CONTAMINANT EXPOSURE AFFECTS GENE EXPRESSION MARKERS IN THE CYSTEINE METABOLISM OF *CHIRONOMUS TEPPERI*

Submitted by

Katherine Joanna Jeppe

Submitted in fulfillment of the degree of Doctor of Philosophy

October 2015

School of BioSciences 4

Faculty of Science

The University of Melbourne

ABSTRACT

In ecotoxicology, organisms are often exposed to contaminated water or sediment to investigate the toxicity of these aspects of the environment. The results of these exposures are intended to predict responses in the ecosystem. However, these tests often employ a single and sometimes tolerant organism and have been criticized for lacking environmental relevance. The aim of this thesis is to improve toxicity tests that use the Australian species *Chironomus tepperi* by developing sensitive and rapid gene expression biomarkers of metal exposure. The pathway chosen for the development of these biomarkers is cysteine metabolism. Cysteine is central to several stress responses and is only produced by transsulfuration of methionine. Hence, it potentially offers informative, specific and consistent biomarkers of metal exposure.

Nine genes involved in the cysteine metabolism were identified in *C. tepperi*, using databases of sequences in related dipteran species. Initially, the expression of these genes was assessed under 24 hour water exposures to different metals. It was found that all of the genes considered respond to metal exposure and that the metals tested induce different responses in gene expression profiles. These results provided the basis for further testing. A pulse water exposure was then investigated to establish if these biomarkers responded to both past and present pollution. Gene expression responses correlated with glutathione-S-transferase (GST) activity after the 24 hour exposure and with metallothionein (MT) concentration after the 96 hour depuration. These results highlighted the need to know exposure time and duration to accurately assess gene expression responses.

Expression of these genes was then investigated in sediment exposures in the laboratory and the field. Gene expression and metabolomic profiles in laboratory-bred *C. tepperi* were investigated after copper exposure in sediment of a field-based microcosm. These responses were then compared

to indigenous macroinvertebrate community responses and population responses of *Potamopyrgus antipodarum* and *Physa acuta*. This experiment demonstrated that gene expression and metabolomic responses were sensitive and correlative markers of copper exposure and that these markers respond at concentrations that caused a decrease in sensitive macroinvertebrate abundances. Finally, a 5 day toxicity test was performed to assess expression profiles of cysteine metabolism genes in *C. tepperi* after exposure to a simple metal-pesticide mixture as well as the first exposure to field sediments. Expression changes were then compared to whole organism endpoints traditionally used in these tests. This chapter established the usefulness of gene expression in a standard toxicity test, and reinforced the sensitivity of this technique while it also highlighted the complex nature of mixture toxicity.

This research is an initial step toward the development of gene expression biomarkers of cysteine metabolism in *C. tepperi* to be incorporated into sediment toxicity testing. Within limitations, gene expression in cysteine metabolism can provide a powerful addition to a multiple lines-of-evidence approach, particularly if used in conjunction with other cellular biomarkers such as metabolomics, MT concentration or GST activity. This thesis therefore contributes baseline scientific information that is critical if this biomarker is to be useful for toxicity testing.

DECLARATION

This is to certify that:

    i.   The thesis comprises only my original work towards the PhD

    ii.   Due acknowledgement has been made in the text to all other material used

    iii.   The thesis is less than 100 000 words in length, excluding tables, maps, bibliographies and appendices

Katherine Joanna Jeppe

October 2015

PREFACE

This thesis comprises two results chapters (chapter 3 and 4), one scientific paper and one submitted paper.

The published paper is:

Katherine J. Jeppe, Melissa E. Carew, Sara Long, Siu F. Lee, Vincent Pettigrove, Ary A. Hoffmann (2014) Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 162,1-6. (Chapter 2)

The content is my own work and co-authorship represents a supervisory role of the other authors. Other authors provided scientific advice and training in techniques, assisted with statistical analyses, and reviewed the manuscript before submission.

The submitted paper is:

Katherine J. Jeppe, Melissa E. Carew, Vincent Pettigrove and Ary A. Hoffmann (submitted) Toxicant mixtures in sediment alter gene expression in the cysteine metabolism of *Chironomus tepperi. Environmental Toxicology and chemistry* (Chapter 5)

The content is my own work and co-authorship represents a supervisory role of the other authors. Other authors provided scientific advice and training in techniques, assisted with statistical analy ses, and reviewed the manuscript before submission.

Dr. Sara Long   Dr. Siu F. Lee

ACKNOWLEDGMENTS

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CHAPTER 1 – GENERAL INTRODUCTION

Aquatic environments are often threatened by anthropogenic activities, such as habitat degradation, altered hydrology and aquatic pollution. It is important to develop tools that enable authorities to monitor and detect impacts of anthropogenic activities before serious environmental damage occurs. Recent developments in molecular technologies provide opportunities to achieve this. This thesis explores how genetic biomarkers can be employed to monitor aquatic ecosystems. Biomarkers are developed to measure the expression of several genes involved in cysteine metabolism in context with components and metabolites in the same pathway. Once developed these biomarkers are tested using both lab-based water and sediment toxicity tests (OECD, 2004; US-EPA, 1996) and a field-based microcosm technique (Pettigrove and Hoffmann, 2005). The microcosm technique used in Chapter 4 has previously been used to investigate the impacts of urban sediments (Carew et al., 2007), agricultural sediments (Sharley et al., 2008; Townsend et al., 2009) and road runoff (Pettigrove et al., 2007). By considering several genes involved in cysteine metabolism this thesis hopes to develop contextual biomarkers of contamination exposure and to comment on how these techniques could apply in the future, with particular attention to the methods employed in this thesis.

1.1 Ecotoxicology

Ecotoxicology investigates the fate and effects of contaminants in the environment and aims to predict, detect, monitor and control the impacts of contaminants in complex ecosystems (Forbes and Forbes, 1994; Moriarty, 1983). Anthropogenic activities cause contaminants to enter the aquatic environment, which poses an increasing problem with continued urbanization and industrialization. Large-scale production of chemicals can cause waterways to be contaminated with many known and novel chemicals. This

can result in routine chemical analyses not always predicting toxicity (Chapman, 1995; Giesy and Hoke, 1991).

In aquatic environments chemicals often occur as pulse events, where episodic pollutants from intermittent events, such as agricultural spraying, sewer spills, storm events and runoff from industrial and construction sites, can present short bursts of high level contamination to aquatic ecosystems (e.g. Brent and Herricks, 1998; Hosmer et al., 1998). Over time many chemicals introduced to waterways bind to suspended particles, settle and accumulate in the sediment. Thus, sediment contamination often occurs at orders of magnitude higher concentrations and is more persistent over time than water contamination in the same system (Burton, 1991).

The interaction of multiple environmental factors (e.g. hydrology, temperature, hardness) with sediment contaminants combined with ecosystem heterogeneity can make studying ecosystem ecotoxicology highly specific and costly (Forbes and Forbes, 1994). Due to these difficulties, the risk of contaminants to ecosystems is assessed using laboratory-based approaches or field-based approaches, with each approach providing advantages and disadvantages.

1.2 Laboratory-based ecotoxicology

Laboratory-based approaches reduce the ecosystem into manageable fragments investigating one or a few aspects of ecosystem threat and use the observed responses as indicators of higher ecological effect (Chapman, 1995; O'Brien and Keough, 2014). These tests offer tight control over environmental conditions and exposure regimes allowing researchers to investigate specific factors of concern (OECD, 2004; US-EPA, 1996).

Laboratory-based tests often employ a single and sometimes tolerant organism to measure one or a few responses usually at the level of the individual and below. Investigating these low-level changes can link exposure to toxicity and in the case of biomarker responses can provide information on the type of chemical and its mode of action (Beaty et al., 1998; Bundy et al.,

2009). Laboratory-based tests are often run as a battery, with several tests on organisms from different groups (e.g. fish, crustacean, insect, algae). This approach can provide rapid and quantifiable evidence of adverse impacts of an exposure to some aspect of an ecosystem (Ireland and Ho, 2005; Nogueira et al., 2015). The use of multiple species from different groups is intended to reduce uncertainty caused by selecting a single surrogate species and to assist in extrapolation to complex ecosystem. However, while this addresses the single species problem, the individual and biomarker level responses themselves are rarely linked to ecologically relevant changes in populations, communities or ecosystems (Ingersoll et al., 2005; O'Brien and Keough, 2014; Sulmon et al., 2015; Wang et al., 2004). Without this link it is possible that the responses observed, while giving information on exposure, may not represent any fitness costs to organisms *in situ* (Lam, 2009).

1.3 Field-based ecotoxicology

Field-based assessments investigate ecosystem changes under anthropogenic pressure. These assessments can be field surveys of communities, such as rapid bioassessment, or micro/mesocosm experiments (Chessman, 1995; Pettigrove and Hoffmann, 2005). These approaches give an overview of ecosystem responses at the population, community or ecosystem level relative to reference/control conditions. Rapid biossessments are widely used in Australia (Chessman, 1995) and abroad (Armitage et al., 1983). However, causal relationships between environmental factors and observed responses cannot be established with field surveys alone (Norton et al., 2003). Furthermore, true replication of sites used in field surveys is impossible so temporal or pseudo-replication is often used. Field-based microcosm and mesocosm experiments have been developed to increase understanding of causality and allow replication. These techniques isolate a smaller, less complex environment and, while not achieving identical conditions to the environment, they are considered more environmentally realistic than laboratory-based tests (Dzialowski et al., 2014). These tests can allow the assessment of several indigenous species, assessing population and community responses. In microcosms, indigenous species can be more

sensitive than laboratory-bred species, adding further ecological relevance to the results obtained from the field-based experiment.

1.4 Biomarkers in ecotoxicology

Biomarkers originated from human toxicology where they proved to be useful measures of human exposure to specific chemicals or as early warning indicators of specific diseases or syndromes (Timbrell, 1998). Defined as the identification of specific molecular, biochemical, physiological and behavioral changes in populations of animals and plants following pollutant exposure, biomarkers show promise for use in ecotoxicology and ecological risk assessment (Depledge and Fossi, 1994). Biomarkers offer tools to investigate class-specific exposure on biota and can indicate whether organisms have been or are being exposed to certain chemicals, which may result in impairments of the ecosystem (Forbes et al., 2006).

There are well-established criteria for biomarker development (Huggett et al., 2002; McCarthy and Shugart, 1990; Peakall, 1994), with current research focused on evaluating biomarker responses with respect to these criteria. Many aspects of subindividual responses are investigated by current ecotoxicological research and show potential as markers of contaminant exposure and toxic effects of exposure (e.g. Arini et al., 2015; Arora and Kumar, 2015; Loayza-Muro et al., 2013). Studies in this area generally identify one or a group of potential biomarkers and measure their response under different stress scenarios (e.g. Martin-Folgar et al., 2015; Nair and Chung, 2015; Park et al., 2009; Planello et al., 2015; Planello et al., 2008). These studies investigating gene expression, often measure genes of interest using quantitative polymerase chain reaction (qPCR) (e.g. Hook et al., 2014b). This approach is cost effective and can be targeted to specific genes of interest; however, it does require specific genes to be previously identified. Recently, advances in 'omics technologies such as next-generation sequencing (NGS), High performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) and High performance Gas Chromatography-Mass Spectrometry (HPGC-MS), have assisted in identifying cellular responses to contaminants in non-model

biota during exposure (Bundy et al., 2009; Hook, 2010; Lemos et al., 2010; Marinkovic et al., 2012a; Marinkovic et al., 2012b; Nair et al., 2011; Snell et al., 2003). These 'omic technologies allow changes to be examined at the cellular level, such as through the transcriptome or metabolome. This allows a holistic view of cell response from which specific responses to contaminants can be identified and explored. 'Omic techniques have great potential to investigate untargeted molecular pathways and discover new biomarkers that can be used to design cost-effective and specific assays. Gene expression measured by untargeted NGS can provide a wide and semi-quantitative view of expression changes in an organism.

For ecological assessment it is important to link biomarkers to an adverse outcome at a biological level of organization relevant to risk assessment (Forbes et al., 2006). To address this challenge adverse outcome pathways (AOPs) have been proposed as conceptual frameworks to include biomarkers in ecotoxicological and ecological risk assessment (Ankley et al., 2010). Gene expression is the first response of organisms that are exposed to stress and can be a rapid and sensitive biomarker. When genes are expressed, enzyme and protein production is initiated. While changes in expression often correlate with changes in protein, metabolite or enzyme levels, this is not always the case. Therefore, it is often not clear if a change in gene expression can be linked with adverse outcomes for the individual or population considered. For this reason, considering gene expression in the context of a reasonably well-understood pathway with related components such as metabolites in the same system could allow development of a robust, sensitive and rapid biomarker suitable for an AOP.

For this thesis, desirable criteria for biomarker performance have been defined as sensitive, specific, robust, reproducible and cost effective. It is also considered important to determine if the biomarker indicates a toxic effect so the biomarker can be considered for inclusion in an AOP.

1.5 Cysteine metabolism

This thesis considers gene expression in the context of the cysteine metabolism pathway, which plays a central role in an organism's detoxification after exposure (Jones et al., 2004; Sugiura et al., 2005). The pathway begins with the essential amino acid methionine, which is obtained from food. The demethylation and remethylation of methionine are essential for cellular repair and function (Chen et al., 2010; James et al., 2002). Cysteine is produced from methionine by the transsulfuration pathway, via the intermediates homocysteine and cystathionine. Cysteine can only be produced in this way and is required for several proteins, including metallothionein (MT), that protect cells from metal exposure. Cysteine is also a precursor for glutathione (GSH) and is involved in several antioxidant responses (Hughes et al., 2009). Several genes regulate cysteine metabolism and its intermediates and the transcription of these genes can report on dynamics of the cysteine metabolic pathway, which in turn can inform how an organism responds to exposure.

1.6 *Chironomus tepperi* as a test species

Species used in laboratory and microcosm tests are limited to those that can survive and reproduce in laboratory conditions or colonize field-based microcosms (Kimball and Levin 1985). Test organisms should be selected based on their importance to the ecosystem, but for practical reasons only a few surrogate species are used (Burton, 1991; Chapman, 1995). Chironomidae are a species-rich family of non-biting midges that satisfies these criteria. They are ubiquitous and have adapted to most habitats from terrestrial to marine and polar to tropical (Armitage et al., 1995). These taxa are functionally diverse and play an important role in aquatic ecosystems. They consume detritus and provide an important food source for several vertebrate and invertebrate groups. Chironomidae have been shown to have a wide range of sensitivities to contaminants and are often used in environmental monitoring (Carew et al., 2007; Cortelezzi et al., 2011).

In the laboratory, the most commonly used genus of Chironomidae is *Chironomus*. They are reasonably easy to culture and spend most of their

relatively short life cycle (20 to 28 days) as larvae exposed to the sediment (ASTM, 1997; OECD, 2004). In Australia there is an endemic species *Chironomus tepperi* that is used in laboratory-based toxicity tests. The sensitivity of *C. tepperi* is comparable to other *Chironomus* species, with a 96 hour LC50 of around 5.7 mg/L cadmium in water (Béchard et al., 2008; Fargasova, 2001). This makes *C. tepperi* a fairly tolerant laboratory species compared to indigenous species, so tests including biomarkers rather that mortality endpoints could make *C. tepperi* a better surrogate for more sensitive groups.

1.7 Linking species response to environmental condition

It is common for ecotoxicological experiments to be limited to either the laboratory or the field. Combining both field and laboratory methods provides a 'multiple lines-of-evidence' approach to ecosystem assessment. This enables strengths and weaknesses of different approaches to be addressed. For example, causality can be established with biomarker and individual responses and environmental relevance can be established with field-based deployments and assessments. (Heiskary and Bouchard, 2015; Kellar et al., 2014).

1.8 Thesis aims and overview

This thesis reports on the development of gene expression biomarkers for cysteine metabolism in *C. tepperi* intended for use in toxicity testing. Gene expression biomarkers for cysteine metabolism were developed and tested using criteria described in Chapter 1.4:

1) Biomarkers were tested for their sensitivity and relation to population impacts by comparing gene expression responses to whole organism endpoints (survival and dry weight) commonly measured in toxicity tests.

2) Biomarkers were tested for rapid response, robustness and reproducibility after predetermined exposure times in water and sediment toxicity tests, in the laboratory and in the field.

3) Gene expression biomarkers were also compared with other components of the cysteine metabolism to further assess the sensitivity and cost effectiveness compared to other potential biomarkers. This also provided the added advantage of placing gene expression in a physiological context.

In this way, the work described here focuses on developing a robust and informative biomarker for use in toxicity testing.

This thesis is divided into four experimental chapters and a conclusion chapter as described below.

Chapter 2 - Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium

Aim: To develop primers that amplify several genes involved in the cysteine metabolism of *C. tepperi* and to investigate the expression profile of these genes under sublethal exposure to different metals in water. Primers were developed for 8 genes involved in cysteine metabolism, encoding enzymes involved in the remethylation cycle (S-*adenosylmethionine synthetase; SAM* and *S-adenosylhomocysteine hydrolase; SAH*), the transsulfuration pathway (*cystathionine-γ-lyase*; *CγL*, *cystathionine-β-synthase*; *CβS*, *γ-glutamylcysteine synthase*; *GCS*, and *glutathione synthetase*; *GS*) and two resulting stress responses (*glutathione S-transferase delta 1*; *GSTd1* and *metallothionein*; *Mtn*). This chapter intended to establish if the target genes can be amplified and if they respond differently to different metals.

Chapter 3 – Gene expression and cysteine metabolism components in *Chironomus tepperi* alter after pulse exposure to cadmium

Aim: To understand the response of cysteine metabolism gene expression biomarkers in *C. tepperi* under pulse exposure of cadmium and to observe if gene expression, glutathione-S-transferase activity and metallothionein concentration are correlated directly after exposure and after depuration in

uncontaminated water. Contamination of waterways often occurs as highly contaminated pulses and if a biomarker is to be used in the field it is important to understand how it responds to present and past exposure. This chapter is intended to identify which genes demonstrate a rapid response to pollution exposure and if any retain evidence of past contamination. Furthermore, this research investigates if gene expression can be linked to population level impacts, which could indicate a toxic effect.

Chapter 4 – Multilevel bioindicators of copper toxicity – linking community impacts with individual responses

Aim: To compare bioindicators of copper toxicity at several levels of organization in laboratory-bred and indigenous species under field-based conditions. This chapter compares indigenous community responses in a field-based microcosm to population, individual and biomarker responses of laboratory-bred species: *C. tepperi*, *Potamopyrgus antipodarum* and *Physa acuta* in the field*.* These species were added to the microcosms for a known time period and bioindicators commonly measured in toxicity tests (survival, dry weight, reproduction and biomarkers) were measured alongside indigenous macroinvertebrate community response. This allowed the comparison of the sensitivities of these different approaches to copper toxicity. Biomarkers were measured in *C. tepperi*, through metabolomics and gene expression in the cysteine metabolism, to allow the type of copper exposure to be established for *C. tepperi.*

Chapter 5 – Toxicant mixtures in sediment alter gene expression in the cysteine metabolism of *Chironomus tepperi*

Aim: To investigate how expression profiles of cysteine metabolism genes in *C. tepperi* respond in a standard toxicity test involving a metal-pesticide mixture in sediment and for the first time demonstrate this technique with field mixtures. This chapter is intended to compare gene expression profile responses to whole organism endpoints traditionally used in these tests. This

chapter reinforces the sensitivity of this technique while also highlighting the complexities of measuring gene expression in real-world mixtures.

Chapter 6- General Discussion

This chapter summarizes the main findings and provides recommendations for further research.

CHAPTER 2 – GENES INVOLVED IN CYSTEINE METABOLISM OF *CHIRONOMUS TEPPERI* ARE REGULATED DIFFERENTLY BY COPPER AND BY CADMIUM

Abstract

Freshwater invertebrates are often exposed to metal contamination, and changes in gene expression patterns can help understand mechanisms underlying toxicity and act as pollutant-specific biomarkers. In this study the expression of genes involved in cysteine metabolism are characterized in the midge *Chironomus tepperi* during exposure to sublethal concentrations of cadmium and copper. These metals altered gene expression of the cysteine metabolism differently. Both metals decreased *S-adenosylhomocysteine hydrolase* expression and did not change the expression of *S-adenosylmethionine synthetase*. Cadmium exposure likely increased cystathionine production by upregulating *cystathionine-β-synthase* expression, while maintaining control level cysteine production via *cystathionine-γ-lyase* expression. Conversely, copper downregulated *cystathionine-β-synthase* expression and upregulated *cystathionine-γ-lyase* expression, which in turn could diminish cystathionine to favor cysteine production. Both metals upregulated glutathione related expression (*γ-glutamylcysteine synthase* and *glutathione synthetase*). Only cadmium upregulated *metallothionein* expression and *glutathione S-transferase d1* expression was upregulated only by copper exposure. These different transcription responses of genes involved in cysteine metabolism in *C. tepperi* point to metal-specific detoxification pathways and suggest that the transsulfuration pathway could provide biomarkers for identifying specific metals.

2.1 Introduction

Gene expression patterns are being increasingly used to identify exposure to stressful environmental conditions. In aquatic environments, they are used to examine how organisms respond to toxicants and are favored because they have the potential to identify sublethal stress that can lead to environmental degradation (Adams et al., 1989; Galay-Burgos et al., 2003). As a result, the US EPA now accepts gene expression data as part of a weight-of-evidence approach for environmental assessment (Van Aggelen et al., 2010). Several studies have investigated transcriptomic responses to pollution in freshwater biota (e.g. Hook et al., 2008; Marinkovic et al., 2012a) and for these data to be fully interpreted the underlying mechanisms causing changes in the transcriptome of studied species need to be understood.

Cysteine metabolism plays a central role in detoxification against environmental stressors (Jones et al., 2004; Sugiura et al., 2005) (Figure 2.1). Demethylation and remethylation of methionine is essential to cellular repair and function (Chen et al., 2010; James et al., 2002). Cysteine can only be produced via the transsulfuration pathway and is required for proteins, including metallothionein (MT), that protect cells directly from metal stress. Cysteine is also a precursor for glutathione (GSH) and is involved in several antioxidant responses (Hughes et al., 2009). Several genes regulate cysteine metabolism and its intermediates, the transcription of these genes can report on dynamics of the cysteine metabolic pathway, which in turn can inform how an organism responds to stressors.

The Chironomidae (Diptera), particularly species from the genus *Chironomus,* are commonly used in toxicological testing as several species can be cultured in the laboratory (for example: Dawson et al., 2000; Stevens, 1993; Watts and Pascoe, 2000). Furthermore, *Chironomus* larvae tend to live on or in the sediments and are exposed to hydrophobic toxicants in sediment (Burton, 1991). *Chironomus riparius* (Chironomidae) is a standard test organism (OECD, 2004; US-EPA, 1996) and *C. tentans* is widely used in toxicity testing

(Lee and Choi, 2009; Martinez et al., 2003; Rakotondravelo et al., 2006). The transcriptome of *C. riparius* was recently investigated (Marinkovic et al., 2012a), although only the responses of a few genes to different pollutants have been described in these species (Lee et al., 2006; Park et al., 2009; Park and Kwak, 2008; Planello et al., 2010). Neither of these species occurs in Australia, however *Chironomus tepperi* (Skuse) is widespread in the Australian mainland, can be easily cultured in the laboratory, and occurs in metal-contaminated habitats. While *C. tepperi* is used in toxicological testing, there is little sequence information and no gene expression data yet available for this species.

In this study, we isolate eight genes involved in the cysteine metabolism of *C. tepperi* using sequence alignments of conserved genes from other chironomid and dipteran species. The genes investigated encode enzymes involved in the remethylation cycle (*S-adenosylmethionine synthetase; SAM* and *S-adenosylhomocysteine hydrolase; SAH*), the transsulfuration pathway (*cystathionine-γ-lyase; CγL, cystathionine-β-synthase*; *CβS*, *γ-glutamylcysteine synthase*; *GCS,* and *glutathione synthetase*; *GS*) and two representing resulting stress response proteins (*glutathione S-transferase delta 1*; *GSTd1* and *metallothionein*; *Mtn*). The transcriptional responses of these genes were characterized after 24 hours exposure to two sublethal concentrations of copper (Cu: 0.05 mg/L and 0.5 mg/L) and cadmium (Cd: 0.25 mg/L and 0.5 mg/L). These metals were chosen for their likely different modes of action, as copper is an essential and cadmium is a non-essential metal. We show that these metals induce different changes in gene expression regulating for cysteine metabolism intermediates and products.

2.2 Materials and Methods

Organism

Chironomus tepperi were cultured from a stock originating from Yanco Agricultural Institute in New South Wales as described previously in Townsend et al. (2012). Briefly, larvae were maintained in culture medium similar to Martin's solution (Martin et al., 1980) which contained reverse osmosis water with 0.12mM $NaHCO_3$, 0.068mM $CaCl_2$, 0.083mM $MgSO_4$, 0.86mM NaCl, 0.015mM KH_2PO_4, 0.089mM $MgCl_2$ and 0.1% (w/v) iron chelate) at $21^oC \pm 1^oC$ and a 16:8 h light:dark photoperiod. Ethanol-rinsed tissue paper was used as artificial substrate in the culture tanks and larvae were fed ground tropical fish flakes (Tetramin ®) three times per week with a ration of 0.25 g per tank. Larvae that hatched from eggs were reared to third instar (9 days old) in aerated culture medium and fed three times a week until the start of exposures.

Exposure experiments

Five replicate beakers of ten larvae were exposed to cadmium chloride ($CdCl_2$) and cupric chloride ($CuCl_2$) in 100 mL culture medium. The surface area of the beaker ensured that each larva had a surface of 5.6 cm^2 to ensure overcrowding did not occur. Exposure concentrations were sublethal as defined by previous acute toxicity tests performed by the authors (Table 2.1). Total metals were determined by ICP-MS (ALS Global) and were within 10% of the nominal concentrations. Exposure concentrations are expressed as proportions of the concentration leading to 50% mortality (LC_{50}), to account for varied induction potentials for each metal (Yoshimi et al., 2009). Larvae were exposed for 24 h to culture medium only (control) or culture medium with low or high sublethal concentrations of Cu and Cd under culture conditions (Table 2.1). No food, substrate or aeration was provided and no mortality occurred during exposure. After 24 h, the ten larvae were pooled, blot dried, snap frozen in liquid nitrogen and stored at -80 ºC.

RNA extraction

Larval specimens were homogenized in liquid nitrogen and total RNA was isolated using an RNeasy® Mini kit (Qiagen Cat. no. 74106), following the manufacturer's protocol for animal tissue. Extracted RNA was treated with RNase-free DNase (Qiagen Cat. no. 79254) and eluted in 50 µL RNase-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and quantified using a Nanodrop (Thermo Scientific). Only RNA samples with a 260/280 ratio between 1.9 and 2.1 were used for cDNA synthesis.

Reverse transcription

Total RNA (2 µg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. no. M1705) following the manufacturer's protocol. The cDNA was then checked for gDNA contamination using a pair of *ribosomal protein L11* primers that flank a 345 bp intron. The expected cDNA amplicon was 466 bp, whereas the amplification of the 811 bp product would indicate the presence of gDNA (Martinez-Guitarte et al., 2007). Template cDNA was then stored at -20 ºC.

Isolation and sequencing of C. tepperi homologues

Literature and online databases were searched to collect sequences for cysteine metabolism genes present in species closely related to *C. tepperi*. Multiple species alignments were performed to identify conserved regions using ClustalW function in Molecular Evolutionary Genetics Analysis (MEGA) 5.0 (http://www.megasoftware.net/) (Larkin et al., 2007). Primers were then designed in these regions using Primer3Plus (www.bioinformatics.nl/primer3plus/). Directly sequenced amplicons from *C. tepperi* were then searched against protein databases NCBI (http://www.ncbi.nlm.nih.gov/) and FlyBase (http://flybase.org/) to ensure the correct gene sequences were amplified. Primers used to identify each gene sequence are displayed in Supplementary file 1 (Appendix 1). Gene sequences longer than 200 bp were submitted to GenBank and primers suitable for real-time PCR – fragment length 60–150 bp, optimal T_m 65 ºC and

primer length 19–26 bp – were designed within the sequence (Supplementary file 2 Appendix 1). The final qPCR amplicons were sequenced and searched against FlyBase and NCBI to ensure the correct product was amplified. Alignment of identification and qPCR amplicons, including primer locations, are displayed in Supplementary file 3 (Appendix 1).

Gene expression analysis

Gene expression was measured using quantitative real-time PCR (qPCR), with gene-specific primers. Quantitative real-time PCR was performed with the Roche LightCycler® 480 (Roche Applied Science, USA) in triplicate 10 µL reactions containing the components described by Takahashi et al. (2010). The following conditions were used: 10 minutes at 95 ºC, followed by 50 cycles of 10 seconds at 95 ºC, 15 seconds at 58 ºC, and 15 seconds at 72 ºC followed by a melting curve analysis from 65 ºC to 95 ºC to ensure a single product was formed.

Statistics

Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann et al., 2011). Differences in relative normalized expression of target genes between exposed and unexposed samples were assessed using ANOVA after verifying homogeneity of variances with Levene tests. Significance of ANOVA tests was evaluated after Dunnett's post hoc tests for multiple comparisons to control exposures. All Normalized data was analyzed using SPSS Statistics 19 (IBM Inc.). These results were compared to the conventional $2^{-\Delta\Delta Ct}$ method to estimate relative gene expression, against the geometric mean expression of housekeeping genes *Actin* (*Act*) and *Ribosomal protein 18S* (*Rp18S*), which did not alter significantly between treatments. Both techniques displayed similar trends, so only NORMAGene analyzed results are displayed.

2.3 Results

Gene sequence identification

All gene sequences identified with the exception of *Mtn* showed homology to target proteins when subjected to a BLASTx search against the *Drosophila melanogaster* annotated protein database (www.ncbi.nim.nih.gov). Amino acid alignments are displayed in Supplementary file 4 (Appendix 1). The *SAH* sequence top hit was CG9977 (GenBank accession no. **NP647746**) responsible for adenosylhomocysteine hydrolase activity, with 88% identity and an expect value (E) of $61e^{-62}$. The fragment included the AdoHcyase domain (E = $1.43e^{-41}$). For *SAM* sequence, the top hit was S-adenosylmethionine synthetase, isoform B (GenBank accession no. **NP722599**) with 87% identity (E = $7e^{-28}$). The fragment included the S-AdoMet_synt_N conserved domain (E = $9.71e^{-18}$).

The *CβS* sequence top hit was cystathionine β-synthase isoform A (GenBank accession no. **NP608424**), with 67.5% identity and E = $6.134e^{-28}$. The fragment included the CBS_like domain (E = $9.56e^{-18}$). For *CγL* sequence, the top hit was Eip55E (GenBank accession no. **NP611352**) responsible for cystathionine γ-lyase activity, with 51% identity and E = $6e^{-19}$. The amplified fragment included the CGS_like domain (E = $2.09e^{-16}$).

The *GCS* sequence top hit was gamma-glutamylcysteine synthetase (GenBank accession no. **AAF66980**), with 76% identity and E = $6e^{-92}$. The fragment included the GCS domain (E = $6.38e^{-55}$). For *GS* sequence, the top hit was glutathione synthetase (GenBank accession no. **ABN58452**), with 67% identity (E = $9e^{-26}$). The fragment included the PLN02977 domain (glutathione synthetase) (E = $2.63e^{-20}$).

The *GSTd1* sequence top hit was glutathione S-transferase D1 (GenBank accession no. **AAB26519**), with 71% identity and E = $2e^{-20}$. The fragment included the GST_C_Delta_Epsilon domain (E = $1.21e^{-12}$). For *Mtn* sequence, the top hit for *D. melanogaster* was MtnC isoform A (GenBank accession no. **NM142625**), with 32.5% identity and E = 0.13. Due to the weak E value, the

BLAST search was widened to include other dipterans. Homology to *Chironomus riparius Mtn* (GenBank accession no. **ADZ54163)** was found, with 85% identity and $E = 1e^{-7}$. The fragment included the Mettallothio_5 domain ($E = 2.3e^{-3}$).

Cysteine metabolism during metal exposure

Expression of genes involved in the cysteine metabolism of *C. tepperi* after exposure to Cu and Cd are displayed in Figure 2.2. For *SAH* expression, the controls, high and low metal exposure differed (ANOVA: $F_{(4, 22)} = 3.34$, $P = 0.032$). Dunnett's t post hoc tests indicated that high metal exposure downregulated *SAH* expression 0.2-fold for Cu ($P = 0.009$) and 0.5-fold for Cd ($P = 0.034$) when compared to the controls. However, low concentration exposures did not change the expression of *SAH* (Cu: $P = 0.3$, Cd: $P = 0.07$) (Figure 2.2a). For the expression of *SAM*, there was no significant difference between treatments ($F_{(4, 19)} = 2.1$, $P = 0.15$) (Figure 2.2b). Although high metal exposure suggested downregulation, this pattern was not significant due to variability in the control expression.

The expression of *CβS* differed between treatments ($F_{(4, 22)} = 8.41$, $P = 0.005$). Dunnett's t test indicated that *CβS* expression upregulated over 2-fold at both low and high concentrations of Cd (low: $P = 0.001$, high: $P = 0.008$). High Cu exposure downregulated *CβS* expression 0.2-fold compared to controls ($P = 0.036$) while low Cu did not change *CβS* expression ($P = 0.87$) (Figure 2.2c). Expression of *CγL* also differed between treatments ($F_{(4, 22)} = 9.11$, $P = 0.007$). Dunnett's t showed that *CγL* expression was upregulated 1.5-fold during high Cu exposure ($P = 0.01$) but unchanged by low Cu ($P = 0.88$) or by Cd exposure (low: $P = 0.31$, high: $P = 0.22$) (Figure 2.2d).

For *GCS* expression, the controls, high and low metal exposure differed ($F_{(4,22)} = 28.9$, $P < 0.001$). Dunnett's t indicated that *GCS* expression was upregulated 2.5-fold following high Cu exposure ($P = 0.006$) while low Cu

caused no change ($P = 0.39$). Cadmium exposure upregulated *GCS* expression at both low (1.5 fold, $P = 0.003$) and high (2-fold, $P < 0.001$) concentrations (Figure 2.2e). For the expression of *GS,* there was also a significant difference between treatments ($F_{(4, 22)} = 26.73$, $P < 0.001$). Dunnett's t test indicated that exposure to Cd upregulated *GS* expression 3-fold (low: $P = 0.018$) and 4-fold (high: $P = 0.005$) compared to controls. Exposure to high Cu upregulated *GS* expression 3-fold ($P = 0.008$), while low Cu caused no change ($P = 0.21$) (Figure 2.2f).

The expression of *GSTd1* was significantly different between treatments ($F_{(4,25)} = 19.30$, $P < 0.001$). Dunnett's t tests showed that this significance was caused by Cu high exposure upregulating *GSTd1* expression 5-fold ($P < 0.001$). Cd (low: $P = 0.64$, high: $P = 0.94$) and low Cu ($P = 0.36$) exposure did not change *GSTd1* expression relative to controls (Figure 2.2g). Finally, the expression of *Mtn* was significantly different between treatments ($F_{(4,23)} = 19.49$, $P < 0.001$). Dunnett's t-test indicated that Cd exposure upregulated *Mtn* expression at both low ($P < 0.001$) and high ($P < 0.001$) concentrations. However, Cu did not significantly change *Mtn* expression (low: $P = 0.79$, high: $P = 0.99$) (Figure 2.2h).

2.4 Discussion

Here we have successfully isolated and characterized gene sequences involved in the cysteine metabolism of *C. tepperi.* The results suggest that cysteine metabolism is regulated differentially under copper and cadmium exposure. These changes in regulation often correlate with changes in protein or enzyme levels. In animals, the methionine/transsulfuration pathway is the only route for the production of cysteine (Rao et al., 1990; Sugiura et al., 2005). Furthermore, cysteine is an essential substrate to all potential sulfur-based ligands and hence for GSH and several proteins used for metal detoxification such as GST and MT. This pathway is controlled by several enzymes, for which the gene sequences were isolated and quantified in this study. The regulation of these enzymes often determines the level of transsulfuration intermediates, which are a composite function of each step within the pathway.

The enzymes encoded by *SAM* to *SAH* are essential for cellular methylation and remethylation (James et al., 2002). The downregulation of *SAH* expression during high metal exposure is likely to reduce the amount of homocysteine available for transsulfuration, as well as reducing methylation capacity of the cell.

The reaction catalyzed by $C\beta S$ allows the cell to convert sulfur within the methionine pool to cystathionine via homocysteine and serine, while $C\gamma L$ is involved in the generation of cysteine from cystathionine. In the current study, upregulation of $C\beta S$ expression was observed during Cd exposure. Here, Cd is likely to increase the production of cystathionine in the cell and maintain the flux of cysteine for incorporation into proteins or used for GSH production. As observed in previous studies (Hughes et al., 2009), the equilibrium favors cystathionine build up in the cell. Combined with the downregulation of *SAH* expression, this response under Cd exposure is expected to diminish homocysteine stocks of the cell.

In contrast during high Cu exposure, upregulation of *CγL* expression and downregulation of *CβS* expression was observed. This could result in increased flux of cysteine into GSH synthesis and protein production, diminishing the cystathionine pool. Cd exposure may therefore increase the cystathionine pool while diminishing the homocysteine pool, while Cu exposure may diminish the cystathionine pool to maintain or increase the cysteine pool. We are unaware of other studies documenting the impact of copper on cysteine metabolism. Despite upstream differences in *CβS* and *CγL* expression, both metals upregulated *GCS* and *GS* expression, hence cysteine is likely being converted into GSH via γ-glutamylcysteine. During Cd exposure, *Mtn* expression was upregulated, while Cu caused no change. The upregulation of *Mtn* expression suggests a protective response to Cd through production of metal sequestration proteins. This response would further diminish the cysteine pool under Cd exposure compared to Cu exposure. However, Cu exposure led to upregulation of *GSTd1* expression, suggesting a possible protective response through GST antioxidant proteins. These responses rely on the production of GSH from cysteine, reliant on *GCS* and *GS* expression that was upregulated during both Cd and Cu exposure. It is likely under Cd exposure that the upregulation of *GCS* and *GS* expression is linked to another antioxidant protein, possibly another member of the GST family, glutathione peroxidase or reductase (Li et al., 2009).

Cysteine metabolism genes showed differential expression after Cu and Cd exposure. During Cd exposure, the likely decrease in homocysteine metabolite pool and increase of cystathionine pool (by downregulation of *SAH* and upregulation of *CβS*) could be important to downstream sequestration and antioxidant response to mitigate the adverse biological effects of cadmium exposure. During Cu exposure, a likely increase flux of cysteine and diminishing of cystathionine pool (by downregulation of *CβS* and upregulation of *CγL* expression) seems indicative of a different antioxidant response (i.e. *GSTd1*). In this way cysteine metabolism appears to respond differently to essential and non-essential metal exposure.

While changes in expression often correlate with changes in protein or enzyme levels, in some cases they do not. Quantifying resulting proteins and metabolites, considering that metal exposure may also impact other cellular functions, would therefore complement these transcriptional data. The results nevertheless suggest that gene expression associated with cysteine metabolism can provide markers of metal stress. Additional insights into this pathway are likely to emerge when patterns of gene regulation are linked to changes in protein activity and metabolic pathway flux. It would also be worthwhile examining levels of metals and patterns of gene expression in *C. tepperi* exposed to contaminated sediments in the field.

