mean of two reference genes (EF1α and RPL13A). The geometric mean was used to normalize relative quantity (MNRQ) was calculated for each gene of grouped 7 biological replicates from the site. The expression level of all the genes was compared with that of expression in fish from Portsea.

**Table 5.2 Primers used for quantification of the mRNA expression by Real-time PCR**

| Gene | Oligo name | Primer DNA Sequence (5'→3') |
|---|---|---|
| MTF1 (KU313702) | qMTF1F | AGTGTGATGTGCAAGGCT |
| | qMTF1R | GATTCACAGTTGAATGTCTTGCCCGTG |
| FPN1 (KU313700) | qFPN1F | CAGAACAGTTGCGTCATC |
| | qFPN1R | GGTCAGAATCCATCCATTG |
| TF (KU313704) | qTFF | CCATTAGAGTGCCAGAAT |
| | qTFR | TCTCATCCATTGCTCATT |
| Ferritin (KU313699) | qFerritinF | TTGAGAAGAACGTGAACC |
| | qFerritinR | CCAGGAAATCGCACATAT |
| MT (KP893712) | qMTF | ATCCGGCTGCACCAAATG |
| | qMTR | GTTTACTGACAGCAGGTGGT |
| RPL13α (KU313703) | qRPL13F | ATCTTCTGGAGGACTGTCA |
| | qRPL13R | GAACACCTTCAGCCTCTC |
| D1 (KP893709) | qD1F | ACAGATCCTGGTTCAGAA |
| | qD1R | ATACTTCACGGCAGACAT |
| D2 (KP893710) | qD2F | GCACTCAACTCCAAAGTAG |
| | qD2R | ACCAGGTGACACATTAGT |
| TTR (KP893711) | qTTRF | AGGTCCATAATCTCATCAC |
| | qTTRR | CTCATCTTCCCAGTTAGC |
| TRα (KP893714) | qTRαF | CCATCCAGAAGAACCTCCA |
| | qTRαR | GTTGCGGGTGATCTTGTC |
| TRβ (KP893715) | qTRβF | TAAGCCTGAGGATATTGG |
| | qTRβR | TTTGTAAACTGACTGAAGG |
| CYP1A (KP893713) | qCYP1AF | ATGACAAGGACAACATTC |
| | qCYP1AR | ATCTGACATCTGGACATT |
| EF1α (KP893716) | qEF1αF | TTGGAGTCAACAAGATGG |
| | qEF1αR | GATGTAGGTGCTCACTTC |
| GRP78 (KU313701) | qGRP78F | TGAAGAAGTCTGACATTG |
| | qGRP78R | GCCATTGAATAACTCCTT |
| Diablo/SMAC1 (KU323388) | qSMAC1F | CAGAGGAGGATTCATATC |
| | qSMAC1R | AAGTTGACAGCATTTATC |
| Diablo/SMAC2 (KU323389) | qSMAC2F | CTGTGTGCTATACCTTTC |
| | qSMAC2R | TGAACTGTCTGTAACCAA |

### 5.3.3 Liver histology

Liver from 96 sand flathead were assessed for histology. The samples were dehydrated by standard procedures, and embedded in paraffin. The samples were sectioned at 5µm on microtome (Microm HM340, Heidelberg, Germany), mounted on microscope slides and dried in an incubation oven at 37°C for overnight. Sections were stained with hematoxylin and eosin stain (H & E) and observed under microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

### 5.3.4 Statistical analysis

One-way ANOVA followed by a Tukey post hoc test was carried out to assess differences between polluted sites and the reference site. The data of gene expression were shown as the geometric means normalized relative quantity (MNRQ) ± 95% confidence interval. The Pearson's correlation coefficient was used to assess the relationships between relative gene expression after all samples were pooled together. The correlation was carried out using GraphPad Prism version 6.0 (GraphPad software Inc). The prevalence of liver histological conditions from sampling locations was expressed as percentage of fish affected. The difference in the prevalence of liver lesions and MMCs in fish from different sites were assessed using the Pearson Chi-square test (IBM SPSS statistics 23.0). Differences were considered statistically significant at $P < 0.05$.

## 5.4 Results

### 5.4.1 Gene expression and relationships between gene transcripts

Hepatic expression levels of TRβ mRNA were significantly up-regulated in flathead sampled at St Leonards, Corio Bay, Mordialloc and Hobsons Bay compared to the reference site (Portsea)

($P < 0.05$, Fig 5.2A). TRα mRNA levels were significantly higher in flathead from Corio Bay ($P < 0.05$) and Mordialloc ($P < 0.05$) compared to fish from the reference site (Fig 5.2A). TTR mRNA expression was significantly lower in fish from Corio Bay than that from the reference site ($P < 0.05$, Fig 5.2A). There was no significant difference in the expression levels of D1 and D2 between St Leonards, Corio Bay, Mordialloc or Hobsons Bay compared to the reference site (Portsea) for (Fig 5.2A).

For the iron homeostasis related genes, the expression levels of FPN1 mRNA were significantly higher in flathead from Corio Bay than fish from the reference site ($P < 0.05$, Fig 5.2B). There was no significant difference in hepatic mRNA levels of MTF1, Ferritin, TF and MT in flathead from St Leonards, Corio Bay, Mordialloc or Hobsons Bay compared to the reference Portsea (Fig 5.2B).
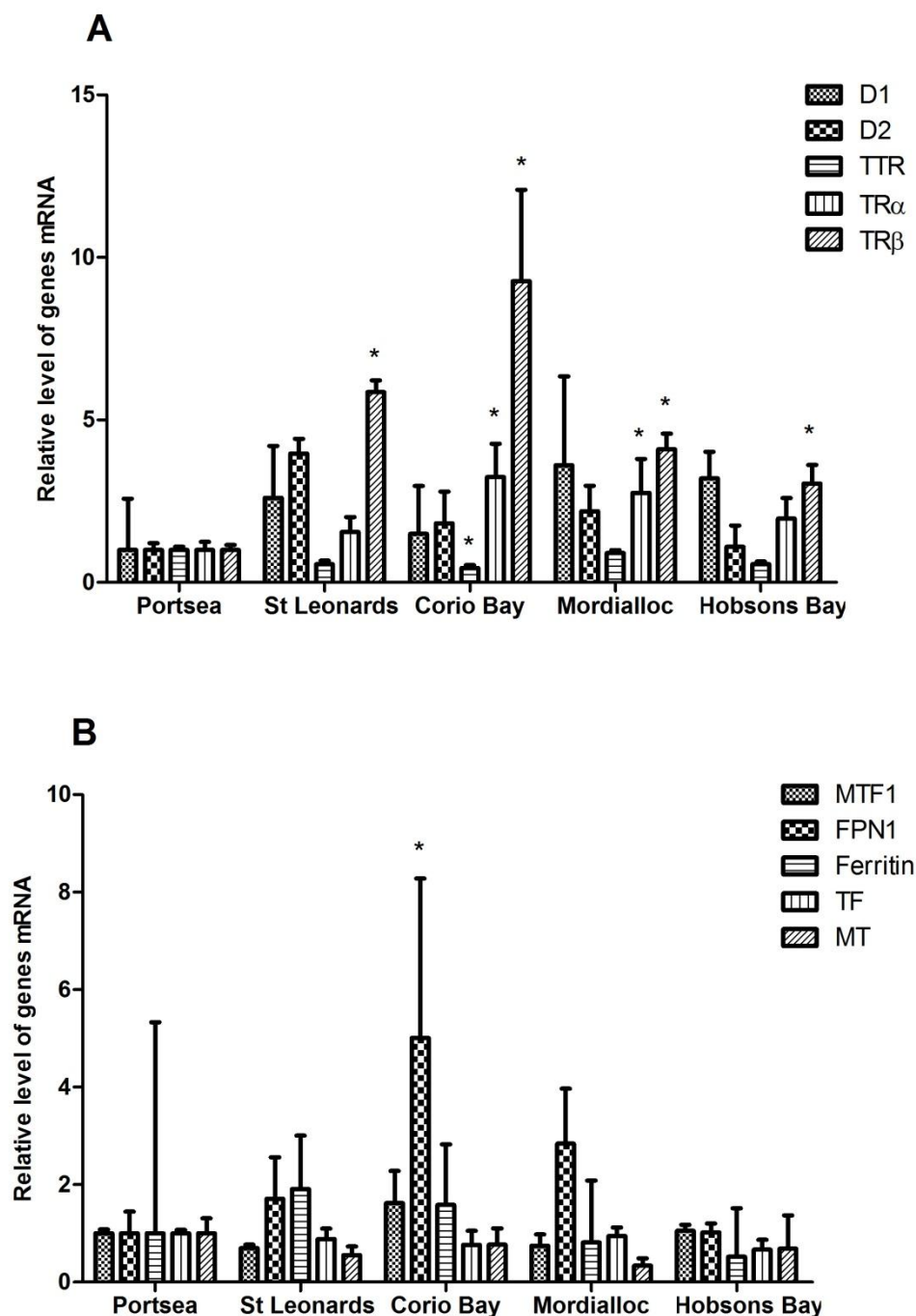
**Figure 5.2** Hepatic mRNA levels of thyroid related genes (A) and metal metabolism related genes (B) relative to mean of two reference genes (EF1α and RPL13) analyzed by qPCR in flathead captured at five sites of Port Phillip Bay. The values are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval. Significant difference between means and fish from reference site (Portsea) is indicated with an asterisk ($P < 0.05$)

The relative level of Diablo/SMAC1 mRNA was increased in liver of flathead from St Leonards (205 fold), Corio Bay (30 fold), Mordialloc (404 fold) and Hobsons Bay (43 fold) compared to Portsea ($P < 0.05$, Fig 5.3A). There was no significant difference between the fish from Portsea and those from other four sites for the expression levels of Diablo/SMAC2 (Fig 5.3B). Similarly CYP1A hepatic transcriptional levels were not significantly different in flathead at different sites (Fig 5.3C). For GRP78, mRNA levels were significantly down-regulated in fish from St Leonards and Corio Bay compared to those from reference site ($P < 0.05$, Fig 5.3D)
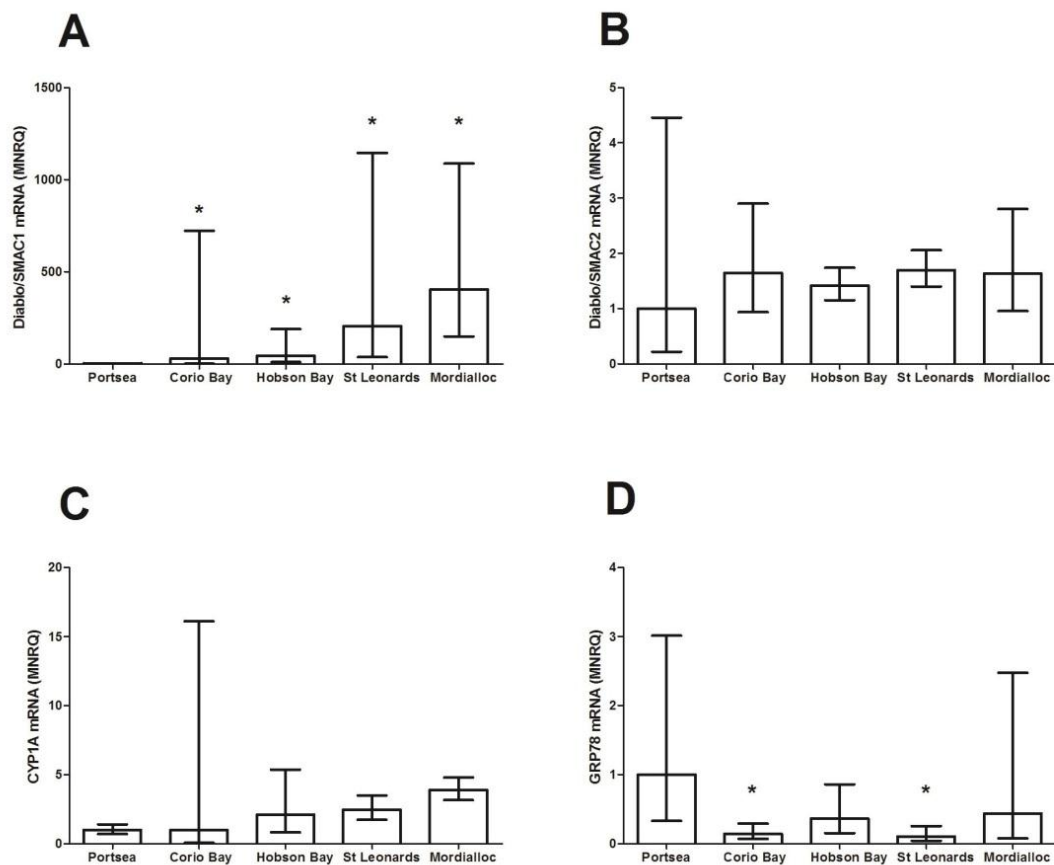


**Figure 5.3** Hepatic mRNA levels of Diablo/SMAC1 (A), Diablo/SMAC2 (B), CYP1A (C) and GRP78 (D) relative to mean of two reference genes (EF1α and RPL13) analyzed by qPCR in flathead captured at five sites of Port Phillip Bay. The values are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval. Significant difference from fish from reference site is indicated with an asterisk (P < 0.05)

