Sub-lethal impacts of coal mine wastewater: Exploring behavioural responses in native aquatic organisms

by

Chantal M. Lanctôt
BSc (Hons), MSc

A thesis
submitted in
fulfillment of the
requirement for the degree of
Doctor of Philosophy

Central Queensland University
School of Medical and Applied Sciences
Australia

April 2016

Abstract

Coal mining represents a primary economic activity in Australia, but wastewater resulting from the extraction and refinement of coal can present risks for aquatic ecosystems. It is, therefore, imperative that the impacts of coal mine water releases on aquatic ecosystems are fully understood. The broad objectives of this thesis are to provide information on the potential toxicity and sub-lethal effects of coal mine wastewater on a range of native macroinvertebrate, amphibian, and fish species that are directly relevant to Central Queensland. In particular, the project evaluates the use of behavioural analysis as a tool for monitoring possible sub-lethal effects of wastewater releases on these organisms.

In the first instance, acute and sub-chronic experiments were performed to compare the sensitivities of various locally relevant aquatic invertebrates and vertebrate species, to coal mine wastewater from two dams (CMW1 and CMW2) located at an open-cut mine in Central Queensland. Two local invertebrate species, the planarian *Dugesia* sp. and the cladoceran *Daphnia carinata*, were identified as good candidates for monitoring water quality and toxicity risks in local mine-affected regions. Though acute lethality was not observed in *Hypseleotris compressa*, *Pseudomugil signifier and Limnodynastes peronii*, fish and tadpoles both exhibited morphological effects following relatively short-term exposures. Due to their fast and apparently sensitive sub-lethal responsiveness, the fish *H. compressa* and the amphibian *L. peronii* were selected to investigate behavioural endpoints following exposure to coal mine wastewater.

In tests with fish, empire gudgeons (*H. compressa*) were exposed to coal mine wastewater and swimming activities and movement patterns were assessed using both visual (*i.e.*, EthoVision® XT video-tracking software) and non-visual (*i.e.*, Multispecies Freshwater Biomonitor®) commercial behavioural monitoring systems. Results demonstrated a range of alterations to behaviour and swimming performance with exposure to sub-lethal mine water dilutions. These endpoints proved more sensitive than developmental or morphological responses and were observable following very limited exposure timeframes (1 h to 15 d), thereby demonstrating the value of considering behavioural responses as sub-lethal toxicity outcomes in fish exposed to complex wastewaters.

Striped marsh frog (*L. peronii*) tadpoles were also exposed to coal mine wastewater to investigate effects on swimming behaviour, and was paired with assessment of survival, growth and development, and metal(loid) bioconcentration in exposed individuals. Overt toxicity (mortality) was high in wastewater from one dam (CMW2), and tadpoles exposed to effluent from the other dam (CMW1) suffered delayed development, and had increased hepatosomatic index values. Exposure to CMW1 and CMW2 resulted in increased activity and altered swimming performance, and animals exhibited significantly higher levels of Se, Co and As in hepatic tissue and tails compared to controls ($p \leq 0.001$).

This study represents a starting point in developing our understanding of sub-lethal behavioural responses in aquatic vertebrates exposed to coal mine wastewater. Whole effluent studies offer a much closer representation of natural exposure scenarios than studies investigating single contaminants, but interpretation can be extremely challenging due to the multitude of possible interactions. Results of this project reveal how variable physico-chemical properties of wastewater from disparate sources, and differential species sensitivities, can ultimately result in diverse toxicological outcomes, highlighting the importance of site-specific toxicity evaluations using locally relevant species. Nevertheless, this research addresses important gaps in the literature, by expanding on the limited number of existing studies that have explored sub-lethal impacts of coal mining on aquatic organisms. Behavioral endpoints show real promise as tools for monitoring wastewater discharges using native fish and amphibian species, but more research is necessary to identify responsible compounds and response thresholds, and to understand the relevance of the observed effects for populations in natural receiving environments.

Keywords: Ecotoxicology; whole effluent toxicity; sub-lethal effects; coal mine wastewater; behaviour; fish; amphibian; macroinvertebrate; bioconcentration; Australia

Acknowledgements

First and foremost, I would like to thank my supervisors, Drs. Scott Wilson, Larelle Fabbro, Steven Melvin and Frederic Leusch, for their support and guidance throughout the course of my PhD.