2.5 Figures
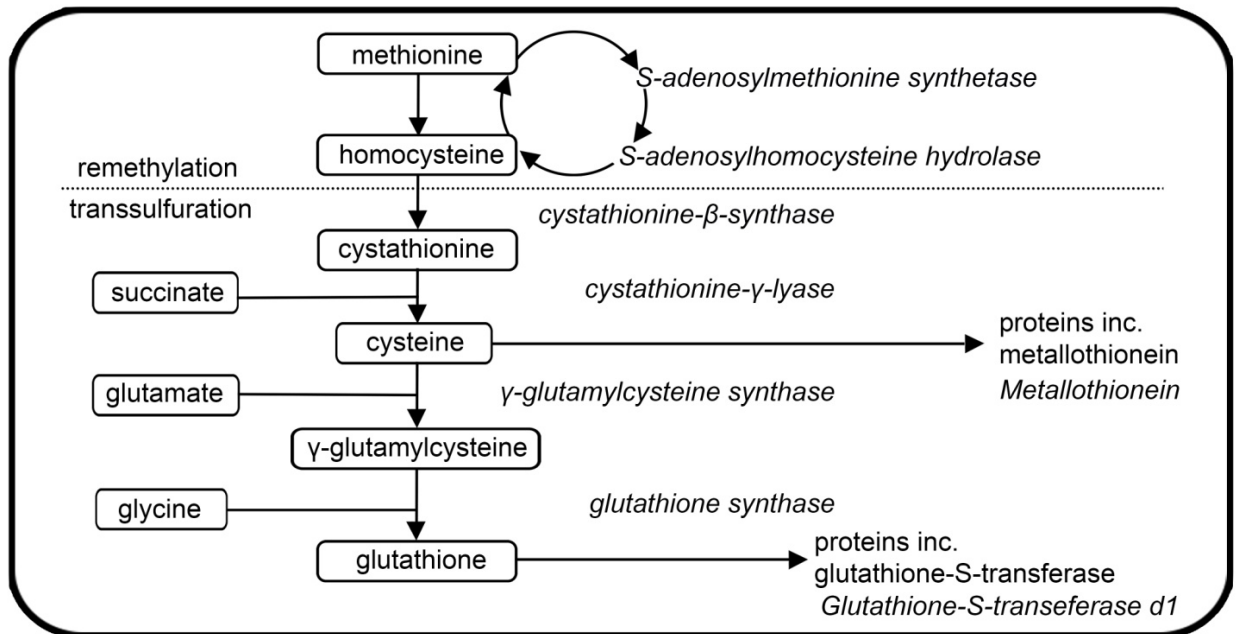
Figure 2.1 Predicted cysteine metabolism of *Chironomus tepperi* adapted from Hughes et al (2009) (based on information from Nemapath49). The pathway is essential for metal defense proteins and antioxidant response. Cysteine and intermediates are produced via the demethylation and transsulfuration of the essential amino acid methionine. Genes regulating this pathway that were quantified in *C. tepperi* are indicated in italic text.
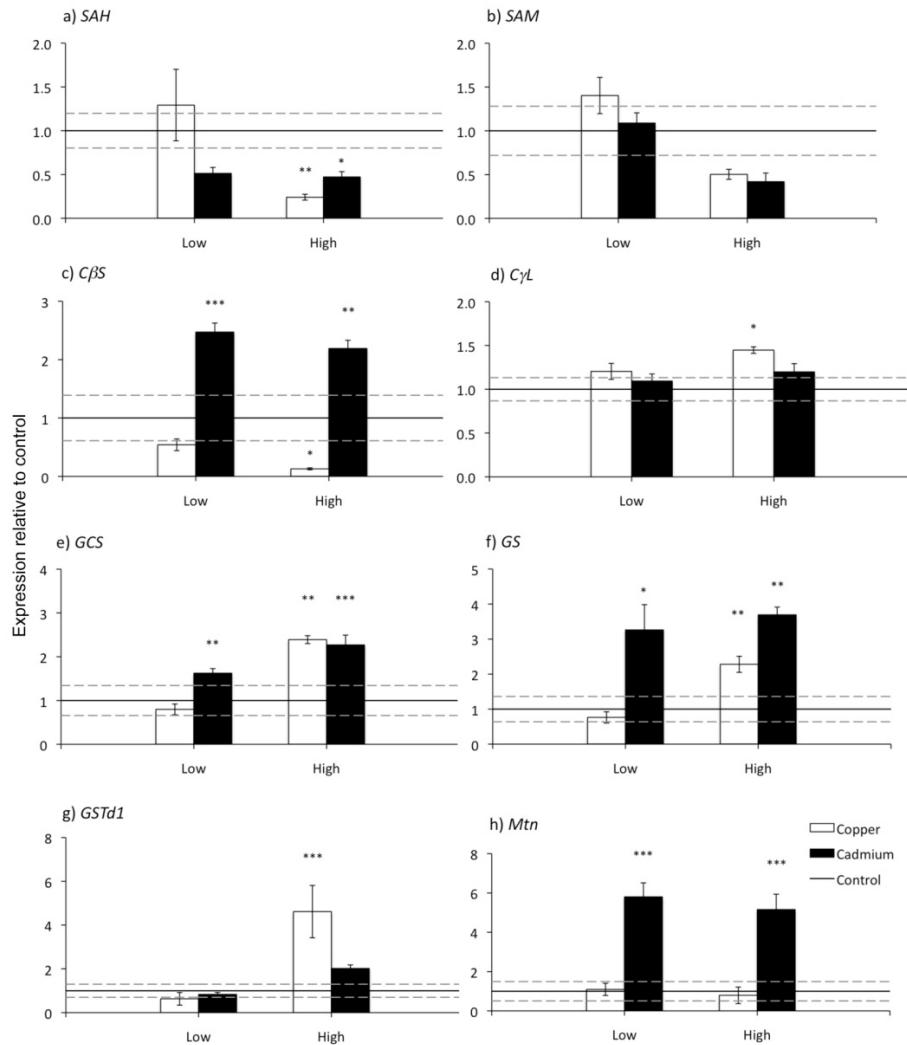
Figure 2.2 Expression profile of *Chironomus tepperi* genes after 24 h exposure to high and low sublethal concentration of copper (n = 5) and cadmium (n = 5): a) *S-adenosylmethionine synthetase* (*SAM*), b) *S-adenosylhomocysteine hydrolase* (*SAH*), c) *cystathionine-β-synthase* (*CβS*), d) *cystathionine-γ-lyase* (*CγL*), e) *γ-glutamylcysteine synthase* (*GCS*), f) *glutathione synthetase* (*GS*), g) *glutathione S-transferase delta 1* (*GSTd1*), h) *metallothionein* (*Mtn*). Expression was measured using RT qPCR and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to control exposures (black line) ± SEM (grey dashed lines). Significance was determined with a one-way ANOVA, equal variance was established with Levene test and Dunnett's t (2-sided) post hoc tests were used to establish significant difference from control exposures. Probability is displayed as * < 0.05, ** <0.01 and *** <0.001. Error bars display SEM.

2.6 Tables

Table 2.1 Sublethal exposure concentrations used for 24 h metal exposures of *Chironomus tepperi*, calculated as percent of lethal concentration of 50% mortality (LC50) from 96 hour water exposures. LC50 and confidence intervals were calculated with Probit analysis (Minitab Inc. version 16)

Contaminant	Low exposure concentration mgL^{-1} (percent of LC_{50})	High exposure concentration mgL^{-1} (percent of LC_{50})	LC_{50} mgL^{-1} (95% confidence interval)
Copper ($CuCl_2$)	0.05 (1)	0.5 (10)	4.51 (2.69 – 8.3)
Cadmium ($CdCl_2$)	0.25 (5)	0.5 (10)	5.72 (5.44 – 6.01)

CHAPTER 3 – GENE EXPRESSION AND CYSTEINE METABOLISM COMPONENTS IN *CHIRONOMUS TEPPERI* ALTER AFTER PULSE EXPOSURE TO CADMIUM

Abstract

Toxicity caused by contaminants entering aquatic environments through pulse events is difficult to assess as the duration, magnitude, and frequency of exposure is often unknown. Biomarkers may be useful to assess the impacts of pulse events as they can detect exposure in aquatic animals. This study was designed to determine if gene expression and components of cysteine metabolism could identify a pulse exposure. The expression of nine genes involved cysteine metabolism in *Chironomus tepperi* was assessed after a 24 hour exposure to cadmium (low: 0.1 and high: 0.5 mg/L) and also after a 96 hour depuration in uncontaminated water. Expression profiles of these genes were compared with glutathione-S-transferase (GST) activity and metallothionein (MT) concentration, and also with survival and emergence occurring after the 96 hour depuration. High cadmium exposure significantly decreased emergence of *C. tepperi*. Of the nine genes measured, seven altered expression after 24 hour exposure to high cadmium, with six of these genes also responding to low cadmium exposure. This response correlated well with GST activity, which increased after exposure; however, MT concentration did not respond immediately after exposures. After 96 hour depuration, high cadmium exposed larvae retained the direction and magnitude of expression for three genes, two genes returned to control levels and two genes upregulated after initial downregulation. The activity of GST reduced but was still slightly higher than control levels, and MT concentration increased. After 96 hour depuration of low cadmium only metallothionein gene expression remained upregulated. This research demonstrates that gene expression and components in the cysteine metabolism respond after depuration only in the high treatment where reduced fitness was observed. This suggests that a toxicity response as well as an exposure response is occurring.

3.1 Introduction

Anthropogenic contaminants often enter aquatic environments in episodic pollution events. Agricultural spray events, sewer spills, storm events and runoff from industrial and construction sites can present short bursts of contaminants to aquatic ecosystems. The duration, magnitude, and frequency of exposure to contaminants determines toxicity to aquatic organisms (Brent and Herricks, 1998; Diamond et al., 2006). Thus, pulse exposures to episodic contamination causes toxicity that can differ to that observed under constant exposure (e.g. Bearr et al., 2006; Hosmer et al., 1998).

Pulse events are often not detected by intermittent water chemistry surveys and even if they are detected, their impacts are difficult to quantify. Recently, biomarkers have become a popular tool in environmental monitoring for identifying exposure and, in some cases, the toxic effects of exposure. Biomarkers could respond to past exposure and be used to identify past pulse exposures (Alvarado et al., 2005), increasing their utility in toxicity testing and environmental monitoring.

Cysteine metabolism plays a central role in detoxification through the production of metallothionein and glutathione (Jones et al., 2004; Sugiura et al., 2005). We have previously reported the response of several genes regulating cysteine metabolism in *Chironomus tepperi* (Diptera) under the influence of waterborne copper or cadmium after a 24 exposure (Jeppe et al., 2014). The different transcriptional responses detected in that work suggest that this pathway could provide informative biomarkers of metal exposure. However, while changes in gene expression often correlate with changes in protein or enzyme levels, this is not always the case. Therefore, in this study we also measure metallothionein (MT) and glutathione-S-transcriptase (GST), an enzyme involved in further detoxification of the product glutathione to xenobiotic substances.

CHAPTER 3

Chironomus tepperi is a common toxicity test organism in southern Australia. Similar to *C. riparius* and *C. tentans* assessed in the northern hemisphere, it has a relatively short life cycle (20 to 28 days) and breeds well in the laboratory (ASTM, 1997; OECD, 2004). The 96 hour LC50 of *C. tepperi* for cadmium in water is around 5.7 mg/L, which makes it sensitive compared to some other *Chironomus* species (Béchard et al., 2008; Fargasova, 2001). However, *Chironomus* species are reasonably tolerant compared to other species such as *Hyalella azteca*, which has an LC50 for cadmium in the µg/L range (US-EPA, 2001). Nevertheless, *Chironomus* species might still be useful surrogates for sensitive groups if changes in biomarkers at non-lethal concentrations could also be used in laboratory tests.

The aim of this study was to investigate gene expression responses of cysteine metabolism in *C. tepperi* after a 24 hour pulse exposure to cadmium and after a 96 hour depuration period. Expression profiles of these genes were compared with GST and MT and with whole organism endpoints (survival and emergence) after depuration. This study therefore tests whether biomarkers in cysteine metabolism provide a useful way of detecting pulse events.

3.2 Materials and Methods

Organism

Chironomus tepperi were cultured from a stock, (donated by Mark Stevens, originating from Yanco Agricultural Institute in New South Wales) as described previously in Townsend et al. (2012). Briefly, larvae were maintained in culture medium similar to Martin's solution (Martin et al., 1980) which contained reverse osmosis water with 0.12 mM $NaHCO_3$, 0.068 mM $CaCl_2$, 0.083 mM $MgSO_4$, 0.86 mM NaCl, 0.015 mM KH_2PO_4, 0.089 mM $MgCl_2$ and 0.1% (w/v) iron chelate) at $21^{\circ}C \pm 1^{\circ}C$ and a 16:8 hour light:dark photoperiod. Ethanol-rinsed tissue paper was used as artificial substrate in the culture tanks and larvae were fed ground tropical fish flakes (Tetramin ®) three times per week with a ration of 0.25 grams per tank.

Exposure experiments

Larvae that hatched from egg masses were reared to third instar (9 days old) in 500 mL per egg mass of aerated culture medium and fed three times a week. At 9 days old, larvae were exposed for 24 hours to culture medium only (control) or culture medium with 0.1 mg/L (low) or 0.5 mg/L (high) cadmium chloride (Cd) with ethanol-rinsed tissue paper as substrate under culture conditions. Four replicate 2L beakers were used for each treatment. After the 24 hour exposure, subsamples were taken for gene expression, MT and GST assays. The remaining larvae were then transferred to uncontaminated culture medium and fresh substrate for a 96 hour depuration, after which subsamples were again taken for gene expression, MT and GST assays. After depuration, emergence commenced and adults were collected and counted as they emerged. Depending on tissue requirements for each biomarker, ten or twenty larvae were rinsed in reverse osmosis water, blot dried and pooled in pre-weighed microcentrifuge tubes from each replicate at each time point. Larvae were then snap frozen in liquid nitrogen and stored at -80 ºC for analysis.

Gene expression analysis

RNA extraction

A subset of 10 larvae from each replicate were homogenized in liquid nitrogen and total RNA was isolated using a High Pure RNA Tissue Kit (Roche, Cat. no. 12033674001), following the manufacturer's protocol. Extracted RNA was eluted in 100 µL RNase-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and was quantified using a Nanodrop (Thermo Scientific). All RNA samples had a 260/280 ratio between 1.9 and 2.1 and were suitable for cDNA synthesis.

Reverse transcription

Total RNA (2 µg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. no. M1705) following the manufacturer's protocol. The cDNA was then checked for gDNA contamination using a pair of *ribosomal protein L11* primers that flank a 345 bp intron. The expected cDNA amplicon was 466 bp, whereas the amplification of the 811 bp product would indicate the presence of gDNA (Martinez-Guitarte et al., 2007). Template cDNA was then stored at -20 ℃.

Gene expression analysis

Genes isolated in the cysteine metabolism, their primers and amplicon sequences, have been described previously (Jeppe et al., 2014). With the exception of *thioredoxin glutathione reductase* (*TGR*), added for this study. The primers used for qPCR amplification of *TGR* were Forward 5′-ACGGATGGGAACTCGAGAAGCC-3′ and Reverse 5′-TCACGCAAATCAACTCTTGTCACCC-3′. The identified sequence containing this fragment was submitted to GenBank (GenBank accession: KJ996072). The *TGR* amplicon showed strong homology to its target protein when subjected to a BLASTx search against the *Drosophila melanogaster* annotated protein database (www.ncbi.nim.nih.gov). The top hit for *TGR* was thioredoxin reductase-1 splice variant sharing 69% identity ($E = 2e^{-11}$). The fragment included the *TRG* domain ($E = 1.56e^{-07}$).

Gene expression analysis

Gene expression was measured using quantitative real-time PCR (qPCR), with gene-specific primers. Quantitative real-time PCR was performed with the Roche LightCycler® 480 (Roche Applied Science, USA) in 10 μL reactions containing the components described by Takahashi et al. (2010). The following conditions were used: 10 minutes at 95 ºC, followed by 50 cycles of 10 seconds at 95 ºC, 15 seconds at 58 ºC, and 15 seconds at 72 ºC followed by a melting curve analysis from 65 ºC to 95 ºC to ensure a single product was formed.

Glutathione S-transferase assay

A subset of 10 larvae were prepared for GST assays following the methods of Ballesteros et al. (2009). Larvae were homogenized in ice-cold buffer consisting of 0.1 M phosphate (Sigma-Aldrich), pH 6.5, 20% v/v glycerol (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich) and 1.4 mM dithioerythritol (Sigma-Aldrich) at a ratio of 1:5 w/v, using a Mixermill, (Retsch, MM 300, Retsch GmbH, Haan, Germany) for 90 seconds, until a smooth homogenate was obtained. The samples were then centrifuged at 14,000g for 20 minutes at 4°C (Eppendorf 5804R, Eppendorf, Hamburg, Germany). Supernatants were the decanted, snap frozen in liquid nitrogen and stored at -80°C until analysis. Activity was measured using a Synergy 2 microplate reader (Biotek Instruments, USA) with modified method based on published protocols (Booth et al., 2007; Habig et al., 1974). The activity of GST was determined following the conjugation of reduced glutathione (GSH) (Sigma-Aldrich) and 1-chloro-2, 4-dinitrobenzene (CDNB) (Sigma-Aldrich) at 340 nm (millimolar extinction coefficient: 9.6), an increase in absorbance over time was observed. The final volume in each well was 200 μL. The concentration of Lowry in each sample was measured using a modified method and GST activity expressed as μmol/min/mg protein (Lowry et al., 1951).

Metallothionein assay

The concentration of MT was measured in a subset of 20 *C. tepperi* larvae using a Synergy 2 microplate reader (Biotek Instruments, USA). The Micro MT spec kit (Ikzus Environment®, Genoa, Italy) was used according to the manufacturers protocol. The kit performs chemical determination of cysteine residues by Ellman's reaction (Viarengo et al., 1997). Endogenous thiols (such as glutathione or free cysteine) do not interfere with the assay (Ungherese et al., 2011). Briefly, *C. tepperi* tissue was homogenized in 160 µl solution A+B using a Mixermill, (Retsch, MM 300, Retsch GmbH, Haan, Germany) for 90 seconds, until a smooth homogenate was obtained. The samples were then centrifuged at 14,000 × g for 5 minutes at 4°C. After a supernatant was obtained, MTs were precipitated with ice-cold alcohol (absolute ethanol). The precipitated MT sample was suspended in 50 µl solution D. The absorption was read at 405 nm with a standard curve as per kit instructions. The concentration of MT (nanomole MT) per gram of tissue was calculated as per the equation in the Micro MT spec kit instructions, concentration was then expressed as nmol/mg protein using the concentration of protein in each sample, measured using a modified method (Lowry et al., 1951).

Statistical analysis

Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann et al., 2011). Differences in relative normalized expression of target genes between treatments were analyzed using R (version 3.2.1, The R Foundation for Statistical Computing Platform). An overview of gene expression in the cysteine metabolism was obtained using principal component analysis (PCA) (princomp[stats]) with a matrix of the 3 cadmium concentrations and 2 time points. Survival and emergence data were arcsine transformed prior to analysis. All data were tested for normality and equal variance using Kolmogorov-Smirnov (ks.test[stats]) and Levene's tests (leveneTest[car]). Significant differences between treatments

were the established with an ANOVA (aov[stats]) with Dunnett's *post hoc* test (glht[multcomp]) to compare between treatments. If data were not normal, significant difference was established with Kruskal Wallis (kruskal.test[stats]). The P values were adjusted for multiple comparisons using the Benjamini Hochberg correction.

3.3 Results

Survival and emergence

Survival and emergence results are displayed in Figure 3.1. For survival, the control, low and high treatments differed (ANOVA: $F_{(6,2)}$ = 18.41, P =0.003). Dunnett's t *post hoc* indicated that high treatment reduced survival to 80% (P = 0.005)(Figure 3.1a). Total emergence also differed between treatments (ANOVA: $F_{(6,2)}$ = 11.24 P = 0.009), with Dunnett's t *post hoc* indicating high Cd significantly reduced emergence to 40% (P = 0.013)(Figure 3.1b).

Gene expression in the cysteine metabolism

Gene expression profiles in the cysteine metabolism were investigated after 24 hour exposure and following the 96 hour depuration. A PCA of gene expression after exposure and depuration of *C. tepperi* to cadmium is displayed in Figure 3.2. Cadmium treatments separated along two axes with PC1 explaining 46.9% variance and PC2 explaining 21.8% variance. After exposure both low and high cadmium treatments separated from the control, however after depuration, only the high treatment separated clearly from the control group.

Individual expression profiles of each gene measured are displayed in Figure 3.3. For *S-adenosylmethionine synthetase* (*SAM*), cadmium treatments significantly altered expression after exposure and marginally altered expression after depuration (ANOVA: $F_{(2,9)}$= 12.93, P = 0.005 and $F_{(2,9)}$= 4.72, P = 0.07, respectively)(Figure 3.3a). *SAM* expression was downregulated 0.5--fold in the high treatment after exposure (P = 0.001) but upregulated 1.5-fold in the high treatment after depuration (marginal P = 0.070) when tested with Dunnett's t-test relative to the control. *S-adenosylhomocysteine hydrolase* (*SAH)* expression altered between treatments after exposure and after depuration (ANOVA: $F_{(2,9)}$= 9.74, P = 0.007 and $F_{(2,9)}$= 7.49, P = 0.026, respectively)(Figure 3.3b). After exposure, *SAH* expression was downregulated 0.6-fold in low treatment (marginal P = 0.055) and 0.3-fold in

the high treatment (P = 0.003). After depuration, *SAH* expression was upregulated 2-fold in the high treatment (P = 0.024).

For *cystathionine-β-synthase* (*CβS*) expression, treatments were significantly different after exposure ($F_{(2,9)}$= 10.87, P = 0.006)(Figure 3.3c). After exposure, *CβS* expression upregulated 2-fold in low cadmium treatment (P = 0.002) and 1.6-fold in the high cadmium treatment (marginal P = 0.058). However, after depuration *CβS* expression returned to control levels ($F_{(2,9)}$= 1.19, P = 0.382). For *cystathionine-γ-lyase* (*CγL*) expression, there was a significant difference between treatments after exposure ($F_{(2,9)}$= 5.033, P = 0.037)(Figure 3.3d). Dunnett's t-test indicated that *CγL* expression downregulated 0.75-fold in the high treatment (P = 0.026). However, after the depuration *CγL* expression had returned to control levels ($F_{(2,9)}$= 2.72, P = 0.145).

The expression of *metallothionein* (*Mtn*) differed after exposure and after depuration ($F_{(2,9)}$= 34.10, P < 0.001 and $F_{(2,9)}$= 13.64, P = 0.005) (Figure 3.3e). After exposure, *Mtn* expression was upregulated in both low and high cadmium treatments, at 8- and 3.5-fold compared to the control (P < 0.001 and P = 0.053, respectively). After depuration *Mtn* expression was still upregulated 3 to 3.5-fold in both cadmium treatments (P = 0.001 and P = 0.007, respectively).

The expression of *γ-glutamylcysteine synthase* (*GCS*) differed significantly after exposure to cadmium and after the depuration ($F_{(2,9)}$= 97.49, P < 0.001 and $F_{(2,9)}$= 53.98, P < 0.001)(Figure 3.3f). After exposure, *GCS* expression was upregulated 4.5-fold in the low treatment (P < 0.001) and 3.5-fold in the high treatment (P < 0.001). After depuration, *GCS* was upregulated 3.5-fold in the high treatment (P < 0.001), however, in the low treatment *GCS* expression had returned to control levels (P = 0.954). For *glutathione synthetase* (*GS*), cadmium significantly altered expression after exposure ($F_{(2,9)}$= 5.80, P = 0.024)(Figure 3.3g). After exposure, *GS* expression was upregulated 2-fold in both cadmium treatments (P = 0.022 and P = 0.041, respectively). After

the depuration, *GS* expression did not differ between treatments ($F_{(2,9)}$= 3.59, *P* = 0.071).

Expression of *glutathione-S-transferase d1* (*GST*) did not differ between treatments after exposure or depuration ($F_{(2,9)}$= 0.57, *P* = 0.587 and $F_{(2,9)}$= 0.82, *P* = 0.473, respectively)(Figure 3.3h). For *thioredoxin glutathione reductase* (*TGR*) expression, treatments were significantly different between after exposure and after depuration ($F_{(2,9)}$= 55.6, *P* <0.001 and $F_{(2,9)}$= 30.83, *P* < 0.001, respectively)(Figure 3.3i). After exposure, *TGR* was upregulated 5-fold in the low cadmium treatment (*P* < 0.001) and 4-fold in the high treatment (*P* < 0.001). After depuration, *TGR* expression was upregulated in the high cadmium treatment (*P* < 0.001), however in the low treatment expression had returned to control levels (*P* = 0.48).

Glutathione S-transferase

The activity of GST was significantly different between treatments after exposure ($F_{(2,10)}$= 6.87, *P* = 0.026), but not after depuration ($F_{(2,10)}$= 1.49, *P* = 0.26)(Figure 3.4a). Dunnett's *post hoc* indicated that GST activity increased 1.5-fold in the high cadmium treatment after exposure (*P* = 0.008) and 1.25-fold after depuration although this was not significant (*P* = 0.33).

Metallothionein

The concentration of MT was not significantly different between treatments after exposure or after depuration ($F_{(2,10)}$= 0.05, *P* = 0.943 and Kruskal-Wallis: χ^2= 2.49, *P* = 0.288, respectively)(Figure 3.4b) even though MT concentration increased 1.25-fold in low cadmium and 1.5-fold in high cadmium after depuration.

3.4 Discussion

In this study, gene expression in the cysteine metabolism of *C. tepperi* altered after exposure to cadmium. After depuration, recovery was observed in the low cadmium treatment but the high cadmium treatment remained different from the control for several genes. These responses correlated with GST activity after exposure and with MT concentration after depuration although changes in these components were not as sensitive or rapid as changes in gene expression. Both GST and MT are regulated by several genes in most species and it is possible that other regulating genes did not respond to exposure, thus diminishing production relative to that expected given the expression levels of the measured genes. Under high exposure, the continued response in gene expression after depuration occurred at a time when there was reduced emergence. This indicates that a pulse may only be detected with this approach when the stress is severe enough to affect emergence. Furthermore, exposure to 24 hour pulse of 10% LC50 cadmium concentration was not expected to cause such severe fitness effects, highlighting the need for longer tests to establish an organism's sensitivity (Jager et al., 2006).

The pulse exposure to cadmium caused a rapid change in gene expression and these results correlated well with results in Chapter 2 (Jeppe et al., 2014). After exposure to cadmium, gene expression was similar to that observed previously, with the exception of *CγL,* which downregulated in high cadmium. In addition the upregulation of *TGR* was not previously documented and occurred at both concentrations. The increased expression of *GCS*, *GS* and *TGR* indicated an antioxidant response after exposure and this was supported by increased activity of GST (although only significant in high treatment). Conversely, expression of the *GST* gene measured here did not alter under cadmium exposure, although in Chapter 2 it responded to copper (Jeppe et al., 2014). There are over ten *GST* genes in related *Chironomus* species and it is possible that the different genes are metal or contaminant specific (Li et al., 2009; Nair and Choi, 2011). This family of genes should be considered in more detail if they are to be used as biomarkers in *C. tepperi*.

Upregulation of *Mtn* expression did not correlate with increased MT concentration immediately after exposure. Gene expression and concentration of metallothionein has been shown to correlate in studies considering individual tissues and longer exposures than used here (Knapen et al., 2007; Scarino et al., 1991). It is possible that although gene expression increased after 24 hour exposure, an increase in MT takes longer to become evident. A time-course comparison of metallothionein gene expression and concentration could help to clarify the likely timeframe involved.

After removal of cadmium for a 96 hour depuration, the expression of all genes in the low cadmium treatment returned to control levels, except *Mtn,* which was still upregulated. This continued upregulation of *Mtn* is not unexpected, as cadmium is often depurated more slowly than other metals (Arini et al., 2015; Shuhaimi-Othman and Pascoe, 2007; Yap et al., 2003). While no difference in emergence was observed in the low cadmium treatment, the response of *Mtn* could indicate ongoing cadmium response. Further investigation is needed to see if this response can be linked to changes in MT concentration or whole organism responses other that emergence. Increased MT levels could make animals more resistant to future exposures to toxic levels of metals (Alvarado et al., 2005; Viarengo et al., 2000). The upregulation of *SAM* and *SAH* after depuration in high cadmium could represent cellular recovery and repair, which may have been inhibited during exposure. Depuration from high cadmium exposure also retained upregulation of *GCS* and *TGR,* demonstrating that an antioxidant response is still required. This was also supported by a slight increase in GST activity.

Gene expression in control treatments after exposure differed to expression in these treatments after depuration. If gene expression is to be a widely used biomarker technique, developmental changes in gene expression should be understood and appropriate controls developed, particularly given the different expression patterns in 11 day old larvae compared to 16 day old larvae.

The continued response of several biomarkers following high cadmium exposure after depuration may indicate toxicity, as reduced emergence occurred under this treatment. No biomarkers except metallothionein gene expression responded after depuration in low cadmium treatments, suggesting that cysteine metabolism biomarkers in *C. tepperi* can recover from low concentration exposures. This recovery from low pulse exposure indicates that this technique, with the exception of *Mtn,* does not respond to past exposure as it does to present exposures, particularly if the organism is not suffering toxicity from the past exposure. This shows a promising link between gene expression and population impacts, as expression was only altered after depuration if toxicity occurred. It is also encouraging that non-toxic pulse exposures would not be identified by this technique after depuration. These biomarkers appear to be rapid to respond to exposure and likely to indicate a toxic effect.

In summary, this research demonstrates the sensitivity of expression biomarkers for identifying exposure in organisms. The recovery of gene expression at low cadmium concentrations suggests that many gene expression changes are temporary and may not indicate the presence of a past pulse of contamination, but the expression of metallothionein represents an exception. Persistent gene expression changes may also highlight toxicity, but larval age and exposure time must be known to correctly interpret expression responses. Long exposure times may be required to observe strong responses in downstream components and processes.

3.5 Figures
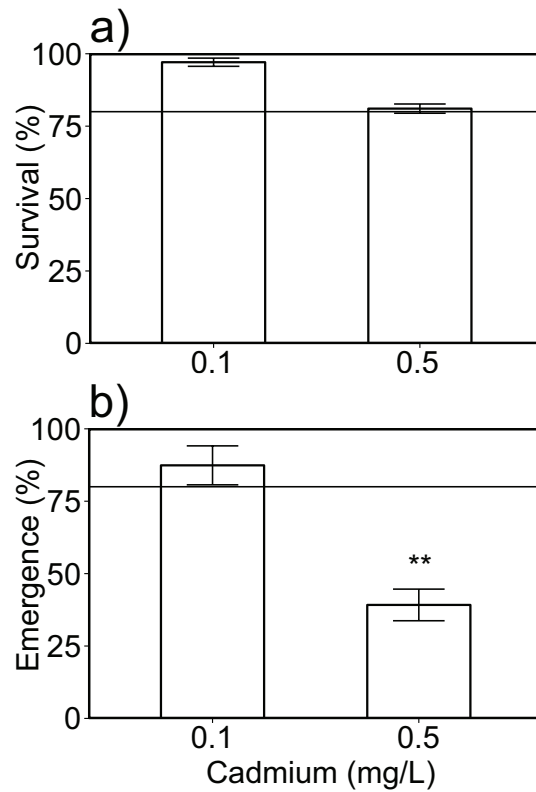
Figure 3.1 a) Survival and b) emergence of *Chironomus tepperi* after a 15 d water exposure including a 24 h pulse exposure control (0 mg/L), low (0.1 mg/L) or high (0.5 mg/L) cadmium chloride concentration. Significance was established with ANOVA and Dunnett's post hoc ** $P < 0.01$. Error bars represent the standard errors of the mean.

Figure 3.2 Principal components analysis (PCA) scores plots of 9 *Chironomus tepperi* cysteine metabolism genes after 24 hour exposure to cadmium in water and 96 hour depuration.
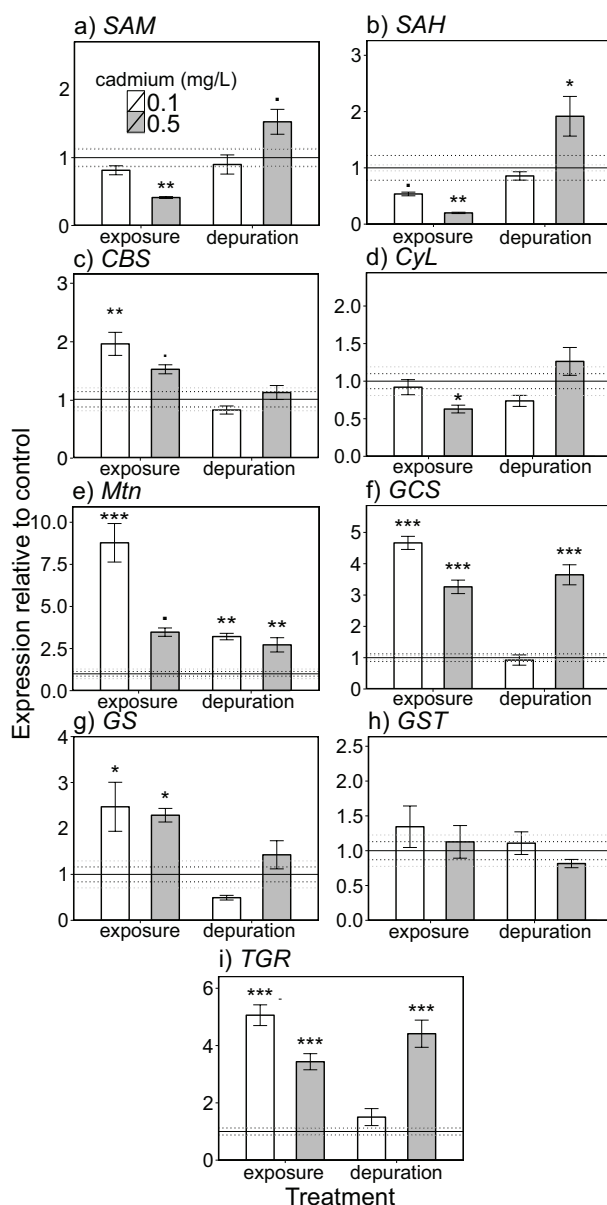
Figure 3.3 Expression profile of *Chironomus tepperi* cysteine metabolism genes after 24 hour exposure to 2 concentrations (0.1 and 0.5 mg/L) of cadmium in water and after a 96 hour depuration: a) *S-adenosylmethionine synthetase* (*SAM*), b) *S-adenosylhomocysteine hydrolase* (*SAH*), c) *cystathionine-β-synthase* (*CβS*), d) *cystathionine-γ-lyase* (*CγL*), e) *metallothionein* (*Mtn*) f) *γ-glutamylcysteine synthase* (*GCS*), g) *glutathione synthetase* (*GS*), and h) *glutathione-S-transferase d1* (*GST*), i) *thioredoxin glutathione reductase* (*TGR*). Expression was measured using RT qPCR and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to control exposures (black line) ± standard errors of the mean (black dashed lines). Significance was determined by ANOVA and Dunnett's *post hoc* (*P < 0.05, ** P < 0.01, ***P < 0.001). Error bars indicate standard errors of the mean.

Figure 3.4 Quantification of a) Glutathione-S-transferase (GST) activity and b) Metallothionein (MT) concentration after 24 hour exposure to 2 concentrations (0.1 and 0.5 mg/L) of cadmium in water and a 96 hour depuration. Data are displayed relative to control exposures (black line). Error bars indicate standard errors of the mean.

CHAPTER 4 - MULTILEVEL BIOINDICATORS OF COPPER TOXICITY – LINKING COMMUNITY IMPACTS WITH INDIVIDUAL RESPONSES

Abstract

Sediment pollution can be toxic and often contributes to aquatic ecosystem decline. To isolate the nature of sediment toxicity, various tests using laboratory-based species have been developed. However, these tests are often criticized for lacking environmental relevance or for failing to act as early warning indicators of broader scale impacts. To meet these criticisms, studies are required that assess multiple toxicity endpoints at several levels of organization, in laboratory-bred and indigenous species. This study considers such a test for sediment copper toxicity in a field-based microcosm. Community level responses of indigenous invertebrates were classified into three groups: sensitive, moderate and tolerant genera, based on prior knowledge. The survival of these groups in the microcosms relative to reference sediment based on EC50 (effect dose of 50% change) was 75, 249 and 713 mg/kg copper, respectively. These community level responses were compared with population, individual and subindividual level responses in 3 laboratory-bred species. For laboratory-bred species, juvenile production in *Potamopyrgus antipodarum* was the most sensitive population level response (EC50: 121 mg/kg), while juvenile production in *Physa acuta* was more tolerant (EC50: 298 mg/kg). Subindividual responses of gene expression and metabolomic changes in *Chironomus tepperi* were the most sensitive laboratory-bred response and were evident at 60 mg/kg, matching sensitive community level responses. For copper, it appears that subindividual responses provide the most sensitive measure for laboratory-bred species, responding at concentrations where the survival of sensitive indigenous macroinvertebrates was declining. These responses also offered information on copper metabolism, indicating direct effects due to exposure in *C. tepperi.* Furthermore, metal specific responses were observed and pathways of interest for further investigation of copper response were identified.

4.1 Introduction

Sediment pollution is widespread and can adversely affect aquatic ecosystems because many aquatic organisms rely on sediment for food and protection (Burton, 1991; Giesy and Hoke, 1991). Hydrophobic contaminants accumulate in sediment over time and contamination often occurs at concentrations that are orders of magnitude higher than water in the same system. The toxic effect of sediment pollution needs to be isolated from confounding factors such as habitat and hydrology when investigating the causes of environmental degradation (Marshall et al., 2010).

A number of approaches have been developed to assess the toxicity of sediments. A common approach is to use laboratory-based bioassays, which investigate toxicity to one or a few laboratory-bred species under standard conditions. These tests allow researchers to control the age and exposure time of organisms, and they help to establish causality between sediment and toxicity by controlling other factors. However, they are often criticized for lacking environmental relevance (Ingersoll et al., 2005; Wang et al., 2004). Changes in laboratory-based bioassays are most often observed at the individual level (dry weight, biomarker changes) and more rarely at the population level (reproduction, F1 responses) (Burton, 1991; Chapman, 1995; Forbes and Forbes, 1994). These responses are used as 'bioindicators' and are assumed to be indicators of broader scale impacts (O'Brien and Keough, 2014; Picado et al., 2007). However, it is rarely shown that bioindicators reflect responses at other levels of biological organization or that the response at the individual level is related to higher-order biological changes to the population and community (Forbes et al., 2006; O'Brien and Keough, 2014). Bioindicators need to be linked with higher-order biological changes to establish environmental relevance (Beketov and Liess, 2012; Rasmussen et al., 2013)

A field-based microcosm approach developed by Pettigrove and Hoffmann (2005) can be used to isolate the effects of sediment pollution from other factors while considering the response of indigenous species communities in

the field. This approach exposes aerial colonizing macroinvertebrates to a simplified ecosystem with different sediment contaminants, which can give a more environmentally realistic assessment of sediment impact than laboratory-based tests (Dzialowski et al., 2014). This approach has been successfully used to investigate the impacts of urban sediments (Carew et al., 2007; Pettigrove and Hoffmann, 2005), agricultural sediments (Sharley et al., 2008; Townsend et al., 2009) and road runoff (Pettigrove et al., 2007). This field-based microcosm approach provides a useful platform for field validation and comparison of bioindicators, because it is a fully replicated and controlled experiment.

To provide environmental relevance of toxicity tests, field deployments of laboratory species have also been used (e.g. Kellar et al., 2014; Morales-Casellesa et al., 2008). These tests measure traits that are commonly monitored in laboratory experiments (e.g. by measuring survival, dry weight, biomarker changes) but increased environmental relevance is provided through the fact that exposure occurs in the field.