Statistically significant positive correlations between expression of Diablo/SMAC1 and CYP1A (r = 0.6812, $P < 0.0001$, Fig 5.4A), TRβ and D2 (r = 0.6481, $P < 0.0001$, Fig 5.4B), TRβ and TRα (r = 0.5198, $P = 0.0016$, Fig 5.4C) were found in this study. A significant negative correlation was observed between the mRNA expression of GRP78 and Diablo/SMAC1 (r = -0.3563, $P = 0.0386$, Fig 5.4D).
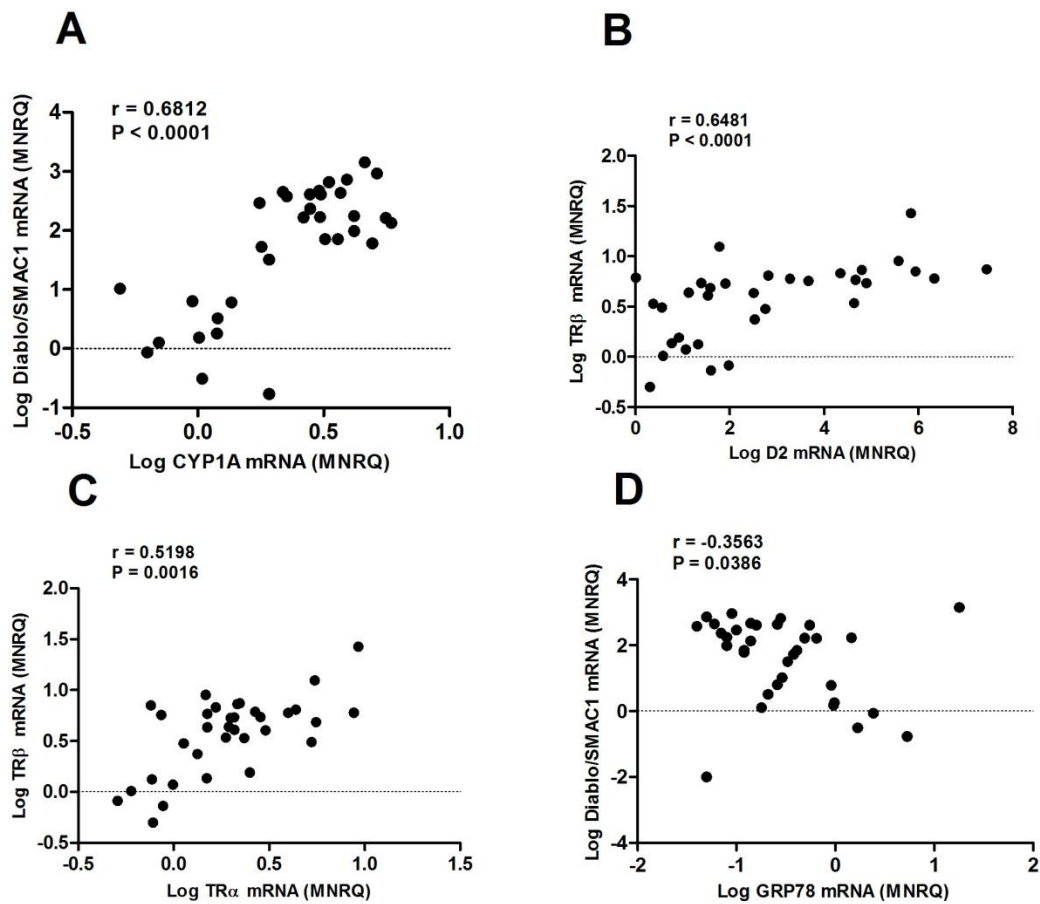


**Figure 5.4 Correlation between different genes expression in sampled fish.** Positive correlations were observed between Diablo/SMAC1 and CYP1A (A) (r = 0.6812, $P < 0.0001$), D2 and TRβ (B) ( r = 0.6481, $P < 0.0001$). TRβ and TRα (C) (r = 0.5198, $P = 0.0016$) and a negative correlation between Diablo/SMAC1 and GRP78 (D) (r = -0.3562, $P = 0.0386$).

### 5.4.2 Liver histology

The prevalence of three types of major lesions and MMCs is shown in Table 5.3. No significant difference was found in the prevalence of MMCs among the different sites ($\chi^2 = 3.458$, *d.f.* = 5, *P* = 0.630). MMCs were relatively common in flathead liver. The MMCs were characterized by clusters of golden or dark brown pigmented cells and occasionally were surrounded with a thin layer of fibrous tissue and lymphocytes (Fig 5.5A). The prevalence of MMCs was 69% in fish from St Leonards, 67% in fish from Hobsons Bay, 61% in fish from Mordialloc, 58% in fish from Clifton Springs, 47% in fish from Corio Bay, and 44% of fish from the reference site. For inflammation, clusters of lymphocytes occurred around bile duct, MMCs, granulomas and blood vessels (Fig 5.5B). Moreover, diffused lymphocytes were found infiltrating the stroma of hepatocytes. There was no significant difference in the prevalence of inflammation among the different sites ($\chi^2 = 7.293$, *d.f.* = 5, *P* = 0.200). The prevalence of inflammation was 71% in fish caught at Corio Bay and 61% at Hobsons Bay, while it affected only 25% of fish sampled at Clifton Springs. Most granulomas in flathead liver were formed by eosinophilic material encapsulated with fibrous tissue (Fig 5.5C). The prevalence of granulomas varied significantly in fish from different sites ($\chi^2 = 18.68$, *d.f.* = 5, *P* = 0.002). This type of granuloma was observed frequently, particularly in fish from St Leonards (69%) and Portsea (44%). Bile duct fibrosis was characterized by proliferation of connective tissue surrounding bile ducts (Fig 5.5D). No significant difference in prevalence of this lesion was observed in fish from different sites ($\chi^2 = 6.428$, *d.f.* = 5, *P* = 0.267). It affected 50% of fish from Portsea and Hobsons Bay, 31% of fish from St Leonards, 42% of fish from Clifton spring, 47% of fish from Corio Bay but only 17% of fish from Modialloc.

**Table 5.3 The prevalence (%) of liver histological conditions in 96 flathead sampled at six locations from Port Phillip Bay. BF:** Bile duct fibrosis. **MMCs:** Melano-macrophage centres.

| Location | n | MMCs | Inflammation | BF | Granuloma |
|---|---|---|---|---|---|
| Clifton Springs | 12 | 58 | 25 | 42 | 17 |
| St Leonards | 13 | 69 | 46 | 31 | 69 |
| Corio Bay | 17 | 47 | 71 | 47 | 24 |
| Mordialloc | 18 | 61 | 44 | 17 | 11 |
| Hobsons Bay | 18 | 67 | 61 | 50 | 17 |
| Portsea (reference) | 18 | 44 | 44 | 50 | 44 |
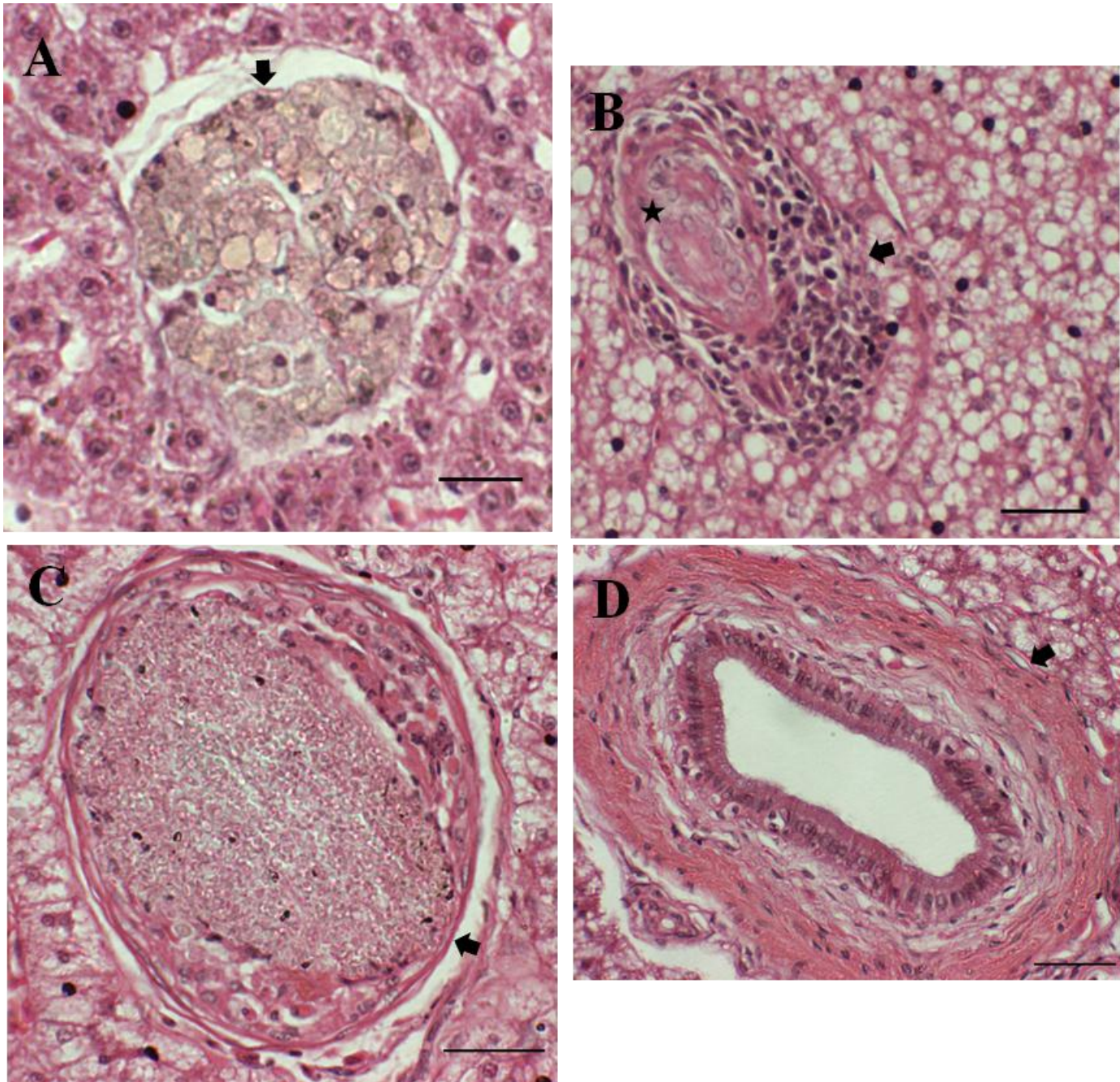
**Figure 5.5 Overview of sand flathead liver histology.** (A) Melano-macrophage centre (arrow). (B) Inflammation (arrow) occurred around bile duct (star). (C) Granuloma (arrow). (D) Bile duct fibrosis (arrow). Bar (A, B, C, D) = 25 µm.

## 5.5 Discussion

### 5.5.1 Thyroid hormone related genes

Thyroid hormone receptor genes (TRα and TRβ) transcripts were up-regulated in sand flathead from most of the sites, which were previously reported to be polluted (Nicholson *et al.*, 1994; Phillips *et al.*, 1992). Levels of TRα and TRβ mRNA were consistently elevated in flathead from Corio Bay and Mordilloc. It is generally accepted that thyroid disruption occurs at several points of thyroid hormone pathway including the synthesis of thyroid hormone, the metabolism of thyroid hormone, the transportation of thyroid hormone and the receptors of thyroid hormone (Patrick, 2009). In the present study, genes involved in thyroid hormone metabolism (D1 and D2); transportation (TTR) and thyroid hormone receptors (TRα and TRβ) were examined in flathead liver. Throughout the thyroid hormone pathway, the thyroid hormone receptor genes appeared to be the most affected in sand flathead from Port Phillip Bay. The up-regulation of thyroid hormone receptor genes is most likely due to the strong thyroid disrupting effects of predominant pollutants such as PCBs, PAHs and organochlorine pesticides in the sampled sites (Maher and Aislabie, 1992; Phillips *et al.*, 1992; Smith *et al.*, 1991). Previous studies have shown that these pollutants have strong thyroid disrupting effects in fish (Brown *et al.*, 2004). For instance, thyroid disrupting effects of PCBs have been assessed in rainbow trout (*Oncorhynchus mykiss*) and results indicated that PCBs were associated with the changes of plasma thyroid hormone level (Leatherland and Sonstegard, 1978, 1980). Although the precise mechanisms of how PCBs disrupt thyroid hormone system remain unknown, it is commonly accepted that these pollutants could alter intracellular thyroid hormone availability or act directly

on the thyroid hormone receptors (Darras, 2008). In our study, increased transcripts of thyroid hormone receptor genes were found in flathead from of the sites which were previously reported as contaminated (Phillips *et al.*, 1992). Therefore, it is reasonable to hypothesise that up-regulation of thyroid hormone receptor genes are associated with organic pollutants in Port Phillip Bay.

TRβ gene appeared to be more sensitive than TRα in the liver of flathead. In the present study, TRβ mRNA was up-regulated in flathead from all the sites previously reported as polluted (Phillips *et al.*, 1992), while TRα mRNA was only increased in fish from two of those sites. Moreover, there was a strong positive correlation between these two genes when all samples were pooled together. This suggests TRα gene has similar expression trend as TRβ gene in the flathead from Port Phillip Bay. For fish TRα and TRβ genes, the difference may due to TRα expression is organ or tissue-specific (Li *et al.*, 2009). The higher sensitivity of TRβ than TRα to the pollutants exposure was observed in our previous study on flathead from a metal polluted estuary (Chapter 2). Hepatic TRβ transcripts were increased in flathead with higher arsenic concentrations in muscles, while the mRNA of TRα was not induced. These results suggest measurement of TRβ hepatic mRNA could serve as biomarker to identify the thyroid disrupting chemicals in sand flathead.

The mRNA of TTR gene was significantly decreased in fish from Corio Bay compared to reference site. A number of studies found that organic pollutants exposure such as polybrominated diphenyl ethers (PBDEs) could result in down-regulation of TTR gene in the embryo, larvae and adult of zebrafish (*Danio rerio*) (Chen *et al.*, 2012; Yu *et al.*, 2010; Yu *et al.*, 2011). Port Phillip Bay was reported to have the highest of total PBDEs concentration in

Australian aquatic environments (Symons *et al.*, 2006). The anthropogenic, geological and physical factors of Corio Bay may contribute the higher accumulation of such pollutants in this area than the reference site. Therefore, it is reasonable to hypothesise that the decrease of TTR gene mRNA level in flathead may be due to the high level of organic pollutants such as PBDEs exposure in Corio Bay.

There was no significant difference for transcripts of D1 and D2 gene in flathead, indicating the transcripts of these two genes were not impacted by pollutants. A positive correlation was found between mRNA of D2 gene and TRβ. This would imply a similar expression pattern of these two genes in fish liver. In contrast to our results, a field study focused on walleye (*Sander vitreus*) showed that D1 gene transcripts were not changed in fish from a polluted site compare to fish from the reference site, whereas D2 gene mRNA level was increased in fish from polluted site (Picard-Aitken *et al.*, 2007). Hepatic mRNA of D2 gene was not modified in killifish (*Fundulus heteroclitus*) from a polluted estuary (Meyer *et al.*, 2005). Previous studies in rainbow trout (*Oncorhynchus mykiss*) and killifish suggested the hepatic D1 and D2 mRNA levels could be influenced by thyroid hormone ($T_3$ or $T_4$) level (Bres *et al.*, 2006; Garcia *et al.*, 2004) Therefore, additional endpoints such as measurement of plasma thyroid hormone level may be helpful to interpret the regulations of D1 and D2 gene in fish from polluted water.