I gratefully acknowledge the Queensland Resource Council Coal Minesite Rehabilitation Trust Fund Scholarship, the Queensland resources industry, the Queensland State Government, the Central Queensland University Postgraduate Research Award and *in-kind* industry support that allowed me to undertake this project.

My sincere thanks to the environmental team at the studied mine, especially Ian and Stacey, for their support and assistance with the organization and collection of mine water. I would also like to thank Dr. William Bennett for his help with the metal analysis of my tissue samples.

Special thanks to my past and present colleagues at the Gladstone campus and Smart Water Research Centre for your friendship and support. It was a pleasure getting to know all of you.

Finally, to my partner, friends and family, thank you for your constant support and encouragement.

Statement of Originality

The work contained in this thesis has not been previously submitted either in whole or in part for a degree at CQUniversity or any other tertiary institution. To the best of my knowledge and belief, the material presented in this thesis is original except where due reference is made in text.

[redacted]

Chantal Lanctôt

Acknowledgement of papers included in this thesis

Included in this thesis are papers in Chapters 3, 4, 5 and 6, which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details for these papers including all authors are:

Chapter 3:

Lanctôt, C., Wilson, S. P., Fabbro, L., Leusch, F. D. L., Melvin, S. D. (2016). Comparative sensitivity of aquatic invertebrate and vertebrate species to wastewater from an operational coal mine in Central Queensland, Australia. *Ecotoxicology and Environmental Safety* **129**, 1–9.

Chapter 4:

Lanctôt, C. M., Melvin, S. D., Fabbro, L., Leusch, F. D. L., and Wilson, S. P. (2016). Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (*Hypseleotris compressa*). *Ecotoxicology and Environmental Safety* **127**, 36–42.

Chapter 5:

Lanctôt, C. M., Melvin, S. D., Leusch, F. D. L., Wilson, S., and Fabbro, L. (2016). Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. *Chemosphere* **144**, 1560–1566.

Chapter 6:

Lanctôt, C., Bennett, W., Wilson, S. P., Fabbro, L., Leusch, F. D. L., Melvin, S. D. (2016). Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater. *Aquatic Toxicology* **173**, 218–227.

Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each paper.

[redacted]

Chantal Lanctôt

Copyright Statement

[redacted]

Chantal Lanctôt

Table of Contents

List of Figures

List of Tables

Chapter 1
Introduction

1.1 Background

1.1.1 Coal mining in Queensland

Coal mining represents an important economic activity in many parts of the world. Large-scale open-cut (surface) mining started in Australia in the 1960s and by the mid-1980s Australia had become the world's largest coal exporter (Höök et al. 2010). Today, Australia remains the largest exporter of coking coal used for metal production (IEA 2012). Coal is mined in all six Australian states, however, Queensland and New South Wales encompass the majority of the black coal deposits, whereas the southern regions around Victoria hold the majority of brown coal (Scott et al. 2010). The Bowen Basin in Central Queensland contains the largest black coal reserve, covering an area of approximately 75,000 km^2 (Figure 1-1). In 2012, a total of 47 operational coal mines in the Bowen Basin produced over 190 million tonnes of saleable coal (DNRM 2013). Other major land-uses in the Bowen Basin area include coal seam gas extraction and agriculture and over 80% of this area has been cleared of the natural vegetation for grazing (Douglas et al. 2008).

The Bowen Basin overlaps with the Fitzroy Basin catchment. This is the second largest drainage system in Australia covering an area of 144,000 km^2 (Douglas et al. 2005), and consisting of several sub-catchments: the Mackenzie, Isaac, Nogoa, Comet, Dawson, Callide and Connors Rivers. These rivers converge into the Fitzroy River, which discharges into the Coral Sea at Keppel Bay. The Fitzroy Basin area is subject to extremely variable sub-tropical to tropical climate, which is dominated by climate extremes including flood and drought. Rivers and streams throughout this area are mainly ephemeral and the flow is highly dependent on rainfall. During the dry season, rivers and streams often experience little or no flow, and many dry out completely. A barrage located in Rockhampton regulates the natural flow of the Fitzroy River and separates the saline water from the upstream freshwater. During high rainfall and flood events, large volumes of freshwater flow into the downstream estuarine section of the river, which have an important influence on the southern Great Barrier Reef (Douglas et al. 2008).