Bioindicators at the individual and subindividual level, such as changes in dry weight, metabolism, proteins and gene expression have been widely used in toxicity testing including sediment testing (Forbes et al., 2006; Huggett et al., 2002; McCarthy and Shugart, 1990; Peakall, 1994). These markers can potentially be rapid and sensitive, as well as reflecting the direct effects of exposure rather than indirect effects. For example, metabolite changes that occur under direct copper exposure are different from those that occur during starvation (Bundy et al., 2008; Warne et al., 2001). As copper has fungicide, insecticide and herbicide properties, it could cause direct toxicity or starvation in macroinvertebrates depending on the concentration and species considered. Measuring the organism's metabolic profile could potentially discern which exposure path is occurring, particularly if there is information on the pathways involved in toxin responses (e.g. Tuffnail et al., 2009). The cysteine metabolism pathway plays a central role in exposure detoxification and several metabolites and genes involved in this pathway have been shown to respond to metal exposure (Figure 4.1)(Hughes et al., 2009; Jeppe et al.,

2014; Long et al., 2015; Sugiura et al., 2005). Cysteine is produced from methionine by the transsulfuration pathway, via the intermediates homocysteine and cystathionine. Cysteine can only be produced in this way and is required for several proteins, including metallothionein, which protects cells from metal exposure. Cysteine is also a precursor for glutathione, which is involved in several antioxidant responses (Hughes et al., 2009). Measuring metabolites and gene expression in the cysteine metabolism of laboratory species could provide a sensitive bioindicator of exposure. However, ideally it needs to be shown that these responses are related to higher-order biological changes in populations or communities and that these responses can be detected above general cellular homeostasis under fluctuating field conditions.

This study combines a field-based microcosm with field deployment of laboratory species, so bioindicators at several levels of biological organization can be assessed and compared. Community responses of indigenous invertebrates in a field-based microcosm were assessed alongside field deployment of three laboratory-bred species: two snails, *Potamopyrgus antipodarum* and *Physa acuta*, and one chironomid, *Chironomus tepperi.* These species have been used previously in laboratory- and field-based toxicity testing (e.g. Choung et al., 2010; Duft et al., 2003; Kellar et al., 2014; Ma et al., 2014; Seeland et al., 2013; Stevens et al., 2003). Population level responses of survival and reproduction were measured in *P. antipodarum* and *P. acuta*. Individual level responses of dry weight and biomarkers, through metabolomics and gene expression in the cysteine metabolism, were measured for *C. tepperi*.

The aim of this research is to compare these different approaches to measuring copper toxicity and compare their sensitivities. Copper concentrations where bioindicators responded were compared to assess the sensitivity of bioindicators in laboratory-bred species to indigenous community responses. The freshwater microcosm technique provided the
 platform to compare these responses under environmentally relevant conditions.

4.2 Materials and Methods

Sediment spiking

Sediment was collected from an uncontaminated wetland (Glynns wetland, Warrandyte, 30 km east of Melbourne); this wetland was also used to measure indigenous community responses (see below). Sediment was sieved to 63 μm and allowed to settle for 24 hours. Then it was spiked using methods from Simpson et al. (2005) with adjustments to be 'dilution pH 7' spiking, whereby a high-spike sediment stock (6000 mg/kg copper) was diluted to improve binding and equilibration of metal to sediment (Hutchins et al., 2008). Briefly, the sediment stock was spiked and the pH adjusted in a nitrogen atmosphere. The spiking solution was made up with deoxygenated water, added to sediment, mixed vigorously and rolled on a bottle roller for 2 to 3 hours. After 24 hours the pH was adjusted to 8 with 1 M NaOH and sediments were equilibrated for 25 days, rolling for 2 to 3 hours, 3 times a week. The copper (as $CuCl_2$ solution) used to spike was analytical reagent grade. After equilibration, the stock sediment was diluted to reach five exposure concentrations of copper (62.5, 125, 250, 500 and 750 mg/kg). These concentrations are chosen to span the range of environmentally relevant concentrations (Gardham et al., 2014; Vinot and Pihan, 2005). The highest concentration found in literature of field sediments was 740 mg/kg (excluding mine impacted sites where copper partitioning can be different from historically contaminated sediments (Gardham et al., 2014; Rios et al., 2008)).

Sediment from each treatment was taken at the start and end of the experiment for chemical analysis of total copper through ICP-AES (ALS: www.alsglobal.com/). The concentrations detected did not differ from the expected concentrations by more than 10% so the expected concentrations are reported. The reference sediment had a background copper concentration of 11 mg/kg and is reported as expected concentration of 0 mg/kg as no copper was added.

Microcosm experiment

The field-based microcosm experiment followed the methods of Pettigrove and Hoffmann (2005). The experiment was set up at the reference wetland (Glynns wetland, Warrandyte). This site has been used for previous microcosm experiments and supports a variety of chironomid and other aerial invertebrate species (Bahrndorff et al., 2006; Pettigrove and Hoffmann, 2005). Each treatment had five replicate microcosms, except the unspiked reference treatment, which had twenty replicates because this treatment provided a common baseline for comparing toxicity. Each microcosm consisted of a 20 litre polypropylene plastic tub (Starmaid International, Melbourne) containing 500 grams of treatment sediment and 15 litres < 64 µm sieved reference site water. Each copper treatment had 5 replicate microcosms. Microcosms were arranged in a randomized block design on floating rafts close to the littoral zone and submerged vegetation (Colombo et al., 2013). The rafts were spaced approximately 1 m apart and anchored parallel to the shoreline. Seven rafts were used, 6 rafts held 8 microcosms and last raft held 7 microcosms giving 55 microcosms. Replicates from different treatments were arranged randomly within each raft.

The rafts were partly submerged so the water level in the microcosms was at approximately the same level as that in the wetland. This ensures that, while there was no cross-contamination between microcosm and wetland water, water in the microcosm was maintained at a temperature comparable to that in the wetland. Polypropylene nets with 2 cm^2 mesh were placed over the microcosms to allow colonization by small aerial invertebrates while excluding large predatory invertebrates that could overwhelm the microcosms. The microcosm experiment ran for 7 weeks during which the replicates were colonized by indigenous invertebrates.

After 7 weeks of colonization the microcosms were processed on site. Each microcosm was sieved through a 250 um screen to recover indigenous macroinvertebrates, which were stored in ethanol for later identification.

Members of Chironomidae were the most common taxa and were identified to genus. Other taxa were identified to family, or lower where possible.

Deployment of laboratory species

Using the design described above, microcosms were used to assess the toxicity responses of three laboratory test species in the field. As this was a novel process in the microcosm approach, 'no animal' controls were included to investigate if the presence of laboratory species affected indigenous colonization. The 'no animal' controls consisted of 5 extra replicate microcosms of reference and 250 mg/kg copper treatments with no laboratory species added.

The laboratory species added to the microcosm were two snail species (*P. acuta* and *P. antipodarum*), which were added in week one and exposed for 6 weeks. For *P. antipodarum,* 40 individuals were added to each microcosm. For *P. acuta*, which is a larger animal (15 to 20 mm shell height), 5 individuals were added to each microcosm to stop the snails overwhelming the microcosms. Five days before the experiment was concluded, 40 five-day old *Chironomus tepperi* larvae were added to simulate a standard five-day toxicity test (OECD, 2004).

At conclusion of the experiment, when each microcosm was sieved, the added laboratory species were retrieved and their survival recorded. A subset of 30 *C. tepperi* was snap frozen on dry ice for biomarkers. Surviving *P. acuta* were stored in ethanol and *P. antipodarum* were anesthetized in 2.5% $MgCl_2$ before storing in ethanol for later assessment of embryo production as described by Schmitt C. et al. (2006). Surviving juveniles of both species, egg mass production for *P. acuta* and embryo production for *P. antipodarum* were recorded under the microscope when indigenous macroinvertebrate were identified.

The *C. tepperi* that were not snap frozen for biomarkers were stored in ethanol for larvae dry weight. This was measured by drying for 24 hours at 60 ºC. This has previously been determined to achieve constant weight (CAPIM unpublished data). Due to the taxonomic similarities of *Chironomus* species and the possible presence of colonizing individuals as well as added laboratory species, the identity of *C. tepperi* was confirmed on dry ice under the microscope before dry weight or biomarker analysis. No *C. tepperi* were found in the 'no animal' controls and no replicate returned more than 40 animals so it was assumed that all *C. tepperi* in the experiment were deployed laboratory individuals.

Gene expression analysis of C. tepperi

RNA extraction

RNA was isolated with High Pure RNA Tissue Kit (Roche, Cat. no. 12033674001) according to the manufacturer's protocol with minor adjustments. The *C. tepperi* larvae added to each replicate were homogenized at 6,800 rpm using a Precellys bead-mill attached to a Cryolys cooling unit (Bertin Technologies, France), pre-chilled with liquid nitrogen, at 0 C° in 300 µL lysis buffer. The homogenate was then transferred and the beads rinsed with a further 150 µL lysis buffer before extracting as per the kit protocol. Extracted RNA was eluted in 50 µL RNase-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and was quantified on a Take3 plate (BioTek). All RNA samples had a 260/280 ratio between 1.9 and 2.1 and were suitable for cDNA synthesis.

Reverse transcription

Total RNA (0.5 µg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. no. M1705), following the manufacturer's protocol. The cDNA was then checked for gDNA contamination using a pair of *ribosomal protein L11* primers as described in Chapter 2 (Martinez-Guitarte et al., 2007). Template cDNA was diluted in 100 µL DPEC water and stored at –20 ºC.

Gene expression analysis

Genes isolated in the cysteine metabolism, their primers and amplicon sequences are described in Chapter 2 (Jeppe et al., 2014), with the exception of *thioredoxin glutathione reductase* (*TGR*), which was added as in Chapter 3. Gene expression was measured using quantitative real-time PCR (qPCR), with gene-specific primers. Quantitative real-time PCR was performed with the Roche LightCycler® 480 (Roche Applied Science, USA) in 10 µL reactions containing the components described by Takahashi et al. (2010). The following conditions were used: 10 minutes at 95 ºC, followed by 50 cycles of 10 seconds at 95 ºC, 15 seconds at 58 ºC, and 15 seconds at 72 ºC followed by a melting curve analysis from 65 ºC to 95 ºC to ensure a single product was formed.

Metabolomic assessment of C. tepperi

Metabolite extraction

Tissue for metabolite extraction was transferred into pre-weighed and pre-cooled 2 mL lysing tubes (containing 1.4 mm ceramic lysing beads (Bertin Technologies, France)) on dry ice and tissue weight was recorded. Metabolites were extracted using a modified Bligh-Dyer extraction method (Bligh and Dyer, 1959). Samples were homogenized at 6,800 rpm using a Precellys bead-mill attached to a Cryolys cooling unit (Bertin Technologies, France), pre-chilled with liquid nitrogen, at -10ºC in ice-cold 330 µL methanol and 110 µL deionized-distilled water containing internal standards (140 µM $^{13}C5$- ^{15}N-Valine and 14 µM $^{13}C6$- Sorbitol). Following homogenization, 110 µL ice-cold chloroform was added to each tube and the solutions were mixed thoroughly and metabolites further extracted at 2ºC for 15 minutes in a shaker. The tubes were then centrifuged at 14,000 g at 0ºC for 5 minutes. Supernatant was transferred to a fresh centrifuge tube and a further 220 µL deionized-distilled water was added to obtain a ratio of 3:3:1 (methanol:water:chloroform). This was mixed and centrifuged at 14,000 g at 0ºC for 5 minutes. The upper phase was collected into a fresh microcentrifuge

tube and aliquots taken for LC-MS analysis.

Metabolomic analysis and data processing

Analysis of amine containing metabolites was performed by LC-MS according to Boughton et al. (2011). Briefly, 10 µL of the extract was buffered by the addition of 70 µL borate buffer, pH 8.8 containing oxidizing and reducing agents (200mM boric acid (Univar), 10 mM Tris(2-carboxyetgyl)-phosphine (TCEP, Sigma Aldrich), 1 mM Ascorbic acid (Sigma Aldrich), pH 8.8 (adjusted with 2M NaOH) and 7.14 µM of 13C3-L-Alanine (Sigma Aldrich) as the internal standard. The reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, 20 µL, 10 mM stock in 100% acetonitrile, SAFC Sigma Aldrich) was added to the mixture and incubated for 10 minutes at 55ºC in a thermo mixer followed by centrifugation at 0ºC for AQC derivatization (Boughton et al., 2011). The supernatant was transferred to HPLC vials for LC-MS analysis. The samples (4 µL) were injected onto a 120 SB-C18 Poroshell 2.1 x 100 mm, 2.7 µm column (Agilent Technologies) using an Agilent 1200-HPLC and analysed using Agilent 6460 Triple Quad MS.

Amine containing compounds were quantified by multiple reaction monitoring (MRM) using a fragmentor voltage range of 76-140V, collision energy range of 9 to 25 V and collision gas (N2) at 10 litres per minute. MRMs for most amine compounds are as described in Boughton et al. (2011).

Statistical analysis

All statistical analysis was conducted 3.2.1 (The R Foundation for Statistical Computing 2015).

Indigenous macroinvertebrate community

Multi-response permutation procedure (MRPP) was conducted (mrpp[vegan]) to test for heterogeneity in genera composition and abundance between concentrations of copper. All community data was log transformed before analysis but did not follow a normal distribution even after transformation. Common taxa were classified into 3 levels of sensitivity. For chironomid

genera, these classifications were based on previous data from microcosms and field sites in the Greater Melbourne Area (Pettigrove, 2006; Melissa Carew unpublished data). For other macroinvertebrate taxa, SIGNAL 2.0 scores were used to classify sensitivity (Chessman, 2003). The response of total macroinvertebrate abundance, number of taxa and each sensitivity category of genera to copper spiked microcosms was modeled with a 3 parameter log-logistic model (drc[drm]). The effect dose of 50% change (ED50) was calculated for comparison between bioindicators.

Added laboratory species

The survival data of added species were arcsine transformed and dry weight data were log transformed before analysis. The response of each bioindicator to copper spiked microcosms was modeled with a 3 parameter log-logistic model and the ED50 calculated (drc[drm]).

Gene expression of C. tepperi

Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann et al., 2011). An overview of gene expression in the cysteine metabolism was obtained using principal component analysis (PCA) (princomp[stats]) with a matrix of the 6 copper concentrations. Differences in relative normalized expression of target genes between copper exposed and unexposed samples were then assessed using ANOVA (aov[stats]) after verifying normality and homogeneity of variances with Shapiro-Wilk (shapiro.test[stats]) and Levene's tests (leveneTest[car]) respectively. Significance of ANOVA tests was evaluated after Dunnett's post hoc tests for multiple comparisons to reference exposures (glht[multcomp]). The P values were adjusted for multiple comparisons using the Benjamini Hochberg correction.

Metabolomic assessment

Prior to statistical analysis all metabolomic data were median normalized to account for differences between samples. Following this, the data were log transformed to account for the heteroscedastic nature of metabolomics data. Metabolites known to be associated with the cysteine metabolism were selected from the LC-MS data (Figure 4.1). Differences in normalized concentrations of these metabolites between copper exposed and unexposed samples were compared using ANOVA (aov[stats]) after verifying normality and homogeneity of variances. Significance of ANOVA tests was evaluated with Dunnett's post hoc tests for multiple comparisons to reference exposures (glht[multcomp]). The *P* values were adjusted for multiple comparisons using the Benjamini Hochberg correction.

Significant amine containing metabolites not involved in cysteine metabolism were identified with ANOVA (Benjamini Hochberg corrected *P* value <0.05) and their variance investigated with PCA (PcaPlots[MetabolomicsAustraliaR]) between copper concentrations and the direction of change identified with boxplots.

4.3 Results

Indigenous macroinvertebrate community

A total of 17,302 invertebrates from 45 taxa colonized the 55 microcosms, with 94.5% of individuals belonging to the family Chironomidae. Abundances, number of microcosms inhabited and sensitivity classification of common taxa are presented in Table 4.1. Genus composition between treatments was not significantly different when tested by an MRPP test (A = -0.003, *P* = 0.563). The average number of taxa did not differ between treatments (data not shown). However, the number of individuals declined with increasing copper and a log-logistic model indicated that 50% of individuals were lost at 133 mg/kg (EC50) copper (Figure 4.2a, Table 4.2). The 'no animal' controls did not differ significantly from the same concentration treatments with animal added so all treatments were pooled by concentration for further analysis. For sensitivity-classified groups of genera, abundance varied between copper treatments. Sensitive taxa displayed an EC50 of 75 mg/kg (Figure 4.2b, Table 4.2), moderately sensitive taxa displayed an EC50 of 249 mg/kg (Figure 4.2c, Table 4.2), while tolerant taxa displayed an EC50 of 713 mg/kg copper (Figure 4.2d, Table 4.2). However, there are high errors associated with the tolerant species EC50 and these species may survive higher concentrations of copper than this experiment investigated (Table 4.2).

Added Potamopyrgus antipodarum

The survival of adult *P. antipodarum* decreased with increasing copper and had an EC50 of 364 mg/kg (Figure 4.3a, Table 4.2). Reproduction was also affected, with juvenile production (EC50: 121 mg/kg) and embryo production (EC50: 172 mg/kg) decreasing at lower concentrations than adult survival (Figure 4.3c, 3e, Table 4.2). Furthermore, juveniles and embryos were not present in the 750 mg/kg treatment and embryos were not present in surviving adults in the 500 mg/kg treatment.

Added Physa acuta

The survival of *P. acuta* decreased slightly in the two highest concentrations of copper with a predicted EC50 of 823 mg/kg (Figure 4.3b, Table 4.2).

Reproduction of *P. acuta* declined with increasing copper with juveniles (EC50: 298 mg/kg) responding at a lower concentration than egg mass production (EC50: 428 mg/kg) or adult survival (Figure 4.3d, f, Table 4.2). The low number of individuals combined with the reasonably high tolerance of *P. acuta* limited power to detect patterns in these data (Table 4.2). However, as juveniles occurred at high numbers (150 to 350 individuals) in low copper treatments, deploying more adults into the microcosms could overwhelm other species.

Added Chironomus tepperi

Individual bioindicators

The survival of *Chironomus tepperi* was not affected by copper exposure (data not shown). However, larval dry weight decreased with increasing copper with an EC50 of 238 mg/kg (Figure 4, Table 4.2).

Gene expression

Gene expression profiles in the cysteine metabolism were investigated in *C. tepperi*. The PCA showed that copper influenced gene expression profiles (Figure 4.5). Copper treatments separated along two axes PC1 (52.3.5% variance explained) and PC2 (21.7% variance explained). All copper treatments separated from the reference with the 500 mg/kg treatment displaying the largest separation from the reference (Figure 4.5a). When considered independently, the lowest copper treatments (60 and 125 mg/kg) separated from the reference PC1 (41.1% variance explained) and PC2 (28.5% variance explained) with variance increasing with Cu concentration (Figure 4.5b).

Individual expression profiles of each gene measured are displayed in Figure 4.6. For *S-adenosylmethionine synthetase* (*SAM*), copper treatments significantly altered expression (ANOVA: $F_{(5,17)}$= 3.73, P = 0.018). The expression of *SAM* was upregulated between 3- and 5-fold in all copper treatments, however; this was only significant in the 125 mg/kg (P = 0.022) and 500 mg/kg treatments (P = 0.004) when tested with Dunnett's t-test

relative to the reference treatment (Figure 4.6a). For *S-adenosylhomocysteine hydrolase* (*SAH*), copper significantly altered expression ($F_{(5,17)}$= 3.04, *P* = 0.038). Upregulation of *SAH* expression was evident in 125, 250 and 500 mg/kg treatments, with 500 mg/kg being significantly different from the reference (*P* = 0.023). In the 750 mg/kg treatment *SAH* expression had returned to reference levels (Figure 4.6b).

The expression of *cystathionine-β-synthase* (*CβS*) did not significantly change due to copper exposure ($F_{(5,17)}$= 1.79, *P* = 0.169) (Figure 4.6c). For *cystathionine-γ-lyase* (*CγL*), expression altered significantly between treatments ($F_{(5,17)}$= 3.92, *P* = 0.015). The expression of *CγL* upregulated with increasing copper, reaching a maximum of 3-fold upregulation in the 500 mg/kg treatment (*P* = 0.004). In the 750 mg/kg treatment upregulation of *CγL* expression had reduced to 2-fold and was not significantly different from the reference (*P* = 0.417)(Figure 4.6d). The expression of *metallothionein* (*Mtn*) differed significantly between exposures ($F_{(5,17)}$= 16.13, *P* < 0.001) (Figure 4.6e). The expression of *Mtn* upregulated 10- to 150-fold in all treatments and was significant in 125 mg/kg treatments and above (125 mg/kg: *P* = 0.007, 250 mg/kg: *P* = 0.03, 500 mg/kg: *P* < 0.001 and 750 mg/kg: *P* = 0.001)(Figure 4.6e).

The expression of *γ-glutamylcysteine synthase* (*GCS*) differed significantly between copper treatments ($F_{(5,17)}$= 6.42, *P* = 0.002) (Figure 4.6f). The expression of *GCS* upregulated in all copper treatments but was only significant in the 500 mg/kg treatment with a 2.5-fold increase (*P* = 0.002) relative to the reference. For *glutathione synthetase* (*GS*), *glutathione-S-transferase d1* (*GST*) and *thioredoxin glutathione reductase* (*TGR*) expression, there was no significant difference between copper treatments ($F_{(5,17)}$= 1.18, *P* = 0.36, $F_{(5,17)}$= 1.94, *P* = 0.139 and $F_{(5,17)}$= 1.55, *P* = 0.228, respectively) (Figure 4.6g, h and 6i, respectively).

Metabolomics

Cysteine metabolism metabolites

From the LC-MS analysis, 9 metabolites involved in cysteine metabolism were selected for consideration in context with gene expression (Figure 4.1, Figure 4.7). The concentration of methionine did not differ between treatments although there was an increase in concentration in 250 mg/kg treatments and above ($F_{(5,18)}$= 1.98, P = 0.135)(Figure 4.7a). For homocysteine, concentration was altered by copper treatments ($F_{(5,18)}$= 5.88, P = 0.002). Homocysteine concentration increased with increasing copper and was significantly different from reference in the 500 mg/kg (P = 0.024) and the 750 mg/kg (P = 0.007) treatments (Figure 4.7b). For serine, cysteine and glutamate, concentration was not altered by copper treatments ($F_{(5,18)}$= 0.72, P = 0.620, $F_{(5,18)}$= 0.94, P = 0.482 and $F_{(5,18)}$= 2.34, P = 0.109, respectively)(Figure 4.7c, d and f). The concentration of lanthionine was significantly altered by copper treatments ($F_{(5,18)}$= 5.37, P = 0.004). However, although lanthionine concentration increased in 250 mg/kg treatments and above, Dunnett's *post hoc* comparisons found no treatment significantly differed from the control (Figure 4.7e). The concentration of γ-glutamylcysteine was marginally altered by copper treatments ($F_{(5,18)}$= 2.51, P = 0.077). Dunnett's *post hoc* comparisons indicated that concentration of γ-glutamylcysteine increased with higher levels of copper and was significantly different from the control in the 500 mg/kg treatment (P = 0.024) (Figure 4.7g). The concentration of glycine was altered by copper treatments ($F_{(5,18)}$= 4.26, P = 0.012). Glycine concentration increased in treatments over 125 mg/kg and Dunnett's *post hoc* indicated that 500 mg/kg (P = 0.084) and 750 mg/kg (P = 0.005) treatments were significantly different from control exposures (Figure 4.7h). The concentration of glutathione did not differ between treatments ($F_{(5,18)}$= 1.10, P = 0.396) (Figure 4.7i).

Other amine containing metabolites

Of the 37 amine containing metabolites identified by LC-MS that were not selected based on their association with cysteine metabolism, 18 altered significantly in copper treatments after Benjamini-Hochberg correction (Table 4.3). A PCA of these metabolites separated primarily along one axis (variance explained 70.33%), with a second axis explaining 9.57% (Figure 4.8a, b). The PCA clearly separated copper treatments from the reference with highest copper concentrations (500 and 750 mg/kg) displaying the largest deviation from the control. Most (11 of 18) of the significantly altered amine containing metabolites increased in concentration in copper exposures of 125 and above (Table 4.3). Asparagine, proline and dihydroxyphenylalanine all increased in all copper treatments compared to the reference, while norepinephrine and kynurenine increased in 250 mg/kg treatments and above. Citrulline was the only amine containing metabolite to display a decrease in concentration, which occurred in 500 and 750 mg/kg copper treatments (Table 4.3).

4.4 Discussion

Indigenous macroinvertebrates

This study investigated responses of invertebrates to copper exposure at several levels of organization, using a field-based microcosm as a platform. This platform allowed the comparison of subindividual, individual, population and community level bioindicators of laboratory and indigenous species under environmentally relevant conditions. The purpose of this comparison was to identify how commonly used toxicity bioindicators relate to community structure and to identify if responses were likely caused by direct or indirect copper exposure.

The indigenous macroinvertebrates that colonized the microcosms displayed a range of copper sensitivities. This demonstrated the diversity of chironomid pollution responses and supports previous research (Carew et al., 2007; Gresens et al., 2007; Mousavi et al., 2003; Pettigrove, 2006). The observed variability in pollution sensitivity is likely a result of genetic resistance, different feeding habits and/or avoidance behavior of different groups. For example species of the *Procladius* genus were tolerant to copper, these species can be carnivorous and feed on other chironomid species (Takacs and Tokeshi, 1993). This habit could allow avoidance of copper in sediment as prey could pre-metabolize it. Furthermore, toxicity of copper could make prey more available, as they could be slowed and burrowing behavior reduced by toxic sediments (Callaghan et al., 2001). Comparatively, species relying on sediment and algae for food such as *Paratanytarsus* or *Chironomus* spp. could experience reduced food supply or direct exposure through ingestion of copper containing sediment (Armitage et al., 1995).

Deployed laboratory species

Whole organism bioindicators

The bioindicators measured in laboratory species displayed different copper sensitivities and hence were of varied value for protecting indigenous invertebrates. The survival of added laboratory species was not an informative

bioindicator of copper toxicity. The short exposure time of *C. tepperi* could explain the difference in survival compared to other species in the *Chironomus* genus, which was classified as sensitive, as earlier instars can be more sensitive to metal toxicity (Gauss et al., 1985; Williams et al., 1986).

Compared with survival, sublethal responses of laboratory species aligned better with indigenous community responses. For snails, *P. antipodarum* was more sensitive than *P. acuta* with number of juveniles being the most sensitive bioindicator. The ED50 for *P. antipodarum* juvenile number aligned with reduced total abundance of indigenous macroinvertebrates and protected moderately sensitive invertebrates. Embryo production of *P. antipodarum* and *C. tepperi* dry weight responded at similar concentrations as moderately sensitive macroinvertebrates; however, from a field perspective , by the time a response was observed in *P. acuta,* moderately sensitive macroinvertebrates would be lost.

Cysteine metabolism

To observe responses in laboratory species at concentrations where survival of sensitive macroinvertebrates was declining, subindividual bioindicators were considered. The cysteine metabolism pathway is displayed in Figure 4.1.Trends in gene expression and metabolite concentrations in the cysteine metabolism of *C. tepperi* were observed at 60 mg/kg copper and above. The upregulation of *SAM* and *SAH* expression are likely responsible for the observed increase in homocysteine under copper exposure. The upregulation of *CγL* could increase the transfer of homocysteine to cysteine via cystathionine and although cysteine and serine levels were not altered, their production and transfer to lanthionine, which was increased, and metallothionein through increased *Mtn* expression, could explain the stable levels of these metabolites. Downstream of cysteine, increased expression of *GCS* corresponded with increased γ-glutamylcysteine concentration and an increase in glycine concentration indicated the beginning of antioxidant responses, although *GS, GST* and glutathione levels were stable. This antioxidant response induced by copper could be metal specific, as previous

studies with zinc found no change in the concentration of intermediates downstream of cystathionine (Long et al., 2015). The expression of *SAH*, *CγL* and *GCS* is upregulated by copper exposure, and contradicts the downregulation of *SAH* and *GCS* and no change in *CγL* that was observed in laboratory exposures to copper spiked sediments (Chapter 5). These differences could be due to field-based conditions, such as fluctuating temperature and reduced food availability that were not considered in laboratory exposures.

Other amine containing metabolites

Amine containing metabolites that were not involved in cysteine metabolism were also affected by copper exposure, with all but citrulline increasing in concentration due to copper exposure relative to the control. This is different from previous research investigating zinc exposure in *C. tepperi* where all significantly altered amine metabolites except for cysteamine decreased after zinc exposure (Long et al., 2015). This highlights the metal specific nature of metabolite responses.

Metabolites that responded to copper exposure were involved in several cellular functions. Ammonia metabolism intermediates were affected (through glutamine, citrulline, arginine and asparagine), as was polyamine biosynthesis (through cadaverine and putrescine) and neurotransmission and modulation (3.hydroxytyramine, 3.methoxytyramine, phenethylamine, tyramine, octopamine and dihydroxyphenylalanine). Tyramine and octopamine can be involved in melanin production (Fuchs et al., 2014). As well as protecting against ultraviolet exposure, melanin can bind to metal ions (Gallas et al., 1999; Szpoganicz et al., 2002). Recently, melanin concentration was found to increase in *C. riparius* exposed to copper (Loayza-Muro et al., 2013). Investigating gene expression and metabolites in this pathway could add to the understanding of *C. tepperi* responses to metal exposure. The increase in pyridoxamine could also be linked to copper levels as it forms complexes with Cu^{2+} and Fe^{2+} as well as being involved in hydroxyl radical scavenging, indicating another possible antioxidant response (Casasnovas et al., 2013).

Of the metabolites responding to copper exposure, no starvation specific metabolites were identified (Tuffnail et al., 2009). This could be due to the short exposure time for laboratory-bred *C. tepperi* or due to species-specific starvation responses (Warne et al., 2001). To discern between starvation and exposure responses in *C. tepperi*, metabolite assessments of starvation exposures need to be conducted. The amine containing metabolites that have responded here offer pathways that could prove useful to further investigate contaminant and toxicity responses in *C. tepperi.*

This research used an integrated approach to detect differences in macroinvertebrate responses under field conditions. Responses to copper exposure were found at all levels of biological organization, with gene expression the most sensitive of the laboratory-based species endpoints. This research demonstrated the need to incorporate subindividual bioindicators in laboratory species to observe responses at concentrations where survival of sensitive macroinvertebrate survival was declining. The subindividual bioindicators measured here offered additional information on copper metabolism, indicating direct exposure in *C. tepperi.* Furthermore, metal specific responses were identified and pathways of interest for further investigation were identified through LC-MS.