Overall, the thyroid hormone receptors and transthyretin were different on transcriptional level in flathead sampled at different sites of Port Phillip Bay. However, further studies including measurement of plasma thyroid hormone level, histological assessment of fish thyroid follicle and chemical analysis as well as controlled laboratory experiments or measurements of pollutant residues in target organs are needed to further assess the risks posed by the pollutants in Port Phillip Bay.

### 5.5.2 Metal metabolism related genes

The metal metabolism related genes including metal-regulatory transcription factor 1 (MTF1), metallothionein (MT), transferrin (TF), ferritin and ferroportin 1 (FPN1) were tested in this study. Among these genes, only the FPN1 mRNA was increased in the fish from Corio Bay compared to fish from reference site. Ferroportin 1 is the only known cellular iron exporter in vertebrates and is essential for iron homeostasis (Donovan *et al.*, 2005). Ferroportin 1 gene (FPN1) mRNA could be induced by transition metal exposures such as cadmium, zinc, manganese, and copper in mammalian cells (Park and Chung, 2009; Troadec *et al.*, 2010). In fish, information on FPN1 in response to metal exposure is scarce. Considering FPN1 is highly conserved (Cianetti *et al.*, 2010), the up-regulation of this gene in flathead may be induced by environmental metal exposure. Oysters and mussels from Corio Bay had a higher level of cadmium accumulation than those from other sites in Port Phillip Bay (Talbot *et al.*, 1976). In addition, flathead is in a relatively high position in the food chain and as a consequence can accumulate high level of pollutants from its diet consisting of fish, crustaceans and molluscs (Parry and Scientific, 1995). As such, it is reasonable to hypothesise that increase of hepatic FPN1 mRNA in flathead could be a result of environmental cadmium exposure in Corio Bay.

In contrast to our previous results for flathead from a metal polluted estuary (Derwent estuary, Tasmania, see chapter 3) MTF1, FPN1 and TF were not changed in flathead from Port Phillip Bay. These different findings are likely related to the fact that fish from Port Phillip Bay were exposed to lower level of metals than fish from Derwent estuary. Fabris *et al.* (2006) reported that trace metal (As, and Hg) concentrations of flathead from Port Phillip Bay were below the

Food Standards Australia and New Zealand food Standards (FSANZ) (Fabris *et al.*, 2006; Gagnon *et al.*, 2016). Hg concentration of flathead from Derwent Estuary however exceeded the FSANZ (Verdouw, 2008).

### 5.5.3 Diablo/SMAC homolog and CYP1A

Diablo/SMAC1 transcripts were significantly increased in the fish from all polluted sites compared to the fish from reference site; however, there was no significant difference for Diablo/SMAC2 gene transcripts. Diablo/SMAC is a mitochondrial released protein and inhibits the inhibitors of apoptosis proteins (IAP) during apoptosis (Du *et al.*, 2000). In fish, several studies showed that Diablo/SMAC was one of the most promising biomarker genes of pollutant exposure (Falciani *et al.*, 2008; Leaver *et al.*, 2010; Williams *et al.*, 2008). The usefulness of Diablo/SMAC homolog gene as a biomarker gene has been further confirmed in follow-up studies in flounder (*Platichthys flesus*) and eelpout (*Zoarces viviparous*) (Asker *et al.*, 2015; Zacchino *et al.*, 2012). Diablo/SMAC homolog has been proposed as a novel molecular biomarker gene of pollutant exposure. In European flounder, a mesocosm study showed the Diablo/SMAC1 gene mRNA increased and CYP1A mRNA was unchanged after exposed to high level of PAH and PCB polluted sediment (Leaver *et al.*, 2010).These results are very similar to our findings in flathead. However, Diablo/SMAC2 gene was more sensitive than Diablo/SMAC1 gene in the liver of male European flounder after Arochlor 1254 and 3-methylcholanthrene injection (Zacchino *et al.*, 2012). The contrasting findings between this and the present study could be due to many factors such as sex difference, species difference and exposure conditions. Detailed mechanisms of Diablo/SMAC homolog response to pollutants in fish liver need to be investigated in future laboratory studies.

In fish, the measurement of cytochrome P450 1A (CYP1A) mRNA has been extensively used as a biomarker for assessing PAHs and PCBs (Goksøyr, 1995). The hepatic gene expression of CYP1A in female flathead failed to reflect exposure to pollutants in the present study. This suggests gender differences may impact the application of CYP1A gene as a biomarker. Similarly, Koenig *et al.* (2013) found there was no difference in hepatic CYP1A transcripts of female fish (*Alepocephalus rostratus*) from two sites with distinct pollutant loads. However, the mRNA of CYP1A gene was increased in male fish from the heavier polluted site (Koenig *et al.*, 2013). These observations suggest CYP1A gene as a biomarker gene may be more suitable in male fish and the gender differences should be assessed before the application of these biomarker genes in environmental monitoring.

A strong positive correlation was found between transcripts of Diablo/SMAC1 and CYP1A. Zacchino et al (2012) reported that Diablo/SMAC homolog and CYP1A genes transcripts were increased in European flounder liver after exposure to Arochlor and 3MC, but no response to PFOA or lindane treatment were found suggesting Diablo/SMAC homolog is similar to CYP1A genes in that it could be activated by planar polyaromatic and polyhalogenated hydrocarbons exposure (Zacchino *et al.*, 2012). In our study, the strong positive relationship between the mRNA of CYP1A and Diablo/SMAC1 may further confirm this assumption.

Overall, Diablo/SMAC1 is a sensitive biomarker gene of pollutant exposure in female flathead. Sex difference should be considered when Diablog/SMAC homolog is used in the environmental monitoring.

### 5.5.4 Glucose-regulated protein 78 (GRP78) gene

GRP78 gene was down-regulated in sand flathead from St Leonards and Corio Bay compared to fish from reference site. A negative correlation was observed between the transcripts of Diablo/SMAC 1 and GRP78. GRP78, a molecular chaperone located in endoplasmic reticulum, plays important role in folding unfolded protein (Sommer and Jarosch, 2002). GRP78 also has caspase regulator activity. It has been reported that GRP78 protects cell from apoptosis by inhibiting caspase 7 activation (Reddy *et al.*, 2003), while Diablo/SMAC is involved in the activation of caspases (Du *et al.*, 2000). GRP78 and Diablo/SMAC genes are important in inhibition/activation apoptosis during cellular stress (Clark *et al.*, 2008). The up-regulation of Diablo/SMAC gene could lead to the down-regulation of GRP78 gene (Clark *et al.*, 2008). Therefore, the down-regulation of GRP78 gene in fish from St Leonards and Corio Bay is likely due to the increase of transcripts of Diablo/SMAC 1 in these fish. The antagonistic relationship between GRP78 and Diablo/SMAC genes has been observed in a microarray sequence study in an Antarctic fish (*Harpagifer antarcticus*) (Clark *et al.*, 2008). The negative correlation between these two genes transcripts in the liver of flathead is consistent with this observation.

### 5.5.5   Liver histopathology

Three major types of non-specific lesions and MMCs were observed in sampled fish from Port Phillip Bay. These hepatic lesions are associated with inflammatory response (presence of granuloma and inflammation) and proliferative change (peribiliary fibrosis) (Fricke *et al.*, 2012). Although it has been suggested non-specific lesions are not major targets for environmental monitoring (Feist *et al.*, 2004), in this study considerable regional differences in terms of prevalence of some histological conditions were present. It has been suggested that higher prevalence of non-specific lesions and MMCs is an indicator of aquatic environmental stressors including environmental contaminants (Fricke *et al.*, 2012).

There was no significant difference in prevalence of MMCs in the liver of flathead among all the sites. Increased prevalence and size of MMCs has been proposed as a biomarker for assessment of environmental pollutants (Agius and Roberts, 2003). For instance, a higher prevalence of MMCs was found in the liver of starry flounder (*Platichthys stellatus*) from a polluted river than those from reference sites (Pierce *et al.*, 1980). In flounder (*Plathichthys flesus*), the highest prevalence of MMCs was found in flounder from heavily contaminated sites (Stentiford *et al.*, 2003). All these observations suggested that the measurement of MMCs in fish liver is a reliable biomarker. In the present study, however, the prevalence of MMCs in flathead liver was not different with regard to research sites, suggesting the density or size of MMCs should be measured in further studies.

The liver inflammation was frequently observed in sampled fish, particularly in fish from Corio Bay and Hobsons Bay. This lesion is commonly found in demersal fish species from polluted waters (Feist *et al.*, 2015). For instance, a field study reported that the prevalence of liver inflammation in sculpin (*Myoxocephalus scorpius*) followed a gradient of exposure to pollutants (Sonne *et al.*, 2014). Inflammation can be induced by toxicants, pathogen infection or injury (Ferrero-Miliani *et al.*, 2007). Corio Bay and Hobsons Bay are heavily impacted by anthropogenic activities, as these two sampling locations are adjacent to Geelong and Melbourne, respectively. Therefore, the pollutants that released from these two densely populated areas may contribute to this lesion.

The highest prevalence of granulomas was observed in fish from St Leonards. The presence of the granuloma implies irritants in fish livers resisted the acute inflammatory response (Adams, 1976). One of the basic functions of granuloma is to destroy these invading agents (Adams, 1976). The aetiology of granulomas is usually difficult to discern (Fricke *et al.*, 2012). The

increased prevalence of this lesion in fish from this location could be due to two possible reasons: (1) In St Leonards, there may be relatively higher level of particular irritants such as environmental chemicals or pathogens, which could evoke the development of granulomas in flathead liver; (2) the flathead inhabiting in this site could be more susceptible to develop granulomas due to presence of some pollutants (Austin, 1998). The trace metal analysis of these flathead showed that fish from the St Lenonards had the highest level of total arsenic in muscle (Gagnon *et al.*, 2016). This may suggest that the higher prevalence of granuloma is associated with environmental arsenic exposure. However, further investigations are required to confirm this causal relationship.

The bile duct fibrosis (peribiliary fibrosis) was also a common lesion. This lesion was frequently found in fish from Portsea and Hobsons Bay. Perbiliary fibrosis is considered to be a non-specific change and is often found in relation to fish age, hepatic metal level, or parasite infection (Bunton *et al.*, 1987; Fricke *et al.*, 2012; Mikaelian *et al.*, 2002). For instance, a previous study reported that high level of accumulation of hepatic copper in white perch (*Morone americana*) was associated with peribiliary fibrosis (Bunton *et al.*, 1987). Mikaelian et al (2002) reported that the grade of peribiliary fibrosis increased with the age and length of Lake Whitefish (*Coregonus clupeaformis*) from contaminated water (Mikaelian *et al.*, 2002). In Baltic eelpout (*Zoarces viviparous*), this type of lesion was usually associated with biliary parasitism, suggesting perbiliary fibrosis is a natural inflammatory response rather than a consequence of pollutant exposure (Fricke *et al.*, 2012). All these studies suggest both biotic and abiotic factors could contribute to this lesion. In this study, no parasite was observed on the liver sections and the reasons of this lesion in flathead were not obvious. Therefore, further studies are needed to identify the aetiology of bile duct fibrosis.

## 5.6 Conclusion

In conclusion, hepatic gene expression analyses and liver histology were assessed in sand flathead from Port Phillip Bay, Victoria, Australian. The transcripts of TRα, TRβ, TTR, Diablo/SMAC1, GRP78 and FPN1 were altered in flathead sampled from different locations, suggesting the regulations of these genes are associated with the location and potentially environmental pollutant exposure. The prevalence of some non-specific hepatic lesions such as granuloma was significant difference in fish from the sites. This indicates hepatic lesion may be caused by environmental stressors including pollutants. Overall data imply Corio Bay was the most affected site among all the sampled sites in Port Phillip Bay. To the best of our knowledge this is the first comprehensive study utilizing sand flathead for molecular and histological biomarkers assessment in Port Phillip Bay.

# Chapter 6 : Summary and general discussion

## 6. 1    Summary of major findings

The aim of this PhD project was to evaluate pathway based genes as potential molecular biomarkers, and to assess the usefulness of histological changes as histological biomarkers in a finfish species, the southern sand flathead, from the southern Australian waters.

### 6. 1.1   Molecular biomarkers

A total of 14 candidate genes were cloned and examined in fish from both the Derwent estuary and Port Phillip Bay. The results of gene expression analysis are summarized in **Table 6.1**. From these results, the following main conclusions can be drawn:

(1). Transthyretin gene (TTR) and thyroid receptor genes (TR$\beta$ and TR$\alpha$) were the most sensitive genes in thyroid hormone pathway. These genes were modified in fish from the Derwent estuary and Port Phillip Bay.

(2). Different pollutants may target different genes in thyroid hormone pathway. In the Derwent estuary, the hepatic mRNA of D2 gene was down-regulated possibly through decreasing the Se bio-availability caused by environmental Hg exposure. However, there were no changes in the expression of this gene in the fish from Port Phillip Bay.

(3). MTF1, TF and FPN1 exhibited higher sensitivity to environmental heavy metal exposure among all the metal homeostasis-related genes.

(4). Diablo/SMAC1 and Diablo/SMAC2 play similar roles during the metal stress, as up-regulations of Diablo/SMAC1 and Diablo/SMAC2 genes were both observed in sand flathead from some heavy metal contaminated sites

(5). Measurement of Diablo/SMAC1 mRNA had greater sensitivity compared to Diablo/SMAC2. The up-regulation of Diablo/SMAC1 was greater than Diablo/SMAC2 in fish from the Derwent estuary, the Diablo/SMAC1 was significantly up-regulated and no regulation of this gene was observed in fish from Port Phillip Bay.

(6). Up-regulation of GRP78 gene may be due to endoplasmic stress caused by environmental metal exposure. GRP78 gene may be involved in Diablo/SMAC mediated apoptosis. The transcript of GRP78 gene was significantly negatively correlated with Diablo/SMAC1 transcript.