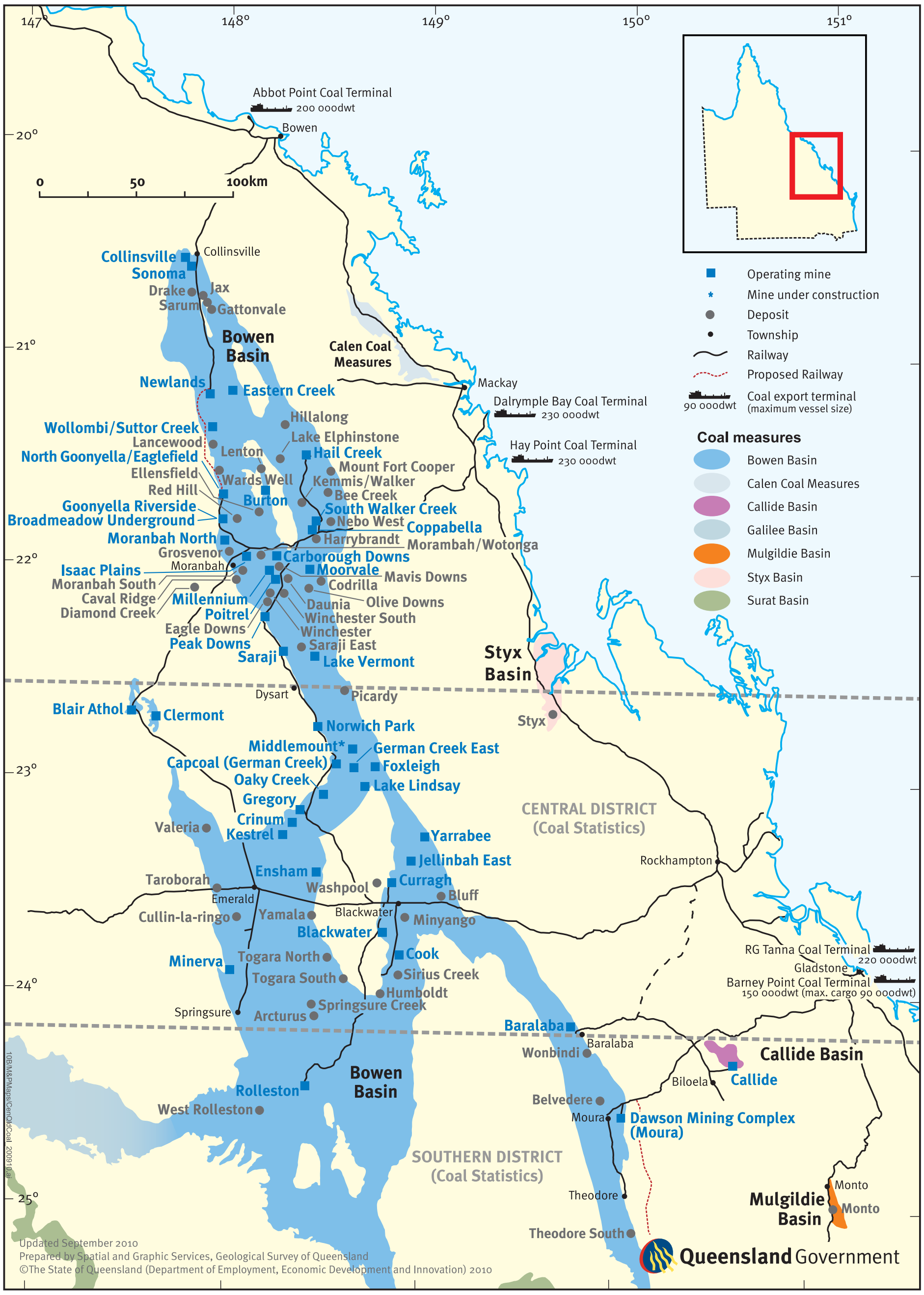

Figure 1-1: Map of the central Queensland illustrating the location of coal mines (blue squares) in the Bowen Basin (Queensland Government 2010).