4.5 Figures
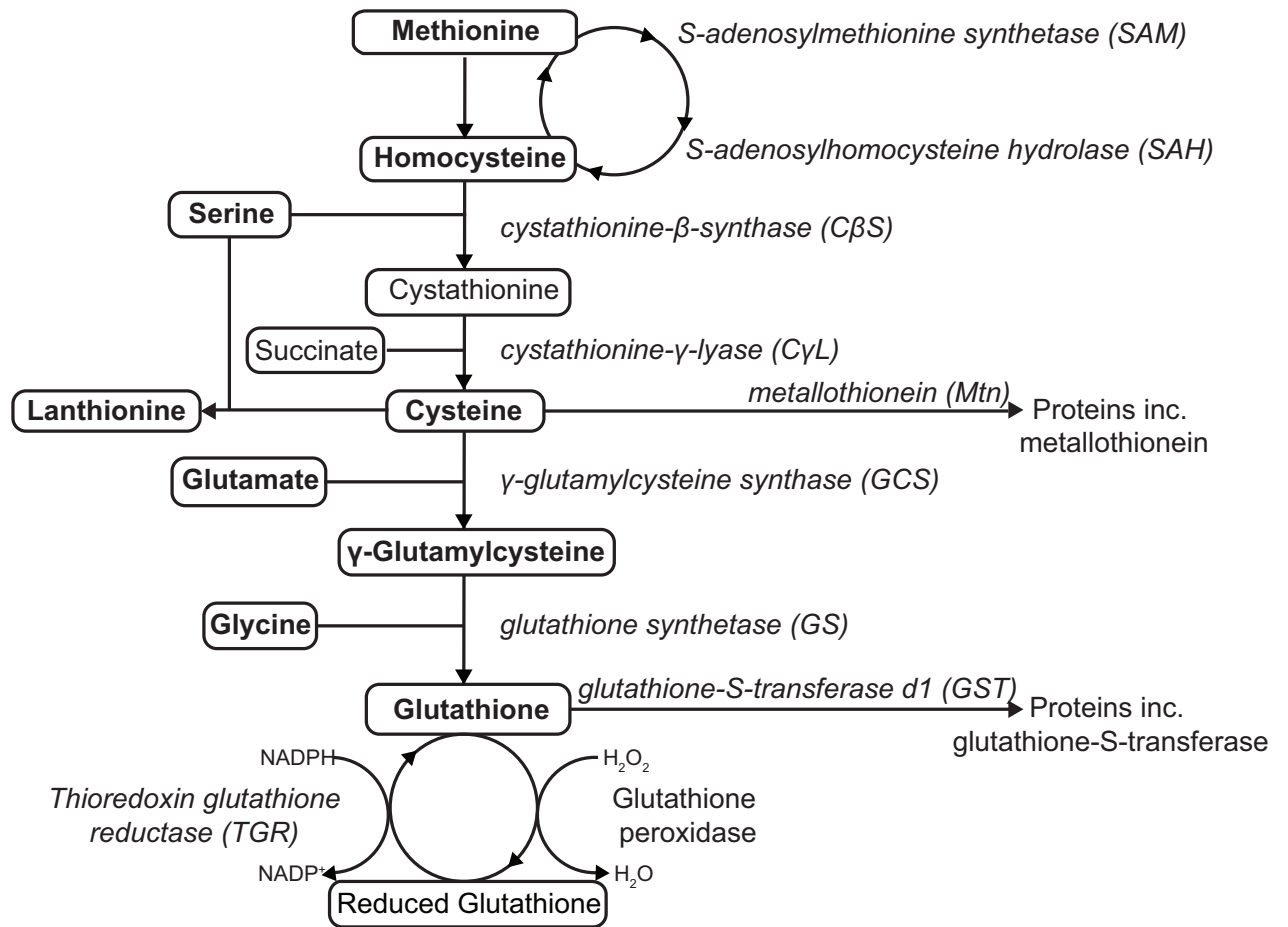
Figure 4.1 Predicted cysteine metabolism of *Chironomus tepperi* adapted from Hughes et al. (2009)(based on information from Nemapath49). Genes that were quantified with qPCR in *C. tepperi* are indicated in italic text and metabolites measured with LC-MS are indicated in bold text.
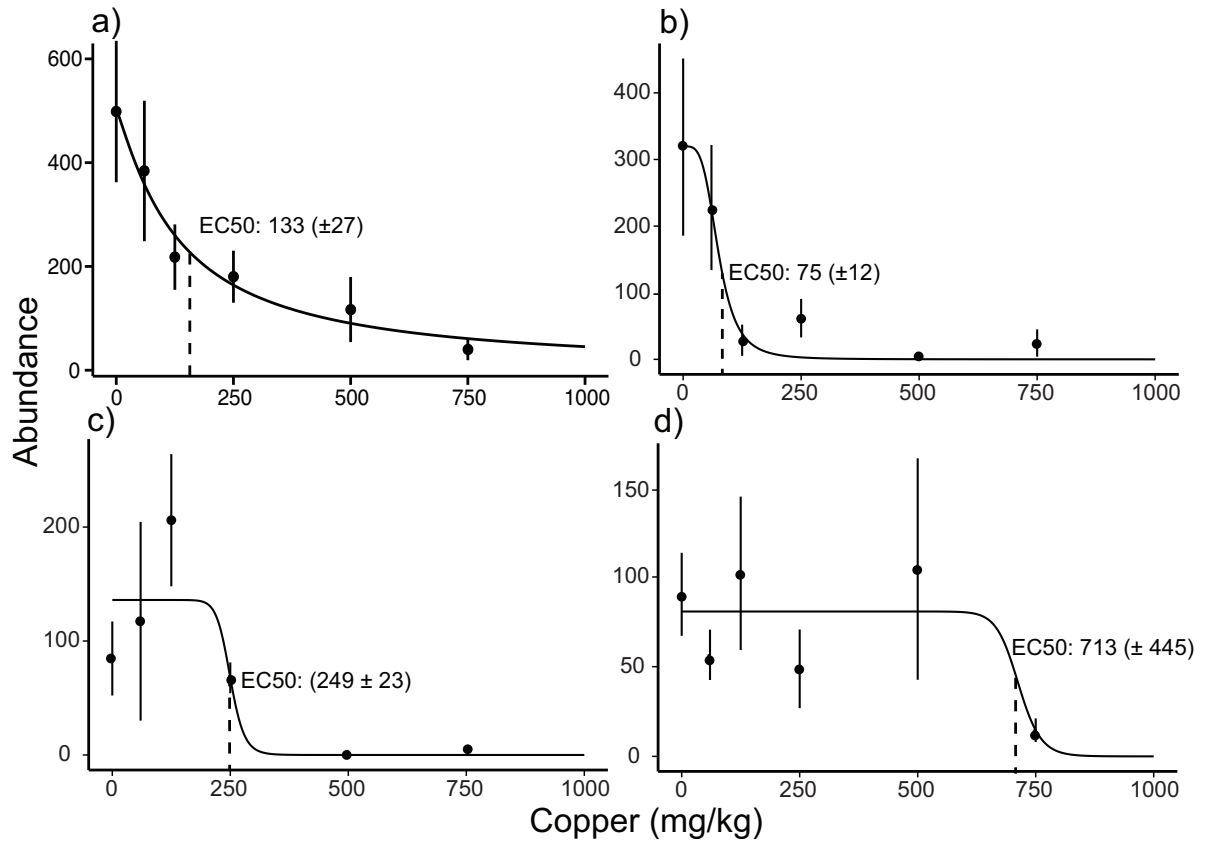
Figure 4.2 The abundance of a) total, b) sensitive, c) moderate and d) tolerant indigenous macroinvertebrates exposed to copper (mg/kg) in spiked microcosms. Lines represent 3-parameter log logistic models of the data with EC50 (± standard errors) indicated by a dashed line. Points represent mean abundance and error bars are standard errors of the mean.
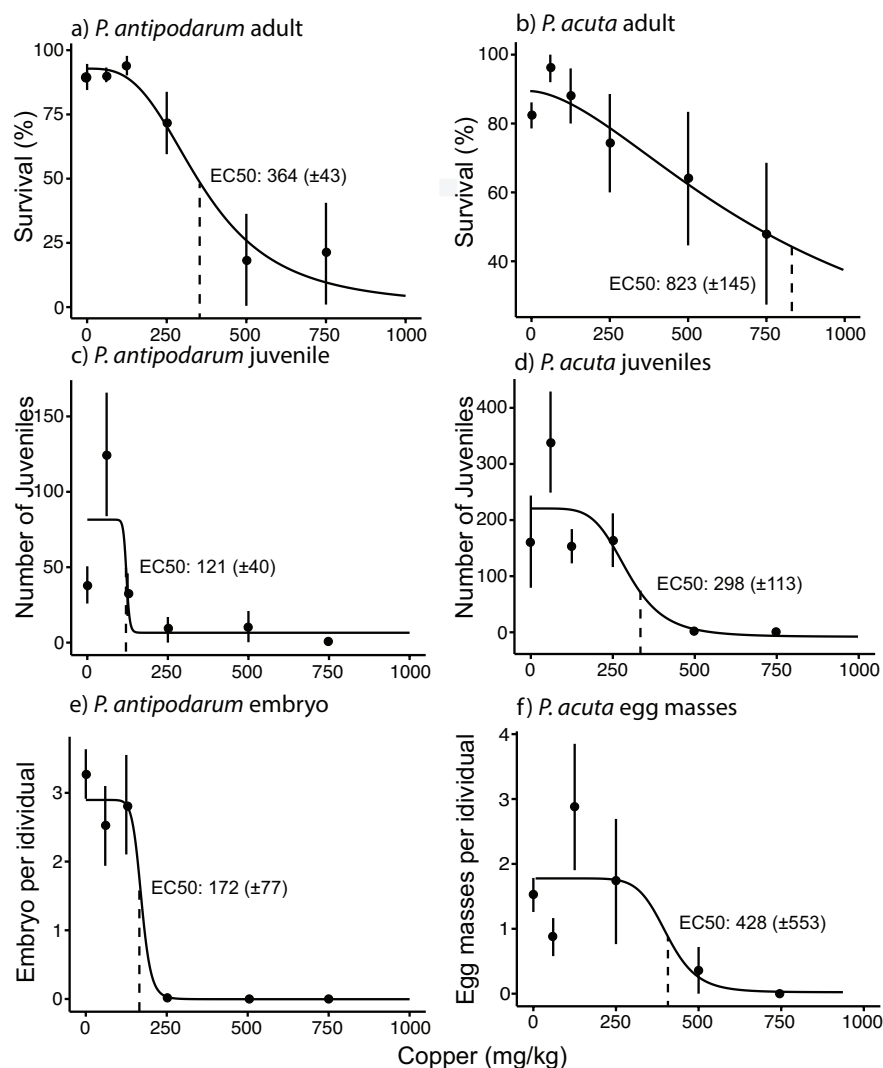
Figure 4.3 The survival of a) *Potamopyrgus antipodarum* and b) *Physa acuta* exposed to copper (mg/kg) in spiked microcosms for 6 weeks. Juvenile production of b) *P. antipodarum* and c) *P. acuta* were measured and d) *P. antipodarum* embryo production and e) *P. acuta* egg mass production per surviving individual are also displayed. Lines represent 3-parameter log logistic models of the data with EC50 (± standard errors) indicated by a dashed line. Points represent mean values for each bioindicator and error bars are standard errors of the mean.
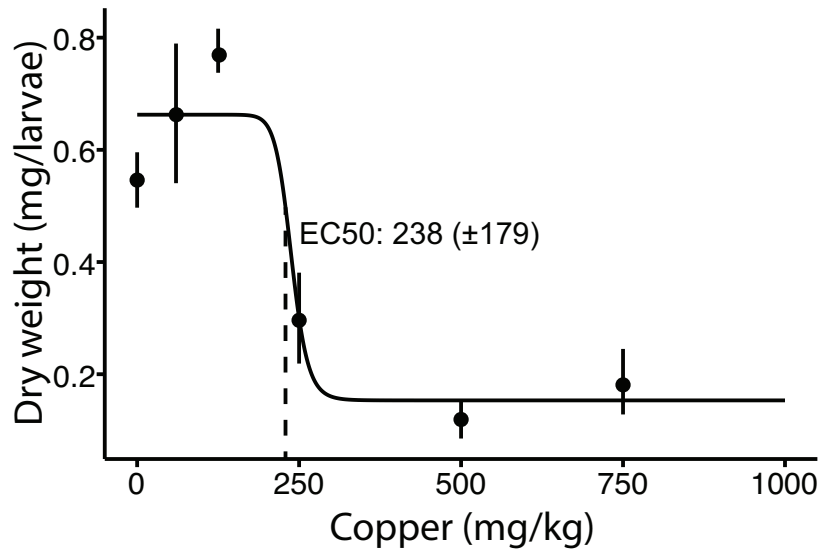
Figure 4.4 Larvae dry weight of *Chironomus tepperi* exposed to copper (mg/kg) in spiked microcosms for 5 days. Lines represent 3-parameter log logistic models of the data with EC50 (± standard errors) indicated by a dashed line. Points represent mean values and error bars are standard errors of the mean.
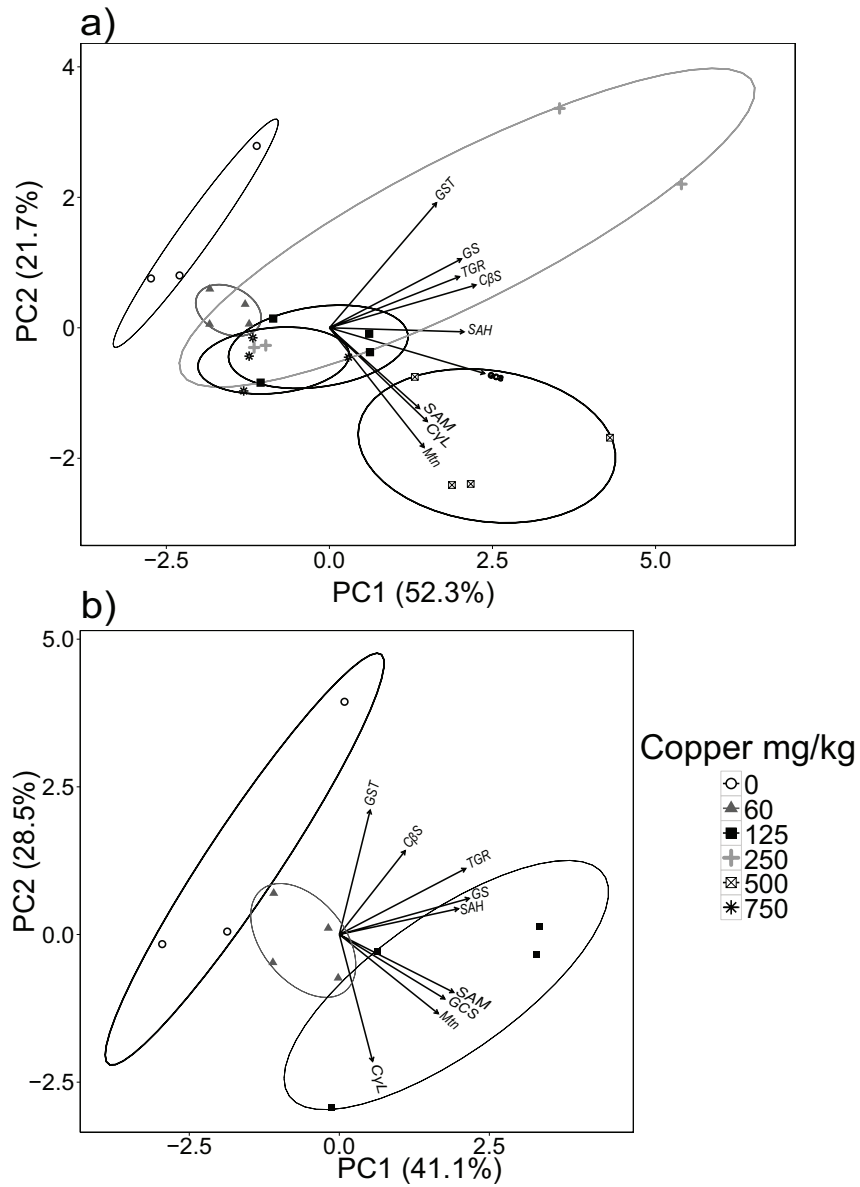
Figure 4.5 Principal components analysis (PCA) scores plots of 9 *Chironomus tepperi* cysteine metabolism genes after 5-day exposure to sediment copper concentrations (mg/kg) in spiked microcosm: a) all copper treatments and b) sublethal copper treatments (n=4, except reference n=3).
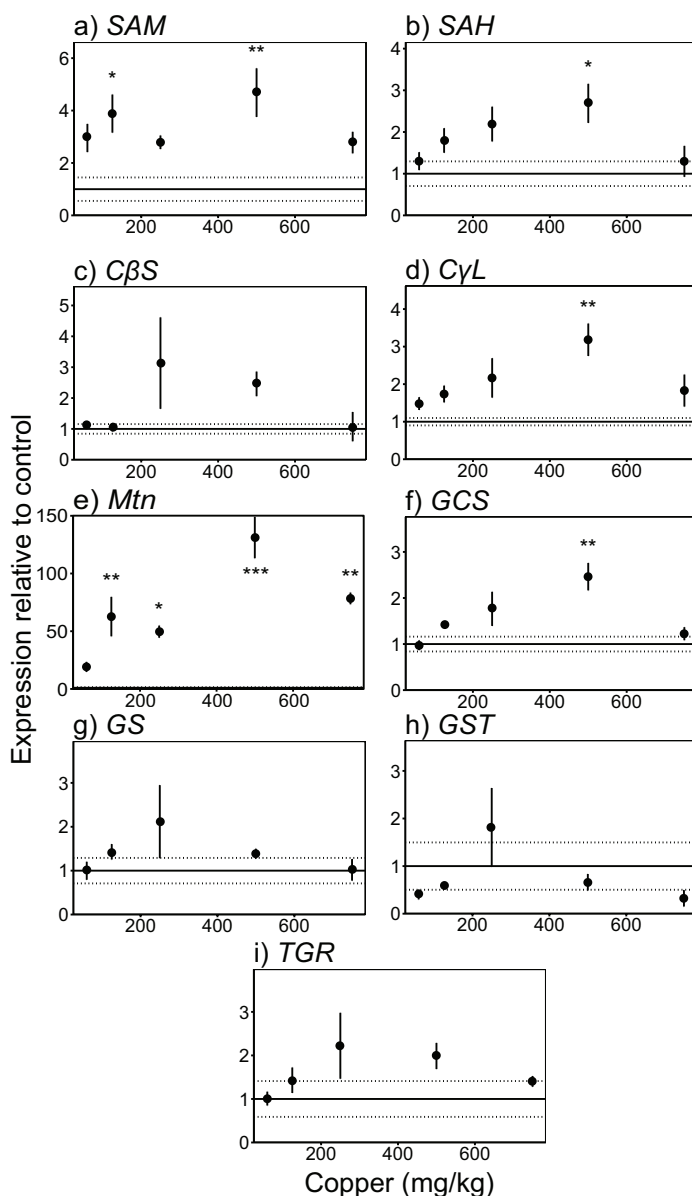
Figure 4.6 Expression profile of *Chironomus tepperi* cysteine metabolism genes after 5-day sediment exposure in copper spiked microcosms: a) *S-adenosylmethionine synthetase* (*SAM*), b) *S-adenosylhomocysteine hydrolase* (*SAH*), c) *cystathionine-β-synthase* (*CβS*), d) *cystathionine-γ-lyase* (*CγL*), e) *metallothionein* (*Mtn*) f) *γ-glutamylcysteine synthase* (*GCS*), g) *glutathione synthetase* (*GS*), and h) *glutathione-S-transferase d1* (*GST*), i) *thioredoxin glutathione reductase* (*TGR*). Expression was measured using RT qPCR and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to control exposures (black line) ± SEM (black dashed lines). Significance was determined with ANOVA and Dunnett's *post hoc* with Benjamini Hochberg adjustment for multiple comparisons (*$P < 0.05$, ** $P < 0.01$). Error bars indicate standard errors of the mean.
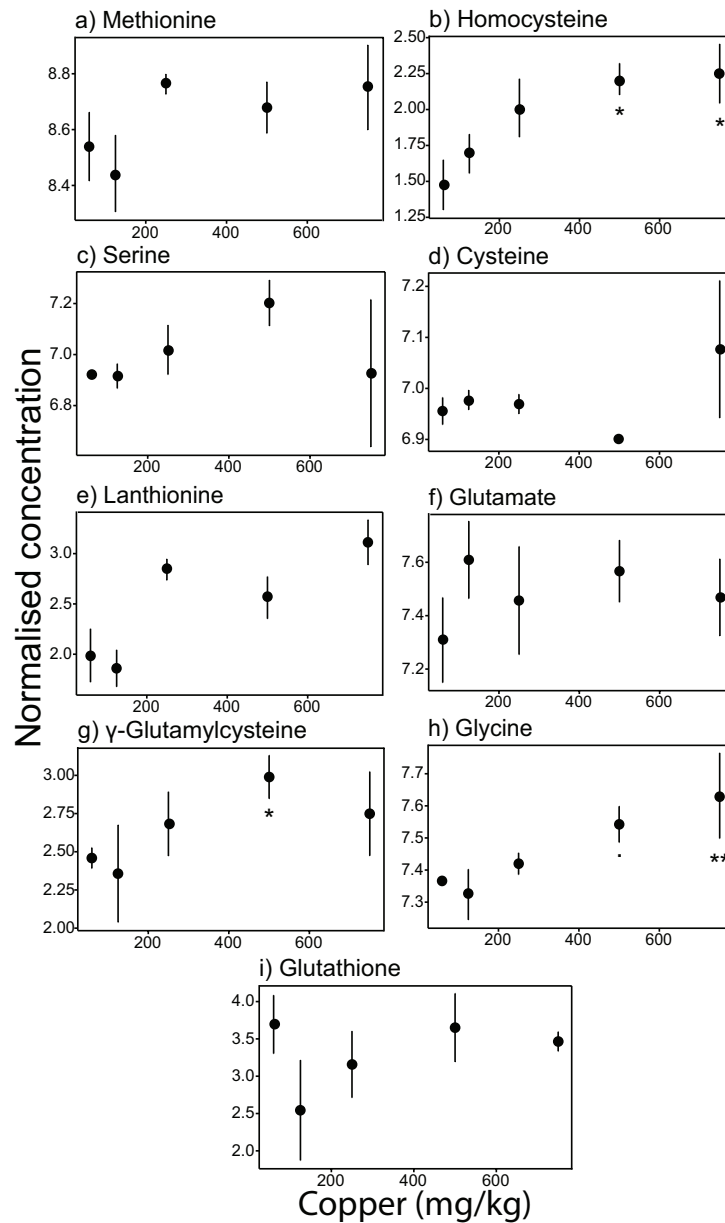
Figure 4.7 Concentration of *Chironomus tepperi* metabolites associated with cysteine metabolism after 5-day sediment exposure in copper spiked microcosms: a) methionine, b) homocysteine, c) serine, d) cysteine, e) lanthionine, f) glutamate, g) γ-glutamylcysteine, h) glycine and i) glutathione. Significance was determined with ANOVA and Dunnett's *post hoc* with Benjamini Hochberg adjustment for multiple comparisons (. *P* < 0.1, *P < 0.05, ** *P* < 0.01). Error bars indicate standard errors of the mean.
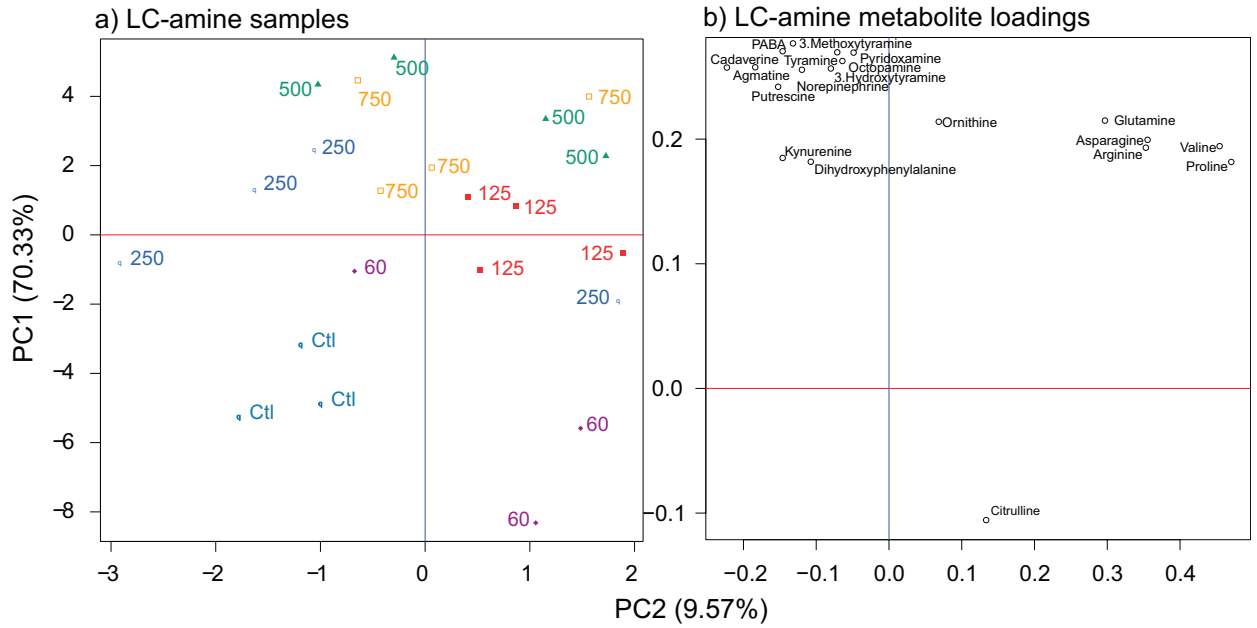
Figure 4.8 Principal components analysis (PCA) scores plots for 19 significant amine

containing metabolites using targeted LC-MS approach displaying a) sample and

b) metabolite loadings (n=4, except 60 mg/kg and reference n=3).

4.6 Tables

Table 4.1 Common taxa representing more than 0.3% of the total abundance
colonizing the microcosm experiment. Taxa are ranked from most to least abundant.

Taxon	Family	Number of individuals	% total abundance	Number of microcosms	Sensitivity ranking	Reference
Paratanytarsus	Chironomidae	5940	35.1	46	sensitive	(Pettigrove, 2006)
Procladius	Chironomidae	3707	21.9	46	tolerant	(Pettigrove, 2006)
Chironomus	Chironomidae	3635	21.5	33	sensitive	Carew unpublished data
Kiefferulus	Chironomidae	2356	13.9	25	moderate	(Pettigrove, 2006)
Cladopelma	Chironomidae	279	1.7	11	moderate	Carew unpublished data
Chaoboridae	Chaoboridae	211	1.2	7	moderate	Carew unpublished data
Parachironomus	Chironomidae	194	1.1	11	tolerant	Carew unpublished data
Berosus	Hydrophilidae	182	1.1	11	moderate	
Polypedilum	Chironomidae	104	0.6	29	tolerant	(Pettigrove, 2006)
Oribatida	Not identified	99	0.6	27	Not known	
Microvelia	Veliidae	48	0.3	27	tolerant	(Chessman, 2003)
Ceratopogonidae	Ceratopogonidae	45	0.3	8	tolerant	(Chessman, 2003)
Ephydridae	Ephydridae	44	0.3	18	tolerant	(Chessman, 2003)

Table 4.2 Results from log-logistic models fit to the different bioindicators measured
in a field-based microcosm displaying median effective dose (ED50).

Bioindicator	ED50	Standard error	Parameter	Value	t-value	*P*-value
Total macroinvertebrates	132.8	26.9	b	1.1	5.2	0.014
			d	502.6	14.3	0.001
			e	132.8	4.9	0.016
Sensitive macroinvertebrates	75.0	11.6	b	16.9	0.1	0.954
			d	136.0	4.6	0.019
			e	249.9	10.7	0.002
Moderately sensitive macroinvertebrates	249.9	23.3	b	3.9	1.8	0.173
			d	319.7	8.5	0.003
			e	75.0	6.5	0.007
Tolerant macroinvertebrates	713.4	445.8	b	30.2	0.1	0.941
			d	80.7	5.9	0.010
			e	713.4	1.6	0.208
C. tepperi dry weight	287.7	109.8	b	2.8	1.3	0.298
			d	0.7	6.6	0.007
			e	287.7	2.6	0.079
P. antipodarum adult survival	364.6	43.1	b	3.0	3.4	0.041
			d	92.7	17.1	<0.001
			e	364.6	8.5	0.003
P. antipodarum juvenile production	121.2	40.3	b	14.5	0.1	0.927
			d	81.5	3.2	0.050
			e	121.2	3.0	0.057
P. antipodarum embryo per individual	171.0	77.5	b	11.8	0.7	0.518
			d	2.9	13.3	0.001
			e	171.0	2.2	0.114
P. acuta adult survival	823.3	145.7	b	1.7	2.7	0.073
			d	89.2	19.1	<0.001
			e	823.3	5.7	0.011
P. acuta juvenile production	298.5	112.7	b	5.3	0.4	0.727
			d	220.1	4.0	0.028
			e	298.5	2.6	0.077
P. acuta egg masses per individual	428.4	553.4	b	8.8	0.1	0.908
			d	1.8	3.8	0.032
			e	428.4	0.8	0.495

Table 4.3 Significant amine-containing metabolites detected in *Chironomus tepperi* larvae following exposure to copper spiked microcosm for 5 days. Significance was assessed with ANOVA and Benjamini Hochberg (BH) adjusted significance levels.

Metabolite	Direction of change	Comparison	df	Sum square	Mean square	F-value	*P* value	BH significance level
Asparagine	increase all treatments	copper	5	2.40	0.48	11.82	<0.001	0.001
		residual	16	0.65	0.04			
3.Hydroxytyramine	increase 125 and above	copper	5	2.32	0.46	7.34	0.001	0.004
		residual	16	1.01	0.06			
3.Methoxytyramine	increase 125 and above	copper	5	2.14	0.43	7.71	0.001	0.004
		residual	16	0.89	0.06			
Octopamine	increase 125 and above	copper	5	2.32	0.46	7.34	0.001	0.004
		residual	16	1.01	0.06			
PABA	increase 125 and above	copper	5	2.86	0.57	8.21	0.001	0.004
		residual	16	1.11	0.07			
Putrescine	increase 125 and above	copper	5	3.76	0.75	7.68	0.001	0.004
		residual	16	1.57	0.10			
Pyridoxamine	increase 125 and above	copper	5	2.46	0.49	7.75	0.001	0.004
		residual	16	1.02	0.06			
Tyramine	increase 125 and above	copper	5	2.93	0.59	8.29	<0.001	0.004
		residual	16	1.13	0.07			
Valine	increase all treatments	copper	5	1.50	0.30	7.34	0.001	0.004
		residual	16	0.65	0.04			
Cadaverine	increase 125 and above	copper	5	2.67	0.53	6.33	0.002	0.007
		residual	16	1.35	0.08			
Agmatine	increase 125 and above	copper	5	2.03	0.41	5.23	0.005	0.014
		residual	16	1.24	0.08			
Norepinephrine	increase 250 and above	copper	5	2.08	0.42	5.30	0.005	0.014
		residual	16	1.25	0.08			
Arginine	increase 125 and above	copper	5	2.59	0.52	5.08	0.006	0.015
		residual	16	1.63	0.10			
Proline	increase all treatments	copper	5	1.42	0.28	5.05	0.006	0.015
		residual	16	0.90	0.06			
Citrulline	decrease 500 and 750	copper	5	2.75	0.55	4.92	0.006	0.015
		residual	16	1.79	0.11			
Glutamine	increase 125 and above	copper	5	4.00	0.80	4.59	0.009	0.019
		residual	16	2.79	0.17			
Dihydroxyphenylalanine	increase all treatments	copper	5	4.40	0.88	4.08	0.014	0.030
		residual	16	3.46	0.22			
Kynurenine	increase 250 and above	copper	5	3.92	0.78	3.98	0.015	0.031
		residual	16	3.15	0.20			

CHAPTER 5 – TOXICANT MIXTURES IN SEDIMENT ALTER GENE EXPRESSION IN THE CYSTEINE METABOLISM OF *CHIRONOMUS TEPPERI*

Abstract

Sediment contamination can pose risks to the environment and sediment toxicity tests have been developed to isolate the impact of sediment from other factors. Mixtures of contaminants often occur in sediments, and traditional endpoints used in toxicity testing, such as growth, reproduction and survival, cannot discern the cause of toxicity from chemical mixtures because of complex interactions. In urban waterways, the synthetic pyrethroid bifenthrin and copper metal are commonly found in mixtures, so this study was designed to investigate how these contaminants cause toxicity in mixtures. To investigate this, *Chironomus tepperi* was exposed to environmentally relevant concentrations of copper and bifenthrin-spiked sediments in a two-way factorial mixture for five days. Growth and expression profiles of cysteine metabolism genes were measured after exposure. Growth was increased in low copper concentrations and decreased at high copper concentrations and was unaffected by bifenthrin exposures. Copper exposures induced possible cellular repair by upregulating *S-adenosylmethionine synthetase* expression and downregulating expression of *S-adenosylhomocysteine hydrolase* and *cystathionine-β-synthase* expression. *Metallothionein* upregulation was also observed. Bifenthrin exposure altered cysteine metabolism to a lesser extent, downregulating *cystathionine-β-synthase* and *γ-glutamylcysteine synthase*. Synergistic, antagonistic and dose dependent interactions were observed and there was evidence of conflicting modes of action and limited substrate production. These findings demonstrate how contextual gene expression changes can be sensitive and specific identifiers of toxicant exposure in mixtures.

5.1 Introduction

Sediments are often highly contaminated because hydrophobic chemicals introduced to waterways bind to suspended particles, settle and accumulate. In this way sediments can reflect past and present contamination at orders of magnitude higher concentrations than water in the same system (Burton, 1991). Many aquatic organisms rely on sediment for protection and food, so sediment toxicity tests using benthic organisms are often included in routine aquatic risk assessment.

Sediment toxicity tests are useful because they standardize experimental conditions and isolate the impact of sediment from factors such as hydrology and habitat (OECD, 2004; US-EPA, 1996). In southern Australia, a common sediment toxicity test organism is the chironomid *Chironomus tepperi.* Similar to *C. riparius* and *C. tentans* assessed in the northern hemisphere (ASTM, 1997; OECD, 2004), it breeds well in the laboratory and spends the majority of its life exposed to sediment (Stevens, 1993). Sediment toxicity tests with this organism traditionally assess mortality and sublethal whole organism endpoints such as reproduction or growth and interpret these endpoints in light of sediment chemistry analyses (e.g. Kellar et al., 2014). However, toxicity from chemical mixtures in field sediments is often difficult to discern because of unpredictable interactions. Mixture interactions occur at various levels. In exposure media, interactions between contaminants can regulate their bioavailable fractions, while at the organism level they may regulate their uptake and depuration processes and their reaction with biological targets (Spurgeon et al., 2010). Synergistic interactions of contaminants can cause toxicity at exposure levels lower than observed in single contaminant exposures (Yang et al., 2004). Chemical analysis of mixtures may not correlate with toxicity because often only part of the mixture associated with toxicity is identified (Donnelly et al., 2004; Eide et al., 2004). Highly contaminated sites are particularly difficult to characterize because of the complexity of the mixtures and changes in chemical composition of sediments over time as deposition and degradation occur (Donnelly et al., 2004). In these cases, it is essential to understand how toxicant specific responses

perform in mixtures, in order to help identify contaminants and improve rapid screening for chemical interactions (Donnelly et al., 2004).

In aquatic monitoring, gene expression is increasingly used to investigate the impact of toxicants on biota. Gene expression has proved useful in identifying sublethal exposure and stress that may lead to population and community impacts (Adams et al., 1989; Galay-Burgos et al., 2003). Several studies have investigated transcriptomic responses to pollution in freshwater biota (e.g. Hook et al., 2008; Marinkovic et al., 2012a). However, whole transcriptome techniques are still not cost effective for routine toxicity testing. An alternative approach involves measuring transcript levels in a well-studied stress response pathway. Cysteine metabolism plays a central role in detoxification against environmental stress (Jones et al., 2004; Sugiura et al., 2005). Previously, we reported the response of several genes regulating cysteine metabolism under the influence of waterborne copper or cadmium (Jeppe et al., 2014 (Chapter 2)). The different transcriptional responses of genes observed implicated metal-specific detoxification pathways and suggested that gene expression profiles in this pathway could potentially provide informative biomarkers. However, contaminant mixtures can influence gene expression differently to individual contaminant exposures (Hook et al., 2008; Spurgeon et al., 2010). These mixtures can act additively, synergistically, or influence expression patterns in idiosyncratic ways

This study was designed to investigate how expression profiles of cysteine metabolism genes in *C. tepperi* respond in a standard toxicity test involving a metal-pesticide mixture, and to compare these expression profile responses to whole organism endpoints traditionally used in these tests. This study investigated the expression profiles of nine genes involved in cysteine metabolism of *C. tepperi*. The genes encode enzymes involved in the remethylation cycle (*S-adenosylmethionine synthetase; SAM* and *S-adenosylhomocysteine hydrolase; SAH*), the transsulfuration pathway (*cystathionine-γ-lyase; CγL, cystathionine-β-synthase*; *CβS, γ-glutamylcysteine synthase*; *GCS-CAT, glutathione synthetase*; *GS* and

thioredoxin glutathione reductase; TGR) and two resulting stress response products (*glutathione S-transferase delta 1*; *GSTd1* and *metallothionein*; *Mtn*). The expression of these genes has previously been characterized under metal exposure in water and this system displayed potential for investigating mixture toxicity (Jeppe et al., 2014 (Chapter 2); Long et al., 2015).

5.2 Materials and Methods

Spiking of sediment

Sediment was collected from Glynns wetland, an uncontaminated wetland 30 km east of Melbourne (OC 2.2%). Sediment was sieved to 63 μm and allowed to settle for 24 hours. It was then spiked using methods from Simpson et al. (2005), with adjustments to be 'dilution pH 7' spiking, whereby a high-spike sediment stock was diluted to improve binding and equilibration of metal to sediment (Hutchins et al., 2008). Sediment stock was spiked and pH adjusted in a nitrogen atmosphere. Spiking solutions were made up with deoxygenated water and added to sediment and then sediment was mixed vigorously and rolled on a bottle roller for 2 to 3 hours. After 24 hours the pH was adjusted to 8 with 1 N NaOH and sediments were equilibrated for 25 days, rolling for 2 to 3 hours 3 times a week. The copper (Cu) used to spike was analytical reagent grade; because it has been shown that pesticide formulation affects toxicity (e.g.Schlenk et al., 2012), we used a commercially available formulation of bifenthrin (Bif) to be environmentally relevant (Hovex ant granules). After equilibration, the stock sediment was diluted and mixed in a two-way factorial design to generate three exposure concentrations of each contaminant: low (Cu 44, Bif 0.032 mg/kg), medium (Cu 176, Bif 0.063 mg/kg,) or high (Cu 308, Bif 0.023 mg/kg). Based on preliminary data, these concentrations were sublethal and expected to induce stress. Hence they were also expected to induce genetic profile changes linked to toxicity. Concentrations of Cu were based on sediment quality guidelines (ANZECC/ARMCANZ, 2000; MacDonald et al., 2000). Low Cu treatment concentration was above the TEC guideline (31.6 mg/kg), medium Cu was above the PEC and ISQG-low guidelines (149 mg/kg and 80 mg/kg, respectively) and high Cu was above the ISQG-high guideline (270 mg/kg). There are currently no sediment quality guidelines for Bif so concentrations were based on observed field concentrations and preliminary laboratory exposures. Sediment from each treatment was taken for chemical analysis at the start of the exposure. Total Cu was analyzed through ICP-AES (ALS: *www.alsglobal.com/)* and Bif was analyzed by method NR47 using GC-MS/MS (NMI laboratories: http://www.measurement.gov.au/)

Site comparison

A comparison of sites was undertaken to investigate if field-collected sediments caused similar responses to those observed in the spiked mixture experiment. Three sites known to have moderate synthetic pyrethroid (SP) and metal contamination were selected from within the greater Melbourne area. The top two centimeters of sediment was collected from each site, filtered to 63 µm and allowed to settle for 24 hours. Metal and pesticide concentrations were then determined as mentioned above. A metal contamination quotient was calculated for the sites from MacDonald et al. (2000) as:

$$PECq= \sum (\frac{M/PECm}{n(M)})$$

Where M is the metal measured, PECm is the consensus-based probable effect concentration (PEC) for metal (M) and n(M) is the total number of metals measured. Synthetic pyrethroid contamination was represented as the sum of bifenthrin and permethrin concentrations (SP mg/kg) (Table 5.1).

Organism exposures

Chironomus tepperi larvae for experiments were maintained at CAPIM (University of Melbourne, Australia), and had been originally collected from Yanco, New South Wales in March 2014 (courtesy of Mark M. Stevens). The culture was maintained under a 16:8 hour light dark cycle at 20 ± 1 ºC in aerated culture medium (0.07 mmol $CaCl_2$, 0.1 mmol NaCl, 0.08 mmol $MgSO_4$, 0.12 mmol $NaHCO_3$, 0.01 mmol KH_2PO_4 and 0.01 µmol $FeCl_3$). Larvae that hatched from eggs were reared to 2nd instar (five days old) in aerated culture medium on ethanol-rinsed tissue paper and were supplemented with 10 mg ground commercial fish food three times a week (TetraMin, Germany).

Five replicate 600 mL beakers were used for mixture treatments and three replicates were used for individual contaminant treatments. Each beaker contained 140 grams of treatment sediment and 200 mL culture medium, and was allowed to settle for 24 hours prior to exposure (ASTM, 1997; OECD,

2004). A 70% water change was then performed and twenty 5-day old larvae were added to each replicate. Larvae were exposed for five days, water was renewed and larvae were fed on days one and three. Dissolved oxygen, pH, ammonia and electric conductivity were measured at each water renewal. After exposures, larvae were removed from sediment using a 125 μm sieve and rinsed in deionized water. A subset of 10 larvae was snap frozen for gene expression analysis and the remaining larvae were stored in 70% ethanol for dry weight analysis.

RNA extraction

RNA was isolated with High Pure RNA Tissue Kit (Roche, Cat. no. 12033674001) according to the manufacturer's protocol with some small adjustments. The larvae from each replicate were homogenized at 0 C° in 300 μL lysis buffer using a cryomill (Precellys 24, Bertin Technologies). The homogenate was transferred and the beads rinsed with a further 150 μL lysis buffer before extracting as per the kit protocol. Extracted RNA was eluted in 50 μL RNase-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and was quantified on a Take3 plate (BioTek). All RNA samples had a 260/280 ratio between 1.9 and 2.1 and were suitable for cDNA synthesis.

Reverse transcription

Total RNA (2 μg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. no. M1705), following the manufacturer's protocol. The cDNA was then checked for gDNA contamination using a pair of *ribosomal protein L11* primers that flank a 345 bp intron. The expected cDNA amplicon was 466 bp, whereas amplification of the 811 bp product would indicate the presence of gDNA (Martinez-Guitarte et al., 2007). Template cDNA was diluted to 400 μL and stored at –20 ºC.

Gene expression analysis

Genes isolated in the cysteine metabolism, their primers and amplicon sequences have been described previously (Jeppe et al., 2014 (Chapter 2)), with the addition of *thioredoxin glutathione reductase* (*TGR*), which was described in Chapter 3.

Gene expression was measured using quantitative real-time PCR (qPCR), with gene-specific primers. Quantitative real-time PCR was performed with the Roche LightCycler® 480 (Roche Applied Science, USA) in 10 µL reactions containing the components described by Takahashi et al. (2010). The following conditions were used: 10 minutes at 95 ºC, followed by 50 cycles of 10 seconds at 95 ºC, 15 seconds at 58 ºC, and 15 seconds at 72 ºC followed by a melting curve analysis from 65 ºC to 95 ºC to ensure a single product was formed.

Statistical analysis

Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann et al., 2011). Differences in relative normalized expression of target genes between treatments were analyzed using R (version 3.2.1, The R Foundation for Statistical Computing Platform). All survival, growth and gene expression data were found to be normal with equal variance using Kolmogorov-Smirnov (ks.test[stats]) and Levene's tests (leveneTest[car]) with the exception of the site sediments. For the spiked mixture experiment, an overview of gene expression in the cysteine metabolism was obtained using principal component analysis (PCA) (princomp[stats]) with a matrix of the nine genes and 16 treatment groups. Gene expression differences between contaminants were tested with factorial ANOVAs (aov[stats]) considering Cu, Bif and the interaction of Cu and Bif. Treatments with significant interactions between contaminants were assessed for antagonism, synergism or idiosyncratic trends by comparing mean values of each treatment. Due to multiple comparisons of treatments involving nine

genes, only differences at P = 0.005 were considered significant based on a Bonferroni correction. Parabolic regression lines were added where significant treatment effects were detected to illustrate changes in expression with levels of Cu. For the preliminary site comparison, sediments from sites were compared to the control with a Kruskal-Wallis rank sum test.

5.3 Results

Survival and growth

Survival of *C. tepperi* after Cu, Bif or mixture exposure did not differ significantly between treatments (ANOVA: $F_{(15,53)}$ = 0.67, P = 0.803) (Figure 5.1A). Exposures were therefore sub-lethal and growth and gene expression results were not confounded by differences in survival. Dry weight of larvae differed significantly with Cu exposure ($F_{(3,53)}$ = 25.70, P < 0.001) but was unaffected by Bif ($F_{(3,53)}$ = 0.62, P = 0.608) with no interaction between these factors ($F_{(9,53)}$ = 1.37, P = 0.225). Dunnett's t test revealed that low Cu led to a marginally non-significant increase in larval dry weight by 5 to 10 mg (P = 0.072), while high Cu significantly reduced larval dry weight by 25 to 30 mg (P < 0.001) compared to control exposures and irrespective of Bif concentration (Figure 5.1B).

Cysteine metabolism

The PCA of all treatments showed that individual Bif treatments and low Cu treatments were not different from the control (Figure 5.2). In contrast, medium and high Cu treatments separated primarily along PC1 (74.1% variance explained) with variance increasing with Cu concentration. This analysis also suggested that the presence of medium and high Bif in mixtures reduced Cu-driven variation.

Individual expression profiles of each gene are displayed in Figure 5.3 and results of the factorial ANOVA are displayed in Table 5.2. For *SAM* expression, Cu and Bif concentrations both had significant effects on expression and an interaction between treatments was evident (P < 0.001) (Figure 5.3A, Table 5.2). When Cu was the only contaminant and present at a high concentration, *SAM* expression upregulated 2-fold. When no Cu was present, Bif caused a small downregulation (0.1-fold change) and the interaction of Cu and Bif was antagonistic. Low and medium Bif exposure reduced the extent to which *SAM* expression was upregulated compared to the controls under high Cu to 1.5-fold and 1.25-fold respectively. Exposure to

high Cu and Bif mixture caused *SAM* expression to be downregulated by 0.5-fold. For the expression of *SAH*, Cu exposure had a significant effect ($P < 0.001$), while Bif did not ($P = 0.11$). However, Bif and Cu displayed a synergistic interaction ($P = 0.004$) (Figure 5.3B, Table 5.2). Under high Cu exposure, *SAH* expression downregulated to 0.6-fold and this downregulation increased to 0.3-fold with increasing Bif.

For the expression of *CβS*, Cu concentration had a significant effect ($P < 0.001$) and Bif did not ($P = 0.416$) (Figure 5.3C). However, the interaction between Cu and Bif was significant ($P < 0.001$) and antagonistic. While *CβS* downregulated with both Cu (0.8-fold) and Bif (0.4-fold), individuals exposed to a mixture of Cu and Bif showed a return to control expression levels. For the expression of *CγL*, there was no significant difference below the Bonferroni corrected *p*-value of 0.005 (Cu ($P = 0.032$) or Bif ($P = 0.141$) and no interaction ($P = 0.756$)) (Figure 5.3D). The expression of *Mtn* differed between exposures (Cu: $P < 0.001$, Bif: $P < 0.001$ and Cu:Bif: $P < 0.001$) (Figure 5.3E, Table 5.2). Under medium and high Cu exposure, *Mtn* expression was upregulated over 10-fold compared to controls. For Bif exposure, *Mtn* expression was downregulated between 0.6- and 0.8-fold. While upregulation of *Mtn* was still evident at medium and high Cu in the presence of Bif, the magnitude of upregulation was lowered to 8-fold under medium Bif and lowered to 5-fold under high Bif exposure, indicating an antagonistic interaction (Figure 5.3E).

The expression of *GCS* differed under Cu ($P < 0.001$) and Bif ($P < 0.001$) exposure (Figure 5.3F) with no significant interaction between the contaminants ($P = 0.052$). Under high Bif exposure, *GCS* expression downregulated 0.7-fold irrespective of low or medium Cu. The expression of *GCS* also downregulated 0.8-fold under medium and low Cu exposure irrespective of Bif, but under high Cu expression it returned to control levels. For *GS* expression, there was no significant difference between treatments based on the Bonferroni adjusted *p*-value of 0.005 (Cu ($P = 0.108$), Bif ($P = 0.141$), interaction effect ($P = 0.015$)) (Figure 5.3G).

For the expression of *GST*, there was no significant difference based on the Bonferroni adjusted *p*-value of 0.005 (Cu (P = 0.024), Bif (P = 0.144) and interaction (P = 0.165)) (Figure 5.3H). For the expression of *TGR*, only Cu treatments differed significantly (Cu: P = 0.001, Bif: P = 0.088 and Cu:Bif: P = 0.167) (Figure 5.3I). Under low and medium Cu exposure, *TGR* expression downregulated 0.8-fold; however, under high Cu (particularly in the presence of Bif) expression was not significantly different from control levels.