**Table 6.1 Summary of biomarker candidate gene sequences cloned from sand flathead.**

| Name | Abrev. (Accession No.) | Derwent estuary | Port Phillip Bay |
|---|---|---|---|
| Thyroid hormone receptor β | TRβ (KP893715) | Up-regulation | Up-regulation |
| Ferroportin-1 | FPN1 (KU313700) | Up-regulation | Up-regulation |
| Direct IAP binding protein with low P*I*-1 | Diablo/SMAC1 (KU323388) | Up-regulation | Up-regulation |
| Direct IAP binding protein with low P*I*-2 | Diablo/SMAC2 (KU323389) | Up-regulation | No regulation |
| Metal regulatory transcription factor 1 | MTF1 (KU313702) | Up-regulation | No regulation |
| Transferrin | TF (KU313704) | Up-regulation | No regulation |
| Metallothionein-2 | MT (KP893712) | Up/No -regulation | No regulation |
| Transthyretin | TTR (KP893711) | Up-regulation | Down-regulation |
| Glucose regulated protein 78 | GRP78 (KU313701) | Up-regulation | Down-regulation |
| Deiodinase-2 | D2 (KP893710) | Down-regulation | No regulation |
| Thyroid hormone receptor α | TRα (KP893714) | No regulation | Up-regulation |
| Deiodinase-1 | D1 (KP893709) | No regulation | No regulation |
| Ferritin M chain | Ferritin (KU313699) | No regulation | No regulation |
| Cytochrome P4501A | CYP1A (KP893713) | No regulation | No regulation |

## 6. 1.2   Histological biomarkers

Gills and liver were the main focus for the investigation of potential histology biomarkers. Both organs were investigated in sand flathead from the Derwent estuary (Chapter 3 and 4). The results indicated gills were sensitive to environmental stressors, and multiple factors could contribute to gill pathologies observed. In general, the liver is a more reliable organ for

histological assessment in the demersal fish (Feist *et al.*, 2004). Therefore, only liver histology was used in flathead from Port Philip Bay (Chapter 5).

**6.1.2.1 Gill histology**

Flathead gill histology was reported in Chapter 3. The summary of the major findings is given in Table 6.2. The gill histology results highlighted the complex interactions among pathogens, environmental stressors and flathead. Although it is difficult to directly link any gill histological conditions to the concentrations of a specific pollutant, significant associations were observed such as the increased prevalence of telangiectasis in association with waterborne pollution and the negative correlation between gill parasites and Hg concentration in fish. These findings are useful for future studies of using flathead gill histology as a monitoring tool.

**Table 6.2 Summary of gill histological assessment**

| Gill histological conditions | Biomarkers | Interpretation |
|---|---|---|
| Hyperplasia and lamellar fusion | No | Environmental metal exposure and gill flukes may be the major contributing factors |
| Epitheliocystis | No | Intracellular bacterial infection (Nowak and Clark, 1999) |
| Telangiectasis | Potential biomarker | It has been proposed as a biomarker and is associated with waterborne pollution (Stentiford *et al.*, 2003) |
| Deformed filament | No | This lesion was only observed in fish from the reference site and may be associated with parasites infections |
| Absence of gill flukes | Potential biomarker | The toxicity of contaminants are causing the decrease in gill fluke numbers |

**6.1.2.2 Liver histology**

Liver histology was assessed in flathead from both Derwent estuary (Chapter 4) and Port Phillip Bay (Chapter 5). The summary of the results is given in Table 6.3. MMCs and three major types of non-specific lesions were observed. These non-specific lesions including bile duct fibrosis, inflammation and granulomas. It has been suggested that these lesions are of lesser importance for environmental monitoring purposes (Feist *et al.*, 2004). The major target lesions such as early non-neoplastic toxicopathic lesions, foci of cellular alteration, benign neoplasms and malignant neoplasms were not observed in flathead liver from both the Derwent estuary and Port Phillip Bay. This may suggest that flathead inhabit in these two areas were not significantly influenced by the environmental pollutants exposure.

**Table 6.3 Summary of liver histological assessment**

| Histological conditions | Derwent estuary | Port Phillip Bay |
|---|---|---|
| MMCs | No significant difference | No significant difference |
| Bile duct fibrosis | Increased prevalence | No significant difference |
| Inflammation | Increased prevalence | No significant difference |
| Granulomas | No significant difference | Increased prevalence |
| Parasites | No significant difference | Not observed |

The increased prevalence, density and size of MMCs in fish liver have been proposed as biomarkers of environmental pollutants exposure (Agius and Roberts, 2003). In this project, the prevalence of flathead liver MMCs was not significantly associated with locations in both Derwent estuary (Chapter 4) and Port Phillip Bay (Chapter 5). The usefulness of MMCs as a histological biomarker needs to be further evaluated in future studies that should focus on measurement of density and size of MMCs.

Three major types of liver lesions are associated with proliferative change (bile duct fibrosis) and inflammatory response (granuloma and inflammation). Fish from most of heavy polluted sites of Derwent estuary had higher prevalence of bile duct fibrosis and inflammation compared to fish from reference site (Chapter 4). High pollutant load is suspected to be the reason for the increased prevalence of these two lesions. In Port Phillip Bay (Chapter 5), the highest prevalence of granuloma was observed in fish with highest total As concentrations. There was no significant difference in prevalence of other lesions.

## 6. 2    Limitations of this research

### 6.2.1    Weakness in interpretations of thyroid hormone status

It is difficult to know whether sand flathead thyroid disruption occurred only on the basis of gene expression results. Measurements of other higher levels of biological response including the thyroid hormone level change and the morphological changes of thyroid follicles are required to investigate thyroid function (Schnitzler *et al.*, 2011). A recent study reported that a simple, sensitive and reproducible methodology was developed to determine the thyroid hormone level in teleost fishes (Noyes *et al.*, 2014). This novel technique integrated with thyroid hormone related-gene expression analysis would provide bigger picture in terms of effects of pollutants on fish thyroid function in the future study.

### 6.2.2    The inconsistent results of MT gene expression

MT has been regarded as one of traditional biomarker gene and has been extensively examined in different fish species (Cheung *et al.*, 2004; George *et al.*, 2004; Lam *et al.*, 1998; Tom *et al.*, 2004; Viarengo *et al.*, 1999). In this research, the expression analysis of this gene was described in Chapter 2, Chapter 3, and Chapter 5. The results of the Derwent estuary study (2013) showed

the mRNA of this gene was slightly up-regulated in fish from Kingston Beach North compared to those from other sites (Chapter 2). However, there was no regulation observed for this gene in fish from the same sampling sites of Derwent estuary in 2014 (Chapter 3) and from different sites of Port Phillip Bay (Chapter 5). These data indicated that the measurement of MT hepatic mRNA is not a reliable biomarker in flathead. The lack of relationship between the expression of this traditional biomarker gene and the environmental heavy metals exposure could be due to the following reasons: 1), the response sensitivity of MT gene may differ among different fish species. For instance, it has been reported that hepatic MT mRNA levels were sensitive to copper, zinc and cadmium exposures in tilapia (*Tilapia mossambica*) but not in carp (*Cyprinus carpio*) (Lam *et al.*, 1998). 2), different isoforms of MT gene might respond to environmental heavy metal exposure differently in the same species. There are two MT isoforms in teleosts, MT-1 and MT-2 (also referred as MT-A and MT-B), and these two isoforms exhibit different extent of sensitivity to metal exposure (Sigel *et al.*, 2009). For example, it has been reported that MT-1 gene was more sensitive to Zn and Cd exposure than MT-2 in rainbow trout (*Oncorhynchus mykiss*) (Zafarullah *et al.*, 1990). This phenomenon was also observed in the Antarctic icefish (*Chionodraco hamatus*) in which MT-1 mRNA was preferentially accumulated in response to Cd, whereas MT-2 was expressed constitutively (Carginale *et al.*, 1998). The MT-1 gene appears to be a better biomarker gene for heavy metal exposure in fish. The partial MT gene mRNA sequence of flathead shares high similarities with MT-2 of *Siniperca chuatsi* (Identities 91%), implying this MT gene belongs to MT-2 family. The cloning of MT-1 gene in flathead was not successful in this project. This is because the coding region of MT genes is relatively short (around 180 bp) and these two isoforms share high similarities. It was very difficult to isolate

MT-1 through the degenerate primers PCR cloning in this project. Future work however should validate the usefulness of MT-1 as biomarker gene.

### 6.2.3 The high variability of ferritin gene expression

The ferritin gene reported in this project encodes a middle chain subunit. The expression analysis of ferritin gene was reported in Chapter 3 and Chapter 5 and there were large individual variations for the expression of this gene. This suggested that this ferritin gene is not a suitable biomarker gene. However, it is noteworthy that teleost possesses three types of ferritin subunits, namely heavy (H), middle (M) and light (L) chain subunit, and genes of these subunits are expressed organ-specific (Mignogna *et al.*, 2002). For example, in Atlantic salmon (*Salmo salar*), the H chain subunit mainly expressed in liver, spleen and heart, while the M chain mRNA exclusively expressed in gonad (Andersen *et al.*, 1995). Among all these ferritin subunits, the gene of H-chain subunit showed a high degree of response sensitivity to environmental stressors. It has been reported that two ferritin heavy chain subunits (H1 and H3) are highly sensitive to As treatment in zebrafish liver (Xu *et al.*, 2013). In rainbow trout, H1, H2 and H3 genes could be induced by low temperature (Yamashita *et al.*, 1996). These observations might suggest ferritin heavy chain subunit genes are more sensitive than middle subunit genes. Therefore, the usefulness of H chain genes as biomarkers should be validated in future studies.

### 6.2.4 Influence of sex difference on gene expression

Female sand flathead are more often captured than male in the field. For instance, there were only 25% of male fish sampled in Port Phillip Bay. In Chapter 5, only the female fish were used for gene expression, and female fish appeared to be unsuitable for the gene expression analysis of Diablo/SMAC 2 and CYP1A. Due to the inadequate number of males sampled, it was difficult to evaluate the influence of sex difference on the gene expression in this research. Therefore, a

large number of fish are needed to ensure enough males are collected. This would help to assess the sex-related differences in the application of these candidate genes.

### 6.2.5    The relationship between trace metal concentrations and biomarkers

Fish muscle was used for trace metal analysis and liver was selected for gene expression analysis. The correlations between hepatic gene transcripts and muscle heavy metal concentrations were described in Chapter 2. Previous studies reported that marine fish liver accumulated relatively higher level of heavy metals than muscle (Al-Yousuf *et al.*, 2000; Canli and Atli, 2003). This is probably because the metal-binding proteins such as metallothioneins are more abundant in fish liver (Ploetz *et al.*, 2007). Future studies should focus on measurements of pollutants in the fish liver rather than muscle which may be easier to identify the potential causal relationship between pollutants and gene transcripts.

### 6.2.6    The quantifications of histological conditions

The prevalence of histological conditions was measured and described in Chapter 3, Chapter 4 and Chapter 5. The severity of histological conditions was also measured for some lesions. For instance, the epithelial hyperplasia and complete fusion of secondary lamellae were observed in all the sampled fish from the Derwent estuary. The severity of this lesion was semi-quantified as follows: 1 (mild) = affected 1 - 25 % of filaments; 2 (moderate) = affected 26 -50 % of filaments; 3 (marked) = affected 51 - 75 % of filaments; 4 (extreme) = affected 76 - 100 % of filaments. The severity of liver inflammation was quantified using the mean percentage tissue affected per image (10 images per section). The results indicated there was no significant difference for the severity of these two lesions among sampling sites (data not shown). While severity of some lesions such as epithelial hyperplasia and complete fusion of secondary lamellae was semi-quantified, a more accurate assessment of severity such as percentage of affected area could

allow greater insight into the potential effects of pollution. A more comprehensive assessment of severity was outside the scope of this project. More accurate severity information that will provide the extent of effect on individual animals should also be included in future studies as this could help to elucidate subtle effects of pollution that are possibly not observed using prevalence date alone.

## 6. 3    Recommendations for further research

### 6.3.1    *In vitro* and *in vivo* experiments are needed to confirm the major findings

The data presented in this research were mainly based on field studies. These results reflected the actual effects of environmental pollutants on sand flathead in the real world. All the major findings should be further confirmed under laboratory conditions. In particular, the exact mechanisms of action of pollutant on the candidate biomarker genes are needed to be elucidated. *In vitro* fish cell assays are considered to be fast and cost-effective methods to further examine the responses of biomarker genes to single or mixed chemicals (Bols *et al.*, 2005; Schirmer, 2006). However, the cellular metabolism of *in vitro* assays is sometimes hard to represent the original tissues or organ or whole animal. This is due to the absence of systemic components and specific cellular interactions in the *in vitro* microenvironment (Mothersill and Austin, 2003). Therefore, *in vivo* experiments are also needed to combine with *in vitro* assays in future studies to bridge the gaps between laboratory and field studies.

### 6.3.2    Caged fish should be combined with flathead in future field study

For the field study, the smaller size laboratory bred fish species should be considered for use as caged fish combined with wild caught fish to investigate the effects of environmental pollutants. The study of the caged fish after short-term exposure to environmental pollution at different

research sites could offer several advantages in evaluating environmental status, 1), it is easier for experimental set-up in the field. 2), the caged fish could eliminate some confounding factors such as migration. 3), the data obtain from the caged fish would be helpful to link with observed effects in wild fish to chronic exposure to environmental pollution (Roberts *et al.*, 2005).

### 6.3.3   Further work is needed to develop the biochemical biomarkers

It is important to have the comprehensive data across multiple levels within an ecosystem when assessing the environmental status (Allen and Moore, 2004). The data represented in this research have covered the effects of pollutants on molecular and organ or tissue levels. However, the influences of pollution on the cellular or subcellular level remain unknown. Therefore, further studies are needed to measure the biochemical biomarkers such as the activities of EROD and ECOD that exhibited high reliability in sand flathead (Brumley *et al.*, 1995; Gagnon and Holdway, 2002; Holdway *et al.*, 1994). Combining the data from molecular, biochemical and histological biomarkers would provide a clearer picture of the state of environment.