1.1.2 Environmental Monitoring and Research Activities

Extensive water quality monitoring, data collection and reporting have been and currently are being carried out in the Fitzroy Basin by various government agencies, universities, water providers, natural resource management groups, mine operators and other stakeholders. Several studies have focused on monitoring water quality of the Fitzroy Basin and have provided extensive data on flows, sediment loads, physical and chemical water quality characteristics, and assemblages of aquatic flora and fauna (*e.g.*, Noble et al. 1996a; 1996b; DEHDNR 1999; DERM 2001; FAB 2008; 2009; DERM 2009; 2010; 2011a; 2011b). A summary of water quality parameters common to the Fitzroy Basin along with discharge limits and guidelines are presented in Table 1.1.

Table 1-1: Water quality parameters and guidelines relevant for the Fitzroy catchment.

Parameters	Environmental values (Min - Max)[a]	Trigger values[c]	Release limits[b]
pH Value	6.7 - 9.8	6.5 - 8.0	6.5 - 9.0
Electrical Conductivity (µS/cm)	70 - 1125	125 - 2200	10000
Suspended Solids (mg/L)	1.0 - 1730	10	
Turbidity (NTU)	0 - 2654	50	
Sulfate as SO_4 (mg/L)		350	250
Fluoride (µg/L)			2000
Ammonia as N (µg/L)		20	1000
Nitrate as N (µg/L)		700	7600
Total Nitrogen (µg/L)	130 - 2760	500	
Total Phosphorus (µg/L)	20 - 1800	50	
Aluminium (µg/L)	<0.05 - 2900	55	1014
Arsenic (µg/L)		24 (As^{III}) 13 (As^{V})	
Cadmium (µg/L)		0.2	
Chromium (µg/L)		1.0 (Cr^{VI})	
Copper (µg/L)	<0.3 - 16000	1.4	5.6
Cobalt (µg/L)			
Nickel (µg/L)		11	
Lead (µg/L)		3.4	
Zinc (µg/L)	<0.01 - 1400	8	17
Manganese (µg/L)	<0.01 - 90	1900	
Selenium (µg/L)		11	10
Silver (µg/L)		0.05	
Uranium (µg/L)			1
Vanadium (µg/L)			10
Boron (µg/L)		370	370
Iron (µg/L)	<0.01 - 4200		1200
Mercury (µg/L)		0.6	
Hydrocarbons C_6 - C_9 (µg/L)			20
Hydrocarbons C_{10} - C_{36} (µg/L)			100

a. Water quality conditions of the Fitzroy Basin (including the lower Dawson, lower Mackenzie and Fitzroy Rivers) measured in 1964–2013 (DEHP 2015).

b. Trigger values based on Queensland Water Quality Guidelines (DEHP 2009) and ANZECC (2000) guidelines for the protection of 95% of species in slightly to moderately disturbed systems.

c. Site-specific Environmental Authority release limits and trigger levels (Oct. 2015).

There are also several research programs currently being carried out by university researchers. These include prior research at CQUniversity focusing on the environmental impacts of waters being discharged from coal mines, the development of tools to assess the health of ephemeral streams affected by mining, as well as the aquatic ecology and health of the Fitzroy River system (Noble et al. 1996a; Flint et al. 2012). In recent years, there has been considerable focus directed towards understanding the risks associated with elevated salinity, resulting in a comprehensive report into the effects of increased salinity from coal mine water discharge on streams in the Fitzroy Catchment (Prasad et al. 2012). For this reason, researchers at the University of Queensland have focused their research on the impacts of sulfate in saline mine water discharge, as well as the overall effects of saline mine discharge, on microbial communities and biogeochemical cycling in the Fitzroy Basin (Vink et al. 2009; Vink and Robbins 2012). Despite the multitude of research efforts focused on understanding the environmental impacts of mining activities in the Fitzroy Basin, there has been a very limited focus directed at understanding the potential for sub-lethal effects resulting from exposures to coal mine wastewater.

As outlined, the major focus of monitoring efforts in the Fitzroy Basin has been largely directed towards assessing primary water quality and identifying thresholds of acute toxicity. There have been almost no studies investigating the chronic toxicological effects of coal mining discharge on native aquatic vertebrates (*i.e.*, fish, amphibians) relevant to the Fitzroy Basin (Hart et al. 2008). As such, there is an urgent need for a broad assessment of potential sub-lethal and longer-term toxicological effects in a range of aquatic organisms inhabiting these waters. Traditionally, this type of investigation would focus predominantly on acute and chronic toxicity bioassays to determine median lethal concentrations (LC50s), and on direct effects of chronic exposures on growth, development and reproduction. More recently, the focus of many broad toxicity evaluations has expanded to include sub-lethal responses that hold relevance for influencing higher-level biological interactions (Groh et al. 2015). This type of approach may therefore prove extremely informative for understanding the potential for coal mining operations in the Bowen Basin to adversely influence sensitive aquatic organisms.