Site comparison

The three sites with moderate metal and synthetic pyrethroid contamination were investigated for four genes showing responses in the spiked mixture experiment. For all sites, the expression of *SAM* did not differ from control levels (Kruskal-Wallis χ^2 = 0.39, df = 1, P = 0.53) (Figure 5.4A). For the expression of *SAH* and *CβS,* all sites downregulated around 0.75-fold (χ^2 = 7.7, df = 1, P < 0.01) (Figure 5.4B) and 0.5-fold (χ^2 = 6.2, df = 1, P < 0.05) (Figure 5.4C), respectively. The expression of *Mtn* was significantly upregulated in all sites by about 5-fold (χ^2 = 8.0, df = 1, P < 0.005) (Figure 5.4D).

5.4 Discussion

This study showed how mixtures influence stress-induced gene expression in *C. tepperi* after a 5-day sediment toxicity test, and compared these gene expression changes to a commonly used sublethal whole organism endpoint. The dry weight of *C. tepperi* larvae indicated a small degree of hormesis in low Cu exposures and toxicity in high Cu exposures based on guidelines for toxicity tests (ASTM, 1997; OECD, 2004). Using these endpoints, it would have been concluded that Cu was beneficial at low exposures and was moderately toxic at high exposures, and that the Bif concentrations investigated in this study were not toxic. However, changes in gene expression profiles altered at medium and high exposure concentrations. This suggests that gene expression responses occurred before a reduction in dry weight was observed, and that gene expression could provide an early signal of pollution exposure and associated stress.

For the Cu-only exposures, four genes displayed consistent and near linear dose response expression patterns. The upregulation of *SAM* expression and downregulation of *SAH* expression at medium and high Cu exposures respectively suggested increased cellular methylation, which is important for cell function and repair (James et al., 2002). The downregulation of *CβS* expression at medium and high Cu exposures is likely to inhibit CβS substrate production from homocysteine, which in turn increases the homocysteine available for *SAM* (and cellular methylation) but also slows the downstream production of cystathionine (Hughes et al., 2009; Rao et al., 1990). Furthermore, the upregulation of *Mtn* observed during medium and high Cu exposures suggested protective metal sequestration through MT production which is likely to further diminish the cysteine pool in the cell (Banni et al., 2007). The downregulation of *GCS* and *TGR* were evident only in low Cu treatments and these responses may need more investigation to establish if they are repeatable and informative of Cu modes of action.

Exposure in Bif-only treatments caused fewer overall changes in gene expression of cysteine metabolism of *C. tepperi.* Only downregulation of *SAM,*

Mtn and *GCS* expression was observed following Bif exposures. The downregulation of these genes could prevent protective responses and increase sensitivity by limiting substrate production along this pathway (Pin-Lan et al., 2007).

Interactions between the effects of Cu and Bif on the expression of cysteine metabolism genes were common and varied depending on which gene was considered. Strong antagonism was observed in *SAM* expression. The presence of Bif in the mixture limited *SAM* and hence SAM substrate production, which could in turn limit the cellular methylation required for Cu metabolism. Furthermore, the synergistic downregulation of *SAH* expression is indicative of the pathway being driven toward cellular methylation as SAM availability is limited. The antagonism between Cu and Bif observed in *CβS* expression was idiosyncratic; while both contaminants individually downregulated *CβS* expression, in medium and high mixtures *CβS* expression returned to control levels. Superficially, this result implies a neutralization of each contaminant by the other. However, it could also indicate increased demand for downstream products, and investigating related pathways could reveal implication of this interaction. The antagonism between Cu and Bif observed in *Mtn* expression indicates possible limitation of MT production under Bif exposure, which could reduce the protective metal response of the cell and increase sensitivity to Cu in mixtures (Hoffman et al., 2015).

Of the four genes that displayed consistent and near linear dose response expression patterns under Cu-only exposures, only two (*Mtn* and *SAH*) retained this pattern in the presence of Bif. This highlights the need to understand gene expression under mixture toxicity if gene expression is to be used as an indicator of exposure to specific pollutants in field sediments. Results from the site comparison were consistent with expectations from the spiked mixture experiment. The expression profiles of the sites investigated aligned well with the medium Cu and medium Bif mixture treatments, which could be expected given the levels of metal and synthetic pyrethroids present in the field sediments.

This study has demonstrated that gene expression can identify exposure in mixtures at concentrations that are sub-lethal. Gene expression analysis may therefore be a valuable tool for environmental monitoring, because it can detect exposure before traditional whole organism endpoints. Gene expression analysis can also identify dominant toxicants in mixed chemical exposures and may indicate links to toxicological mechanisms that lead to interaction effects between contaminants.

In the present study we have confirmed the suitability of several *C. tepperi* genes as molecular biomarkers of exposure to Cu and Bif in a mixture. The study showed that gene expression responses occur at lower concentrations than whole organism endpoints. Furthermore, the results identified several expression interactions that should be considered when looking at contaminants in mixtures.

5.5 Figures
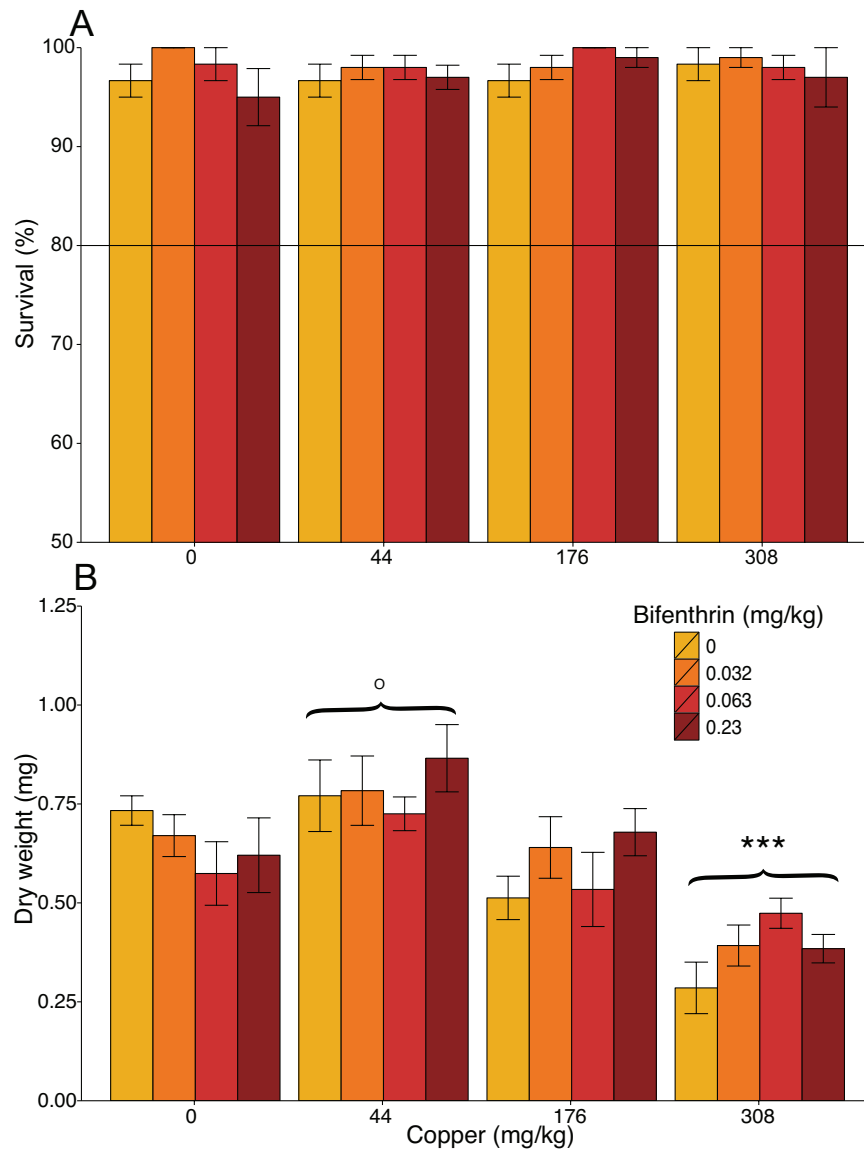
Figure 5.1 A) Survival and B) dry weight of *Chironomus tepperi* larvae after exposure different concentrations of copper and bifenthrin (n = 3) and mixtures of both (n = 5). Treatment effects were tested with ANOVA (***P < 0.001 and o marginal non-significance). Error bars indicate standard errors of the mean.
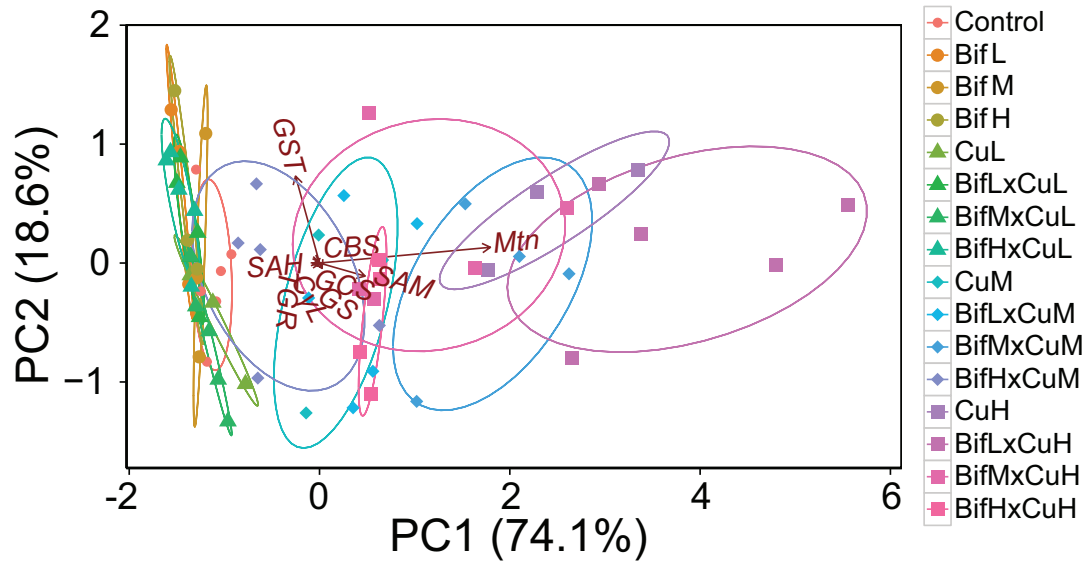
Figure 5.2 Principal components analysis (PCA) scores plots of 9 *Chironomus tepperi* cysteine metabolism genes after 5-day exposure to 16 treatments of copper (Cu) and bifenthrin (Bif) in two-way factorial mixture of low (L), medium (M) and high (H) concentrations (single treatments n=3, mixture treatments n = 5).
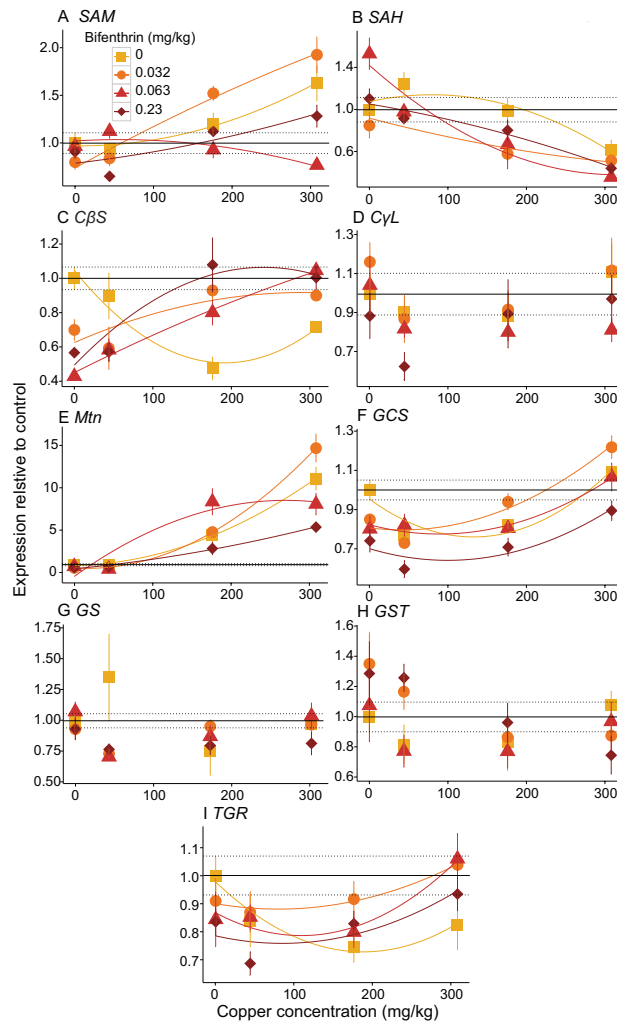
Figure 5.3 Expression profile of *Chironomus tepperi* cysteine metabolism genes after 5-day sediment exposure copper and bifenthrin (n = 3) and mixtures of both (n = 5): A) *S-adenosylmethionine synthetase* (*SAM*), B) *S-adenosylhomocysteine hydrolase* (*SAH*), C) *cystathionine-β-synthase* (*CβS*), D) *cystathionine-γ-lyase* (*CγL*), E) *metallothionein* (*Mtn*) F) *γ-glutamylcysteine synthase* (*GCS-CAT*), G) *glutathione synthetase* (*GS*), H) *glutathione S-transferase delta 1* (*GST*), and I) *thioredoxin glutathione reductase* (*TGR*). Expression was measured using RT qPCR and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to control exposures (black line) ± standard error of the mean (black dashed lines). Parabolic trend lines are displayed for genes with significant responses to either contaminant or an interaction between the contaminants. Significance was determined with a factorial ANOVA. Error bars indicate standard errors of the mean.
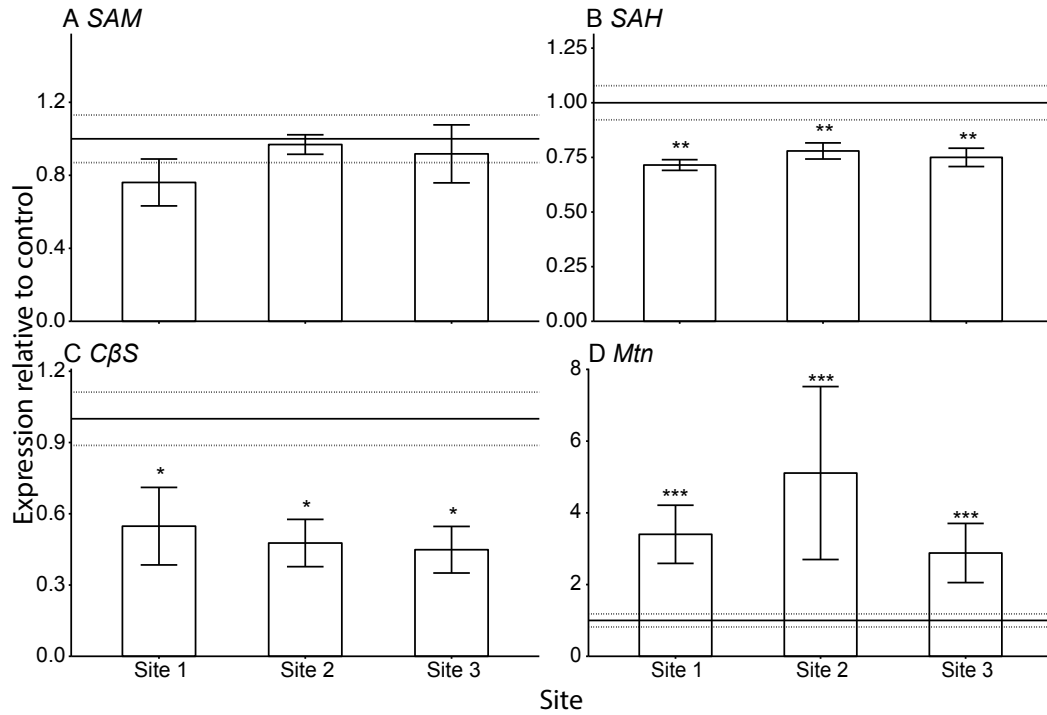
Figure 5.4 Expression of A) S-adenosylmethionine synthetase (SAM), B) S-adenosylhomocysteine hydrolase (SAH), C) cystathionine-β-synthase (CβS), and D) metallothionein (Mtn) in three field sediments. Sites were contaminated with moderate levels of metal and synthetic pyrethroids as indicated in Table 1. Site differences were tested with Kruskal-Wallis tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

5.6 Tables

Table 5.1 Probable Effect Concentration Quotient (PECq) for metals and sum of synthetic pyrethroid concentrations (SPs) in site sediments. A PECq of more than 1 implies toxic metal concentrations. No guideline values are available for SPs.

Site	PECq	SP (mg/kg)
Site 1	0.31	0.177
Site 2	0.54	0.122
Site 3	0.64	0.117

Table 5.2 Significance of copper (Cu) and bifenthrin (Bif) on *Chironomus tepperi* cysteine metabolism genes after 5-day sediment exposure to copper and bifenthrin (n = 3) and mixtures of both (n = 5) as assessed by a factorial ANOVA. Significant differences after Bonferroni adjustment ($P < 0.005$) are indicated in bold text.

Gene	Contaminant	Df	Mean square	F-value	*P value*
SAM	Bif	3	0.54	12.81	**<0.001**
	Cu	3	0.94	22.38	**<0.001**
	Cu:Bif	9	0.39	9.26	**<0.001**
	residual	51	0.04		
SAH	Bif	3	0.09	2.11	0.111
	Cu	3	1.31	29.98	**<0.001**
	Cu:Bif	9	0.14	3.21	**0.004**
	residual	51	0.04		
CβS	Bif	3	0.03	0.97	0.416
	Cu	3	0.31	10.56	**<0.001**
	Cu:Bif	9	0.21	7.28	**<0.001**
	residual	51	0.03		
CγL	Bif	3	0.11	1.88	0.144
	Cu	3	0.18	3.18	0.032
	Cu:Bif	9	0.04	0.63	0.762
	residual	51	0.06		
Mtn	Bif	3	26.96	8.15	**<0.001**
	Cu	3	327.22	98.96	**<0.001**
	Cu:Bif	9	24.26	7.34	**<0.001**
	residual	52	3.31		
GS	Bif	3	0.16	1.90	0.141
	Cu	3	0.38	2.13	0.108
	Cu:Bif	9	0.02	2.58	0.015
	residual	52	0.01		
GCS	Bif	3	0.08	14.37	**<0.001**
	Cu	3	0.09	34.90	**<0.001**
	Cu:Bif	9	0.11	2.05	0.052
	residual	51	0.04		
GST	Bif	3	0.14	1.89	0.143
	Cu	3	0.26	3.43	0.024
	Cu:Bif	9	0.12	1.52	0.165
	residual	52	0.08		
TPx	Bif	3	0.04	2.30	0.088
	Cu	3	0.11	6.01	**0.001**
	Cu:Bif	9	0.03	1.52	0.167
	residual	51	0.02		

CHAPTER 6 - GENERAL DISCUSSION

This thesis reports for the first time on the development and testing of gene expression biomarkers of cysteine metabolism in *C. tepperi*, with intended use in toxicity testing. In total, nine genes were identified and characterized with all showing potential for identifying exposure to metals under different exposure conditions (Chapter 2). In Chapter 3, gene expression profiles altered after a pulse exposure to cadmium. After depuration from this exposure, several genes remained responsive in high exposures but in low exposures all genes except *Mtn* returned to control levels. These findings verified those observed in Chapter 2, demonstrating that gene expression responses were rapid and repeatable and by linking gene expression in cysteine metabolism with GST activity and MT production. Furthermore, prolonged gene expression changes linked with population level responses. This chapter also highlighted the importance of exposure time and organism age when considering gene expression responses.

Chapter 4 investigated gene expression in copper spiked microcosms in the field. This enabled comparison of organism responses at the community, population, individual and biomarker levels, confirming that gene expression is a sensitive marker in *C. tepperi*. *Chironomus tepperi* gene expression changes occurred at concentrations where community responses in sensitive indigenous macroinvertebrates were observed. Furthermore, gene expression and metabolomic profiles indicated that direct copper exposure was occurring in *C. tepperi* with cysteine metabolism and other copper response pathways altered by exposure. Chapter 5 investigated the interaction of chemical mixtures using sediment spiked with copper and bifenthrin. For the first time this work tested these gene expression biomarkers with field mixtures. Several, but not all, gene expression responses were repeatable between different experiments, highlighting the complexities of measuring gene expression as biomarkers in ecotoxicology.

Based on the criteria defined in Chapter 1 gene expression in the cysteine metabolism is a promising biomarker of exposure that could be included in an adverse outcome pathway (AOP). Gene expression was sensitive, rapid and the response of several genes was reproducible between similar experiments. However, two genes tested were not consistently reproducible, the expression of CγL was different in Chapters 2 and 3 when exposed to 0.5 mg/L cadmium in water. In Chapter 3, CγL expression was downregulated but in Chapter 2 it was unchanged in this treatment and the reason for this variability is unknown at this stage. Similarly, between Chapters 4 and 5 the expression of *SAH* responded differently under similar concentrations of copper in sediment. In Chapter 4, *SAH* expression was unchanged (but trending toward upregulation) at 250 mg/kg copper but in Chapter 5 expression was downregulated at 300 mg/kg copper. These inconsistencies in *SAH* expression could be due to differences larval developmental stages caused by temperature variation in the field-based experiment compared to laboratory-based experiment, which was maintained at 21 °C. However these differences are caused, these genes should be considered with caution in future studies and only used to support other aspects of the cysteine metabolism.

These gene expression biomarkers also demonstrated a link to individual and population toxicity in several experiments, which is promising for future work to include these genes in an AOP (Kramer et al., 2011). The one exception to this is in Chapter 3, where the expression of *Mtn* was upregulated after a pulse exposure that did not cause mortality. While this does not necessarily mean that toxicity was not occurring, investigation into the links between *Mtn* expression and individual or population impacts is needed before these biomarkers can be considered in an AOP.

Overall, *C. tepperi* is a fairly tolerant laboratory species presenting an LC50 of 5.7 mg/L for cadmium in water, which is similar to some other *Chironomus* species but other species such as the amphipod *Hyalella azteca* have LC50s in the µg/L range (US-EPA, 2001). In sediment toxicity tests using moderately contaminated sediments, *C. tepperi* often does not show predictive

correlations with contamination for growth or emergence endpoints (CAPIM unpublished data). Furthermore, this species can respond to water quality parameters, such as pH and electrical conductivity when exposed to moderate sediment contamination (Hale et al., 2014). Hence, using gene expression can offer a more sensitive and specific measure of the response to moderate sediment contamination than mortality and growth measures. Developing biomarkers, such as gene expression, is essential if *C. tepperi* is to be to be used as an indicator of sediment toxicity in the future. If incorporated appropriately into a multiple lines-of-evidence approach, the biomarkers developed in this thesis could provide a powerful tool to identify contaminant exposure and toxicity at lower concentrations than current *C. tepperi* tests.

The biomarkers developed here can inform on metal exposure and would be a useful addition to sediment toxicity testing. However, to incorporate this technique into routine toxicity tests, responses of other pathways and to other contaminant groups need to be considered. Multiple pathways could be identified rapidly and efficiently using next-generation sequencing (NGS) or untargeted metabolomics techniques. A battery of sublethal exposures could identify multiple pathways potentially responding to specific contaminants (e.g. Hook et al., 2014b; Marinkovic et al., 2012a). The genes and metabolites of interest in identified pathways could then be tested, as cysteine metabolism pathway has been tested in this thesis, to identify strengths and limitations. It is likely that to acquire enough useful genes in enough pathways to identify common contaminant exposure, a large number of pathways should be identified from initial untargeted screening. This is now achievable with high throughput technologies and there has been much relevant work in other species for several contaminants, such as synthetic pyrethroids (e. g. Connon et al., 2009; Shi et al., 2011), metals (e. g. Hook et al., 2014a; Long et al., 2015; Regier et al., 2013), hydrocarbons (e. g. Hook et al., 2010) and other contaminants (Marinkovic et al., 2012a). While some studies have identified genes of interest and validated their response with qPCR (e. g. Hook et al., 2014b; Regier et al., 2013), to date it appears no study has used gene expression in standard toxicity testing. To achieve this, it is necessary to

validate expression of several genes in several pathways, so that toxicity of field sediments can be confidently predicted.

To validate the response of the cysteine metabolism and other potential stress response pathways (such as melanin production or ammonia metabolism identified in Chapter 4), *C. tepperi* could be exposed to a range of moderately contaminated field sediments (at least 30 sites) in a 5-day toxicity test. Sites would be selected that contain contaminants of interest at several concentrations in mixtures. The response of genes and metabolites in each pathway of interest could then be measured through qPCR and targeted metabolomics to allow rapid assessment of key intermediates in standard toxicity tests. To further strengthen gene expression as a biomarker technique, developmental changes in gene expression should be understood. Daily 'omics assessment through larval development could help explain why 11 day old larvae displayed different gene expression profiles to 16 day old larvae, as was observed in Chapter 3.

If comprehensive coverage of contaminant responses cannot be achieved through targeted gene and metabolomic techniques alone, other cellular biomarkers, such as contaminant specific proteins, could be incorporated. For example, well-studied proteins such as acetylcholinesterase for organophosphate pesticides (Arora and Kumar, 2015; Bocquene and Galgani, 1991) or carboxylesterase for synthetic pyrethroids (Heidari et al., 2005) could be used alongside the gene expression and metabolomic responses to expand the coverage of cellular responses to contamination.

If gene expression is included in sediment toxicity testing, after the initial development and primer design, the additional cost and effort would be small (about $70 per site and 1 week per 50 sites turnaround time). For this small investment a large amount of additional information on exposure and toxicity could be gained. Furthermore, not every site would need to be assessed for gene expression analysis. This technique would be most effective where moderate contamination occurs but effects are sublethal (to help identify causal factors) or where an effect is suspected but no sublethal effects are

apparent (to see if exposure to toxins has nevertheless occurred). In contrast, assessments of highly contaminated sites would benefit more from a technique like toxicity identification evaluation (Kellar et al., 2014; US-EPA, 2007).

There were several limitations to measuring gene expression biomarkers that were highlighted in this research. For *C. tepperi,* controlling organism age and exposure duration is critical if gene expression profiles are to be interpreted correctly. These issues limit the use of this technique to either laboratory-based sediment toxicity tests or to field deployments of laboratory-species. It is evident that gene expression may not be appropriate for assessing responses in field-collected organisms, where age and duration of exposure are unknown.

This research is the first step towards developing gene expression biomarkers of cysteine metabolism in *C. tepperi*, which can be incorporated into an AOP and wider sediment toxicity testing. If the limitations are understood, gene expression in cysteine metabolism will provide a powerful new tool to complement sediment toxicity tests and be part of multiple lines-of-evidence approaches. Gene expression biomarkers are an emerging tool to assess and prioritize the impact of contaminants. Their use in environmental monitoring will be facilitated by decreasing cost and increasing capability to gather and interpret gene expression data. This thesis contributes essential scientific information critical in the on-going development gene expression biomarkers.

APPENDIX 1: SUPPLEMENTARY MATERIAL

Supplementary file 1 Primers used to identify potential stress gene homologues in *Chironomus tepperi*. Including species used, GenBank accession numbers of alignment sequences and references.

Gene	Alignment spp.	GenBank Accession #	Primers 5'- 3'	Fragment size (bp)	Reference
ribisomal protein 18S	*C. riparius* [a]	DQ657920	F – GTTCGGAAGTTGATTATTGTCCAG R – ACGCGAGCGGAACTCACGCAAC	200	Park & Kwark (2008)
actin	*C. tentans* [a] *C. riparius* *C. yoshimatsui*	DQ176317 EU808012 AB070370	F – GATGAAGATCCTCACCGAACG R – CCTTACGGATATCAACGTCGC	201	Lee et al. (2006)
S- adenosylmethionine synthetase	*C. tentans* [b] *D. melanogaster* *A. aegypti*	DV188273 NM164356 XM001649241	F – ACAAGATCCAAATGCCAAAGTTGC R – ATCCAAACATCAAACCCTGATCG	281	
S-adenosylhomocysteine hydrolase	*D. melanogaster* [b] *A. aegypti* *C. riparius*	FBgn0015011 XM001651001 SRR496839.941385.2	F – AAATGCCTGGAATTATGACTCTTC R – CATTAAATGCGTTGCATCACCAC	336	
cystathionine-β-synthase	*D. melanogaster* [b] *A. aegypti*	NM1345080 XM001659193	F – ACACTGGAATCGGTTTAGCAATGG R – CTGTACCGTCATAATGAGCCAAAGG	264	
cystathionine-γ-lyase	*C. quinquefastiatus* *C. riparius* *C. tentans* [b] *A. aegypti*	XM001863008 SRR496839.8434444.2 DV182348 XM001848042	F – TAGAAAACATGCCTTGCATCACTCG R – GACTTTCATCGATGGATTTGTTGG	287	
γ-glutamylcysteine synthase	*C. quinquefastiatus* *C. riparius* *C. duplex* [b] *C. riparius* *D. melanogaster*	AY431633 SRR496839.34039.2 AY490748 NM080266 JQ762262	F – CGCAAATCTCTATTTTCCCAGACG R – GCGCCAATAAATGATCGATACCTC	576	
glutathione synthetase	*C. tentans* *C. duplex* [b]	DV184943 AY490750 AY490749	F – AATGGAGTGCCCGATTGATG R – TAGCCTTCAGGATTTTTAAGCACC	244	
glutathione S-transferase delta 1	*C. tentans* [a]	FJ851365	F – CTCCATGCCGTGCTGTCCA R – CGTAGCCAGGAACTGTTGATTTG	549	Li et al. (2009)
metallothionein	*D. melanogaster* [b]	NM1442625	F – ACCAATCTTGCGCGCCAAGG R – TGCAACAGTTCGTTGCAGCAG	137	
ribosomal protein L11	*C. riparius* [a]	EF179385	F – AGATCCCGTAAGCTTTGCC R – CATACTTCTGTTGGAACC	cDNA 466 gDNA 811	Martinez-Guitarte et al (2007)

[a] Primers used directly on *C. tepperi*
[b] Sequences aligned and primers designed for *C. tepperi*

Supplementary file 2 Primers used in real-time PCR to amplify cysteine metabolism genes in *Chironomus tepperi*. Including closest related species, protein homology as by BlastX(%) and GenBank accession number of resulting amplicons

Gene	Protein homology (%)	Related species	Primer 5´ - 3´	Fragment size (bp)	GenBank accession no.
actin	100	*Chironomus yoshimatsui* mRNA for actin, partial cds **AB070370**	F – CGCTGCTGCATCAACCTCAC R – GATTCTGGGCAACGGAAACG	93	Too short to be submitted see alignment below
S-adenosylmethionine synthetase	95	S-adenosylmethionine synthetase, isoform L [*Drosophila melanogaster*] **NP001259791**	F – CCAAAAGTTGCATGTGAAACTGTAAGC R – TTGATGTGATTTCACCACACAATAGG	66	Too short to be submitted see alignment below
S-adenosylhomocysteine hydrolase	89	Putative adenosylhomocysteinase 3 [*Crassostrea gigas*] **EKC42316**	F – GCATTGGGTGCATCTGTTCG R – CCGCGCCATGCAAATACAC	107	**KF740474**
cystathionine-β-synthase	82	related to CYS4-cystathionine beta-synthase [*Sporisorium reilianum SRZ2*] **XP002411237**	F – GCAATGGCATGTGCTGTTCG R – TTTCAGCACCAAGAGCTTTCAAGAC	100	**KF740475**
cystathionine-γ-lyase	72	*Bdellovibrio bacteriovorus* str. Tiberius, complete genome (cystathionine gamma lyase feature) **CP002930**	F – GGGAATGGAAGTGATTTTTGTCG R – TTGTTGGCGTTTCCAACCAC	98	**KF740476**
glutathione synthetase	95	*Chironomus duplex* clone 2 GS gene, partial cds **AY490750**	F – CAAGCATTAGCAAAGCCTGGAATC R – TTGTGATTTCATCCTCATCAGTCAAG	61	Too short to be submitted see alignment below
γ-glutamylcysteine synthase	98	*Chironomus riparius* gamma-glutamylcystein synthase mRNA, complete cds **JQ762262**	F – TGCGACGTGGAGAGAAGGTG R – CCCATTCCAAAACCCATTGC	130	**KF740473**
glutathione S-transferase delta 1	89	*Chironomus tentans* glutathione S-transferase (GSTd1) mRNA, complete cds **FJ851365**	F – GCTATGGAAACTGCAATGGGATTC R – TCAGCAACAGTGAGGCTATCACC	83	Too short to be submitted see alignment below
metallothionein	81	*Chironomus riparius* metallothionein mRNA, complete cds **HQ260607**	F – ACCAATCTTGCGGCGCCAAGG R – TGCAACAGTTCGTTGCAGCAG	137	Too short to be submitted see alignment below

Supplementary file 3 Alignments and primer locations for identification and amplification of cysteine metabolism genes in *C. tepperi*.

Forward primers are indicated by green background and reverse primers with orange background. Identification primers were designed

from closely related species and occur outside amplification primers.

S-adenosylmethionine synthetase

Species																								
	---	--A	CAA	GAT	CCA	AAT	GCC	AAA	GTT	GCA	TGT	GAA	ACT	GTA	AGC	AAA	ACT	GGA	ATG	ATC	CTA	TTG	TGT	GGT	GAA	ATC
Chironomus tepperi	CTC	AG.	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...
Chironomus riparius	CTG	AG.	...	...	...	...	...	...	...	...	...	..G	...	...	..T	..G	...	...	...	..T	...	..C	...	...	...	...
Drosophila melanogaster	CTG	AAG	..G	...	...	..G	...	...	...	...	...	...	..G	..C	...	...	...	...	...	...	..G	..C	...	..C	...	...
Ripitortus pedestris	TTA	AG.	...	..T	G..	...	..G	...	...	...	...	...	...	GC.	..G	...	...	..G	...	..T	T..	..G	...	...	..G	...
Modiolus americanus	CTG	AA.	...	..C	...	...	..G	...	...	..C	...	..C	...	..CT	...	...	...	...	...	..T	..G	...	..A	...	..G	...
Bombus terrestris	TTA	AA.	...	...	G..	...	...	..A	...	...	..T	..T	...	..CT	...	...	..A	...	..A	...	T.G	..A	...	...	..G	...
Acyrthosiphon pisum	CTG	AA.	...	G.C	...	..T	..A	...	...	..C	A..	...	...	..C.	...	..C	...	...	..A	T.T.	C.C	...	..A	...	..T	...
Tubulanus polymorphus	TTG	AAG	...	..T	.TG	...	..C	...	...	..T	...	...	AC.	...	...	...	..C	...	...	T..	G.A	...	...	...	..T	...