## 6. 4   Concluding statement

This research has expanded understanding of adverse effects of pollution on transcriptional and histological level in an indicator species. The gene expression results suggested TTR and TRβ are potential biomarker genes on thyroid pathway; metal-homeostasis related genes including MTF1, TF and FPN1 may be sensitive and specific biomarker genes to environmental heavy metal exposure; Diablo/SMAC 1 is a promising biomarker gene for cellular damage caused by pollutants. In terms of histological biomarkers, the increased of telangiectasis and absence of gill flukes seemed to be associated with environmental pollution. Some non-specific liver lesions such as bile duct fibrosis and inflammation could generally reflect environmental stressors

including pollutants. Future investigations would be valuable to further confirm these findings under controlled laboratory conditions.

# References

Abdel-Moneim, A.M., Al-Kahtani, M.A., Elmenshawy, O.M., 2012. Histopathological biomarkers in gills and liver of *Oreochromis niloticus* from polluted wetland environments, Saudi Arabia. Chemosphere 88, 1028-1035.

Abernathy, C.O., Thomas, D.J., Calderon, R.L., 2003. Health effects and risk assessment of arsenic. J Nutr 133, 1536S-1538S.

Adams, D.O., 1976. The granulomatous inflammatory response. A review. Am J Pathol 84, 164-192.

Agius, C., 1980. Phylogenetic Development of Melano-Macrophage Centers in Fish. Journal of zoology 191, 11-31.

Agius, C., 1981. Preliminary studies on the ontogeny of the melano-macrophages of teleost haemopoietic tissues and age-related changes. Developmental and comparative immunology 5, 597-606.

Agius, C., Roberts, R.J., 2003. Melano-macrophage centres and their role in fish pathology. Journal of fish diseases 26, 499-509.

Ahmed, M.K., Habibullah-Al-Mamun, M., Parvin, E., Akter, M.S., Khan, M.S., 2013. Arsenic induced toxicity and histopathological changes in gill and liver tissue of freshwater fish, tilapia (*Oreochromis mossambicus*). Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 65, 903-909.

Al-Bairuty, G.A., Shaw, B.J., Handy, R.D., Henry, T.B., 2013. Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 126, 104-115.

Al-Yousuf, M., El-Shahawi, M., Al-Ghais, S., 2000. Trace metals in liver, skin and muscle of *Lethrinus lentjan* fish species in relation to body length and sex. Science of The Total Environment 256, 87-94.

Alazemi, B.M., Lewis, J.W., Andrews, E.B., 1996. Gill damage in the freshwater fish *Gnathonemus petersii* (family: Mormyridae) exposed to selected pollutants: An ultrastructural study. Environmental Technology 17, 225-238.

Allen, J.I., Moore, M.N., 2004. Environmental prognostics: is the current use of biomarkers appropriate for environmental risk evaluation? Marine Environmental Research 58, 227-232.

Amiard-Triquet, C., Amiard, J.-C., Rainbow, P.S., 2012. Ecological biomarkers: indicators of ecotoxicological effects. CRC Press.

An, L., Hu, J., Yang, M., Zheng, B., Wei, A., Shang, J., Zhao, X., 2011. CYP1A mRNA expression in redeye mullets (*Liza haematocheila*) from Bohai Bay, China. Mar Pollut Bull 62, 718-725.

Andersen, O., Dehli, A., Standal, H., Giskegjerde, T.A., Karstensen, R., Rorvik, K.A., 1995. Two ferritin subunits of Atlantic salmon (*Salmo salar*): cloning of the liver cDNAs and antibody preparation. Mol Mar Biol Biotechnol 4, 164-170.

Andrews, G.K., 2001. Cellular zinc sensors: MTF-1 regulation of gene expression, Zinc Biochemistry, Physiology, and Homeostasis. Springer, pp. 37-51.

Arellano, J.M., Storch, V., Sarasquete, C., 1999. Histological changes and copper accumulation in liver and gills of the Senegales sole, *Solea senegalensis*. Ecotoxicol Environ Saf 44, 62-72.

Arosio, P., Levi, S., 2002. Ferritin, iron homeostasis, and oxidative damage. Free radical biology & medicine 33, 457-463.

Asker, N., Almroth, B.C., Albertsson, E., Coltellaro, M., Bignell, J.P., Hanson, N., Scarcelli, V., Fagerholm, B., Parkkonen, J., Wijkmark, E., 2015. A gene to organism approach–assessing the impact of environmental pollution in eelpout (*Zoarces viviparus*) females and larvae. Environmental Toxicology and Chemistry.

Asker, N., Kristiansson, E., Albertsson, E., Larsson, D.G., Forlin, L., 2013. Hepatic transcriptome profiling indicates differential mRNA expression of apoptosis and immune related genes in eelpout (*Zoarces viviparus*) caught at Goteborg harbor, Sweden. Aquat Toxicol 130-131, 58-67.

Austin, B., 1998. The effects of pollution on fish health. J Appl Microbiol 85 Suppl 1, 234S-242S.

Ayling, G.M., Wilson, K.C., Ratkowsky, D.A., 1975. Sand flathead (*Platycephalus bassensis*), an indicator species for mercury pollution in Tasmanian waters. Marine Pollution Bulletin 6, 142-144.

Baatrup, E., 1991. Structural and functional effects of heavy metals on the nervous system, including sense organs, of fish. Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology 100, 253-257.

Barregard, L., Lindstedt, G., Schutz, A., Sallsten, G., 1994. Endocrine function in mercury exposed chloralkali workers. Occup Environ Med 51, 536-540.

Bayne, B.L., 1985. The Effects of stress and pollution on marine animals. Praeger.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of fish diseases 22, 25-34.

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., Ron, D., 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2, 326-332.

Besselink, H.T., van Beusekom, S., Roex, E., Vethaak, A.D., Koeman, J.H., Brouwer, A., 1996. Low hepatic 7-ethoxyresorufin-O-deethylase (EROD) activity and minor alterations in retinoid and thyroid hormone levels in flounder (*Platichthys flesus*) exposed to the polychlorinated biphenyl (PCB) mixture, Clophen A50. Environ Pollut 92, 267-274.

Beyer, J., 1996. Fish biomarkers in marine pollution monitoring. Evaluation and validation in laboratory and field studies.

Bhattacharya, T., Bhattacharya, S., Ray, A.K., Dey, S., 1989. Influence of industrial pollutants on thyroid function in *Channa punctatus* (Bloch). Indian J Exp Biol 27, 65-68.

Bleau, H., Daniel, C., Chevalier, G., vanTra, H., Hontela, A., 1996. Effects of acute exposure to mercury chloride and methylmercury on plasma cortisol, T3, T4, glucose and liver glycogen in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 34, 221-235.

Bloom, H., Ayling, G.M., 1977. Heavy metals in the Derwent Estuary. Environmental Geology 2, 3-22.

Boehlert, G.W., Mundy, B.C., 1988. Roles of behavioral and physical factors in larval and juvenile fish recruitment to estuarine nursery areas, American Fisheries Society Symposium, pp. 1-67.

Bols, N., Dayeh, V., Lee, L., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. Biochemistry and molecular biology of fishes 6, 43-84.

Bres, O., Plohman, J.C., Eales, J.G., 2006. A cDNA for a putative type III deiodinase in the trout (*Oncorhynchus mykiss*): influence of holding conditions and thyroid hormone treatment on its hepatic expression. Gen Comp Endocrinol 145, 92-100.

Brown, S.B., Adams, B.A., Cyr, D.G., Eales, J.G., 2004. Contaminant effects on the teleost fish thyroid. Environmental toxicology and chemistry / SETAC 23, 1680-1701.

Brumley, C.M., Haritos, V.S., Ahokas, J.T., Holdway, D.A., 1995. Validation of Biomarkers of Marine Pollution Exposure in Sand Flathead Using Aroclor-1254. Aquatic Toxicology 31, 249-262.

Bruno, D.W., Nowak, B., Elliott, D.G., 2006. Guide to the identification of fish protozoan and metazoan parasites in stained tissue sections. Dis Aquat Organ 70, 1-36.

Brusle, J., Anadon, G., 1996. The structure and function of fish liver. Fish morphology, 77-93.

Bryan, G., 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. Proceedings of the Royal Society of London B: Biological Sciences 177, 389-410.

Bucke, D., Feist, S.W., 1993. Histopathological Changes in the Livers of Dab, *Limanda limanda* (L). Journal of fish diseases 16, 281-296.

Bucke, D., Vethaak, A., Lang, T., Mellergaard, S., 1996. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. International Council for the Exploration of the sea.

Bunton, T.E., Baksi, S.M., George, S.G., Frazier, J.M., 1987. Abnormal hepatic copper storage in a teleost fish (*Morone americana*). Veterinary pathology 24, 515-524.

Burri, L., Strahm, Y., Hawkins, C.J., Gentle, I.E., Puryer, M.A., Verhagen, A., Callus, B., Vaux, D., Lithgow, T., 2005. Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane. Molecular biology of the cell 16, 2926-2933.

Canli, M., Atli, G., 2003. The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. Environmental Pollution 121, 129-136.

Carginale, V., Scudiero, R., Capasso, C., Capasso, A., Kille, P., di Prisco, G., Parisi, E., 1998. Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish, which exhibits no basal metallothionein protein but high endogenous mRNA levels. Biochemical Journal 332, 475-481.

Chen, Q., Yu, L., Yang, L., Zhou, B., 2012. Bioconcentration and metabolism of decabromodiphenyl ether (BDE-209) result in thyroid endocrine disruption in zebrafish larvae. Aquat Toxicol 110-111, 141-148.

Chen, W.Y., John, J.A., Lin, C.H., Lin, H.F., Wu, S.C., Lin, C.H., Chang, C.Y., 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquat Toxicol 69, 215-227.

Cheung, A.P., Lam, T.H., Chan, K.M., 2004. Regulation of Tilapia metallothionein gene expression by heavy metal ions. Mar Environ Res 58, 389-394.

Cho, Y.S., Lee, S.Y., Kim, K.Y., Bang, I.C., Kim, D.S., Nam, Y.K., 2008. Gene structure and expression of metallothionein during metal exposures in *Hemibarbus mylodon*. Ecotoxicol Environ Saf 71, 125-137.

Cianetti, L., Gabbianelli, M., Sposi, N.M., 2010. Ferroportin and erythroid cells: an update. Adv Hematol 2010.

Ciarrocca, M., Tomei, F., Caciari, T., Cetica, C., Andre, J.C., Fiaschetti, M., Schifano, M.P., Scala, B., Scimitto, L., Tomei, G., Sancini, A., 2012. Exposure to arsenic in urban and rural areas and effects on thyroid hormones. Inhalation toxicology 24, 589-598.

Clark, M.S., Fraser, K.P.P., Burns, G., Peck, L.S., 2008. The HSP70 heat shock response in the Antarctic fish *Harpagifer antarcticus*. Polar Biology 31, 171-180.

Cohen-Lehman, J., Dahl, P., Danzi, S., Klein, I., 2010. Effects of amiodarone therapy on thyroid function. Nature reviews. Endocrinology 6, 34-41.

Corsolini, S., Romeo, T., Ademolla, N., Greco, S., Focardi, S., 2002. POPs in key species of marine Antarctic ecosystem. Microchemical Journal 73, 187-193.

Cuklev, F., Gunnarsson, L., Cvijovic, M., Kristiansson, E., Rutgersson, C., Bjorlenius, B., Larsson, D.G., 2012. Global hepatic gene expression in rainbow trout exposed to sewage effluents: a comparison of different sewage treatment technologies. The Science of the total environment 427-428, 106-114.

Damek-Poprawa, M., Sawicka-Kapusta, K., 2003. Damage to the liver, kidney, and testis with reference to burden of heavy metals in yellow-necked mice from areas around steelworks and zinc smelters in Poland. Toxicology 186, 1-10.

Daniel, S., Richard, H., Scott, S., Michael, H.D., William, B., 2008. Biomarkers, The Toxicology of Fishes. CRC Press, pp. 683-731.

Darras, V.M., 2008. Endocrine disrupting polyhalogenated organic pollutants interfere with thyroid hormone signalling in the developing brain. Cerebellum 7, 26-37.

Dauvin, J.C., 2008. Effects of heavy metal contamination on the macrobenthic fauna in estuaries: the case of the Seine estuary. Mar Pollut Bull 57, 160-169.

Davey, J.C., Nomikos, A.P., Wungjiranirun, M., Sherman, J.R., Ingram, L., Batki, C., Lariviere, J.P., Hamilton, J.W., 2008. Arsenic as an endocrine disruptor: arsenic disrupts retinoic acid receptor and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. Environ Health Perspect 116, 165-172.

Di Giulio, R.T., Hinton, D.E., 2008. The Toxicology of Fishes. CRC Press.

Donovan, A., Lima, C.A., Pinkus, J.L., Pinkus, G.S., Zon, L.I., Robine, S., Andrews, N.C., 2005. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metabolism 1, 191-200.

dos Santos, D.C., da Matta, S.L., de Oliveira, J.A., dos Santos, J.A., 2012. Histological alterations in gills of *Astyanax aff. bimaculatus* caused by acute exposition to zinc. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 64, 861-866.

Du, C.Y., Fang, M., Li, Y.C., Li, L., Wang, X.D., 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102, 33-42.

Dunier, M., Siwicki, A.K., 1993. Effects of Pesticides and Other Organic Pollutants in the Aquatic Environment on Immunity of Fish a Review. Fish & Shellfish Immunology 3, 423-438. Duruibe, J.O., Ogwuegbu, M.O.C., Egwurugwu, J.N., 2007. Heavy metal pollution and human biotoxic effects. International Journal of Physical Sciences 2, 112-118.

Eisler, R., 2004. Arsenic hazards to humans, plants, and animals from gold mining, Reviews of environmental contamination and toxicology. Springer, pp. 133-165.

El-Shahawi, M.S., Hamza, A., Bashammakh, A.S., Al-Saggaf, W.T., 2010. An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. Talanta 80, 1587-1597.

Ellingsen, D.G., Efskind, J., Haug, E., Thomassen, Y., Martinsen, I., Gaarder, P.I., 2000. Effects of low mercury vapour exposure on the thyroid function in chloralkali workers. J Appl Toxicol 20, 483-489.

Elsenhans, B., Schümann, K., Forth, W., 1991. Toxic metals: interactions with essential metals. Nutrition, Toxicity, and Cancer,(Rowland IR, ed). Boca Raton, FL: CRC Press Inc, 223-258.