Following major flooding events of 2008, the Queensland government released two reports detailing the potential consequences of mine releases and the cumulative impacts of mining on water quality in the Fitzroy Basin (Hart et al. 2008; DERM 2009). The cumulative impact assessment released by the Queensland Department of Environment and Resource Management (DERM) concluded that mining discharge into the Fitzroy Basin catchment is not protective of the downstream environment, and that data is extremely limited and therefore inadequate for quantifying cumulative impacts (DERM 2009). One specific area where information was particularly deemed insufficient involved the assessment of potential chronic and sub-lethal effects of coal mine wastewater on native aquatic organisms. One particular area of emerging interest in sub-lethal toxicology testing involves examining the potential for key environmental contaminants, such as mine wastewater, to influence behavioural responses in aquatic organisms inhabiting receiving environments (Melvin and Wilson 2013; Bae and Park 2014). Recent advancements in behavioural toxicology, and the knowledge that animal behaviours often correlate well with endpoints of broader ecological relevance (Sarria et al. 2011; Groh et al. 2015), mean that novel approaches to toxicity testing can and should be developed.

This thesis investigates the use of novel approaches to toxicity testing using innovative computer-based behavioural analysis, to assist the mining industry with toxicological monitoring of wastewater discharges and the rapid identification of unacceptable levels of contamination in natural waterways receiving coal mine water releases. Techniques for behavioural analysis are not particularly new to environmental toxicology, but recent evidence indicates that they may be extremely beneficial tools for broad sub-lethal toxicity evaluations (Melvin and Wilson 2013). This has sparked considerable international interest in behavioural toxicology, and it is expected that this approach will provide new insights on how animals might respond to coal mine wastewater. Despite the fact that behavioural analysis represents a novel tool in the assessment of sub-lethal toxicity associated with coal mine wastewater, it is anticipated that the methodologies developed through this research will yield effective monitoring strategies that are fast, sensitive and ecologically relevant to the local environment. Given the relatively low volumes of wastewater commonly released by mining operations, gaining information regarding sensitive sub-lethal endpoints and developing tools to assess these endpoints is critical, as this will (i) help to ensure that discharge limits provide thorough protection of the downstream environment and (ii) ensure that approved limits are being met.

1.2 Thesis objectives

The key objective of this thesis is to provide information regarding the potential for toxicity and sub-lethal effects of coal mine wastewater on a range of native aquatic organisms of Central Queensland. Information will be broadly applicable to coal mining operations in the Bowen Basin and throughout Central Queensland, and the concepts developed will be broadly applicable to toxicity testing of wastewater discharges. Specific knowledge gaps will be filled regarding the chronic and sub-lethal effects of coal mine wastewater on development, fundamental behaviours, and bioaccumulation in native aquatic vertebrate communities in the receiving environment.

The specific objectives of thesis were to:

1) review the existing literature describing the environmental impacts of coal mining wastewater;

2) compare sensitivities of locally relevant aquatic invertebrates and vertebrate species to coal mine wastewater to identify candidate species for effective monitoring of water quality and toxicity risk in Central Queensland;

3) investigate behavioural responses of native organisms to coal mine wastewater, to evaluate its potential use for evaluating sub-lethal effects associated with wastewater releases on freshwater ecosystems; and

4) investigate potential associations between toxicological outcomes and metal(loid) bioconcentration in exposed individuals.

1.3 Thesis outline

The thesis is presented as individual papers, each addressing at least one of the objectives listed above.

Chapter 1 (this chapter) presents a brief introduction to coal mining and its environmental impacts in Australia. The chapter also broadly outlines existing knowledge gaps related to the toxicity of coal mine wastewater and defines the objectives and structure of the thesis.

Chapter 2 presents a comprehensive literature review of the environmental impacts of coal mining, with a particular emphasis on the effects of coal mine wastewater to aquatic biota, thereby addressing objective 1. Throughout this review, the need for research on potential sub-lethal and longer-term toxicological effects of coal mine wastewater in local species is clearly illustrated, reinforcing the rationale for the thesis. Specific knowledge gaps were addressed through a series of experiments presented in Chapters 3 to 6.