Species																							
Chironomus tepperi	ACA	TCA	AAA	GGA	TTT	GTT	GAT	TAT	CAA	AGA	ATT	GTC	CGT	GAT	ACT	GTT	CAT	ATT	GGA	TAC	GAT	GAC	TCT	TCA	AAA
Chironomus riparius	..	..G	...	...	...	...	...	...	...	..A	...	..G	...	..A	...	..C	...	...	...	...	...	...	..C	..A	...
Drosophila melanogaster	..G	..T	...	..G	G.G	..C	..C	...	..C	...	..G	...	...	G..	..G	...	...	...	..C	..C	...	..C	..C	...	..G
Ripitortus pedestris	...	..G	...	...	G..	...	..G	...	..G	..AG	G..	..T	..A	...	..A	C.G	...	..T	..T	..T	..C	...	..C	...	...
Modiolus americanus	...	...	...	..CT	AA.	...	..C	...	..C	...	G..	..C	...	G..	..A	...	..G	..A	..T	..T	...	...	G..	...	...
Bombus terrestris	...	...	..A	CT	G.G	...	...	..A	...	G..	A.T	..T	..G	...	..A	...	..G	..A	..A	..T	...	...	..T	..A	...
Acyrthosiphon pisum	...	..C	...	CC	AA.	...	..C	...	...	G.A	..T	...	..A	...	..C	...	..C	..C	...	..C	..C	...	..C	...	...
Tubulanus polymorphus	..A	..G	..T	AAC	...	...	..C	..TG	...	A..	A.A	...	...	AC.	...	..G	A.C	...	G..	..G	..A	...	G.C	..A	..T

Species																					
Chironomus tepperi	GGT	TTT	GAT	T--	AAA	ACA	TGT	AGT	ATG	GTC	GTC	AAT	GTT	GAC	CAA	AAA	CCA	AAT	ATT	GCA	GTG
Chironomus riparius	...	...	...	AT	AAA	ACA	TGT	AGT	GTT	ATG	GTC	AAT	..A	...	..A	AGC	CCA	AAT	..T	..C	...
Drosophila melanogaster	...	...	..C	GG	AAG	ACA	CTT	AGT	CTT	GTA	GTC	ATC	...	GAC	..A	TCG	CCC	AAC	..T	..T	..C
Ripitortus pedestris	...	...	...	AC	CGG	ACT	TGT	AGT	GTA	CTG	GTA	GCA	..A	GAG	CAG	AGC	CCA	GAT	ATC	..T	..C
Modiolus americanus	...	..A	...	AC	AAA	ACA	TGC	AAT	TTG	GTA	ATT	GCT	..G	GAA	CAA	AGT	CCA	GAC	ATT	..A	G..
Bombus terrestris	...	...	..C	TC	AAG	CTT	TGC	AGT	CTG	GTG	CTT	GCG	..A	GCC	CCA	TCA	CCA	AAT	..T	..G	..T
Acyrthosiphon pisum	...	...	..C	AC	ACA	TGT	TGC	TCG	ATG	GTG	CTT	GCT	..C	GAT	CCA	TCT	CCA	AAT	..C	..C	..T
Tubulanus polymorphus	...	..T	...	AC	AAG	TCC	TGT	AAT	TCG	GTC	CTA	GTA	G..	GCC	GAA	AGT	CCC	GAT	..T	GCA	..C

S-adenosylhomocysteine hydrolase

The page contains a multiple nucleotide sequence alignment of the *S-adenosylhomocysteine hydrolase* gene across the following species, with dots indicating identity to the reference sequence and conserved/variable codon positions highlighted:

- *Chironomus tepperi*
- *Chironomus*
- *Drosophila melanogaster*
- *Ceratitis capitata*
- *Leptinotarsa decemlineata*
- *Apis florea*
- *Bombus impatiens*

Cystathionine-beta-synthase

Chironomus tepperi
Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Culex quinquefasciatu
Aedes aegypti

Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Culex quinquefasciatu
Aedes aegypti

Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Culex quinquefasciatu
Aedes aegypti

Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Culex quinquefasciatu
Aedes aegypti

Cystathionine-γ-lyase

Chironomus tepperi
Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Meleagris gallopavo
Anolis carolinensis

Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Meleagris gallopavo
Anolis carolinensis

Chironomus tepperi
Chironomus riparius

γ-Glutamylcysteine synthase

Drosophila melanogaster
Meleagris gallopavo
Anolis carolinensis

Chironomus tepperi
Chironomus riparius
Drosophila melanogaster
Meleagris gallopavo
Anolis carolinensis

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius

Chironomus tepperi
Chironomus duplex
Chironomus riparius

Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Chironomus tepperi
Chironomus duplex
Chironomus riparius
Aedes aegypti
Drosophila melanogaster

Glutathione synthetase

Chironomus tepperi
Chironomus duplex
Drosophila melanogaster
Drosophila mojavensis
Bombus terrestris
Apis florea

Chironomus tepperi
Chironomus duplex
Drosophila melanogaster
Drosophila mojavensis
Bombus terrestris
Apis florea

Chironomus tepperi
Chironomus duplex
Drosophila melanogaster
Drosophila mojavensis
Bombus terrestris
Apis florea

Chironomus tepperi
Chironomus duplex
Drosophila melanogaster
Drosophila mojavensis
Bombus terrestris
Apis florea

Metallothionein

Chironomus tepperi
Chironomus tentans
Chironomus riparius
Drosophila melanogaster
Chironomus tepperi
Chironomus tentans
Chironomus riparius
Drosophila melanogaster

Glutathione S-transferase delta 1

Chironomus tepperi
Chironomus tentans
Ceratitis capitata
Episyrphus balteatus
Drosophila melanogaster
Chironomus tepperi
Chironomus tentans
Ceratitis capitata
Episyrphus balteatus
Drosophila melanogaster
Chironomus tepperi
Chironomus tentans
Ceratitis capitata
Episyrphus balteatus
Drosophila melanogaster

Supplementary file 4 Amino acid alignments of cysteine metabolism genes in *C. tepperi*

S-adenosylhomocysteine hydrolase

```
Chironomus tepperi          ?MALRKRASDDKPLKNAKIVGCTHVNAQTAVLIETLAALGASVRWAACNIYSTQNEVAAALAESGFSVFAWRGETEEDFWWCIDKCVNAENWQPNMILLDDGGDA?
Chironomus riparius          I...........................................................................................................
Drosophila melanogaster      II..K..AE.....D.........I........VE.....................IPI.......R..............................T
Ceratitis capitata           I....A....D......I........VE...........................ADIPI.......R....S.....................T
Leptinotarsa decemlineata    I................I...................L..............A..................D................T
Apis florea                  I......AE.............I...........VH..Q...............HA.YPI........A....................T
Bombus impatiens             I.....A.................I...........VH..Q...............HA.YPI.......A...................T
```

S-adenosylmethionine synthetase

```
Chironomus tepperi           ?QDPNAKVACETVSKTGMILLCGEITSKGFVDYQRIVRDTVKHIGYDDSSKGFD?
Chironomus riparius          R.....................................E....E.........Y
Drosophila melanogaster      K.................A............AV...KV..E..Q..........Y
Riptortus pedestris          R...D.........T...........AV...KV..E..N..............Y
Modiolus americanus          K..........L.....VA.......AN...TVI....Q........A....Y
Bombus terrestris            K..D..........T..........AV......K.................F
Acyrthosiphon pisum          K...D........T..........AN......V...I.............Y
Tubulanus polymorphus        K.....M....ST.....V.......NAN...KVI...I..........AR....Y
```

Cystathionine-beta-synthase

```
Chironomus tepperi           ?GLAMACAVRGYRCIIVLPEKNSDEKVNVLKALGAEIIRTRTEARFDEPDSLVAVAQRLQKEIPNSVIINQYTNSGNPLAH
Chironomus riparius          I...................................................A............................
Drosophila melanogaster      I.....K..K.....M...M.N...SA.RT...K....P..AY.S.EG.IY...Q..R.T..IV.D..R.A.......
Culex quinquefasciatu        I...LGA..K..K.V.M...M.N...DT.Q....V..PN..S..S.EG.I.....R.R..A...............
Aedes aegypti                I...LGA..K..K.V..M...M.N...DT.Q.......V..PN..S..H.EG.I.........R.....A.........
```

Cystathionine-γ-lyase

```
Chironomus tepperi           A QTAIISTLKTGDVIITGDDVYGGTNRLFRNLAVNMGMEVIFVDMTDVSNLEKAMKDNVKLVWLETPTN?
Chironomus riparius          ...................................................R.....E...............P
Drosophila melanogaster      .T..VLTM.SS..H..M.........I.QV.TRL.ISAT...P.KLDLIKSSI.PET.....I.S....P
Meleagris gallopavo          .TLN.THL..A..T..CM.........Y.QQI..K..LK....C.KLKC..A.ITPET....I......P
Anolis carolinensis          .TLN.AHL..A..N.VCT.........Y.SKM.SD..LKTV...C.KLES.DA.ITPQT....I......P
```

γ-Glutamylcysteine synthase

```
Chironomus tepperi           HVYMDAMGFGMGNCCLQLTFQACNINEARYLYDQLTPLCPIMLAFTAASPIYRGYLITDIDCRNNVISASVDCRTMEERGLAPLIKENQFRINKSRYDS1DSVLSENGEKYNDVPLLYNEEDYEKLRKGGID
Chironomus riparius          .........................................................................L.....................................................
Drosophila melanogaster      V.LL.........C.........T...R....A....L.........................A....K..D.K.............DQQK...A.........PE.A......T.D.KV.QR.VE....
Chironomus duplex            ------?.............................................................S..........K...........................K...E.....
Aedes aegypti                .H.........C.............T..T...M....L.......A...........V.........K...EV..NDR...Y..............PA.......VLD.KL.NR..E.N..
```

Glutathione synthetase

Chironomus tepperi	?KCPSIHYHLAGTKKKVQQALAKPGILKRFLTDEDEITRVKEIFTGLYSLDKEEGGDKVVEMVLKNPEG*
Chironomus duplex	I..........................R.........K..E...................D.........Y
Drosophila melanogaster	I..................Q.AV.E..IN.PE..KA.GK............DN.A.NASY..A.RT..RF
Drosophila mojavensis	I..........Q.........Q.AV.E..IN.PE.MKA.GK.........M.DN.V.NANY..AMRT..KF
Bombus terrestris	I......Q..........S..Y.NV.NVL.K.DNT.KQIQ.V.A.....EFNNEAEVIIDNAMSD.KKY
Apis florea	I..................S..Y.NA.NI..K.DNK.KQIQ.VYA.....EFNNEAEEIINNAIS..KKY

Metallothionein

Chironomus tepperi	LGCKCCSQNCTCKDQSCGQGCQCAQGCKCPCVTGSKDNCCKNCC?
Chironomus riparius	M.........................KE..............K
Chironomus tentans	M.......................K...D......N.........K
Drosophila melanogaster	MV..G.GT..K.Q.TK..DN.A.N.D....V.KN.P...Q...SK*-

Glutathione S-transferase delta 1

Chironomus tepperi	?YPQLFAKAPADPEKFKAMETAMGFFNSFLEKSKYAAGDSLTVADISLVATVSSYDA
Chironomus tentans	Y...................L..T...G.........................
Ceratitis capitata	Y....V.........MY.KI.FE.L.T..DG...V...QV....HAIL.SI.TF.V
Episyphus balteatus	Y...I.............K..E.F..LDI..NGQQ....E.......A.....T.EV
Drosophila melanogaster	Y...V...........A..KI.A.FE.L.T...GQD..............A.......TFEV

APPENDIX 2: ADDITIONAL PUBLICATION

This is a paper I contributed to during my candidature.

I helped conduct the experiments and measured gene expression in larvae for comparison with metabolomic profiles investigated by Sara Long and Metabolomics Australia.
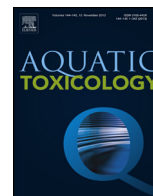
A multi-platform metabolomics approach demonstrates changes in energy metabolism and the transsulfuration pathway in *Chironomus tepperi* following exposure to zinc

Sara M. Long [a],[*], Dedreia L. Tull [b], Katherine J. Jeppe [a],[c], David P. De Souza [b], Saravanan Dayalan [b], Vincent J. Pettigrove [c], Malcolm J. McConville [b], Ary A. Hoffmann [a],[d]

[a] *Centre for Aquatic Pollution, Identification and Management (CAPIM), School of BioSciences, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, 3052, Australia*
[b] *Metabolomics Australia, Bio21 Molecular Science and Biotechnology Institute, 30 Flemington Road, Parkville, 3052, Australia*
[c] *Centre for Aquatic Pollution, Identification and Management (CAPIM), School of BioSciences, The University of Melbourne, 3010, Australia*
[d] *School of BioSciences, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, 3052, Australia*

ABSTRACT

Measuring biological responses in resident biota is a commonly used approach to monitoring polluted habitats. The challenge is to choose sensitive and, ideally, stressor-specific endpoints that reflect the responses of the ecosystem. Metabolomics is a potentially useful approach for identifying sensitive and consistent responses since it provides a holistic view to understanding the effects of exposure to chemicals upon the physiological functioning of organisms. In this study, we exposed the aquatic non-biting midge, *Chironomus tepperi*, to two concentrations of zinc chloride and measured global changes in polar metabolite levels using an untargeted gas chromatography–mass spectrometry (GC–MS) analysis and a targeted liquid chromatography–mass spectrometry (LC–MS) analysis of amine-containing metabolites. These data were correlated with changes in the expression of a number of target genes. Zinc exposure resulted in a reduction in levels of intermediates in carbohydrate metabolism (*i.e.*, glucose 6-phosphate, fructose 6-phosphate and disaccharides) and an increase in a number of TCA cycle intermediates. Zinc exposure also resulted in decreases in concentrations of the amine containing metabolites, lanthionine, methionine and cystathionine, and an increase in metallothionein gene expression. Methionine and cystathionine are intermediates in the transsulfuration pathway which is involved in the conversion of methionine to cysteine. These responses provide an understanding of the pathways affected by zinc toxicity, and how these effects are different to other heavy metals such as cadmium and copper. The use of complementary metabolomics analytical approaches was particularly useful for understanding the effects of zinc exposure and importantly, identified a suite of candidate biomarkers of zinc exposure useful for the development of biomonitoring programs.

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1. Introduction

Polluted habitats are commonly monitored by measuring a biological response that is sensitive to particular pollutants and representative of other biological responses in the system. While this is a cost-effective approach, as all components of the system do not need to be measured, the identification of a suitable read-out that is both sensitive and ecologically relevant can be challenging (Hankard et al., 2004). Biomarkers are biological responses measured at the level of an individual organism and have been used widely as indicators of pollution exposure that affects a range of species (Di Guilio et al., 1989; Depledge and Fossi, 1994; Brown et al., 2004). Biomarkers can be early warning responses that demonstrate the effects of pollutants on an individual before significant ecosystem impairment occurs.

Well-established biochemical biomarker analyses tend to focus on a particular endpoint where prior knowledge of the mechanism of toxicity (or mode of action) is known. While this is useful in demonstrating exposure to the chemical, it is somewhat limiting

* Corresponding author. Tel.: +61 3 8344 2522; fax: +61 3 8344 2279.
E-mail addresses: hoskins@unimelb.edu.au (S.M. Long), dedreia@unimelb.edu.au (D.L. Tull), k.jeppe@unimelb.edu.au (K.J. Jeppe), desouzad@unimelb.edu.au (D.P. De Souza), sdayalan@unimelb.edu.au (S. Dayalan), vpet@unimelb.edu.au (V.J. Pettigrove), malcolmm@unimelb.edu.au (M.J. McConville), ary@unimelb.edu.au (A.A. Hoffmann).

in that effects on other biochemical pathways and physiological systems may be overlooked. An alternative approach is to use metabolomics. Metabolomics refers to the analysis of low molecular weight metabolites (typically <1500 Da) within a cell, tissue or organism (Bundy et al., 2009) which provides a holistic view of changes underpinning physiological processes affected by a range of conditions including disease (Xia et al., 2013) and exposure to chemicals (Poynton et al., 2011; Jones et al., 2013). Non targeted metabolomic approaches can be used to discover new components and responses in biological systems (Garcia-Reyero and Perkins, 2011) and are readily applicable to non-model organisms (Bundy et al., 2008) as the majority of metabolites are conserved across species (Jones et al., 2013).

Environmental metabolomics characterizes the interactions of living organisms with their environments (Viant, 2007; Jones et al., 2013). Recently, studies have demonstrated that metabolomics approaches can be used to develop a metabolic signature for predicting whole organism toxicity (Hines et al., 2010) and also identifying small metabolite biomarkers of pollutant exposure in aquatic organisms (Taylor et al., 2009, 2010). The predictive nature of metabolomics in biomarker research could have far-reaching applications for monitoring the impacts of stressors on environmental health. Hailemariam et al. (2014) identified three plasma metabolite biomarkers in dairy cows that together were predictive of a diseased state up to 4 weeks prior to clinical symptoms being observed. This approach could be developed for biomonitoring assessments so that changes in a specific suite of metabolites in resident biota (irrespective of taxonomic class) could predict the presence of an individual chemical or class of chemicals prior to effects being seen at higher levels of biological organization. The first step in such a process is to identify candidate metabolite biomarkers that respond to specific chemical stressors.

Chironomids are a species-rich (over 15,000 species worldwide) family of non-biting midges of the Dipteran order (Armitage et al., 1995). They are widespread and abundant in a variety of habitats in waterways globally, and have a variety of roles within an ecosystem (Armitage et al., 1995), including as prey to many invertebrate and vertebrate predators. A chironomid has four life stages: egg, four larval stages (instars – the latter three instars are benthic in nature and so are in direct contact with pollutants that adsorb to the sediment and are also in the pore water), a pupal stage and finally an adult stage capable of flight. Chironomids are used widely in biomonitoring studies worldwide, for example they are one of the macroinvertebrate families used in the assessment of river condition (Armitage et al., 1983; Chessman, 1995) and are also used in standard sediment toxicity tests (OECD, 2004). As a group, chironomids are considered relatively pollution-tolerant. However, within the family there is a range of pollution tolerance levels (Wright and Burgin, 2009; Carew et al., 2011). *Chironomus tepperi* (Skuse) is an Australian chironomid species that is easy to culture in the laboratory (Stevens, 1993) and has a relatively short life cycle (between 17 and 24 days). This species have been used in a number of recent ecotoxicological studies investigating the effects of stressors and chemicals (Stevens et al., 2005; Choung et al., 2010; Heckmann et al., 2011; Townsend et al., 2012; Jeppe et al., 2014).

Zinc is a heavy metal found in waterways in Australia (Marshall et al., 2010; Kellar et al., 2014) and worldwide (Townsend, personal communication). Its presence in water bodies at elevated concentrations is known to have adverse effects on resident biota (Pettigrove and Hoffmann, 2005). Concentrations of zinc detected in urban waterways in Melbourne, Australia, typically range between 0.111 and 3.41 mg/L in industrialized areas (CAPIM, unpublished data). Laboratory studies showed that exposure to zinc at sub-lethal concentrations resulted in detrimental effects on survival, growth and emergence of *C. tepperi* larvae (Engelstad, 2011). Zinc is a physiologically important metal, involved in protein folding and regulation of gene expression (Klaassen et al., 1999); however, when present at elevated concentrations in an organism it can trigger toxic responses including a reduction in growth, oxidative stress responses and DNA repair (Tang et al., 2015).

Previous studies suggested the transsulfuration pathway is affected by metal exposure in a range of organisms from the nematode *Caenorhabditis elegans* (Hughes et al., 2009) to the laboratory rat (Sugiura et al., 2005) and also, more recently, in *C. tepperi* larvae (Jeppe et al., 2014). The transsulfuration pathway is involved in the conversion of the essential amino acid methionine to cysteine, *via* cystathionine. Cysteine plays a role in protecting organisms against metal and oxidative stress as it is incorporated into metallothioniens (MT) (metals such as cadmium, copper and zinc bind to the disulfide bonds of cysteine within the MT) and also can be converted to glutathione. Glutathione is considered the most important non-protein thiol in living systems (Kretzschmar and Klinger, 1990). Hughes et al. (2009) observed a decrease in cystathionine in *C. elegans* exposed to cadmium and suggested that the decrease was likely when cysteine production was favored. The authors suggested that free cysteine levels may be depleted through incorporation into protein and/or conversion to glutathione during cadmium exposure, as the equilibrium will favor end products and reduce levels of cystathionine (Hughes et al., 2009). Jeppe et al. (2014) also observed changes in gene expression in enzymes involved in the production of cystathionine following exposure to copper and cadmium in *C. tepperi* larvae. As far as we are aware, there have been no published data on the effects of zinc exposure on the transsulfuration pathway in *C. tepperi*.

To understand the effects of zinc exposure in chironomids, we used for the first time a multi-platform metabolomics approach. We exposed *C. tepperi* larvae to two sub-lethal concentrations of zinc and used an untargeted GC–MS approach to identify a range of polar metabolites that changed following exposure. Following this, we conducted a targeted analysis that focused on amine-containing metabolites (using LC–MS) that included intermediates from the transsulfuration pathway.

2. Aims

The aims of this study were to develop and optimize methods for extracting chironomid metabolites for quantification using GC–MS, then to investigate the effects of zinc exposure on *C. tepperi* larvae using an integrated suite of analyses involving metabolomics, gene expression and enzyme activity assays, enabling the identification of a novel suite of candidate metabolite biomarkers of zinc exposure in this species.

3. Materials and methods

3.1. Chironomid culture

The *C. tepperi* larvae for this study originated from temporary ponds at Yanco Agricultural Institute in New South Wales (Choung et al., 2010) and from CSIRO South Australia (courtesy of H. Doan and A. Kumar, CSIRO). *C. tepperi* were cultured in plastic tanks ($25 \times 29 \times 15$ cm) and maintained at a constant temperature of $21 \pm 1\,°C$, relative humidity >60% and a 16:8 h light:dark (16:8 h L:D) photoperiod, as described elsewhere (Jeppe et al., 2014). The culture was maintained in aerated aquaria containing roughly shredded ethanol-rinsed tissue paper in artificial water made from a modified version of Martin's solution (reverse osmosis water with 0.12 mM $NaHCO_3$, 0.068 mM $CaCl_2$, 0.083 mM $MgSO_4$, 0.86 mM NaCl, 0.015 mM KH_2PO_4, 0.089 mM $MgCl_2$ and 0.1% (w/v) iron) (Martin et al., 1980). Larvae were fed ground tropical fish flakes (Tetramin®) three times per week with a ration of 0.25 g per

tank. Larvae that hatched from eggs were reared to third instar (11 days old) in aerated culture medium until the start of metal exposures.

3.2. Biomass optimization study

To determine the optimum number of chironomid larvae for metabolite measurements, increasing numbers (10, 20, 30, 40 and 60) of C. tepperi larvae were randomly sampled. Larvae (11 days old) were collected using a Pasteur pipette, rinsed in distilled water, blotted dry on tissue paper and placed into pre-weighed, pre-cooled 2 mL lysing tubes containing 1.4 mm ceramic lysing beads (Bertin Technologies, France) then snap frozen, re-weighed and stored at −80 °C until metabolite extraction. Five larvae were added at random to each tube until the appropriate number of larvae in a tube was reached (n = 3 replicates per group).

3.3. Zinc exposure

3.3.1. Chironomid exposure

C. tepperi 3rd instar larvae (11 days old) were exposed for 24 h to two concentrations of zinc chloride (2 mg/L – low or 20 mg/L – high); controls (artificial water only) were run alongside the zinc treatments. Concentrations were based on a percentage of the acute toxicity concentration (LC50) of zinc chloride to C. tepperi that had previously been determined (LC50 186 mg/L, 95% CI 157–214 – CAPIM, unpublished data) and were known to be sub-lethal, the 'low' dose was 1% LC50 and the 'high' dose was 10% LC50. Ten replicate acid-washed 600 mL glass beakers for each treatment were set up and 15 larvae were randomly placed in each beaker, with 200 mL of test solution in each. The beakers were randomly placed in an incubator with a constant temperature of 21 ± 1 °C and a 16:8 h L:D photoperiod. Neither food nor aeration was provided during the exposure period. One sheet of ethanol-rinsed tissue paper was roughly shredded and placed into each beaker as substrate. At the end of the exposure period, ten larvae from two beakers for each treatment were pooled to make one replicate, so in total there were five replicate samples (of 20 larvae per replicate) for each treatment. The remaining ten larvae from each replicate (five from each beaker) were pooled and separated into two groups, one for oxidative stress enzyme activity determinations and the other for gene expression analysis. A sample of the test water for each treatment was collected at the end of the exposure and analyzed for total zinc concentration by ICP-AES (ALS Global, Scoresby, Victoria).

3.3.2. Sample preparation

At the end of the exposure larvae were immediately rinsed in distilled water, blotted dry on tissue paper and placed into pre-weighed and pre-cooled 2 mL lysing tubes containing 1.4 mm ceramic lysing beads (Bertin Technologies, France) for metabolomics experiments, or into microcentrifuge tubes for enzyme activity and gene expression determinations, in dry ice to stop any further cellular processes occurring. Larvae were frozen within 30 s of being removed from the exposure medium and were stored at −80 °C until analysis.

3.3.3. Metabolite extraction

Metabolites were extracted using a modified Bligh–Dyer extraction method (Bligh and Dyer, 1959). Briefly, samples were pre-weighed and lysed at 6800 rpm using a Precellys bead-mill attached to a Cryolys cooling unit (Bertin Technologies, France), pre-chilled with liquid nitrogen, at −10 °C in ice-cold 200 μL methanol and 500 μL deionised–distilled water containing internal standards (140 μM $^{13}C_5$-^{15}N-Valine and 14 μM $^{13}C_6$-Sorbitol). Following homogenization, 300 μL ice-cold chloroform was added to

each tube, and the solutions were mixed thoroughly and metabolites further extracted at 50 °C for 15 min in a shaker. The tubes were then placed on ice for 15 min, followed by thorough mixing, and centrifuged at 14,000 × g at −10 °C for 15 min. The upper phase was collected into a fresh microcentrifuge tube and aliquots taken for GC–MS and LC–MS analysis.

3.3.4. GC–MS analysis and data processing

Twenty microlitres of the upper phase containing polar metabolites were transferred into a GC–MS microvial insert, evaporated to dryness in vacuo and washed with 50 μL methanol and then dried again in vacuo to ensure absolute dryness. The samples were derivatized online using a Gerstel MPS2 XL autosampler robot (Mülheim an der Ruhr, Germany). Each sample was treated with methoxyamine (Sigma–Aldrich, St. Louis, MO), in pyridine (Sigma–Aldrich, St. Louis, MO), (20 μL of 30 mg/mL, w/v) and the methoximated metabolites derivatized with N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% TMCS (20 μL, Pierce Technologies, Waltham, MA). A 1 μL aliquot was injected into the GC–MS for metabolite analysis. A pooled biological quality control (PBQC) was prepared by pooling 20 μL of the upper phase from all of the chironomid larvae samples extracted, mixed thoroughly and aliquoted into 20 μL aliquots and dried down and derivatized in the same way as the samples prior to injection onto the GC–MS.

GC–MS was performed using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer (Santa Clara, CA) with a Gerstel Autosampler (MPS 2 XL). Gas chromatography was performed using a 30 m J & W Scientific VF-5ms column (plus 10 m Eziguard pre-column Agilent Technologies, Santa Clara, CA) with a 250 μm internal diameter and 0.25 μm film thickness. The injection inlet temperature was 250 °C, the GC–MS interface temperature 280 °C and the ion source temperature 250 °C. The carrier gas, helium, was used at a flow rate of 1 mL min^{-1}. The GC oven temperature started at 35 °C (held for 2 min) and the temperature was ramped by 25 °C min^{-1} to 325 °C (held for 5 min). Mass spectra were recorded at 9.19 scans/s over an m/z range of 50–600.

Agilent GC–MS chemstation .d format files were converted to NetCDF format for analysis using the Metabolomics Australia GC–MS alignment and integration software, PyMS python toolkit (O'Callaghan et al., 2012). The peaks identified by PyMS in the untargeted matrix were then quantitated by automated integration of the area under the peaks using Agilent MassHunter Quantitative analysis software. Using the MassHunter "Compounds at a Glance" feature, the peaks were manually visualized, validated and gap-filled. Any peaks that had not been correctly identified by the software were manually integrated. Following this, the data were exported as an integrated area matrix for statistical analysis.

3.3.5. LC–MS analysis and data processing

LC–MS amine analysis was performed according to Boughton et al. (2011). Briefly, 10 μL of the aqueous polar fraction was diluted 10-fold with 50% methanol in water (v/v). An aliquot (10 μL) of the diluted extract was buffered by the addition of 70 μL of borate buffer, pH 8.8 containing oxidizing and reducing agents 200 mM boric acid (Univar), 10 mM tris(2-carboxyetgyl)-phosphine (TCEP, Sigma–Aldrich), 1 mM ascorbic acid (Sigma–Aldrich), pH 8.8 (adjusted with 2 M NaOH) and 7.14 μM of $^{13}C_3$-L-alanine (Sigma–Aldrich) as the internal standard. AQC reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, 20 μL, 10 mM stock in 100% acetonitrile, SAFC Sigma–Aldrich) was added to the mixture and incubated for 10 min at 55 °C in a thermo mixer followed by centrifugation at 0 °C for AQC derivatization (Boughton et al., 2011). The supernatant was transferred to HPLC vials for LC–MS analysis.

The samples (4 μL) were injected onto a 120 SB-C18 Poroshell 2.1 × 100 mm, 2.7 micron column (Agilent Technologies) using an Agilent 1200-HPLC and analyzed using Agilent 6460 Triple Quad MS. Amine containing compounds were quantified by multiple reaction monitoring (MRM) using a fragmentor voltage range of 76–140 V, collision energy range of 9–25 V and collision gas (N_2) at 10 L/min. MRMs for most amine compounds are as described in Boughton et al. (2011). Additional MRM transitions used were 379–171 for lanthionine-AQC (M^{+1}), 275–171 for lanthionine-bis AQC (M^{+2}), 549.1–171 for lanthionine-bis AQC (M^{+1}), 393.1–171 for cystathionine AQC (M^{+1}), 282.1–171 for cystathionine bis AQC (M^{+2}) and 563.1–171 for cystathionine bis AQC (M^{+1}). LC–MS data was processed using Agilent MassHunter Quantitative software (v5).

3.3.6. Statistical analysis

Prior to statistical analysis all data were normalised to the internal standard and weight to account for differences between samples. Following this, the data were log (natural) transformed to account for the heteroscedastic nature of metabolomics data. Both multivariate and univariate analysis was performed on the integrated area matrix. A principal components analysis (PCA) was done in order to understand the global behavior of metabolites across the samples and the different groups. Following this, a univariate analysis was done to investigate which metabolites were significantly different between the two compared groups. Specifically, the Student's *t*-test was performed with alpha = 0.05. This was followed by correcting for multiple comparisons using the Benjamini–Hochberg method for controlling false discovery rate (Benjamini and Hochberg, 1995) with a false discovery threshold of 0.05.

For the GC–MS analysis, the groups control, zinc high and zinc low had 5, 4 and 5 samples, respectively. Similarly for the LC–MS analysis, each of the above groups had 5 samples each. Due to the small sample sizes, some interesting metabolites may have been ignored after BH adjustment of the *p*-value. We have considered for discussion a few such metabolites (detailed below), especially if they showed changes consistent with other related metabolites in the pathway under consideration.

3.3.7. Metabolite identification

Statistically significant metabolites were identified by spectral matching using a combination of Metabolomics Australia in-house and NIST08 GC–MS mass spectral libraries. All spectral matching was performed using Agilent GC–MS Chemstation data analysis software.

3.3.8. Oxidative stress enzyme assays

3.3.8.1. Sample preparation.
Pooled larvae were prepared for antioxidant enzyme assays following a method described elsewhere (Ballesteros et al., 2009). Briefly, larvae were homogenized in ice cold buffer 0.1 M phosphate buffer (Sigma–Aldrich), pH 6.5, 20% v/v glycerol (Sigma–Aldrich), 1 mM EDTA (Sigma–Aldrich) and 1.4 mM dithioerythritol (Sigma–Aldrich) at a ratio of 1:5 w/v, using a Mixermill, (Retsch, MM 300, Retsch GmbH, Haan, Germany) for 90 s, until a smooth homogenate was obtained. The samples were then centrifuged (Eppendorf 5804R, Eppendorf, Hamburg, Germany) at 14,000 × g for 20 min at 4 °C. Supernatants were decanted, snap frozen in liquid nitrogen and stored at −80 °C until analysis.

3.3.8.2. Enzyme assays.
All enzyme assays were carried out using a Synergy 2 microplate reader (Biotek Instruments, USA). Glutathione S-transferase (GST) activity was measured using a modified method based on published protocols (Habig et al., 1974; Booth et al., 2007) where GST activity was determined following

the conjugation of reduced glutathione (GSH) (Sigma–Aldrich) and 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma–Aldrich) at 340 nm, using a millimolar extinction coefficient of 9.6; an increase in absorbance over time was observed. The final volume in each well was 200 μL. Glutathione reductase (GR) and Glutathione peroxidase (GPx) activity was measured as described elsewhere (Ballesteros et al., 2009), and adapted for use in a microplate reader. Enzyme activity was determined following the consumption of NADPH at 340 nm, using a millimolar extinction coefficient of 6.22; a decrease in absorbance over time was observed. The final volume in each well for both assays was 300 μL. A blank was run on each plate alongside the samples; all samples and blanks were analysed in triplicate. The concentration of protein in each sample was measured using a modified method (Lowry et al., 1951). Enzyme activity is expressed as μmol/min/mg protein.

3.3.8.3. Statistical analysis.
Statistical analyses were performed using the statistical software package Minitab® (statistical software release 16; Minitab, State College, PA, USA). General linear model (GLM) and Tukey *post-hoc* tests were used to test for differences in enzyme activity between treatments. Differences between treatments in the *post-hoc* tests were considered significant if $p < 0.05$. Data were checked to ensure that they conformed to the assumption of homogeneity of variance between groups and normal distribution of residuals.

3.3.9. Gene expression

3.3.9.1. RNA extraction and reverse transcription.
Detailed methods into RNA extraction, reverse transcription, isolation and sequencing of *C. tepperi* homologues and gene expression analysis are presented elsewhere (Jeppe et al., 2014). Briefly, larvae were homogenized in liquid nitrogen and total RNA was isolated using a High Pure Tissue kit (Roche: Cat. No. 12 033 674 001), following the manufacturer's protocol for animal tissue. Extracted RNA was eluted in 50 μL RNase-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and quantified using a Nanodrop (Thermo Scientific). Only RNA samples with a 260/280 ratio between 1.9 and 2.1 were used for cDNA synthesis. Total RNA (2 μg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. No. M1705) following the manufacturer's protocol. The cDNA was then checked for gDNA contamination using a pair of ribosomal protein L11 primers that flank a 345 bp intron. The expected cDNA amplicon was 466 bp, whereas the amplification of the 811 bp product would indicate the presence of gDNA (Planello et al., 2007). Template cDNA was stored at −20 °C for later gene expression analysis.

3.3.9.2. Gene expression analysis.
Gene expression was measured using quantitative real-time PCR (qPCR), with transsulfuration gene primers previously described (Jeppe et al., 2014). The genes measured were those involved in the transsulfuration pathway and were S-adenosylmethionine synthetase (SAM), S-adenosylhomocysteine hydrolase (SAH), cystathionine-β synthase (CβS), cystathionine-γ lyase (CγL), glutamate cysteine ligase (GCL) (previously γ-glutamylcysteine synthase) and glutathione synthase (GS) as well as metallothionien (MT). Quantitative real-time PCR was performed with the Roche LightCycler® 480 (Roche Applied Science, USA) in triplicate 10 μL reactions containing the components described by Takahashi et al. (2010). The following PCR conditions were used: 10 min at 95 °C, followed by 50 cycles of 10 s at 95 °C, 15 s at 58 °C, and 15 s at 72 °C followed by a melting curve analysis from 65 °C to 95 °C to ensure a single product was formed.

3.3.9.3. Data processing.
Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann

et al., 2011). Differences in relative normalized expression of target genes between zinc exposed and unexposed samples were assessed using ANOVA after verifying normality and homogeneity of variances with Shapiro–Wilk and Levene's tests, respectively. Significance of ANOVA tests was evaluated after Dunnett's *post hoc* tests for multiple comparisons to control exposures. All normalized data was analyzed using R (version 3.0.3).

4. Results and discussion

4.1. Biomass optimization experiment

To determine the optimal number and biomass of 3rd instar *C. tepperi* larvae required for the metabolomics study, varying numbers and mass of larvae were extracted and analyzed using a targeted GC–MS approach. Third instar larvae were used in this study as this larval stage shows less variable biomarker responses compared to the more sensitive 2nd instar (Domingues et al., 2007). Additionally, 3rd instar larvae have substantially more biomass than 2nd instars, resulting in fewer individuals being required for analysis. These trials were directed at achieving detection of the highest number of metabolites, whilst also ensuring that detector saturation with more abundant metabolites did not occur. Moreover, it is essential to ensure the optimum biomass is used as the data are used for metabolite normalization prior to analysis (Dieterle et al., 2006). There was a linear association (R^2 of 0.96 – Fig 1a) between chironomid number and weights of the pooled larvae between the three replicates. Over 100 metabolite peaks were detected, of which 30 were identified based on their diagnostic mass spectrum and GC retention time relative to defined standards.

Saturation in metabolite responses was not observed for any of these peaks when increasing numbers of larvae were pooled, up to and including 30 larvae (Fig 1b). Additionally, there was good correlation between larvae number and metabolite concentration. For example Pearson's correlation coefficients for alanine, isoleucine, succinate and phenylalanine were 0.77, 0.92, 0.95 and 0.90, respectively, for 10 through to 60 larvae. Based on these trials, optimal results were obtained using twenty 3rd instar chironomid larvae for each replicate providing low variability in sample weight and good detection and sensitivity of a range of polar metabolites in the GC–MS analyses.

4.2. Zinc treatment

Water quality measurements were taken at the end of the exposure period; pH ranged between 5.8 and 6.5, EC ranged between 207 and 259 μS/cm, and dissolved oxygen ranged between 69 and 79% saturation. The water quality parameters are within the guidelines set by the OECD (2004) for chironomid toxicity tests, and therefore, the differences observed in the present study are not likely to adversely impact the metabolism of chironomids. Total hardness, alkalinity and dissolved major cations (calcium, magnesium, sodium and potassium) did not differ between treatments. The measured concentrations were within 10% of the nominal concentrations and control concentrations of Zn were below the limit of detection (0.01 mg/L). There was no mortality in any of the treatments and there was no difference in the weights of the larvae at termination of the exposure (mean (SD) weights (mg) of 5 replicates of 20 larvae were 136.5 (6.9), 138.74 (11.25) and 135.02 (20.18) for control, zinc low and zinc high, respectively), indicating there
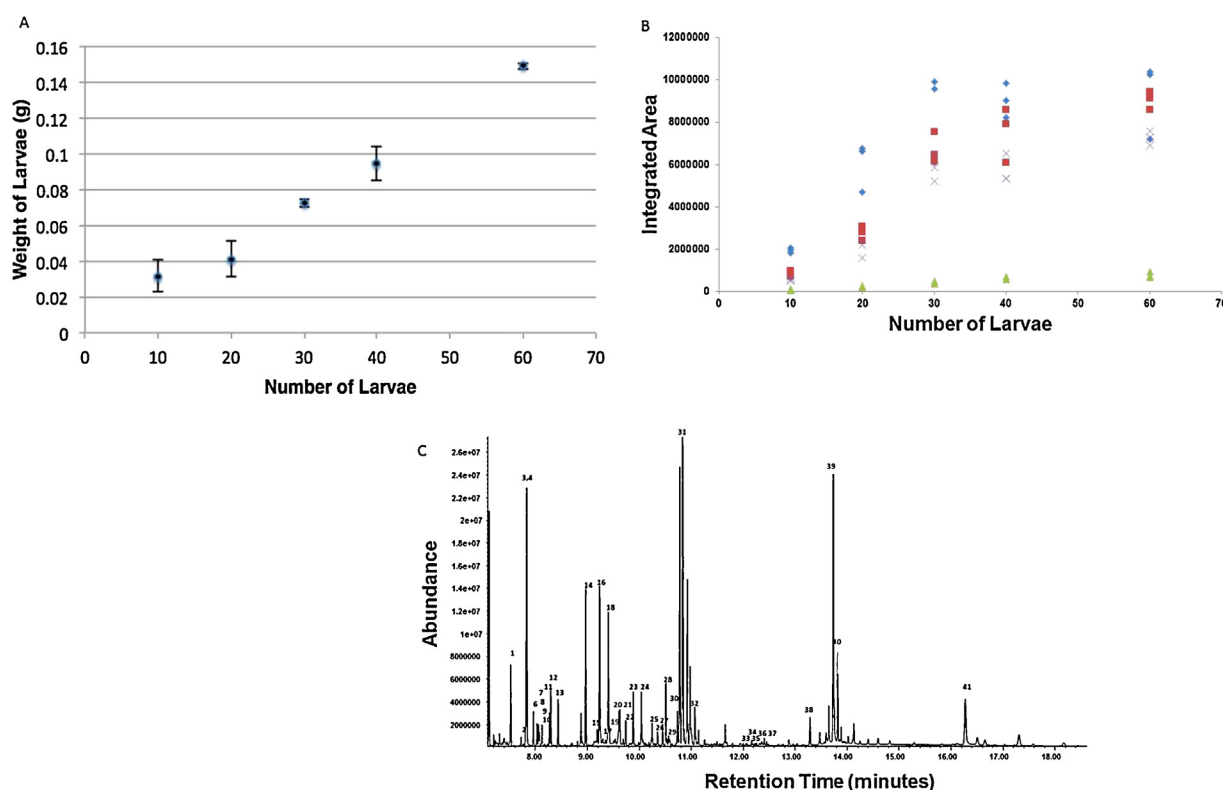

Fig. 1. Scatter plot showing the distribution of weights in relation to number of third instar *Chironomus tepperi* larvae, error bars represent standard deviation (a). Graph showing changes in metabolite concentration against number of third instar (11-day-old) *Chironomus tepperi* larvae using GC–MS targeted analysis; $n = 3$ replicates per treatment. (♦) alanine, (■) isoleucine, (▲) succinate, X phenylalanine (b). A sample GC–MS chromatogram of a control 11-day-old *Chironomus tepperi* larvae. 1: valine, 2: urea, 3: serine, 4: glycerol, 5: leucine, 6: isoleucine, 7: proline, 8: glycine, 9: succinate, 10: glycerate, 11: fumarate, 12: serine, 13: threonine, 14: malate, 15: methionine, 16: 5-oxoproline, 17: cysteine, 18: 2-oxoglutarate, 19: α-ketoglutarate, 20: ornithine, 21: glutamate, 22: phenylalanine, 23: asparagine, 24: lysine, 25: glycerol 3-phosphate, 26: glutamine, 27: 3-phospho glycerate, 28: citrate, 29: citrulline, 30: putrescine, 31: glucose, 32: tyrosine, 33: cystathionine, 34: tryptophan, 35: spermidine, 37: fructose 6-phosphate, 38: glucose 6-phosphate, 39: inosine, 40: maltose, 41: trehalose, 42: trisaccharides (c).

were no overt signs of toxicity and that the concentrations used in the study were sub-lethal. Interestingly, the weights of larvae at the end of the exposure study were higher than in the optimisation study; this is likely a consequence of the age of the larvae at the end of the exposure study (12 days) as well as the fact that they originated from a different stock of larvae as the cultures are replenished annually to reduce inbreeding.

4.2.1. Untargeted GC–MS metabolite screen

As this was the first time a metabolomics approach has been used to investigate the effects of zinc exposure upon *C. tepperi*, we began with an initial broad-screen untargeted approach using GC–MS. This approach enabled a wide range of polar metabolites in central carbon metabolism to be detected that may reflect global changes in the physiological state of the chironomids following exposure to zinc. We used an untargeted analysis, as we did not want to exclude any insect/chironomid-specific metabolites. This was followed by a targeted analysis, using LC–MS, which focused on detecting a specific class of compounds as previous studies had demonstrated amine-containing compounds in the transsulfuration pathway being affected by metal exposure.

To assess metabolic changes due to Zn exposure, chironomids were subjected to an untargeted analysis of polar metabolites using GC–MS. A typical GC–MS chromatogram of *C. tepperi* larvae is shown in Fig. 1c. The ~300 peaks detected in these analyses were aligned using the PyMS tool and specific metabolites identified based on their mass spectrum and GC-retention times. A greater number of peaks were identified in the second study compared to the optimization study as a consequence of improved alignment, identification and quantification software that had become available for the second study. Identified polar metabolites included many amino acids (such as phenylalanine and alanine), other amine-containing compounds (such as lanthionine and cystathionine), mono- and di-saccharides (including trehalose and maltose), glycolytic intermediates (such as glucose 6 - phosphate) and tricarboxylic acid (TCA) cycle intermediates (such as succinate). Additional sugar isomers were provisionally identified based on their characteristic mass spectra and retention time, while a number of other unidentified compounds were not matched to any of our MS libraries, including the National Institute of Standards and Technology (NIST) database and may be dipteran or chironomid-specific metabolites.