Eustace, I., 1974. Zinc, cadmium, copper, and manganese in species of finfish and shellfish caught in the Derwent Estuary, Tasmania. Marine and Freshwater Research 25, 209-220.

Fabris, G., Turoczy, N.J., Stagnitti, F., 2006. Trace metal concentrations in edible tissue of snapper, flathead, lobster, and abalone from coastal waters of Victoria, Australia. Ecotoxicol Environ Saf 63, 286-292.

Fabris, G.J., Monahan, C., Nicholson, G., Walker, T.I., 1992. Total mercury concentrations in sand flathead, *Platycephalus bassensis* Cuvier & Valenciennes, from Port Phillip Bay, Victoria. Marine and Freshwater Research 43, 1393.

Falciani, F., Diab, A.M., Sabine, V., Williams, T.D., Ortega, F., George, S.G., Chipman, J.K., 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquat Toxicol 90, 92-101.

Feist, S.W., Lang, T., Stentiford, G., Köhler, A., 2004. Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. International Council for the Exploration of the Sea.

Feist, S.W., Stentiford, G.D., Kent, M.L., Ribeiro Santos, A., Lorance, P., 2015. Histopathological assessment of liver and gonad pathology in continental slope fish from the northeast Atlantic Ocean. Mar Environ Res 106, 42-50.

Fernandes, C., Fontainhas-Fernandes, A., Rocha, E., Salgado, M.A., 2008. Monitoring pollution in Esmoriz Paramos lagoon, Portugal: liver histological and biochemical effects in *Liza saliens*. Environ Monit Assess 145, 315-322.

Ferrero-Miliani, L., Nielsen, O.H., Andersen, P.S., Girardin, S.E., 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clinical and experimental immunology 147, 227-235.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J.V., Garcia-Santos, S., Monteiro, S.M., Carrola, J., Matos, P., Fontainhas-Fernandes, A., 2007. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesquisa Veterinária Brasileira 27, 103-109.

Fok, P., Eales, J.G., Brown, S.B., 1990. Determination of 3,5,3'-triiodo-L-thyronine (T3) levels in tissues of rainbow trout (*Salmo gairdneri*) and the effect of low ambient pH and aluminum. Fish Physiol Biochem 8, 281-290.

Ford, M.J., 2001. Molecular evolution of transferrin: evidence for positive selection in salmonids. Mol Biol Evol 18, 639-647.

Fournie, J.W., Summers, J.K., Courtney, L.A., Engle, V.D., Blazer, V.S., 2001. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. Journal of Aquatic Animal Health 13, 105-116.

Fracalossi, D.M., Allen, M.E., Nichols, D.K., Oftedal, O.T., 1998. Oscars, *Astronotus ocellatus*, have a dietary requirement for vitamin C. J Nutr 128, 1745-1751.

Freitas, J., Cano, P., Craig-Veit, C., Goodson, M.L., Furlow, J.D., Murk, A.J., 2011. Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay. Toxicol in Vitro 25, 257-266.

Fricke, N.F., Stentiford, G.D., Feist, S.W., Lang, T., 2012. Liver histopathology in Baltic eelpout (*Zoarces viviparus*) A baseline study for use in marine environmental monitoring. Mar Environ Res 82, 1-14.

Gagnon, M.M., Baker, J.K., Long, S.M., Hassell, K.L., Pettigrove, V., 2016. Contaminant concentrations are declining in axial tissue of sand flathead (*Platycephalus bassensis*) collected from an urbanlised catchement (Port Phillip Bay, Australia). Submitted.

Gagnon, M.M., Holdway, D.A., 2002. EROD activity, serum SDH and PAH biliary metabolites in sand flathead (*Platycephalus bassensis*) collected in Port Phillip Bay, Australia. Mar Pollut Bull 44, 230-237.

Galli, P., Crosa, G., Mariniello, L., Ortis, M., D'Amelio, S., 2001. Water quality as a determinant of the composition of fish parasite communities. Hydrobiologia 452, 173-179.

Garcia, G.C., Jeziorski, M.C., Valverde, R.C., Orozco, A., 2004. Effects of iodothyronines on the hepatic outer-ring deiodinating pathway in killifish. Gen Comp Endocrinol 135, 201-209.

George, S., Gubbins, M., MacIntosh, A., Reynolds, W., Sabine, V., Scott, A., Thain, J., 2004. A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platicthys flesus*) subjected to estuarine pollution. Mar Environ Res 58, 571-575.

Gernhöfer, M., Pawert, M., Schramm, M., Müller, E., Triebskorn, R., 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecosystem Stress and Recovery 8, 241-260.

Giari, L., Simoni, E., Manera, M., Dezfuli, B.S., 2008. Histo-cytological responses of *Dicentrarchus labrax* (L.) following mercury exposure. Ecotoxicol Environ Saf 70, 400-410.

Goksøyr, A., 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution, Toxicology in Transition. Springer, pp. 80-95.

Gomon, M.F., Glover, J.C., Kuiter, R.H., 1994. Fishes of Australia's south coast.

Gonzalez, P., Dominique, Y., Massabuau, J.C., Boudou, A., Bourdineaud, J.P., 2005. Comparative effects of dietary methylmercury on gene expression in liver, skeletal muscle, and brain of the zebrafish (*Danio rerio*). Environ Sci Technol 39, 3972-3980.

Goyer, R.A., 1997. Toxic and essential metal interactions. Annu Rev Nutr 17, 37-50.

Green, G., Coughanowr, C., 1997. State of the Derwent Estuary 2003: A Review of Pollution Sources. Loads and Environmental Quality Data From 2003.

Green, G.J., Coughanowr, C., 2003. State of the Derwent Estuary: a review of pollution sources, loads and environmental quality data from 1997 to 2003. Department of Primary Industries, Water and Environment.

Gul, S., Belge-Kurutas, E., Yildiz, E., Sahan, A., Doran, F., 2004. Pollution correlated modifications of liver antioxidant systems and histopathology of fish (Cyprinidae) living in Seyhan Dam Lake, Turkey. Environ Int 30, 605-609.

Gunther, V., Lindert, U., Schaffner, W., 2012. The taste of heavy metals: gene regulation by MTF-1. Biochim Biophys Acta 1823, 1416-1425.

Haasch, M.L., Prince, R., Wejksnora, P.J., Cooper, K.R., Lech, J.J., 1993. Caged and Wild Fish - Induction of Hepatic Cytochrome-P-450 (Cyp1a1) as an Environmental Biomonitor. Environmental Toxicology and Chemistry 12, 885-895.

Harris, G., Scientific, C., 1996. Port Phillip Bay environmental study: final report. CSIRO Canberra.

Harris, H.H., Pickering, I.J., George, G.N., 2003. The chemical form of mercury in fish. science 301, 1203.

He, X., Ma, Q., 2009. Induction of metallothionein I by arsenic via metal-activated transcription factor 1: critical role of C-terminal cysteine residues in arsenic sensing. J Biol Chem 284, 12609-12621.

He, X.Q., Ma, Q., 2011. Metal sensing by MTF1 through its carboxyl-terminal cysteine residues. Faseb Journal 25, 1090.1015.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8, R19.

Holdway, D.A., Brennan, S.E., Ahokas, J.T., 1994. Use of Hepatic Mfo and Blood Enzyme Biomarkers in Sand Flathead (*Platycephalus bassensis*) as Indicators of Pollution in Port Phillip Bay, Australia. Marine Pollution Bulletin 28, 683-695.

Hu, B., Cui, R., Li, J., Wei, H., Zhao, J., Bai, F., Song, W., Ding, X., 2013. Occurrence and distribution of heavy metals in surface sediments of the Changhua River Estuary and adjacent shelf (Hainan Island). Mar Pollut Bull 76, 400-405.

Iavicoli, I., Fontana, L., Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. Journal of toxicology and environmental health. Part B, Critical reviews 12, 206-223.

Ibhazehiebo, K., Iwasaki, T., Kimura-Kuroda, J., Miyazaki, W., Shimokawa, N., Koibuchi, N., 2011. Disruption of thyroid hormone receptor-mediated transcription and thyroid hormone-induced Purkinje cell dendrite arborization by polybrominated diphenyl ethers. Environ Health Perspect 119, 168-175.

Ip, C.C., Li, X.D., Zhang, G., Wai, O.W., Li, Y.S., 2007. Trace metal distribution in sediments of the Pearl River Estuary and the surrounding coastal area, South China. Environ Pollut 147, 311-323.

Jensen, S., Johnels, A.G., Olsson, M., Otterlind, G., 1969. DDT and PCB in marine animals from Swedish waters. Nature 224, 247-250.

Jezierska, B., Lugowska, K., Witeska, M., 2009. The effects of heavy metals on embryonic development of fish (a review). Fish Physiol Biochem 35, 625-640.

Johnson, K.M., Lema, S.C., 2011. Tissue-specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in striped parrotfish (*Scarus iseri*). Gen Comp Endocrinol 172, 505-517.

Jones, B.G., Chenhall, B.E., Debertsion, F., Hutton, A.C., 2003. Geochemical comparisons between estuaries with non-industrialised and industrialised catchments: the Huon and Derwent River estuaries; Tasmania. Australian Journal of Earth Sciences 50, 653-667.

Jones, H., Swadling, K., Butler, E., Macleod, C., 2014. Complex patterns in fish–sediment mercury concentrations in a contaminated estuary: The influence of selenium co-contamination? Estuarine, Coastal and Shelf Science 137, 14-22.

Jones, K.C., de Voogt, P., 1999. Persistent organic pollutants (POPs): state of the science. Environ Pollut 100, 209-221.

Karan, V., Vitorovic, S., Tutundzic, V., Poleksic, V., 1998. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. Ecotoxicol Environ Saf 40, 49-55.

Kelly, B.C., Ikonomou, M.G., Blair, J.D., Morin, A.E., Gobas, F.A., 2007. Food web-specific biomagnification of persistent organic pollutants. science 317, 236-239.

Kendall, S.D., Christensen, M.J., 1997. Selenium deficiency decreases expression of the genes for transthyretin and citrate transport protein in rat liver. Nutr Res 17, 351-362.

Kennish, M.J., 1996. Practical handbook of estuarine and marine pollution. CRC press.

Khan, M.A.K., Wang, F.Y., 2009. Mercury-Selenium Compounds and Their Toxicological Significance: Toward a Molecular Understanding of the Mercury-Selenium Antagonism. Environmental Toxicology and Chemistry 28, 1567-1577.

Kime, D.E., 1995. The Effects of Pollution on Reproduction in Fish. Reviews in Fish Biology and Fisheries 5, 52-95.

Kime, D.E., 2012. Endocrine disruption in fish. Springer Science & Business Media.

Kirubagaran, R., Joy, K.P., 1994. Effects of short-term exposure to methylmercury chloride and its withdrawal on serum levels of thyroid hormones in the catfish *Clarias batrachus*. Bull Environ Contam Toxicol 53, 166-170.

Kitchin, K.T., Wallace, K., 2008. The role of protein binding of trivalent arsenicals in arsenic carcinogenesis and toxicity. J Inorg Biochem 102, 532-539.

Knapen, D., Reynders, H., Bervoets, L., Verheyen, E., Blust, R., 2007. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. Aquat Toxicol 82, 163-172.

Koenig, S., Porte, C., Sole, M., Sturve, J., 2013. Biliary PAH and alkylphenol metabolites, biomarker enzyme activities, and gene expression levels in the deep-sea fish Alepocephalus rostratus. Environ Sci Technol 47, 2854-2861.

Kondoh, M., Inoue, Y., Atagi, S., Futakawa, N., Higashimoto, M., Sato, M., 2001. Specific induction of metallothionein synthesis by mitochondrial oxidative stress. Life Sci 69, 2137-2146.

Lafferty, K.D., 1997. Environmental parasitology: What can parasites tell us about human impacts on the environment? Parasitology Today 13, 251-255.

Lam, K.L., Ko, P.W., Wong, J.K.Y., Chan, K.M., 1998. Metal toxicity and metallothionein gene expression studies in common carp and tilapia. Marine Environmental Research 46, 563-566.

Lam, P.K.S., 2009. Use of biomarkers in environmental monitoring. Ocean & Coastal Management 52, 348-354.

Lam, P.K.S., Gray, J.S., 2003. The use of biomarkers in environmental monitoring programmes. Marine Pollution Bulletin 46, 182-186.

Langdon, J.S., 1986. Haemosiderosis in *Platycephalus bassensis* and *Diodon nicthemerus* in south-east Australian coastal waters. Marine and Freshwater Research 37, 587.

Langlois, D., Cooper, R.J., Clark, N.H., Ratkowsky, D.A., 1987. The Effect of a Mercury Containment Program at a Zinc Smelting Plant on the Mercury Content of Sand Flathead in the Derwent Estuary. Marine Pollution Bulletin 18, 67-70.

Lans, M., Klasson-Wehler, E., Brouwer, A., 1994. Thyroid hormone binding proteins as targets for hydroxylated PCB, PCDD and PCDF metabolites; an overview. Organohalogen Compounds 20, 481-485.

Leatherland, J., Sonstegard, R., 1978. Lowering of serum thyroxine and triiodothyronine levels in yearling coho salmon, *Oncorhynchus kisutch*, by dietary mirex and PCBs. Journal of the Fisheries Board of Canada 35, 1285-1289.

Leatherland, J., Sonstegard, R., 1980. Effect of dietary polychlorinated biphenyls (PCBs) or mirex in combination with food deprivation and testosterone administration on serum thyroid hormone concentration and bioaccumulation of organochlorines in rainbow trout, *Salmo gairdneri*. Journal of fish diseases 3, 115-124.

Leatherland, J.F., 2000. Contaminant-altered thyroid function in wildlife. Taylor & Francis, New York.

Leatherland, J.F., 2005. Reflections on the thyroidology of fishes: from molecules to humankind. Guelph Ichthyology Reviews 2.

Leaver, M.J., Diab, A., Boukouvala, E., Williams, T.D., Chipman, J.K., Moffat, C.F., Robinson, C.D., George, S.G., 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: Absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. Aquatic Toxicology 96, 234-245.

Lee, A.S., 2005. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35, 373-381.

Lehigh Shirey, E.A., Jelaso Langerveld, A., Mihalko, D., Ide, C.F., 2006. Polychlorinated biphenyl exposure delays metamorphosis and alters thyroid hormone system gene expression in developing *Xenopus laevis*. Environ Res 102, 205-214.