Chapter 3 compares the sensitivity of a range of native aquatic invertebrates and vertebrate species exposed to wastewater from an operating coal mine in Central Queensland. This chapter directly addresses objective 2 and represents an important preliminary step for planning the main experimental components of the thesis. The chapter was published in Ecotoxicology and Environmental Safety. Results from this chapter successfully identified several candidate species that were used for subsequent behavioural experimentation, as presented in chapters 4 to 6.

Two different techniques (*i.e.*, visual and non-visual) were employed to monitor sub-lethal behavioural responses in fish and tadpoles, and the resulting data produced three manuscripts (Chapters 4, 5 and 6).

Chapter 4 investigates behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater using the Multispecies Freshwater Biomonitor®, and evaluates the potential applicability of this equipment to monitor wastewater discharges. This chapter addresses objective 3, and was published in Ecotoxicology and Environmental Safety.

Chapter 5 investigates the effects of coal mine wastewater on behavioural responses and swimming performance of empire gudgeons (*H. compressa*), this time using Ethovision® XT video-tracking software, again addressing objective 3. This was published in the journal Chemosphere.

Chapter 6 evaluates the chronic effects of coal mine wastewater on survival, growth, development and behaviour of striped marsh frog tadpoles (*Limnodynastes peronii*), and investigates associations between toxicological outcomes and metal(loid) bioconcentration in exposed individuals. This chapter addresses objectives 3 and 4, and was published in Aquatic Toxicology.

Finally, Chapter 7 provides a summary of the overall findings of this thesis, particularly those acquired through the experimental work (*i.e.*, Chapters 3 to 6), and presents recommendations for further research. References are provided at the end of each chapter.

1.4 References

ANZECC and ARMCANZ, 2000. Australian and New Zealand Guidelines for Fresh and Marine Waters. Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand, Canberra.

Bae, M.-J., and Park, Y.-S. (2014). Biological early warning system based on the responses of aquatic organisms to disturbances: A review. *Science of The Total Environment* **466-467**, 635–649.

DEH and DNR (1999). Testing the Waters. Queensland Government, Brisbane, Australia.

DEHP (2009). Queensland Water Quality Guidelines, Version 3. Department of Environment and Heritage Protection.

DEHP (2015). Draft environmental impact statement, Volume 1 – Chapter 11 Water quality. Department of Environment and Heritage Protection

DERM (2001). Australia-wide assessment of river health: Queensland AusRivAS sampling and processing manual (No.12). Commonwealth of Australia and QLD Department of Natural

Resources and Mines, Canberra and Rocklea.

DERM (2009). A study of the cumulative impacts on water quality of mining activities in the Fitzroy River Basin. Department of Environment and Resource Management.

DERM (2010). Fitzroy Basin Draft Water Resource Plan Environmental Assessment - Stage 2 Assessment report. State of Queensland, Department of Environment and Resource Management.

DERM (2011a). Developing water quality guidelines for the protection of the freshwater aquatic ecosystems in the Fitzroy Basin. Department of Environment and Resource Management.

DERM (2011b). Fish Water Quality Guidelines for Fitzroy Basin Freshwaters. Department of Environment and Resource Management.

DNRM (2013). Queensland coal statistics report for the financial year ended June 2013. Department of Natural Resources and Mine, Queensland Government.

Douglas, G. B., Ford, P. W., Palmer, M. R., Noble, R. M., and Packett, R. J. (2005). Identification of sediment sources in the Fitzroy River Basin and Estuary, Queensland, Australia. Cooperative Research Centre for Coastal Zone, Estuary and Waterway Management.

Douglas, G. B., Ford, P. W., Palmer, M. R., Noble, R. M., Packett, R. J., and Krull, E. S. (2008). Fitzroy River Basin, Queensland, Australia. IV. Identification of flood sediment sources in the Fitzroy River. *Environmental Chemistry* **5**, 243.

FBA (2008). Fitzroy Basin water quality improvement report. Fitzroy Basin Association Inc.

FBA (2009). Priority neighbourhood catchments water quality monitoring program; 2005-09. Fitzroy Basin Association Inc.