4.2.1.1. Changes in polar metabolites following zinc treatment.
To obtain an overview of the effects on chironomids when treated with and without zinc, including at high and low zinc concentrations, a multivariate analysis using principal component analysis (PCA) of the GC–MS data matrix with the ~300 metabolic features was performed. PCA showed that there was separation of the zinc high treatment group from the zinc low and control treatment groups (Fig. 2a), predominantly along PC2.

4.2.1.2. Effects on energy metabolism.
Following multivariate analyses, pairwise comparisons were carried out to identify metabolites that changed significantly between treatments. A total of 30 metabolites were significantly different (with BH-adjusted p values <0.05) in abundance between treatments (control *vs.* zinc low and control *vs.* zinc high – see Table 1a for list of putatively identified metabolites that were significantly different to the controls); there were no metabolites that differed between zinc low and zinc high treatments. We detected changes in metabolites involved in energy metabolism, *i.e.*, glycolysis and the TCA cycle, as well as changes in abundance of unidentified disaccharides and sugars following zinc treatment. There was a decrease in abundance of hexose-phosphates that play a role in multiple pathways including glycolysis, the pentose phosphate pathway and glycogen/trehalose

metabolism. Glucose 6-phosphate (G6P-BH-adjusted p value 0.001 and 0.008, for zinc high and low, respectively) and fructose 6-phosphate (F6P-BH adjusted p value 0.003 and 0.037, for zinc high and low, respectively) had reduced abundance following zinc exposure, irrespective of concentration, and a number of unidentified disaccharides which, when metabolised, can be used as an alternative source of glucose in glycolysis, were also present in lower concentrations following zinc exposure. Interestingly, we observed significant increases in abundance of the TCA cycle intermediates citrate/isocitrate (we could not distinguish citrate from isocitrate as they elute at the same retention time and have a similar mass ion profile – therefore, we have grouped the two metabolites together) (BH-adjusted p value 0.008 and 0.004, for zinc high and low, respectively), and the metabolites malate, fumarate and α ketoglutarate were considered 'trending' toward being increased in abundance compared to controls (Table 1a), along with some unknown metabolites. Additionally, there were significant increases in a small number of metabolites following zinc low exposure compared to controls which included phosphorylated sugars and deoxyhexose sugars. Overall, the GC–MS results indicated zinc exposure resulted in changes in carbohydrate and energy metabolism in *C. tepperi* larvae.

The present study found that there were decreases in some metabolites involved in glycolysis and increases in a number of TCA cycle intermediates, which may be indicative of stimulation of glycolysis and inhibition of the TCA cycle following zinc exposure. As we used steady state metabolomics and so only have a snap-shot of the metabolite concentrations at one time point, we are not able to determine whether changes in metabolite abundance is due to inhibition or activation of the enzyme that catalyses the conversion of the metabolite (and hence an accumulation or depletion of the metabolite) or whether there is increased demand for the metabolites in the pathway. One approach to examine these metabolic changes is to carry out metabolic flux studies using ^{13}C/^{15}N-labelled substrates to understand the mechanisms underlying this process (Saunders et al., 2015).

In general, the basic steps of energy metabolism in insects are no different to that in most other organisms (Klowden, 2007). The conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (FBP) by phosphofructokinase (PFK) is the primary step at which glycolysis is regulated (Mathews et al., 2000); moreover, the activity of PFK is sensitive to the energy status of the cell and also to the levels of intermediates from other metabolic pathways, including citrate (Mathews et al., 2000). Isocitrate dehydrogenase (ICDH) is a control point for the TCA cycle in insects (Nation, 2008) and is involved in the conversion of isocitrate to α-ketoglutarate. It is inhibited by high concentrations of ATP and stimulated by isocitrate, ADP and inorganic phosphate. We hypothesise that the decrease in the early phases of glycolysis observed in the present study may be a result of activation of PFK following zinc exposure due to either zinc directly interacting with PFK or as an indirect effect of reduced ATP concentrations from the inhibition of the TCA cycle. A decrease in ATP results in activation of PFK as there is a demand for energy and, as glycolysis is the first step in energy metabolism by the cell, PFK is activated to produce ATP as well as to produce pyruvate for oxidation in the TCA cycle. Zinc is known to inhibit ICDH in cultured hepatocytes and may interfere with a number of key enzymes in the TCA cycle, resulting in changes in ATP production (Lemire et al., 2008).

We identified a number of disaccharides in the GC–MS analyses that had distinct GC-retention times and mass spectra from commonly reported disaccharides (*i.e.*, trehalose, maltose or sucrose) and were decreased following Zn exposure. We also identified trehalose, which is commonly the major low molecular weight carbohydrate reserve in haemolymph of insects (Bischof, 1995; Bentivegna, 2002; Klowden, 2007). However, this disaccharide was
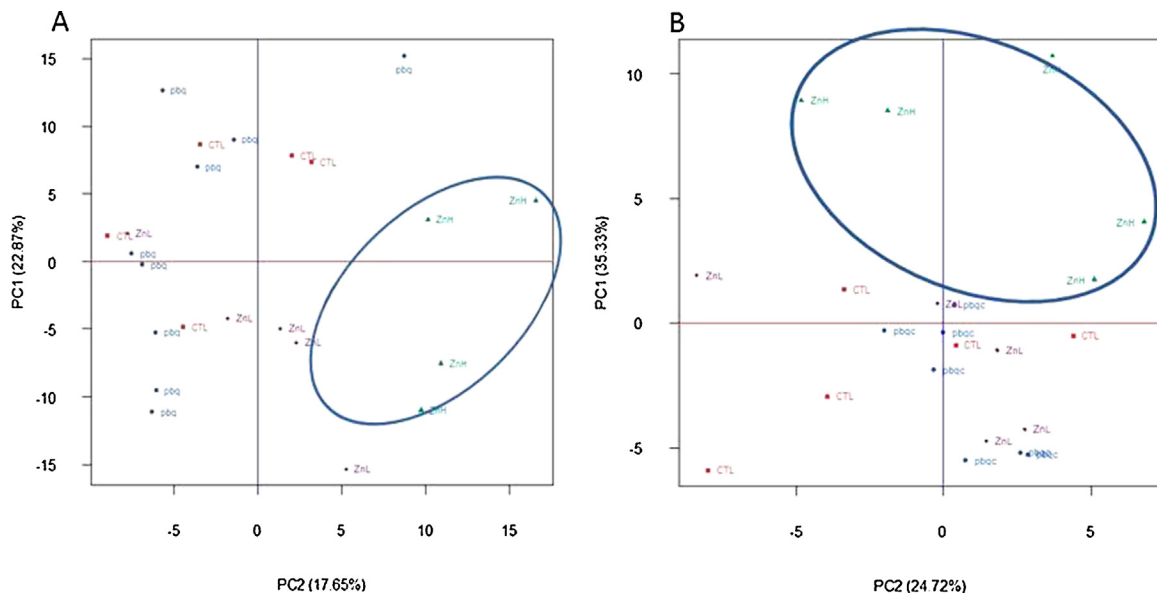
Fig. 2. Principal components analysis scores plots of 299 metabolites measured using GC–MS (a) or 60 amine-containing metabolites measured using a targeted LC–MS approach (b) in 3rd instar *Chironomus tepperi* larvae following 24 h exposure to water only (control), low (2 mg/L) or high (20 mg/L) concentrations of zinc chloride. The high zinc exposure separates from the remaining treatments along PC2 (circled). Zinc high treatment separates along PC1 (circled). $n = 5$ replicates per treatment, except for the high zinc treatment where $n = 4$. (■) – control (CTL), (♦) – Zinc low (ZnL), (▲) – Zinc high (Zn H), (●) – pooled biological quality control (PBQC).

present at low levels in total body extracts of *C. tepperi* larvae (Fig. 1c) and exposure to zinc did not have a significant effect on the abundance of this metabolite. These analyses suggest that other oligosaccharides may be quantitatively more important in non-haemolymph tissues and also responsive to cellular stresses induced by Zn exposure. Disaccharides can be metabolized and enter the glycolytic cycle as another source of glucose for energy metabolism. If zinc exposure results in an up-regulation of gly-colysis, it follows that there would be a decrease in disaccharide abundance to meet the energy requirements of the larvae following exposure.

A number of studies have investigated the effects of metal expo-sure on energy metabolism in invertebrates (Bischof, 1995; Canesi et al., 2001; Bentivegna, 2002; Wang et al., 2010; Emre et al., 2013) and plants (Gutierrez-Carbonell et al., 2013). These stud-ies have tended to find that exposure results in changes in energy metabolism that are consistent with our findings. Interestingly, PFK activity is elevated in the digestive gland of the mussel, *Mytilus gal-loprovincialis*, when exposed to zinc (Canesi et al., 2001) which is in line with our suggestion that a reduction of F6P and G6P are a result of activation of PFK in zinc-exposed *C. tepperi* larvae. Addi-tionally, up-regulation in energy metabolism occurs as a response

to cadmium exposure in earthworms (Wang et al., 2010). Changes in energy metabolism observed in the present study may be related to the increased demand for energy due to upregulation of other pathways in response to zinc exposure, such as production of met-allothionein proteins to detoxify metals (Gillis et al., 2002). If this is the case, a consequence of long term exposure to zinc may be a reduction in growth as energy is being directed into these responses rather than growth. This may account for the decrease in growth of *C. tepperi* larvae following a 5 day exposure to concentrations of zinc similar to those used here (Engelstad, 2011).

4.2.2. Targeted LC–MS metabolite analysis

Amine containing metabolites have previously been shown to be affected by metal exposure in rats and *C. elegans* (Sugiura et al., 2005; Hughes et al., 2009). To determine whether metabolism of amine metabolites were affected by zinc exposure in chirono-mids, we analyzed zinc-treated and control samples using a highly specific and sensitive LC–MS-based targeted metabolite analysis for amine-containing compounds (Boughton et al., 2011). Approx-imately 60 amine-containing metabolites were detected in the chironomids and their identity confirmed by reference to com-mercial standards and development of appropriate MRMs, see

Table 1a

Significant putatively-identified polar metabolites detected in *Chironomus tepperi* larvae following exposure to zinc low (2 mg/L) or zinc high (20 mg/L) compared to controls (water only) for 24 h. Metabolites were considered significant if the BH adjusted p value was <0.05. Metabolites were considered trending toward significance if BH adjusted p value was <0.19 but >0.05 and p value was < 0.05.

Metabolite	Treatment	t-Statistic	p value	BH-adjusted p value	Fold change
Glucose 6-phosphate	Zn High	−14.32	<0.001	0.001	−2.45
Fructose 6-phosphate	"	−8.20	<0.001	0.003	−1.55
Citrate/isocitrate	"	7.20	<0.001	0.008	0.51
α Ketoglutarate	"	3.33	0.021	0.112	0.44
Fumarate	"	2.54	0.040	0.160	0.18
Malate	"	2.40	0.048	0.181	0.09
Citrate/isocitrate	Zn Low	7.40	<0.001	0.004	0.50
Glucose 6-phosphate	"	−6.86	<0.001	0.008	−2.07
Fructose 6-phosphate	"	−4.55	0.003	0.037	−1.18
Glutamate	"	3.50	0.008	0.083	0.13
Malate	"	3.18	0.014	0.121	0.12

Table 1b

Significant amine-containing metabolites detected in *Chironomus tepperi* larvae following exposure to zinc low (2 mg/L) or zinc high (20 mg/L) compared to controls (water only) for 24 h. Metabolites were considered significant if the BH adjusted *p* value was <0.05. Metabolites were considered trending toward significance if BH adjusted *p* value was <0.08 but >0.05 and *p* value was <0.025.

Metabolite	Treatment	*t*-Statistic	*p* value	BH-adjusted *p* value	Fold change
Citrulline	Zn High	−11.91	<0.001	0.002	−1.65
Cadaverine	"	−8.67	<0.001	0.002	−2.02
Asparagine	"	−6.55	<0.001	0.006	−0.48
Lysine	"	−5.77	0.001	0.007	−0.47
Cystathionine	"	−5.63	0.001	0.007	−0.57
Glutamine	"	−5.67	0.001	0.014	−0.43
Serine	"	−3.46	0.010	0.060	−0.30
Lanthionine	"	−3.57	0.009	0.060	−0.40
Methionine	"	−3.26	0.012	0.060	−0.51
2-Aminobutyric acid	"	−3.65	0.012	0.060	−0.69
GABA	"	−3.65	0.012	0.060	−0.69
Cysteamine	"	3.17	0.015	0.064	0.48
Glycine	"	−3.07	0.015	0.064	−0.28
Cystine	"	−3.00	0.024	0.085	−0.58
Cadaverine	Zn Low	−9.37	<0.001	0.001	−1.65
Citrulline	"	−6.69	<0.001	0.005	−1.12

Supplementary Table 1 for the full list of metabolites detected in this analysis. Principal components analysis (PCA) of the amine-containing metabolites showed that there was separation between zinc high and other treatments (Fig. 2b). Similarly to the GC–MS metabolite comparisons, there were no significant differences in metabolites between the two zinc treatments. Pairwise comparisons (*t*-tests) between the controls and zinc treatments revealed that two metabolites were significantly lower in abundance in the zinc-treated larvae compared to controls – citrulline (BH-adjusted *p* values 0.002 and 0.005, for zinc high and low, respectively) and cadaverine (BH-adjusted *p* values 0.002 and 0.001 for zinc high and low, respectively – Table 1b). Additionally, asparagine, lysine, cystathionine, glutamine, serine, lanthionine, methionine, 2 aminobutyric acid, GABA, cysteamine, glycine and cystine were different in zinc high-treated larvae compared to controls (Table 1b). All of these metabolites, except cysteamine, showed lower abundance following zinc exposure compared to controls. These metabolites were also detected using GC–MS; however, they were present in low abundance, and therefore, subtle changes as a result of zinc treatment could not be determined based on the GC–MS data. The LC–MS approach that we employed in this study is targeted and highly sensitive to detecting changes in amine-containing compounds; therefore, we were able to detect subtle changes not apparent through GC–MS.

4.2.2.1. Effects on transsulfuration pathway. This study on the effects of zinc exposure on the metabolism of amine metabolites in chironomids showed that there was a decrease in the abundance of cystathionine (BH-adjusted *p* value 0.007) and methionine (BH-adjusted *p* value 0.06 – Table 1b) (Fig. 3), two of the intermediates of the transsulfuration pathway (Fig. 4), as well as lanthionine, which is also involved in the production of cysteine in eukaryotes (Shinbo, 1988a,b). Additionally, there was a trend toward a decrease in serine and glycine (BH-adjusted *p* values of 0.06 and 0.064, respectively – Table 1b), amino acids involved in the conversion of homocysteine to cystathionine (serine) and conversion of γ-glutamylcysteine to glutathione (glycine). The fact that the levels of these metabolites responded in a similar way indicates that these changes are a true reflection of the effect of zinc upon this pathway.

Our results are consistent with recent studies which reported that the transsulfuration pathway in a range of taxa is affected by metal exposure (Hughes et al., 2009; Jeppe et al., 2014). In particular, Hughes et al. (2009) found that exposure to cadmium resulted

in a decrease in cystathionine abundance in *C. elegans* which they suggested was due to direct inhibition of the enzymes regulating cystathionine concentrations in the cell, namely cystathionine β synthase (CβS) and cystathionine γ lyase (CγL). We examined the expression of the genes encoding these enzymes following zinc exposure and results showed that there was no significant difference in expression of CβS or CγL (*p* > 0.05) in treated compared to control *C. tepperi* larvae (Fig. 5); this indicates a different mechanism of toxicity because (unlike with cadmium) zinc did not inhibit these enzymes. Jeppe et al. (2014) also noted that different patterns of gene expression occurred following copper and cadmium exposure suggesting different modes of toxicity.

Results from our study suggest zinc primarily affects early steps in the transsulfuration pathway in *C. tepperi,* as opposed to the whole of the pathway as observed following cadmium and copper exposure (Jeppe et al., 2014). We did not find changes in any intermediates downstream of cystathionine (namely cysteine and glutathione). This is consistent with the gene expression results which indicated significant changes in demethylation/remethylation of methionine and homocysteine (Fig. 5) and in metallothionien (MT) upregulation following zinc exposure. Unfortunately, we were not able to detect changes in homocysteine abundance following zinc exposure as this metabolite co-eluted with another compound. Further experiments investigating metabolic flux would provide information on the flow of metabolites throughout the pathway.

Hughes et al. (2009) postulated that a decrease in the levels of cystathionine in *C. elegans* in response to cadmium exposure was likely when cysteine production was favored; however, cysteine abundance was not measured in their study. The authors suggested that free cysteine levels may be depleted through incorporation into protein and/or conversion to glutathione during cadmium exposure, as the equilibrium will favor end products and reduce levels of cystathionine (Hughes et al., 2009). Our results show that there were no differences in the abundance of cysteine and there were no significant changes in gene expression of CγL or glutamate cysteine ligase (GCL) (the enzymes that regulate its production and conversion), so it is unclear if this process was occurring. However, we found an upregulation of MT gene expression in *C. tepperi* larvae following exposure to zinc high treatment, which suggests that cysteine could be removed from the system through incorporation into MT; this has also been observed following cadmium exposure (Jeppe et al., 2014). Additionally, there were no differences in either the abundance of intermediates, or expression of genes encoding
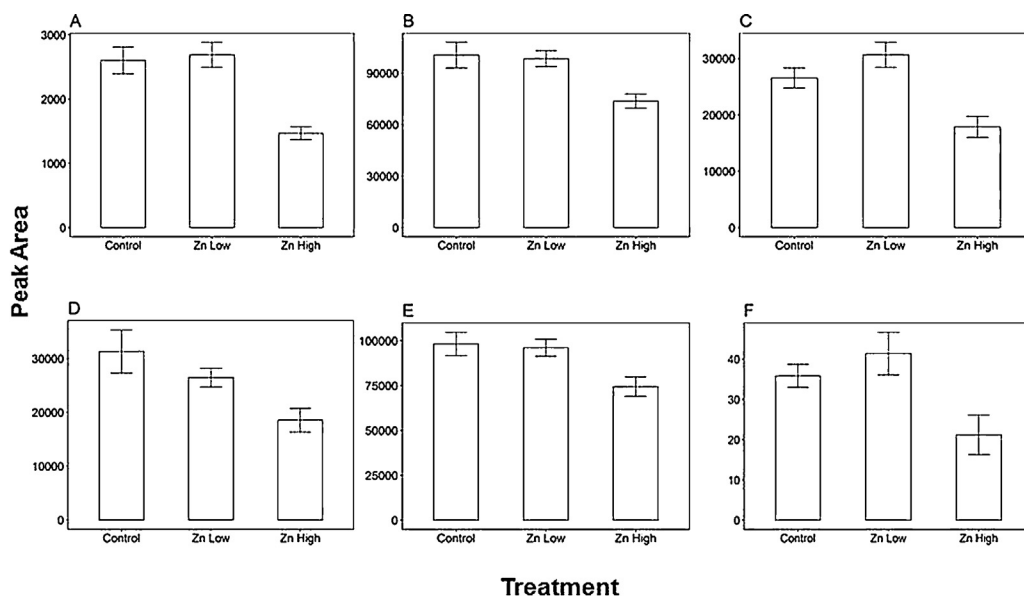
Fig. 3. Bar graphs showing mean (±SEM) peak areas (normalized to weight and internal standard) of significant and 'trending' transsulfuration pathway metabolites detected using LC–MS, in 3rd instar *Chironomus tepperi* larvae following 24 h exposure to water only (control), low (2 mg/L) or high (20 mg/L) concentrations of zinc chloride. $n = 5$ replicates per treatment, except for the high zinc treatment ($n = 4$). (A) cystathionine, (B) serine, (C) lanthionine, (D) methionine, (E) glycine and (F) cystine.

the enzymes involved in the production of the intermediates further down the pathway (*i.e.*, glutathione and γ-glutamyl cysteine). This also suggests that cysteine is being primarily incorporated into MT. Furthermore, there were no differences in glutathione-related antioxidant enzyme responses in zinc-exposed larvae compared to controls. Mean (±SD) enzyme activities (μmol/min/mg protein) were 10.12 (1.26), 9.06 (1.3) and 10.57 (1.94) for control, zinc low and zinc high GST, respectively, 0.96 (0.3), 1.31 (0.34) and 1.50 (0.43) for control, zinc low and zinc high GR, respectively and 0.32 (0.12), 0.41 (0.05) and 0.361 (0.19) for control, zinc low and zinc high GPx, respectively). This suggests that there was no additional demand for glutathione to be utilized in an antioxidant response to zinc exposure. Given the results we have on the transsulfuration pathway, we suggest that the mechanism of toxicity of zinc in *C. tepperi* is different to that of other heavy metals including copper and cadmium.

4.3. Identification of a biomarker profile unique to zinc

One of the aims of the study was to identify a suite of metabolite biomarkers which could be used as a unique fingerprint or profile of zinc exposure in chironomids. These biomarkers could be incorporated into biomonitoring programs to assess the health of biota in waterways. This study has identified that short-term exposure to zinc resulted in a reduction in abundance of the amine-containing metabolites methionine, cystathionine and lanthionine, and G6P and F6P, and increases in citrate/isocitrate, malate, fumarate and α-ketoglutarate abundance in *C. tepperi* larvae, which could prove to be a zinc-specific response. This needs to be investigated further as other studies have shown changes in energy metabolism to be indicative of a general stress response. However, we suspect that a zinc-specific response is characterized by the specific metabolite changes as detailed above. In a similar vein, Hailemariam
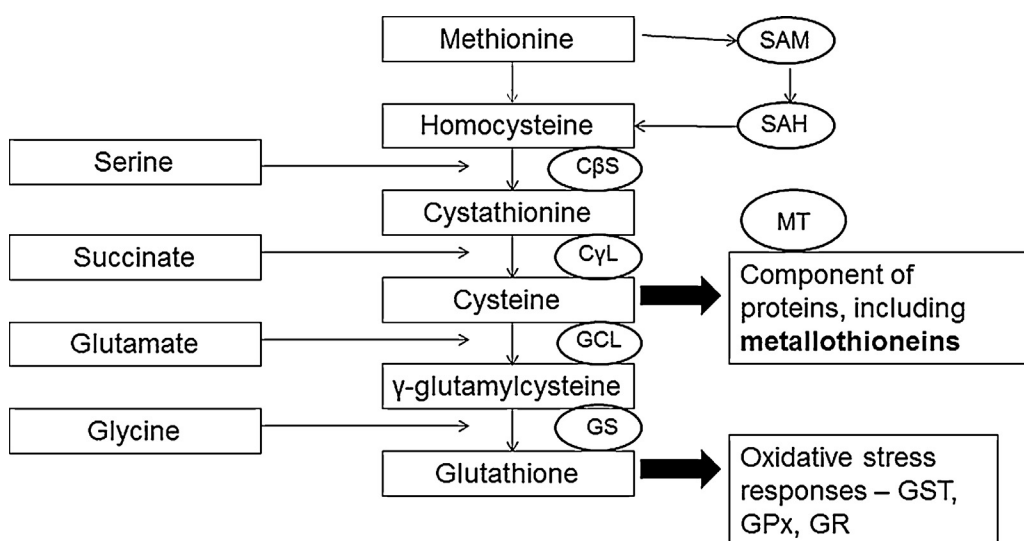

Fig. 4. Metabolites (boxes) and enzymes or proteins (measured through gene expression – circles) involved in the transsulfuration pathway that were measured in the present study. *S*-adenosyl methionine synthetase (SAM), *S*-adenosylhomocysteine hydrolase (SAH), cystathionine β synthase (CβS), cystathionine γ lyase (CγL), glutamate cysteine ligase (GCL), glutathione synthase (GS) and metallothionein (MT).
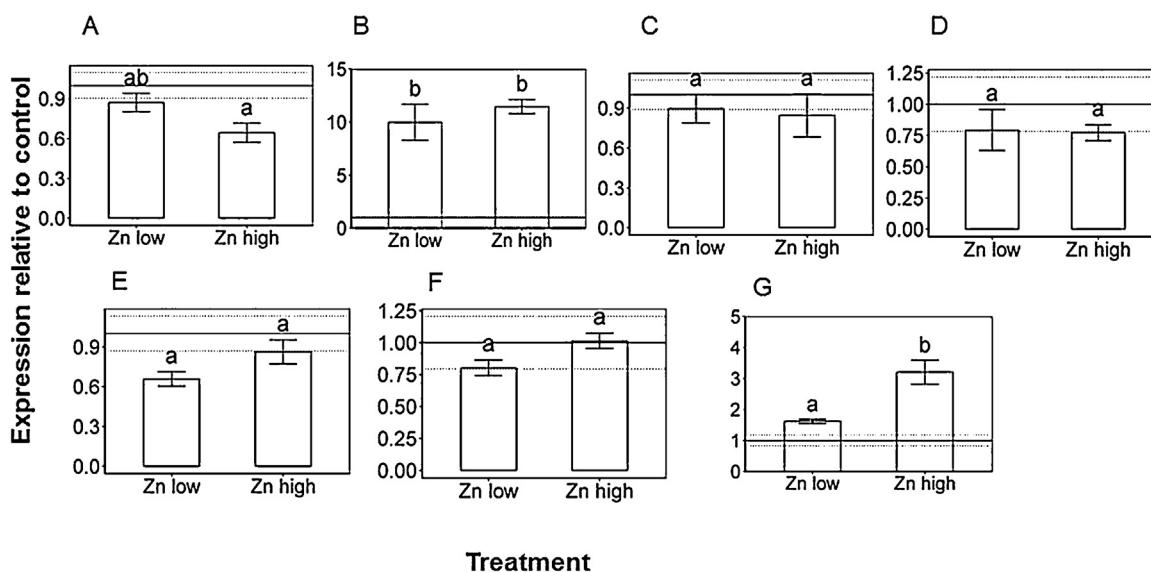
Fig. 5. Mean (±SEM) gene expression relative to controls for *S*-adenosylhomocysteine hydrolase (SAH) (a), *S*-adenosyl methionine synthetase (SAM) (b), cystathionine β synthase (CβS) (c), cystathionine γ lyase (CγL) (d), γ glutamylcysteine synthase (GCS) (also known as glutamate cysteine ligase (GCL)) (e), glutathione synthase (GS) (f) and metallothionein (MT) (g) in 3rd instar *Chironomus tepperi* larvae following 24 h exposure to low (2 mg/L) and high (20 mg/L) concentrations of zinc chloride. Control expression is the solid black line. Letters represent significant difference in expression compared to controls; letters that are different between bars represent significant differences in gene expression between treatments. $n = 5$ replicates.

et al. (2014) identified three metabolite biomarkers in dairy cows that always responded in the same way to a particular diseased state prior to clinical symptoms and suggested that these three metabolites be measured to assess whether cows are in this diseased state. By identifying a handful of metabolites that represent a specific response to zinc exposure, a metabolomics approach could eventually be tailored to provide a cost-effective and sensitive approach to biomonitoring; extraction and loading methods can then be optimized for only targeted metabolites. To explore this further, exposures involving other metals and other classes of chemical (including pesticides and industrial chemicals such as hydrocarbons) and non-chemical stressors (such as nutrient, salinity, temperature and dissolved oxygen) need to be undertaken. Furthermore, to ensure the biomarkers we have identified in this study are metal specific, exposures to chemicals with known modes of action that are not thought to act on this pathway (such as those that activate cytochrome P450 monooxygenase responses or inhibit acetylcholinesterase activity) as well as compounds that are known to affect the pathway (such as hydrogen peroxide) should be carried out to characterize the specificity of responses to different chemical classes.

4.4. Understanding the mechanism of toxicity of zinc

To further explore the mechanism underlying the effects of zinc exposure on the transsulfuration pathway and to understand the relationship of the intermediates in the pathway, detailed measurements of different metabolic fluxes should be carried out. For example, in the present study we did not observe any significant differences in glutathione abundance following zinc exposure. Glutathione is produced from other pathways as well as the transsulfuration pathway; therefore, the observations made in the present study are likely to reflect overall glutathione abundance in *C. tepperi* larvae, rather than transsulfuration pathway-specific effects.

4.5. Using a multi-platform metabolomics approach

As this was the first study to investigate the potential of metabolomics analyses to understand the effects of zinc exposure

in chironomids, we began with an initial broad-screen untargeted approach using GC–MS followed by a targeted analysis, as suggested by Booth et al. (2011). We followed our initial screening with a targeted LC–MS analysis to detect changes in amine-containing compounds and begin to understand the underlying mechanisms of zinc toxicity in *C. tepperi*. GC–MS enabled a wide range of polar metabolites in central carbon metabolism to be detected that may reflect global changes in the physiological state of the chironomids following exposure to zinc. We used an untargeted analysis, as we did not want to exclude any insect or even chironomid-specific metabolites. A number of unidentified metabolites were found to be significantly altered in abundance following zinc exposure. If these are consistently affected in subsequent experiments, identification would be worthwhile. Following the GC–MS screen, we used a targeted analysis (that we had recently developed) that was focused on detecting a specific class of compounds, as recent publications by Sugiura et al. (2005), Hughes et al. (2009) and Jeppe et al. (2014) identified a number of amine-containing intermediates in the transsulfuration pathway as being affected by metals. This approach was ideal for the first assessment of effects of zinc exposure in chironomids using metabolomics to identify a range of potential candidate biomarkers as well as providing us with information on toxicity responses. Additionally, based on an integrated approach consisting of multi-platform metabolomics, gene expression analysis and enzyme activity, we were able to develop a greater understanding of the effects of zinc on *C. tepperi* larvae.

5. Conclusions

This study highlights the usefulness of a multi-platform metabolomics approach to identifying effects of zinc exposure to *C. tepperi* larvae. We have identified a suite of candidate biomarkers that could represent a potential metabolite profile of zinc exposure in biomonitoring programs, as well as identifying the transsulfuration pathway as being affected by zinc exposure. The specificity of these small metabolite biomarkers to zinc and/or metals needs to be explored further by exposing organisms to other stressors. Importantly, metabolomics approaches can be used to gain novel insights into the potential mode of action of heavy metal pollu-

tants and direct further follow-on experiments. By starting with a broad approach and subsequently focussing on key pathways, together with targeted analyses of gene expression, we have started to unravel the involvement of the transsulfuration pathway in toxicity responses involving zinc.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.03.009.

References

Armitage, P., Cranston, P.S., Pinder, L.C.V. (Eds.), 1995. Chapman & Hall, London, UK.

Armitage, P.D., Moss, D., Wright, J.F., Furse, M.T., 1983. The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites. Water Res. 17, 333–347.

Ballesteros, M.L., Wunderlin, D.A., Bistoni, M.A., 2009. Oxidative stress responses in different organs of *Jenynsia multidentata* exposed to endosulfan. Ecotoxicol. Environ. Saf. 72, 199–205.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B (Methodol.) 57, 289–300.

Bentivegna, C.S., 2002. Advancing monosaccharides as biomarkers: Part II: effects of starvation and cadmium in *Chironomous riparius* as detected by fluorophore-assisted carbohydrate-electrophoresis. Aquat. Toxicol. 61, 111–126.

Bischof, C., 1995. Effects of heavy metal stress on carbohydrate and lipid concentrations in the haemolymph and total body tissue of parasitized *Lymantria dispar* L. larvae (Lepidoptera). Comp. Biochem. Physiol. Part C 112, 87–92.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Booth, L.H., Wratten, S.D., Kehrli, P., 2007. Effects of reduced rates of two insecticides on enzyme activity and mortality of an aphid and its lacewing predator. J. Econ. Entomol. 100, 11–19.

Booth, S.C., Workentine, M.L., Weljie, A.M., Turner, R.J., 2011. Metabolomics and its application to studying metal toxicity. Metallomics 3, 1142–1152.

Boughton, B.A., Callahan, D.L., Silva, C., Bowne, J., Nahid, A., Rupasinghe, T., Tull, D.L., McConville, M.J., Bacic, A., Roessner, U., 2011. Comprehensive profiling and quantitation of amine group containing metabolites. Anal. Chem. 83, 7523–7530.

Brown, P.J., Long, S.M., Spurgeon, D.J., Svendsen, C., Hankard, P., 2004. Toxicological and biochemical responses of the earthworm *Lumbricus rubellus* to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. Chemosphere 57, 1675–1681.

Bundy, J.G., Davey, M.P., Viant, M.R., 2009. Environmental metabolomics: a critical review and future perspectives. Metabolomics 5, 3–21.

Bundy, J.G., Sidhu, J.K., Rana, F., Spurgeon, D.J., Svendsen, C., Wren, J.F., Sturzenbaum, S.R., Morgan, A.J., Kille, P., 2008. 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrates, the earthworm *Lumbricus rubellus*. BMC Biol. 6, 25.

Canesi, L., Betti, M., Ciacci, C., Gallo, G., 2001. Insulin-like effect of zinc in *Mytilus* digestive gland cells: modulation of tyrosine kinase-mediated cell signaling. Gen. Comp. Endocrinol. 122, 60–66.

Carew, M.E., Miller, A.D., Hoffmann, A.A., 2011. Phylogenetic signals and ecotoxicological responses: potential implications for aquatic biomonitoring. Ecotoxicology 20, 595–606.

Chessman, B.C., 1995. Rapid assessment of rivers using macroinvertebrates: a procedure based on habitat-specific sampling, family level identification and a biotic index. Aust. J. Ecol. 20, 122–129.

Choung, C.B., Hyne, R.V., Stevens, M.M., Hose, G.C., 2010. A low concentration of atrazine does not influence the acute toxicity of the insecticide terbufos or its breakdown products to *Chironomus tepperi*. Ecotoxicology 19, 1536–1544.

Depledge, M.H., Fossi, M.C., 1994. The role of biomarkers in environmental assessment (2). Invertebrates. Ecotoxicology 3, 161–172.

Di Guilio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. Environ. Toxicol. Chem. 8, 1103–1123.

Dieterle, F., Ross, A., Schlotterbeck, G., Senn, H., 2006. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application of H-1 NMR metabonomics. Anal. Chem. 78, 4281–4290.

Domingues, I., Guilhermino, L., Soares, A.M.V.M., Nogueira, A.J.A., 2007. Assessing dimethoate contamination in temperate and tropical climates: potential use of biomarkers in bioassays with two chironomid species. Chemosphere 69, 145–154.

Emre, I., Kayis, T., Coskun, M., Dursun, O., Cogun, H.Y., 2013. Changes in antioxidative enzyme activity, glycogen, lipid, protein, and malondialdehyde content in cadmium-treated *Galleria mellonella* larvae. Ann. Entomol. Soc. Am. 106, 371–377.

Engelstad, L.E., 2011. Determining the effects of metal exposure on *Chironomus tepperi*. In: Department of Zoology. The University of Melbourne, Melbourne, p. 42.

Garcia-Reyero, N., Perkins, E.J., 2011. Systems biology: leading the revolution in ecotoxicology. Environ. Toxicol. Chem. 30, 265–273.

Gillis, P.L., Diener, L.C., Reynoldson, T.B., Dixon, D.G., 2002. Cadmium-induced production of a metallothionein-like protein in *Tubifex tubifex* (oligochaeta) and *Chironomus riparius* (Diptera): correlation with reproduction and growth. Environ. Toxicol. Chem. 21, 1836–1844.

Gutierrez-Carbonell, E., Lattanzio, G., Sagardoy, R., Rodriguez-Celma, J., Ruiz, J.J.R., Matros, A., Abadia, A., Abadia, J., Lopez-Millan, A.-F., 2013. Changes induced by zinc toxicity in the 2-DE protein profile of sugar beet roots. J. Proteomics 94, 149–161.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hailemariam, D., Mandal, R., Saleem, F., Dunn, S.M., Wishart, D.S., Ametaj, B.N., 2014. Identification of predictive biomarkers of disease state in transition dairy cows. J. Dairy Sci. 97, 2680–2693.

Hankard, P.K., Svendsen, C., Wright, J., Wienberg, C., Fishwick, S.K., Spurgeon, D.J., Weeks, J.M., 2004. Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis. Sci. Total Environ. 330, 9–20.

Heckmann, L.H., Sorensen, P.B., Krogh, P.H., Sorensen, J.G., 2011. NORMA-gene: a simple and robust method for qPCR normalization based on target gene data. BMC Bioinf. 12, 250.

Hines, A., Staff, F.J., Widdows, J., Compton, R.M., Falciani, F., Viant, M.R., 2010. Discovery of metabolic signatures for predicting whole organism toxicology. Toxicol. Sci. 115, 369–378.

Hughes, S.L., Bundy, J.G., Want, E.J., Kille, P., Sturzenbaum, S.R., 2009. The metabolomic responses of *Caenorhabditis elegans* to cadmium are largely independent of metallothionein status but dominated by changes in cystathionine and phytochelatins. J. Proteome Res. 8, 3512–3519.

Jeppe, K.J., Carew, M.E., Long, S.M., Lee, S.F., Pettigrove, V., Hoffmann, A.A., 2014. Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. Comp. Biochem. Physiol. Part C 162, 1–6.

Jones, O.A.H., Maguire, M.L., Griffin, J.L., Dias, D.A., Spurgeon, D.J., Svendsen, C., 2013. Metabolomics and its use in ecology. Aust. Ecol. 38, 713–720.

Kellar, C.R., Hassell, K.L., Long, S.M., Myers, J.H., Golding, L., Rose, G., Kumar, A., Hoffmann, A.A., Pettigrove, V., 2014. Ecological evidence links adverse biological effects to pesticide and metal contamination in an urban Australian watershed. J. Appl. Ecol. 51, 426–439.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmcol. Toxicol. 39, 267–294.

Klowden, M.J., 2007. Physiological Systems in Insects, 2nd ed. Amsterdam, Academic Press.

Kretzschmar, M., Klinger, W., 1990. The hepatic glutathione system – influences of xenobiotics. Exp. Pathol. 38, 145–164.

Lemire, J., Mailloux, R., Appanna, V.D., 2008. Zinc toxicity alters mitochondrial metabolism and leads to decreased ATP production in hepatocytes. J. Appl. Toxicol. 28, 175–182.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marshall, S., Pettigrove, V., Carew, M., Hoffmann, A., 2010. Isolating the impact of sediment toxicity in urban streams. Environ. Pollut. 158, 1716–1725.

Martin, J., Kuvangkadilok, C., Peart, D.H., Lee, B.T.O., 1980. Multiple sex determining regions in a group of related *Chironomus* species (Diptera: Chironomidae). Heredity 44, 367–382.

Mathews, C.K., van Holde, K.E., Ahern, K.G., 2000. Biochemistry, 3rd ed. Addison Wesley-Longman, Inc., San Francisco.

Nation, J.L., 2008. Insect Physiology and Biochemistry, 2nd ed. Boca Raton, Taylor & Francis Group LLC.

O, S., 'Callaghan, DeSouza, D.P., Isaac, A., Wang, Q., Hodkinson, L., Olshansky, M., Erwin, T., Appelbe, B., Tull, D.L., Roessner, U., Bacic, A., McConville, M.J., Likic, V.A., 2012. PyMS: a Python toolkit for processing of gas chromatography–mass

spectrometry (GC–MS) data. Application and comparative study of selected tools. BMC Bioinf. 13, 115.

OECD, 2004. OECD Guidelines for the testing of chemicals. In: Sediment–Water Chironomid Toxicity Test Using Spiked Sediment 218. OECD.

Pettigrove, V., Hoffmann, A., 2005. A field-based microcosm method to assess the effects of polluted urban stream sediments on aquatic macroinvertebrates. Environ. Toxicol. Chem. 24, 170–180.