Levine, S.L., Oris, J.T., 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquatic Toxicology 46, 279-287.

Li, W., Zha, J., Spear, P.A., Li, Z., Yang, L., Wang, Z., 2009. Changes of thyroid hormone levels and related gene expression in Chinese rare minnow (*Gobiocypris rarus*) during 3-amino-1,2,4-triazole exposure and recovery. Aquat Toxicol 92, 50-57.

Liu, J., Kadiiska, M.B., Liu, Y., Lu, T., Qu, W., Waalkes, M.P., 2001. Stress-related gene expression in mice treated with inorganic arsenicals. Toxicological sciences : an official journal of the Society of Toxicology 61, 314-320.

Liu, Y.W., Lo, L.J., Chan, W.K., 2000. Temporal expression and T3 induction of thyroid hormone receptors alpha 1 and beta 1 during early embryonic and larval development in zebrafish, *Danio rerio*. Mol Cell Endocrinol 159, 187-195.

Ma, K.X., Chen, G.W., Shi, C.Y., Cheng, F.F., Dou, H., Feng, C.C., Liu, D.Z., 2014. Molecular characterization of the glucose-regulated protein 78 (GRP78) gene in planarian *Dugesia japonica*. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 171, 12-17.

Maher, W.A., Aislabie, J., 1992. Polycyclic aromatic hydrocarbons in nearshore marine sediments of Australia. The Science of the total environment 112, 143-164.

Malins, D.C., Krahn, M.M., Brown, D.W., Rhodes, L.D., Myers, M.S., McCain, B.B., Chan, S.L., 1985. Toxic chemicals in marine sediment and biota from Mukilteo, Washington: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). J Natl Cancer Inst 74, 487-494.

Mallatt, J., 1985. Fish Gill Structural-Changes Induced by Toxicants and Other Irritants a Statistical Review. Canadian Journal of Fisheries and Aquatic Sciences 42, 630-648.

Manera, M., Serra, R., Isani, G., Carpene, E., 2000. Macrophage aggregates in gilthead sea bream fed copper, iron and zinc enriched diets. Journal of fish Biology 57, 457-465.

Martelli, A., Rousselet, E., Dycke, C., Bouron, A., Moulis, J.M., 2006. Cadmium toxicity in animal cells by interference with essential metals. Biochimie 88, 1807-1814.

Mason, R.P., Reinfelder, J.R., Morel, F.M.M., 1996. Uptake, toxicity, and trophic transfer of mercury in a coastal diatom. Environmental Science & Technology 30, 1835-1845.

Matthiessen, P., Law, R.J., 2002. Contaminants and their effects on estuarine and coastal organisms in the United Kingdom in the late twentieth century. Environmental Pollution 120, 739-757.

McCarthy, J.F., Shugart, L.R., 1990. Biomarkers of environmental contamination.

Mclusky, D.S., Bryant, V., Campbell, R., 1986. The Effects of Temperature and Salinity on the Toxicity of Heavy-Metals to Marine and Estuarine Invertebrates. Oceanogr Mar Biol 24, 481-520.

Mellanen, P., Soimasuo, M., Holmbom, B., Oikari, A., Santti, R., 1999. Expression of the vitellogenin gene in the liver of juvenile whitefish (*Coregonus lavaretus* L. s.l.) exposed to effluents from pulp and paper mills. Ecotoxicol Environ Saf 43, 133-137.

Meyer, J.N., Volz, D.C., Freedman, J.H., Di Giulio, R.T., 2005. Differential display of hepatic mRNA from killifish (*Fundulus heteroclitus*) inhabiting a Superfund estuary. Aquat Toxicol 73, 327-341.

Mieiro, C.L., Bervoets, L., Joosen, S., Blust, R., Duarte, A.C., Pereira, M.E., Pacheco, M., 2011. Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish considerations on tissue and species specific responses. Chemosphere 85, 114-121.

Mignogna, G., Chiaraluce, R., Consalvi, V., Cavallo, S., Stefanini, S., Chiancone, E., 2002. Ferritin from the spleen of the Antarctic teleost *Trematomus bernacchii* is an M-type homopolymer. European Journal of Biochemistry 269, 1600-1606.

Mikaelian, I., de Lafontaine, Y., Harshbarger, J.C., Lee, L.L., Martineau, D., 2002. Health of lake whitefish (*Coregonus clupeaformis*) with elevated tissue levels of environmental contaminants. Environmental toxicology and chemistry / SETAC 21, 532-541.

Moller, H., 1987. Pollution and parasitism in the aquatic environment. Int J Parasitol 17, 353-361.

Mori, K., Yoshida, K., Tani, J., Hoshikawa, S., Ito, S., Watanabe, C., 2006. Methylmercury inhibits type II 5'-deiodinase activity in NB41A3 neuroblastoma cells. Toxicol Lett 161, 96-101.

Mothersill, C., Austin, B., 2003. *In Vitro* Methods in Aquatic Ecotoxicology. Springer Science & Business Media.

Moulis, J.M., 2010. Cellular mechanisms of cadmium toxicity related to the homeostasis of essential metals. Biometals 23, 877-896.

Myers, M., Stehr, C., Olson, O., Johnson, L., McCain, B., 1993. National status and trends program for National Benthic Surveillance Project: Pacific coast. Fish histopathology and relationships between toxicopathic lesions and exposure to chemical contaminants for cycles I to V (1984-88). Technical memo. National Marine Fisheries Service, Seattle, WA (United States). Northwest Fisheries Science Center.

Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., Varanasi, U., 1998. Toxicopathic hepatic lesions in subadult English sole (*pleuronectes vetuls*) from Puget Sound, Washington, USA: relationships with other biomarkers of contaminant exposure. Mar Environ Res 45, 47-67.

Naidu, K.A., Naidu, K.A., Ramamurthi, R., 1983. Histological observations in gills of the teleost *Sarotherodon mossambicus* with reference to mercury toxicity. Ecotoxicol Environ Saf 7, 455-462.

Naldoni, J., Maia, A.A., da Silva, M.R., Adriano, E.A., 2014. *Henneguya cuniculator* sp. nov., a parasite of spotted sorubim *Pseudoplatystoma corruscans* in the Sao Francisco Basin, Brazil. Dis Aquat Organ 107, 211-221.

Navarro, A., Quiros, L., Casado, M., Faria, M., Carrasco, L., Benejam, L., Benito, J., Diez, S., Raldua, D., Barata, C., Bayona, J.M., Pina, B., 2009. Physiological responses to mercury in feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site. Aquat Toxicol 93, 150-157.

Nichols, J.W., Wedemeyer, G.A., Mayer, F.L., Dickhoff, W.W., Gregory, S.V., Yasutake, W.T., Smith, S.D., 1984. Effects of freshwater exposure to arsenic trioxide on the parr-smolt transformation of coho salmon (*Oncorhynchus kisutch*). Environmental Toxicology and Chemistry 3, 143-149.

Nicholson, G.J., Theodoropoulos, T., Fabris, G.J., 1994. Hydrocarbons, Pesticides, Pcb and Pah in Port Phillip Bay (Victoria) Sand Flathead. Marine Pollution Bulletin 28, 115-120.

Nogueira, P., Pacheco, M., Lourdes Pereira, M., Mendo, S., Rotchell, J.M., 2010. Anchoring novel molecular biomarker responses to traditional responses in fish exposed to environmental contamination. Environ Pollut 158, 1783-1790.

Nowak, B.F., Clark, A., 1999. Prevalence of epitheliocystis in Atlantic salmon, *Salmo salar* L., farmed in Tasmania, Australia. Journal of fish diseases 22, 73-78.

Nowak, B.F., Dawson, D., Basson, L., Deveney, M., Powell, M.D., 2004. Gill histopathology of wild marine fish in Tasmania: potential interactions with gill health of cultured Atlantic salmon, *Salmo salar* L. J Fish Dis 27, 709-717.

Noyes, P.D., Lema, S.C., Roberts, S.C., Cooper, E.M., Stapleton, H.M., 2014. Rapid method for the measurement of circulating thyroid hormones in low volumes of teleost fish plasma by LC-ESI/MS/MS. Anal Bioanal Chem 406, 715-726.

Oliva, M., Garrido, M.C., Sales Marquez, D., Gonzalez de Canales, M.L., 2009. Sublethal and lethal toxicity in juvenile Senegal sole (*Solea senegalensis*) exposed to copper: a preliminary toxicity range finding test. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 61, 113-121.

Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. Thyroid 15, 799-813.

Pandey, S., Parvez, S., Ansari, R.A., Ali, M., Kaur, M., Hayat, F., Ahmad, F., Raisuddin, S., 2008. Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. Chem Biol Interact 174, 183-192.

Park, B.Y., Chung, J., 2009. Cadmium increases ferroportin-1 gene expression in J774 macrophage cells via the production of reactive oxygen species. Nutr Res Pract 3, 192-199.

Parry, G.D., Scientific, C., 1995. The distribution, abundance and diets of demersal fish in Port Phillip Bay. CSIRO INRE Port Phillip Bay Environment Study.

Patrick, L., 2009. Thyroid disruption: mechanism and clinical implications in human health. Alternative medicine review : a journal of clinical therapeutic 14, 326-346.

Penglase, S., Hamre, K., Ellingsen, S., 2014. Selenium prevents downregulation of antioxidant selenoprotein genes by methylmercury. Free Radical Biology and Medicine 75, 95-104.

Pereira, S., Pinto, A.L., Cortes, R., Fontainhas-Fernandes, A., Coimbra, A.M., Monteiro, S.M., 2013. Gill histopathological and oxidative stress evaluation in native fish captured in Portuguese northwestern rivers. Ecotoxicol Environ Saf 90, 157-166.

Pérez-Carrera, E., León, V.M.L., Parra, A.G., González-Mazo, E., 2007. Simultaneous determination of pesticides, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in seawater and interstitial marine water samples, using stir bar sorptive extraction–thermal desorption–gas chromatography–mass spectrometry. Journal of Chromatography A 1170, 82-90.

Phillips, D., 1976. The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. II. Relationship of metals in the mussel to those discharged by industry. Marine Biology 38, 71-80.

Phillips, D.J., 1977. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments a review. Environmental Pollution (1970) 13, 281-317.

Phillips, D.J.H., Richardson, B.J., Murray, A.P., Fabris, J.G., 1992. Trace-Metals, Organochlorines and Hydrocarbons in Port Phillip Bay, Victoria a Historical Review. Marine Pollution Bulletin 25, 200-217.

Picard-Aitken, M., Fournier, H., Pariseau, R., Marcogliese, D.J., Cyr, D.G., 2007. Thyroid disruption in walleye (*Sander vitreus*) exposed to environmental contaminants: cloning and use of iodothyronine deiodinases as molecular biomarkers. Aquat Toxicol 83, 200-211.

Pierce, K.V., Mccain, B.B., Wellings, S.R., 1980. Histopathology of Abnormal Livers and Other Organs of Starry Flounder *Platichthys stellatus* (Pallas) from the Estuary of the Duwamish River, Seattle, Washington, USA. Journal of fish diseases 3, 81-91.

Pino, V., Ayala, J.H., Afonso, A.M., Gonzalez, V., 2000. Determination of polycyclic aromatic hydrocarbons in marine sediments by high-performance liquid chromatography after microwave-assisted extraction with micellar media. Journal of chromatography. A 869, 515-522.

Pinto, E., Sigaud-kutner, T., Leitao, M.A., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal–induced oxidative stress in algae1. Journal of Phycology 39, 1008-1018.

Ploetz, D., Fitts, B., Rice, T., 2007. Differential accumulation of heavy metals in muscle and liver of a marine fish,(King Mackerel, *Scomberomorus cavalla* Cuvier) from the Northern Gulf of Mexico, USA. B Environ Contam Tox 78, 134-137.

Poleksic, V., Lenhardt, M., Jaric, I., Djordjevic, D., Gacic, Z., Cvijanovic, G., Raskovic, B., 2010. Liver, gills, and skin histopathology and heavy metal content of the Danube sterlet (*Acipenser ruthenus* Linnaeus, 1758). Environmental toxicology and chemistry / SETAC 29, 515-521.

Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjornsson, B.T., Einarsdottir, I.E., Canario, A.V., Sweeney, G.E., 2001. Thyroid hormones in growth and development of fish. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 130, 447-459.

Power, D.M., Morgado, I., Cardoso, J.C., 2009. Evolutionary insights from fish transthyretin, Recent Advances in Transthyretin Evolution, Structure and Biological Functions. Springer, pp. 59-75.

Qian, Y., Falahatpisheh, M.H., Zheng, Y., Ramos, K.S., Tiffany-Castiglioni, E., 2001. Induction of 78 kD glucose-regulated protein (GRP78) expression and redox-regulated transcription factor activity by lead and mercury in C6 rat glioma cells. Neurotox Res 3, 581-589.

Quiros, L., Pina, B., Sole, M., Blasco, J., Lopez, M.A., Riva, M.C., Barcelo, D., Raldua, D., 2007. Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: laboratory and field studies. Chemosphere 67, 1144-1154.

Rainbow, P.S., 1995. Biomonitoring of heavy metal availability in the marine environment. Marine Pollution Bulletin 31, 183-192.

Ratkowsky, D., Dix, T., Wilson, K., 1975. Mercury in fish in the Derwent Estuary, Tasmania, and its relation to the position of the fish in the food chain. Marine and Freshwater Research 26, 223-231.

Raymond, L.J., Ralston, N.V., 2004. Mercury: selenium interactions and health implications. Seychelles Medical and Dental Journal 7, 72-77.

Reddy, R.K., Mao, C., Baumeister, P., Austin, R.C., Kaufman, R.J., Lee, A.S., 2003. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278, 20915-20924.

Rees, C.B., McCormick, S.D., Vanden Heuvel, J.P., Li, W.M., 2003. Quantitative PCR analysis of CYP1A induction in Atlantic salmon (*Salmo salar*). Aquatic Toxicology 62, 67-78.

Ribeiro, C.A., Vollaire, Y., Sanchez-Chardi, A., Roche, H., 2005. Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. Aquat Toxicol 74, 53-69.

Roberts, A.P., Oris, J.T., Burton, G.A., Clements, W.H., 2005. Gene expression in caged fish as a first-tier indicator of contaminant exposure in streams. Environmental Toxicology and Chemistry 24, 3092-3098.