Planello, R., Martinez-Guitarte, J.L., Morcillo, G., 2007. Ribosomal genes as early targets of cadmium-induced toxicity in *Chironomus riparius* larvae. Sci. Total Environ. 373, 113–121.

Poynton, H.C., Taylor, N.S., Hicks, J., Colson, K., Chan, S., Clark, C., Scanlan, L., Loguinov, A.V., Vulpe, C., Viant, M.R., 2011. Metabolomics of microliter hemolymph samples enables an improved understanding of the combined metabolic and transcriptional responses of *Daphnia magna* to cadmium. Environ. Sci. Technol. 45, 3710–3717.

Saunders, E.C., DeSouza, D.P., Chambers, J.M., Ng, M., Pyke, J., McConville, M.J., 2015. Use of C - 13 stable isotope labelling for pathway and metabolic flux analysis in leishmania parasites. Methods Mol. Biol. 1201, 281–296.

Shinbo, H., 1988a. An enzyme that catalyses the cleavage of lanthionine in the silkworm, *Bombyx mori*. Comp. Biochem. Physiol. Part B 91, 309–314.

Shinbo, H., 1988b. An enzyme that catalyses the synthesis of lanthionine in the silkworm *Bombyx mori*. Comp. Biochem. Physiol. Part B 91, 301–307.

Stevens, M.M., 1993. Larval development in *Chironomus tepperi* (Diptera: Chironomidae) under laboratory conditions. Environ. Entomol. 22, 776–780.

Stevens, M.M., Helliwell, S., Hughes, P.A., 2005. Toxicity of *Bacillus thuringiensis* var. *Israelensis* formulations, Spinosad, and selected synthetic insecticides to *Chironomus tepperi* larvae. J. Am. Mosq. Control Assoc. 21, 446–450.

Sugiura, Y., Kashiba, M., Maruyama, K., Hoshikawa, K., Sasaki, R., Saito, K., Kimura, H., Goda, N., Suematsu, M., 2005. Cadmium exposure alters metabolomics of sulfur containing amino acids in rat testes. Antioxid. Redox Signal. 7, 781–787.

Takahashi, K.H., Rako, L., Takano-Shimizu, T., Hoffmann, A.A., Lee, S.F., 2010. Effects of small Hsp genes on developmental stability and microenvironmental canalization. BMC Evol. Biol. 10, 11.

Tang, S., Wu, Y.G., Ryan, C.N., Yu, S.Y., Qin, G.Q., Edwards, D.S., Mayer, G.D., 2015. Distinct expression profiles of stress defense and DNA repair genes in *Daphnia pulex* exposed to cadmium, zinc and quantum dots. Chemosphere 120, 92–99.

Taylor, N.S., Weber, R.J.M., Southam, A.D., Payne, T.G., Hrydziuszko, O., Arvanitis, T.N., Viant, M.R., 2009. A new approach to toxicity testing in *Daphnia magna*: application of high throughput FT-ICR mass spectrometry metabolomics. Metabolomics 5, 44–58.

Taylor, N.S., Weber, R.J.M., White, T.A., Viant, M.R., 2010. Discriminating between different acute chemical toxicities via changes in the daphnid metabolome. Toxicol. Sci. 118, 307–317.

Townsend, K.R., Pettigrove, V.J., Hoffmann, A.A., 2012. Food limitation in *Chironomus tepperi*: effects on survival, sex ratios and development across two generations. Ecotoxicol. Environ. Saf. 84, 1–8.

Viant, M.R., 2007. Metabolomics of aquatic organisms: the new omics on the block. Mar. Ecol. Prog. Ser. 332, 301–306.

Wang, X., Chang, L., Sun, Z., Zhang, Y., Yao, L., 2010. Analysis of earthworm *Eisenia fetida* proteomes during cadmium exposure: an ecotoxicoproteomics approach. Proteomics 10, 4476–4490.

Wright, I.A., Burgin, S., 2009. Effects of organic and heavy metal pollution on chironomids within a pristine upland catchment. Hydrobiologia 635, 15–25.

Xia, J., Broadhurst, D.I., Wilson, M., Wishart, D.S., 2013. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 9, 280–299.

REFERENCES

Adams, S.M., Shepard, K.L., Greeley, M.S., Jimenez, B.D., Ryon, M.G., Shugart, L.R., McCarthy, J.F., Hinton, D.E., 1989. The use of biomarkers for assessing the effects of pollutant stress on fish. Marine Environmental Research 28, 459-464.

Alvarado, N.E., Buxens, A., Mazon, L.I., Marigomez, I., Soto, M., 2005. Cellular biomarkers of exposure and biological effect in hepatocytes of turbot (Scophthalmus maximus) exposed to Cd, Cu and Zn and after depuration. Aquatic Toxicology 74, 110-125.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry 29, 730-741.

ANZECC/ARMCANZ, 2000. Australian and New Zealand guidelines for freshwater and marine water quality. Australian and New Zealand environment and conservation council/ agriculture and resource management council of Australia and New Zealand, Canberra, ACT.

Arini, A., Gourves, P.Y., Gonzalez, P., Baudrimont, M., 2015. Metal detoxification and gene expression regulation after a Cd and Zn contamination: An experimental study on *Danio rerio*. Chemosphere 128, 125-133.

Armitage, P.D., Cranston, P.S., Pinder, L.C.V., 1995. The Chironomidae. biology and ecology of non-biting midges. Chapman & Hall, London, UK.

Armitage, P.D., Moss, D., Wright, J.F., Furse, M.T., 1983. The performance of a new biological water quality score system based on macroinvertebrates

REFERENCES

over a wide range of unpolluted running water sites. Water Research 17, 333-347.

Arora, S., Kumar, A., 2015. Binary combinations of organophosphorus pesticides exhibit differential toxicity under oxidised and un-oxidised conditions. Ecotoxicology and Environmental Safety 115, 93-100.

ASTM, 1997. Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates, ASTM Annual Book of Standards. American Society for Testing and Materials, Philadelphia, PA.

Bahrndorff, S., Ward, J., Pettigrove, V., Hoffmann, A.A., 2006. A microcosm test of adaptation and species specific responses to polluted sediments applicable to indigenous chironomids (Diptera). Environmental Pollution 139, 550-560.

Ballesteros, M.L., Wunderlin, D.A., Bistoni, M.A., 2009. Oxidative stress responses in different organs of *Jenynsia multidentata* exposed to endosulfan. Ecotoxicology and Environmental Safety 72, 199-205.

Banni, M., Dondero, F., Jebali, J., Guerbej, H., Boussetta, H., Viarengo, A., 2007. Assessment of heavy metal contamination using real-time PCR analysis of mussel metallothionein mt10 and mt20 expression: a validation along the Tunisian coast. Biomarkers 12, 369-383.

Bearr, J.S., Diamond, J., Latimer, H., Bowersox, M., 2006. Effects of pulsed copper exposures on early life-stage Pimephales promelas. Environmental Toxicology and Chemistry 25, 1376-1382.

Beaty, B.J., Black, W.C., Carlson, J.O., Clements, W.H., DuTeau, N., Harrahy, E., Nuckols, J., Olson, K.E., Rayms-Keller, A., 1998. Molecular and genetic ecotoxicologic approaches to aquatic environmental bioreporting. Environmental Health Perspectives 106, 1395-1407.

Béchard, K., Gillis, P., Wood, C., 2008. Acute toxicity of waterborne Cd, Cu, Pb, Ni, and Zn to first-instar *Chironomus riparius* larvae. Archives of Environmental Contamination and Toxicology 54, 454-459.

REFERENCES

Beketov, M.A., Liess, M., 2012. Ecotoxicology and macroecology – Time for integration. Environmental Pollution 162, 247-254.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology 37, 911-917.

Bocquene, G., Galgani, F., 1991. Acetylcholinesterase in marine organisms, instrumental in monitoring the effects of organophosphorous and carbamate pesticides. Inst Oceanographique, Paris.

Booth, L.H., Wratten, S.D., Kehrli, P., 2007. Effects of reduced rates of two insecticides on enzyme activity and mortality of an aphid and its lacewing predator. Journal of Economic Entomology 100, 11-19.

Boughton, B.A., Callahan, D.L., Silva, C., Bowne, J., Nahid, A., Rupasinghe, T., Tull, D.L., McConville, M.J., Bacic, A., Roessner, U., 2011. Comprehensive profiling and quantitation of amine group containing metabolites. Analytical Chemistry 83, 7523-7530.

Brent, R.N., Herricks, E.E., 1998. Postexposure effects of brief cadmium, zinc, and phenol exposures on freshwater organisms. Environmental Toxicology and Chemistry 17, 2091-2099.

Bundy, J.G., Davey, M.P., Viant, M.R., 2009. Environmental metabolomics: a critical review and future perspectives. Metabolomics 5, 3-21.

Bundy, J.G., Sidhu, J.K., Rana, F., Spurgeon, D.J., Svendsen, C., Wren, J.F., Sturzenbaum, S.R., Morgan, A.J., Kille, P., 2008. 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. Bmc Biology 6.

Burton, G.A., 1991. Assessing the toxicity of fresh-water sediments. Environmental Toxicology and Chemistry 10, 1585-1627.

Callaghan, A., Hirthe, G., Fisher, T., Crane, A., 2001. Effect of short-term exposure to chlorpyrifos on developmental parameters and biochemical

REFERENCES

biomarkers in *Chironomus riparius* Meigen. Ecotoxicology and Environmental Safety 50, 19-24.

Carew, M.E., Pettigrove, V., Cox, R.L., Hoffmann, A.A., 2007. The response of Chironomidae to sediment pollution and other environmental characteristics in urban wetlands. Freshwater Biology 52, 2444-2462.

Casasnovas, R., Ortega-Castro, J., Donoso, J., Frau, J., Munoz, F., 2013. Theoretical calculations of stability constants and pK(a) values of metal complexes in solution: application to pyridoxamine-copper(II) complexes and their biological implications in AGE inhibition. Physical Chemistry Chemical Physics 15, 16303-16313.

Chapman, J.C., 1995. The role of ecotoxicity testing in assessing water-quality. Australian Journal of Ecology 20, 20-27.

Chen, N.C., Yang, F., Capecci, L.M., Gu, Z.Y., Schafer, A.I., Durante, W., Yang, X.F., Wang, H., 2010. Regulation of homocysteine metabolism and methylation in human and mouse tissues. FASEB Journal 24, 2804-2817.

Chessman, B.C., 1995. Rapid assessment of rivers using macroinvertebrates - A procedure based on habitat-specific sampling, family level identification and a biotic index. Australian Journal of Ecology 20, 122-129.

Chessman, B.C., 2003. SIGNAL 2 – A Scoring System for Macro-invertebrate ('Water Bugs') in Australian Rivers, Monitoring River Heath Initiative Technical Report no 31, Commonwealth of Australia, Canberra.

Choung, C.B., Hyne, R.V., Stevens, M.M., Hose, G.C., 2010. A low concentration of atrazine does not influence the acute toxicity of the insecticide terbufos or its breakdown products to *Chironomus tepperi*. Ecotoxicology 19, 1536-1544.

Colombo, V., Mohr, S., Berghahn, R., Pettigrove, V.J., 2013. Structural changes in a macrozoobenthos assemblage after imidacloprid pulses in aquatic field-based microcosms. Archives of Environmental Contamination and Toxicology 65, 683-692.

REFERENCES

Connon, R.E., Geist, J., Pfeiff, J., Loguinov, A.V., D'Abronzo, L.S., Wintz, H., Vulpe, C.D., Werner, I., 2009. Linking mechanistic and behavioral responses to sublethal esfenvalerate exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae). BMC Genomics 10, (15 December 2009)-(2015 December 2009).

Cortelezzi, A., Paggi, A.C., Rodriguez, M., Capitulo, A.R., 2011. Taxonomic and nontaxonomic responses to ecological changes in an urban lowland stream through the use of Chironomidae (Diptera) larvae. Science of the Total Environment 409, 1344-1350.

Dawson, T.D., Jenson, J.J., Norberg-King, T.J., 2000. Laboratory culture of *Chironomus tentans* for use in toxicity testing: optimum initial egg-stocking densities. Hydrobiologia 438, 251-256.

Depledge, M.H., Fossi, M.C., 1994. THE ROLE OF BIOMARKERS IN ENVIRONMENTAL ASSESSMENT .2. INVERTEBRATES. Ecotoxicology 3, 161-172.

Diamond, J.M., Klaine, S.J., Butcher, J.B., 2006. Implications of pulsed chemical exposures for aquatic life criteria and wastewater permit limits. Environmental Science & Technology 40, 5132-5138.

Donnelly, K.C., Lingenfelter, R., Cizmas, L., Falahatpisheh, M.H., Qian, Y.C., Tang, Y., Garcia, S., Ramos, K., Tiffany-Castiglioni, E., Mumtaz, M.M., 2004. Toxicity assessment of complex mixtures remains a goal. Environmental Toxicology and Pharmacology 18, 135-141.

Duft, M., Schulte-Oehlmann, U., Weltje, L., Tillmann, M., Oehlmann, J., 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. Aquatic Toxicology 64, 437-449.

Dzialowski, A.R., Rzepecki, M., Kostrzewska-Szlakowska, I., Kalinowska, K., Palash, A., Lennon, J.T., 2014. Are the abiotic and biotic characteristics of

REFERENCES

aquatic mesocosms representative of *in situ* conditions? Journal of Limnology 73, 603-612.

Eide, I., Neverdal, G., Thorvaldsen, B., Arneberg, R., Grung, B., Kvalheim, O.M., 2004. Toxicological evaluation of complex mixtures: fingerprinting and multivariate analysis. Environmental Toxicology and Pharmacology 18, 127-133.

Fargasova, A., 2001. Winter third- to fourth-instar larvae of *Chironomus plumosus* as bioassay tools for assessment of acute toxicity of metals and their binary combinations. Ecotoxicology and Environmental Safety 48, 1-5.

Forbes, V.E., Forbes, T.L., 1994. Ecotoxicology in theory and practice. Springer, London, UK.

Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Environmental Toxicology and Chemistry 25, 272-280.

Fuchs, S., Rende, E., Crisanti, A., Nolan, T., 2014. Disruption of aminergic signalling reveals novel compounds with distinct inhibitory effects on mosquito reproduction, locomotor function and survival. Scientific Reports 4, 5526.

Galay-Burgos, M., Spurgeon, D.J., Weeks, J.M., Sturzenbaum, S.R., Morgan, A.J., Kille, P., 2003. Developing a new method for soil pollution monitoring using molecular genetic biomarkers. Biomarkers 8, 229-239.

Gallas, J.M., Littrell, K.C., Seifert, S., Zajac, G.W., Thiyagarajan, P., 1999. Solution structure of copper ion-induced molecular aggregates of tyrosine melanin. Biophysical Journal 77, 1135-1142.

Gardham, S., Chariton, A.A., Hose, G.C., 2014. Invertebrate community responses to a particulate- and dissolved-copper exposure in model freshwater ecosystems. Environmental Toxicology and Chemistry 33, 2724-2732.

REFERENCES

Gauss, J.D., Woods, P.E., Winner, R.W., Skillings, J.H., 1985. Acute toxicity of copper to three life stages of *Chironomus tentans* as affected by water hardness-alkalinity. Environmental Pollution (Series A) - Ecological and Biological 37, 149-157.

Giesy, J.P., Hoke, R.A., 1991. Bioassessment of the toxicity of fresh-water sediment. International Association of Theoretical and Applied Limnology.

Gresens, S.E., Belt, K.T., Tang, J.A., Gwinn, D.C., Banks, P.A., 2007. Temporal and spatial responses of Chironomidae (Diptera) and other benthic invertebrates to urban stormwater runoff. Hydrobiologia 575, 173-190.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione s-transferases - First enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130-7139.

Hale, R., Marshall, S., Jeppe, K.J., Pettigrove, V., 2014. Separating the effects of water physicochemistry and sediment contamination on *Chironomus tepperi* (Skuse) survival, growth and development: a boosted regression tree approach. Aquatic Toxicology.

Heckmann, L.H., Sorensen, P.B., Krogh, P.H., Sorensen, J.G., 2011. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. BMC Bioinformatics 12, 250.

Heidari, R., Devonshire, A.L., Campbell, B.E., Dorrian, S.J., Oakeshott, J.G., Russell, R.J., 2005. Hydrolysis of pyrethroids by carboxylesterases from *Lucilia cuprina* and *Drosophila melanogaster* with active sites modified by *in vitro* mutagenesis. Insect Biochemistry and Molecular Biology 35, 597-609.

Heiskary, S.A., Bouchard, R.W., 2015. Development of eutrophication criteria for Minnesota streams and rivers using multiple lines of evidence. Freshwater Science 34, 574-592.

Hoffman, A.E., Miles, L., Greenfield, T.J., Shoen, C., DeStefano, M., Cynamon, M., Doyle, R.P., 2015. Clinical isolates of *Candida albicans*,

REFERENCES

Candida tropicalis, and *Candida krusei* have different susceptibilities to Co(II) and Cu(II) complexes of 1,10-phenanthroline. Biometals 28, 415-423.

Hook, S.E., 2010. Promise and progress in environmental genomics: a status report on the applications of gene expression-based microarray studies in ecologically relevant fish species. Journal of Fish Biology 77, 1999-2022.

Hook, S.E., Lampi, M.A., Febbo, E.J., Ward, J.A., Parkerton, T.F., 2010. Hepatic gene expressionin rainbow trout (*Oncorhynchus mykiss*) exposed to different hydrocarbon mixtures. Environmental Toxicology and Chemistry 29, 2034-2043.

Hook, S.E., Osborn, H.L., Golding, L.A., Spadaro, D.A., Simpson, S.L., 2014a. Dissolved and particulate copper exposure induces differing gene expression profiles and mechanisms of toxicity in the deposit feeding amphipod *Melita plumulosa*. Environmental Science & Technology 48, 3504-3512.

Hook, S.E., Osborn, H.L., Spadaro, D.A., Simpson, S.L., 2014b. Assessing mechanisms of toxicant response in the amphipod *Melita plumulosa* through transcriptomic profiling. Aquatic Toxicology 146, 247-257.

Hook, S.E., Skillman, A.D., Gopalan, B., Small, J.A., Schultz, I.R., 2008. Gene expression profiles in rainbow trout, *Onchorynchus mykiss*, exposed to a simple chemical mixture. Toxicological Sciences 102, 42-60.

Hosmer, A.J., Warren, L.W., Ward, T.J., 1998. Chronic toxicity of pulse-dosed fenoxycarb to *Daphnia magna* exposed to environmentally realistic concentrations. Environmental Toxicology and Chemistry 17, 1860-1866.

Huggett, R., Kimerle, R., Mehrle, P.J., Bergman, H., 2002. Biomarkers: Biochemical, physiological, and histological markers of anthropogenic stress. Lewis, Boca Raton, USA.

Hughes, S.L., Bundy, J.G., Want, E.J., Kille, P., Sturzenbaum, S.R., 2009. The metabolomic responses of *Caenorhabditis elegans* to cadmium are largely independent of metallothionein status, but dominated by changes in

REFERENCES

cystathionine and phytochelatins. Journal of Proteome Research 8, 3512-3519.

Hutchins, C.M., Teasdale, P.R., Lee, S.Y., Simpson, S.L., 2008. Cu and Zn concentration gradients created by dilution of pH neutral metal-spiked marine sediment: A comparison of sediment geochemistry with direct methods of metal addition. Environmental Science & Technology 42, 2912-2918.

Ingersoll, C.G., Wang, N., Hayward, J.M.R., Jones, J.R., Jones, S.B., Ireland, D.S., 2005. A field assessment of long-term laboratory sediment toxicity tests with the amphipod *Hyalella azteca*. Environmental Toxicology and Chemistry 24, 2853-2870.

Ireland, D.S., Ho, K.T., 2005. Toxicity tests for sediment quality assessments. Crc Press-Taylor & Francis Group, Boca Raton.

Jager, T., Heugens, E.H.W., Kooijman, S., 2006. Making sense of ecotoxicological test results: Towards application of process-based models. Ecotoxicology 15, 305-314.

James, S.J., Melnyk, S., Pogribna, M., Pogribny, I.P., Caudill, M.A., 2002. Elevation in S-adenosylhomocysteine and DNA hypomethylation: Potential epigenetic mechanism for homocysteine-related pathology. Journal of Nutrition 132, 2361S-2366S.

Jeppe, K.J., Carew, M.E., Long, S.M., Lee, S.F., Pettigrove, V., Hoffmann, A.A., 2014. Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 162, 1-6.

Jones, D.P., Go, Y.M., Anderson, C.L., Ziegler, T.R., Kinkade, J.M., Kirlin, W.G., 2004. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. FASEB Journal 18, 1246-1248.

Kellar, C.R., Hassell, K.L., Long, S.M., Myers, J.H., Golding, L., Rose, G., Kumar, A., Hoffmann, A.A., Pettigrove, V., 2014. Ecological evidence links

REFERENCES

adverse biological effects to pesticide and metal contamination in an urban Australian watershed. Journal of Applied Ecology 51, 426-439.

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. Aquatic Toxicology 82, 163-172.

Kramer, V.J., Etterson, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.T., 2011. Adverse outcome pathways and ecological risk assessment: Bridging to population-level effects. Environmental Toxicology and Chemistry 30, 64-76.

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

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lee, S.-W., Choi, J., 2009. Multi-level ecotoxicity assay on the aquatic midge, *Chironomus tentans* (Diptera, Chironomidae) exposed to octachlorostyrene. Environmental Toxicology and Pharmacology 28, 269-274.

Lee, S.M., Lee, S.B., Park, C.H., Choi, J., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, Chironomidae) larvae exposed to various environmental pollutants: A potential biomarker of freshwater monitoring. Chemosphere 65, 1074-1081.

Lemos, M.F.L., Soares, A., Correia, A.C., Esteves, A.C., 2010. Proteins in ecotoxicology - How, why and why not? Proteomics 10, 873-887.

Li, X.W., Zhang, X., Zhang, J.Z., Starkey, S.R., Zhu, K.Y., 2009. Identification and characterization of eleven glutathione S-transferase genes from the aquatic midge *Chironomus tentans* (Diptera: Chironomidae). Insect Biochemistry and Molecular Biology 39, 745-754.

REFERENCES

Loayza-Muro, R.A., Marticorena-Ruiz, J.K., Palomino, E.J., Merritt, C., De Baat, M.L., Van Gemert, M., Verweij, R.A., Kraak, M.H.S., Admiraal, W., 2013. Persistence of chironomids in metal polluted andean high altitude streams: does melanin play a role? Environmental Science & Technology 47, 601-607.

Long, S.M., Tull, D.L., Jeppe, K.J., De Souza, D.P., Dayalan, S., Pettigrove, V.J., McConville, M.J., Hoffmann, A.A., 2015. A multi-platform metabolomics approach demonstrates changes in energy metabolism and the transsulfuration pathway in *Chironomus tepperi* following exposure to zinc. Aquatic Toxicology 162, 54-65.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Ma, J., Zhou, C., Li, Y., Li, X., 2014. Biochemical responses to the toxicity of the biocide abamectin on the freshwater snail *Physa acuta*. Ecotoxicology and Environmental Safety 101, 31-35.

MacDonald, D.D., Ingersoll, C.G., Berger, T.A., 2000. Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. Archives of Environmental Contamination and Toxicology 39, 20-31.

Marinkovic, M., de Leeuw, W.C., de Jong, M., Kraak, M.H.S., Admiraal, W., Breit, T.M., Jonker, M.J., 2012a. Combining next-generation sequencing and microarray technology into a transcriptomics approach for the non-model organism *Chironomus riparius*. PLoS One 7, e48096.

Marinkovic, M., de Leeuw, W.C., Ensink, W.A., de Jong, M., Breit, T.M., Admiraal, W., Kraak, M.H.S., Jonker, M.J., 2012b. Gene expression patterns and life cycle responses of toxicant-exposed chironomids. Environmental Science & Technology 46, 12679-12686.

REFERENCES

Marshall, S., Pettigrove, V., Carew, M., Hoffmann, A., 2010. Isolating the impact of sediment toxicity in urban streams. Environmental Pollution 158, 1716-1725.

Martin, J., Kuvangkadilok, C., Peart, D.H., Lee, B.T.O., 1980. Multiple sex determining regions in a group of related *Chironomus* species (Diptera: Chironomidae). Heredity 44, 367 - 382.

Martin-Folgar, R., de la Fuente, M., Morcillo, G., Martinez-Guitarte, J.L., 2015. Characterization of six small HSP genes from *Chironomus riparius* (Diptera, Chironomidae): Differential expression under conditions of normal growth and heat-induced stress. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 188, 76-86.

Martinez, E.A., Moore, B.C., Schaumloffel, J., Dasgupta, N., 2003. Morphological abnormalities in *Chironomus tentans* exposed to cadmium- and copper-spiked sediments. Ecotoxicology and Environmental Safety 55, 204-212.

Martinez-Guitarte, J.L., Planello, R., Morcillo, G., 2007. Characterization and expression during development and under environmental stress of the genes encoding ribosomal proteins L11 and L13 in *Chironomus riparius*. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 147, 590-596.

McCarthy, J., Shugart, L., 1990. Biomarkers of environmental contamination. Lewis, Boca Raton, USA.

Morales-Casellesa, C., Martin-Diaz, M.L., Riba, I., Sarasquete, C., DelValls, T.A., 2008. Sublethal responses in caged organisms exposed to sediments affected by oil spills. Chemosphere 72, 819-825.

Moriarty, F., 1983. Ecotoxicology: The Study of Pollutants in Ecosystems Academic Press, London, UK.

Mousavi, S.K., Primicerio, R., Amundsen, P.A., 2003. Diversity and structure of Chironomidae (Diptera) communities along a gradient of heavy metal

REFERENCES

contamination in a subarctic watercourse. Science of the Total Environment 307, 93-110.

Nair, P.M.G., Choi, J., 2011. Identification, characterization and expression profiles of *Chironomus riparius* glutathione S-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. Aquatic Toxicology 101, 550-560.

Nair, P.M.G., Chung, I.M., 2015. Alteration in the expression of antioxidant and detoxification genes in *Chironomus riparius* exposed to zinc oxide nanoparticles. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 190, 1-7.

Nair, P.M.G., Park, S.Y., Choi, J., 2011. Analyses of Expressed Sequence Tags from *Chironomus riparius* using pyrosequencing : Molecular ecotoxicology perspective. Environmental health and toxicology 26, e2011010.

Nogueira, V., Lopes, I., Rocha-Santos, T.A.P., Rasteiro, M.G., Abrantes, N., Goncalves, F., Soares, A.M.V.M., Duarte, A.C., Pereira, R., 2015. Assessing the ecotoxity of metal nano-oxides with potential for wastewater treatment. Environmental Science and Pollution Research International 22, 13212-13224.

Norton, S.B., Rao, L., Suter, G., Cormier, S.M., 2003. Minimizing cognitive errors in site-specific causal assessments. Human and Ecological Risk Assessment 9, 213-229.

O'Brien, A.L., Keough, M.J., 2014. Ecological responses to contamination: A meta-analysis of experimental marine studies. Environmental Pollution 195, 185-191.

OECD, 2004. Sediment-water chironomid toxicity test using spiked sediment 218, OECD Guideline for testing chemicals. Organization for Economic Co-operation and Development, Washington DC, USA, pp. 1-21.

Park, K., Bang, H.W., Park, J., Kwak, I.S., 2009. Ecotoxicological multilevel-evaluation of the effects of fenbendazole exposure to *Chironomus riparius* larvae. Chemosphere 77, 359-367.

REFERENCES

Park, K., Kwak, I.S., 2008. Expression of *Chironomus riparius* serine-type endopeptidase gene under di-(2-ethylhexyl)-phthalate (DEHP) exposure. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 151, 349-354.

Peakall, D.P., 1994. Biomarkers – The way forward in environmental assessment. TEN 1, 55-60.

Pettigrove, V., 2006. The impact of contaminated sediments on the aquatic macroinvertebrates from Greater Melbourne Area, Australia, Centre for Environmental Stress and Adaptation Research. La Trobe University, Melbourne, Australia

Pettigrove, V., Hoffmann, A., 2005. A field-based microcosm method to assess the effects of polluted urban stream sediments on aquatic macroinvertebrates. Environmental Toxicology and Chemistry 24, 170-180.

Pettigrove, V., Marshall, S., Ryan, B., Hoffmann, A., 2007. A field microcosm method to determine the impact of sediments and soils contaminated by road runoff on indigenous aquatic macroinvertebrates, Highway and Urban Environment. Springer, London, UK, pp. 385-398.

Picado, A., Bebianno, M.J., Costa, M.H., Ferreira, A., Vale, C., 2007. Biomarkers: a strategic tool in the assessment of environmental quality of coastal waters. Hydrobiologia 587, 79-87.

Pin-Lan, L., Fan, Y., Ningjun, L., 2007. Hyperhomocysteinemia - association with renal transsulfuration and redox signaling in rats. Clinical Chemistry and Laboratory Medicine 45, 1688-1693.

Planello, R., Herrero, O., Gomez-Sande, P., Ozaez, I., Cobo, F., Servia, M.J., 2015. Ecdysone-related biomarkers of toxicity in the model organism *Chironomus riparius*: Stage and sex-dependent variations in gene expression profiles. PLoS One 10, 20.

Planello, R., Martinez-Guitarte, J.L., Morcillo, G., 2008. The endocrine disruptor bisphenol A increases the expression of HSP70 and ecdysone

REFERENCES

receptor genes in the aquatic larvae of *Chironomus riparius*. Chemosphere 71, 1870-1876.

Planello, R., Martinez-Guitarte, J.L., Morcillo, G., 2010. Effect of acute exposure to cadmium on the expression of heat-shock and hormone-nuclear receptor genes in the aquatic midge *Chironomus riparius*. Science of the Total Environment 408, 1598-1603.

Rakotondravelo, M.L., Anderson, T.D., Charlton, R.E., Zhu, K.Y., 2006. Sublethal effects of three pesticides on activities of selected target and detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera : Chironomidae). Archives of Environmental Contamination and Toxicology 51, 360-366.

Rao, A.M., Drake, M.R., Stipanuk, M.H., 1990. Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. Journal of Nutrition 120, 837-845.

Rasmussen, J.J., Wiberg-Larsen, P., Kristensen, E.A., Cedergreen, N., Friberg, N., 2013. Pyrethroid effects on freshwater invertebrates: A meta-analysis of pulse exposures. Environmental Pollution 182, 479-485.

Regier, N., Baerlocher, L., Munsterkotter, M., Farinelli, L., Cosio, C., 2013. Looking for biomarkers of Hg exposure by transcriptome analysis in the aquatic plant *Elodea nuttallii*, Proceedings of the 16th International Conference on Heavy Metals in the Environment. E D P Sciences, Paris, France.

Rios, C.A., Williams, C.D., Roberts, C.L., 2008. Removal of heavy metals from acid mine drainage (AMD) using coal fly ash, natural clinker and synthetic zeolites. Journal of Hazardous Materials 156, 23-35.

Scarino, M.L., Poverini, R., Dilullo, G., Bises, G., 1991. Metallothionein gene-expression in the intestinal-cell-modulation of messenger-RNA and protein-synthesis by copper and zinc. Biochemical Society Transactions 19, S283-S283.

REFERENCES

Schlenk, D., Lavado, R., Loyo-Rosales, J.E., Jones, W., Maryoung, L., Riar, N., Werner, I., Sedlak, D., 2012. Reconstitution studies of pesticides and surfactants exploring the cause of estrogenic activity observed in surface waters of the San Francisco Bay Delta. Environmental Science & Technology 46, 9106-9111.

Schmitt C., Duft M., Brandelik C., Schulte-Oehlmann U., Oehlmann J., 2006. SOP for testing chemicals Also: proposal for new guideline reproduction test with the prosobranch snail *Potamopyrgus antipodarum* for testing endocrine active chemicals. Johann Wolfgang Goethe University Frankfurt am Main Department of Aquatic Ecotoxicology.

Seeland, A., Albrand, J., Oehlmann, J., Muller, R., 2013. Life stage-specific effects of the fungicide pyrimethanil and temperature on the snail *Physella acuta* (Draparnaud, 1805) disclose the pitfalls for the aquatic risk assessment under global climate change. Environmental Pollution 174, 1-9.

Sharley, D.J., Hoffmann, A.A., Pettigrove, V., 2008. Effects of sediment quality on macroinvertebrates in the Sunraysia region of the Murray-Darling Rivers, Australia. Environmental Pollution 156, 689-698.

Shi, X., Gu, A., Ji, G., Li, Y., Di, J., Jin, J., Hu, F., Long, Y., Xia, Y., Lu, C., Song, L., Wang, S., Wang, X., 2011. Developmental toxicity of cypermethrin in embryo-larval stages of zebrafish. Chemosphere 85, 1010-1016.

Shuhaimi-Othman, M., Pascoe, D., 2007. Bioconcentration and depurationof of copper, cadmium, and zinc mixtures by the freshwater amphipod *Hyalella azteca*. Ecotoxicology and Environmental Safety 66, 29-35.

Simpson, S.L., Batley, G.E., Charlton, A.A., Stauber, J.L., King, C.K., Chapman, J.C., Hyne, R.V., Gale, S.A., Roach, A.C., Maher, W.A., 2005. Handbook for Sediment Quality Assessment. CSIRO, Bangor NSW.

Snell, T.W., Brogdon, S.E., Morgan, M.B., 2003. Gene expression profiling in ecotoxicology. Ecotoxicology 12, 475-483.

REFERENCES

Spurgeon, D.J., Jones, O.A.H., Dorne, J., Svendsen, C., Swain, S., Sturzenbaum, S.R., 2010. Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Science of the Total Environment 408, 3725-3734.

Stevens, M.M., 1993. Larval development in *Chironomus tepperi* (Diptera, Chironomidae) under laboratory conditions. Environmental Entomology 22, 776-780.

Stevens, M.M., Warren, G.N., Braysher, B.D., 2003. Oviposition response of *Chironomus tepperi* to nitrogenous compounds and bioextracts in two-choice laboratory tests. Journal of Chemical Ecology 29, 911-920.

Sugiura, Y., Kashiba, M., Maruyama, K., Hoshikawa, K., Sasaki, R., Saito, K., Kimura, H., Goda, N., Suematsu, M., 2005. Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes. Antioxidants & Redox Signaling 7, 781-787.

Sulmon, C., van Baaren, J., Cabello-Hurtado, F., Gouesbet, G., Hennion, F., Mony, C., Renault, D., Bormans, M., El Amrani, A., Wiegand, C., Gerard, C., 2015. Abiotic stressors and stress responses: What commonalities appear between species across biological organization levels? Environmental Pollution 202, 66-77.

Szpoganicz, B., Gidanian, S., Kong, P., Farmer, P., 2002. Metal binding by melanins: studies of colloidal dihydroxyindole-melanin, and its complexation by Cu(II) and Zn(H) ions. Journal of Inorganic Biochemistry 89, 45-53.

Takacs, V., Tokeshi, M., 1993. The diet of *Procladius signatus* (Diptera: Chironomidae) in Lough Neagh. Irish Naturalists' Journal 24, 282-283.

Takahashi, K.H., Rako, L., Takano-Shimizu, T., Hoffmann, A.A., Lee, S.F., 2010. Effects of small Hsp genes on developmental stability and microenvironmental canalization. BMC Evolutionary Biology 10, 11.

Timbrell, J.A., 1998. Biomarkers in toxicology. Toxicology 129, 1-12.

REFERENCES

Townsend, K.R., Pettigrove, V.J., Carew, M.E., Hoffmann, A.A., 2009. The effects of sediment quality on benthic macroinvertebrates in the River Murray, Australia. Marine and Freshwater Research 60, 70-82.

Townsend, K.R., Pettigrove, V.J., Hoffmann, A.A., 2012. Food limitation in *Chironomus tepperi*: Effects on survival, sex ratios and development across two generations. Ecotoxicology and Environmental Safety 84, 1-8.

Tuffnail, W., Mills, G.A., Cary, P., Greenwood, R., 2009. An environmental H-1 NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation. Metabolomics 5, 33-43.

Ungherese, G., Baroni, D., Bruni, P., Focardi, S.E., Ugolini, A., 2011. Metallothionein induction in the sandhopper *Talitrus saltator* (Montagu)(Crustacea, Amphipoda). Water, Air, & Soil Pollution 219, 343-351.

US-EPA, 1996. Chironomid sediment toxicity test, ecological effect tests guidelines, EPA 712-C-96-313 2nd ed. EPA 600/R-99/064. United States - Environmental Protection Agency, Washington DC, USA.

US-EPA, 2001. Update of ambient water quality criteria for cadmium. U.S. Environmental Protection Agency, Washington D.C.

US-EPA, 2007. Sediment Toxicity Identification Evaluation (TIE) Phases I, II, and III Guidance Document. EPA/600/R-07/080 Office of Research and Development. Washington, DC.

Van Aggelen, G., Ankley, G.T., Baldwin, W.S., Bearden, D.W., Benson, W.H., Chipman, J.K., Collette, T.W., Craft, J.A., Denslow, N.D., Embry, M.R., Falciani, F., George, S.G., Helbing, C.C., Hoekstra, P.F., Iguchi, T., Kagami, Y., Katsiadaki, I., Kille, P., Liu, L., Lord, P.G., McIntyre, T., O'Neill, A., Osachoff, H., Perkins, E.J., Santos, E.M., Skirrow, R.C., Snape, J.R., Tyler, C.R., Versteeg, D., Viant, M.R., Volz, D.C., Williams, T.D., Yu, L., 2010. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: Hurdles, achievements, and future outlook. Environmental Health Perspectives 118, 1-5.

REFERENCES

Viarengo, A., Lafaurie, M., Gabrielides, G.P., Fabbri, R., Marro, A., Romeo, M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Marine Environmental Research 49, 1-18.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Marine Environmental Research 44, 69-84.

Vinot, I., Pihan, J.C., 2005. Circulation of copper in the biotic compartments of a freshwater dammed reservoir. Environmental Pollution 133, 169-182.

Wang, F.Y., Goulet, R.R., Chapman, P.M., 2004. Testing sediment biological effects with the freshwater amphipod *Hyalella azteca*: the gap between laboratory and nature. Chemosphere 57, 1713-1724.

Warne, M.A., Lenz, E.M., Osborn, D., Weeks, J.M., Nicholson, J.K., 2001. Comparative biochemistry and short-term starvation effects on the earthworms *Eisenia veneta* and *Lumbricus terrestris* studied by H-1 NMR spectroscopy and pattern recognition. Soil Biology & Biochemistry 33, 1171-1180.

Watts, M.M., Pascoe, D., 2000. A comparative study of *Chironomus riparius* Meigen and *Chironomus tentans* Fabricius (Diptera : Chironomidae) in aquatic toxicity tests. Archives of Environmental Contamination and Toxicology 39, 299-306.

Williams, K.A., Green, D.W.J., Pascoe, D., Gower, D.E., 1986. The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera, Chironomidae) and its ecological significance for pollution regulation. Oecologia 70, 362-366.

Yang, R.S.H., El-Masri, H.A., Thomas, R.S., Dobrev, I.D., Dennison, J.E., Bae, D.S., Campain, J.A., Liao, K.H., Reisfeld, B., Andersen, M.E., Mumtaz, M., 2004. Chemical mixture toxicology: from descriptive to mechanistic, and going

REFERENCES

on to *in silico* toxicology. Environmental Toxicology and Pharmacology 18, 65-81.

Yap, C.K., Ismail, A., Tan, S.G., Omar, H., 2003. Accumulation, depuration and distribution of cadmium and zinc in the green-lipped mussel *Perna viridis* (Linnaeus) under laboratory conditions. Hydrobiologia 498, 151-160.

Yoshimi, T., Odagiria, K., Hiroshige, Y., Yokobori, S.-i., Takahashi, Y., Sugaya, Y., Miura, T., 2009. Induction profile of HSP70-cognate genes by environmental pollutants in Chironomidae. Environmental Toxicology and Pharmacology 28, 294-301.