Rozsa, L., Reiczigel, J., Majoros, G., 2000. Quantifying parasites in samples of hosts. The Journal of parasitology 86, 228-232.

Sahu, S.N., Lewis, J., Patel, I., Bozdag, S., Lee, J.H., Sprando, R., Cinar, H.N., 2013. Genomic analysis of stress response against arsenic in *Caenorhabditis elegans*. PloS one 8, e66431.

Sanchez, W., Porcher, J.M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. Trac Trend Anal Chem 28, 150-158.

Schirmer, K., 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology 224, 163-183.

Schlenk, D., Wolford, L., Chelius, M., Steevens, J., Chan, K.M., 1997. Effect of arsenite, arsenate, and the herbicide monosodium methyl arsonate (MSMA) on hepatic metallothionein expression and lipid peroxidation in channel catfish. Comparative biochemistry and physiology. Part C, Pharmacology, toxicology & endocrinology 118, 177-183.

Schlenk, D., Zhang, Y.S., Nix, J., 1995. Expression of hepatic metallothionein messenger RNA in feral and caged fish species correlates with muscle mercury levels. Ecotoxicol Environ Saf 31, 282-286.

Schnitzler, J.G., Celis, N., Klaren, P.H., Blust, R., Dirtu, A.C., Covaci, A., Das, K., 2011. Thyroid dysfunction in sea bass (*Dicentrarchus labrax*): underlying mechanisms and effects of polychlorinated biphenyls on thyroid hormone physiology and metabolism. Aquat Toxicol 105, 438-447.

Shao, C.C., Li, N., Zhang, Z.W., Su, J., Li, S., Li, J.L., Xu, S.W., 2014. Cadmium supplement triggers endoplasmic reticulum stress response and cytotoxicity in primary chicken hepatocytes. Ecotoxicol Environ Saf 106, 109-114.

Shen, S., Li, X.F., Cullen, W.R., Weinfeld, M., Le, X.C., 2013. Arsenic binding to proteins. Chem Rev 113, 7769-7792.

Sigel, A., Sigel, H., Sigel, R.K., 2009. Metallothioneins and related chelators. Royal Society of Chemistry.

Silke, J., Meier, P., 2013. Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. Cold Spring Harb Perspect Biol 5, a008730.

Simon, E., Bytingsvik, J., Jonker, W., Leonards, P.E., de Boer, J., Jenssen, B.M., Lie, E., Aars, J., Hamers, T., Lamoree, M.H., 2011. Blood plasma sample preparation method for the assessment of thyroid hormone-disrupting potency in effect-directed analysis. Environ Sci Technol 45, 7936-7944.

Smith, J.D., Bagg, J., Wrigley, I., 1991. Extractable Polycyclic-Hydrocarbons in Waters from Rivers in South-Eastern Australia. Water Research 25, 1145-1150.

Sola, F., Isaia, J., Masoni, A., 1995. Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss*. J Appl Toxicol 15, 391-398.

Sommer, T., Jarosch, E., 2002. BiP binding keeps ATF6 at bay. Developmental cell 3, 1-2.

Sonne, C., Bach, L., Sondergaard, J., Riget, F.F., Dietz, R., Mosbech, A., Leifsson, P.S., Gustavson, K., 2014. Evaluation of the use of common sculpin (*Myoxocephalus scorpius*) organ histology as bioindicator for element exposure in the fjord of the mining area Maarmorilik, West Greenland. Environ Res 133, 304-311.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Feist, S.W., 2009. Site-specific disease profiles in fish and their use in environmental monitoring. Marine Ecology Progress Series 381, 1-15.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Thain, J.E., Feist, S.W., 2010. Effect of age on liver pathology and other diseases in flatfish: implications for assessment of marine ecological health status. Marine Ecology Progress Series 411, 215-230.

Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M., Feist, S.W., 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar Environ Res 55, 137-159.

Stephens, S.M., Alkindi, A.Y.A., Waring, C.P., Brown, J.A., 1997. Corticosteroid and thyroid responses of larval and juvenile turbot exposed to the water-soluble fraction of crude oil. Journal of fish Biology 50, 953-964.

Stride, M.C., Nowak, B.F., 2014. Epitheliocystis in three wild fish species in Tasmanian waters. J Fish Dis 37, 157-162.

Sures, B., 2001. The use of fish parasites as bioindicators of heavy metals in aquatic ecosystems: a review. Aquatic Ecology 35, 245-255.

Symons, R., Stevenson, G., Gaus, C., 2006. Assessment of concentrations of polybrominated diphenyl etherflame retardants in aquatic environments in Australia.

Talbot, V.W., Magee, R.J., Hussain, M., 1976. Cadmium in port Phillip Bay mussels. Marine Pollution Bulletin 7, 84-86.

Tan, S.W., Meiller, J.C., Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. Crit Rev Toxicol 39, 228-269.

Taylor, D.M., Taylor, D.M., Williams, D.R., 1995. Trace element medicine and chelation therapy. Royal society of chemistry.

Thain, J.E., Vethaak, A.D., Hylland, K., 2008. Contaminants in marine ecosystems: developing an integrated indicator framework using biological-effect techniques. Ices J Mar Sci 65, 1508-1514.

Theil, E.C., 1987. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Annu Rev Biochem 56, 289-315.

Thornalley, P.J., Vasak, M., 1985. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta 827, 36-44.

Tom, M., Chen, N., Segev, M., Herut, B., Rinkevich, B., 2004. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. Marine Pollution Bulletin 48, 705-710.

Troadec, M.B., Ward, D.M., Lo, E., Kaplan, J., De Domenico, I., 2010. Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux. Blood 116, 4657-4664.

Usuki, F., Fujita, E., Sasagawa, N., 2008. Methylmercury activates ASK1/JNK signaling pathways, leading to apoptosis due to both mitochondria and endoplasmic reticulum (ER)-generated processes in myogenic cell lines. Neurotoxicology 29, 22-30.

Uysal, K., Emre, Y., Kose, E., 2008. The determination of heavy metal accumulation ratios in muscle, skin and gills of some migratory fish species by inductively coupled plasma-optical emission spectrometry (ICP-OES) in Beymelek Lagoon (Antalya/Turkey). Microchemical Journal 90, 67-70.

Vahter, M., Marafante, E., 1983. Intracellular interaction and metabolic fate of arsenite and arsenate in mice and rabbits. Chem Biol Interact 47, 29-44.

van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13, 57-149.

van Dyk, J.C., Pieterse, G.M., van Vuren, J.H., 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. Ecotoxicol Environ Saf 66, 432-440.

Verdouw, J., 2008. Heavy metal contamination in muscle tissue of four key recreational fish species from the Derwent estuary. University of Tasmania.

Verdouw, J.J., Macleod, C.K., Nowak, B.F., Lyle, J.M., 2011. Implications of Age, Size and Region on Mercury Contamination in Estuarine Fish Species. Water Air Soil Poll 214, 297-306.

Viarengo, A., Burlando, B., Dondero, F., Marro, A., Fabbri, R., 1999. Metallothionein as a tool in biomonitoring programmes. Biomarkers 4, 455-466.

Wada, H., Cristol, D.A., McNabb, F.M., Hopkins, W.A., 2009. Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. Environ Sci Technol 43, 6031-6038.

Walker, T.I., 1982. Effects of length and locality on the mercury content of blacklip abalone, *Notohaliotis ruber* (Leach), blue mussel, *Mytilus edulis* planulatus (Lamarck), sand flathead, *Platycephalus bassensis* Cuvier & Valenciennes, and long-nosed flathead, *Platycephalus caeruleopunctatus* (McCulloch), from Port Phillip Bay, Victoria. Marine and Freshwater Research 33, 553.

Wang, S.L., Xu, X.R., Sun, Y.X., Liu, J.L., Li, H.B., 2013. Heavy metal pollution in coastal areas of South China: a review. Mar Pollut Bull 76, 7-15.

Ward, D.M., Kaplan, J., 2012. Ferroportin-mediated iron transport: expression and regulation. Biochim Biophys Acta 1823, 1426-1433.

Ward, D.M., Nislow, K.H., Folt, C.L., 2010. Bioaccumulation syndrome: identifying factors that make some stream food webs prone to elevated mercury bioaccumulation. Ann Ny Acad Sci 1195, 62-83.

Warnick, S.L., Bell, H.L., 1969. The acute toxicity of some heavy metals to different species of aquatic insects. Journal (Water Pollution Control Federation), 280-284.

Weng, C.Y., Chiou, S.Y., Wang, L., Kou, M.C., Wang, Y.J., Wu, M.J., 2014. Arsenic trioxide induces unfolded protein response in vascular endothelial cells. Arch Toxicol 88, 213-226.

Wenning, R.J., Martello, L., 2013. POPs in Marine and freshwater environments. Environ. Forensics Persistent Org. Pollut 35.

Williams, H.H., Mackenzie, K., 2003. Marine parasites as pollution indicators: an update. Parasitology 126, S27-S41.

Williams, T.D., Diab, A., Ortega, F., Sabine, V.S., Godfrey, R.E., Falciani, F., Chipman, J.K., George, S.G., 2008. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. Aquat Toxicol 90, 83-91.

Wolke, R., 1992. Piscine macrophage aggregates: a review. Annual Review of Fish Diseases 2, 91-108.

Xu, H., Lam, S.H., Shen, Y., Gong, Z., 2013. Genome-wide identification of molecular pathways and biomarkers in response to arsenic exposure in zebrafish liver. PloS one 8, e68737.

Yamano, K., 2005. The role of thyroid hormone in fish development with reference to aquaculture. Jarq-Jpn Agr Res Q 39, 161-168.

Yamashita, M., Ojima, N., Sakamoto, T., 1996. Molecular cloning and cold-inducible gene expression of ferritin H subunit isoforms in rainbow trout cells. J Biol Chem 271, 26908-26913.

Yamashita, Y., Tominaga, O., Takami, H., Yamada, H., 2003. Comparison of growth, feeding and cortisol level in *Platichthys bicoloratus* juveniles between estuarine and nearshore nursery grounds. Journal of fish Biology 63, 617-630.

Yamauchi, K., Ishihara, A., 2009. Evolutionary changes to transthyretin: developmentally regulated and tissue-specific gene expression. Febs J 276, 5357-5366.

Yamauchi, K., Ishihara, A., Fukazawa, H., Terao, Y., 2003. Competitive interactions of chlorinated phenol compounds with 3,3',5-triiodothyronine binding to transthyretin: detection of possible thyroid-disrupting chemicals in environmental waste water. Toxicol Appl Pharmacol 187, 110-117.

Yan, H., Wang, N., Weinfeld, M., Cullen, W.R., Le, X.C., 2009. Identification of arsenic-binding proteins in human cells by affinity chromatography and mass spectrometry. Anal Chem 81, 4144-4152.

Yang, X., An, L., Li, X., 2014. Arsenic trioxide induced endoplasmic reticulum stress in laryngeal squamous cell line Hep-2 cells. Auris Nasus Larynx 41, 81-83.

Yaoita, Y., Brown, D.D., 1990. A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev 4, 1917-1924.

Yasutake, A., Nakamura, M., 2011. Induction by mercury compounds of metallothioneins in mouse tissues: inorganic mercury accumulation is not a dominant factor for metallothionein induction in the liver. The Journal of toxicological sciences 36, 365-72.

Yilmaz, F., Ozdemir, N., Demirak, A., Tuna, A.L., 2007. Heavy metal levels in two fish species *Leuciscus cephalus* and *Lepomis gibbosus*. Food Chem 100, 830-835.

Yin, Z., Jiang, H., Lee, E.S., Ni, M., Erikson, K.M., Milatovic, D., Bowman, A.B., Aschner, M., 2010. Ferroportin is a manganese-responsive protein that decreases manganese cytotoxicity and accumulation. J Neurochem 112, 1190-1198.

Yu, L., Deng, J., Shi, X., Liu, C., Yu, K., Zhou, B., 2010. Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic-pituitary-thyroid axis of zebrafish larvae. Aquat Toxicol 97, 226-233.

Yu, L., Lam, J.C., Guo, Y., Wu, R.S., Lam, P.K., Zhou, B., 2011. Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in zebrafish. Environ Sci Technol 45, 10652-10659.

Zacchino, V., Minghetti, M., Centoducati, G., Leaver, M.J., 2012. Diablo/SMAC: a novel biomarker of pollutant exposure in European flounder (*Platichthys flesus*). Ecotoxicol Environ Saf 79, 176-183.

Zafarullah, M., Olsson, P.E., Gedamu, L., 1990. Differential regulation of metallothionein genes in rainbow trout fibroblasts, RTG-2. Biochim Biophys Acta 1049, 318-323.

Zakin, M.M., 1992. Regulation of transferrin gene expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 6, 3253-3258.

Zhang, F., Degitz, S.J., Holcombe, G.W., Kosian, P.A., Tietge, J., Veldhoen, N., Helbing, C.C., 2006. Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors. Aquat Toxicol 76, 24-36.

Zhang, Y., Lu, R., Liu, W., Wu, Y., Qian, H., Zhao, X., Wang, S., Xing, G., Yu, F., Aschner, M., 2013. Hormetic effects of acute methylmercury exposure on grp78 expression in rat brain cortex. Dose-response : a publication of International Hormesis Society 11, 109-120.

Zhao, L., Xia, Z., Wang, F., 2014. Zebrafish in the sea of mineral (iron, zinc, and copper) metabolism. Front Pharmacol 5, 33.

Zhong, B., Mao, H., Fan, Q., Liu, Y., Hu, Y., Mi, Y., Wu, F., Hu, C., 2014. SiRNA-mediated knockdown of CiGRP78 gene expression leads cell susceptibility to heavy metal cytotoxicity. Gene 552, 219-224.

Zhu, Y., Fan, Q., Mao, H., Liu, Y., Hu, C., 2013. GRP78 from grass carp (*Ctenopharyngodon idella*) provides cytoplasm protection against thermal and $Pb^{2+}$ stress. Fish & shellfish immunology 34, 617-622.

Zoeller, R.T., Tan, S.W., Tyl, R.W., 2007. General background on the hypothalamic-pituitary-thyroid (HPT) axis. Crit Rev Toxicol 37, 11-53.