Flint, N., Rolfe, J., Jones, C., Sellens, C., Rose, A., and Fabbro, L. D. (2012). Technical Review for the Development of an Ecosystem Health Index and Report Card for the Fitzroy Partnership for River Health. Centre for Environmental Management, Central Queensland University, Rochkampton, AU.

Groh, K. J., Carvalho, R. N., Chipman, J. K., Denslow, N. D., Halder, M., Murphy, C. A., Roelofs, D., Rolaki, A., Schirmer, K., and Watanabe, K. H. (2015). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. *Chemosphere* **120**, 778–792.

Hart, B. T., Greenfield, P., and Pascoe, M. (2008). Review of the Fitzroy River Water Quality Issues. Water Science Pty Ltd and Water Studies Centre, Monash University.

Höök, M., Zittel, W., Schindler, J., and Aleklett, K. (2010). Global coal production outlooks based on a logistic model. *Fuel* **89**, 3546–3558.

IEA (2012). Coal information. International Energy Agency.

Melvin, S. D., and Wilson, S. P. (2013). The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. *Chemosphere* **93**, 2217–2223.

Noble, R. M., Duivenvoorden, L. J., Rummenie, S. K., Long, P. E., and Fabbro, L. D. (1996).

Downstream effects of land use in the Fitzroy Catchment. The State of Queensland, Department of Natural Resources.

Noble, R. M., Rummenie, S. K., Long, P. E., Fabbro, L. D., and Duivenvoorden, L. J. (1996). The Fitzroy River catchment-An assessment of the condition of the riverine system. In: 8th Australian Agronomy Conference, Toowoomba.

Prasad, R., Vink, S., Mann, R. M., Nanjappa, V., and Choy, S. C. (2012). Assessing the ecotoxicology of salinity on organisms in seasonally flowing streams in the Fitzroy Catchment. The State of Queensland.

Queensland Government (2010). Central Queensland Coal. The State of Queensland - Department of Employment, Economic Development and Innovation.

Sarria, M. P., Soares, J., Vieira, M. N., Filipe, L., Castro, C., Santos, M. M., and Monteiro, N. M. (2011). Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles. *Chemosphere* **85**, 1543–1547.

Scott, B., Ranjith, P. G., Choi, S. K., and Khandelwal, M. (2010). A review on existing opencast coal mining methods within Australia. *Journal of Mining Science* **46**, 280–297.

Vink, S., and Robbins, S. (2012). Mine water management in variable climate regimes. In: International Mine Water Association Symposium. pp. 20A–20I

Vink, S., Moran, C. J., Golding, S. D., Baublys, K., and Nanjappa, V. (2009). Understanding mine site water and salt dynamics to support integrated water quality and quantity management. *Mining Technology* **118**, 185–192.

Chapter 2

Environmental impacts of coal mine wastewater

Coal mining can result in a wide range of adverse impacts on natural animals and ecosystem health. Broad environmental issues associated with coal-mining operations can include landscape destruction and alteration, air pollution and water pollution (Bian et al. 2010). Importantly, such environmental impacts are not only limited to direct effects of coal excavation, but might also result from the transportation, processing (*i.e.*, crushing, screening and washing), and utilisation of coal (Bian et al. 2010). The diverse consequences of coal mining operations on natural environments highlight a need to develop and improved our understanding of the risks of local mining operations on native organisms. Though all aspects of coal mining can contribute to the deterioration of native ecosystems, this review was focused on water pollution.

Water management is a key environmental issue related to opencast coal mining operations (Vink and Robbins 2012), and water consumption and pollution are two of the key challenges for effective water management. The coal industry requires a constant supply of water for dust suppression, coal washing, as well as cooling systems in power pants. This can leave mining operations with large quantities of wastewater that needs to be managed (Scott et al. 2010; Haibin and Zhenling 2010; Thiruvenkatachari et al. 2011). Mines will often collect and reuse water whenever it is economically feasible, however water reuse must be closely managed as it can lead to increased salinity, acidity, alkalinity or accumulation of other contaminants over time that may impact licensed discharge requirements (Vink et al. 2009; Thiruvenkatachari et al. 2011). The central goal of meeting discharge requirements is to protect downstream aquatic environments and the animals that inhabit them, and it has recently been concluded that this goal is not being effectively realised (DERM 2009), though efforts are being made to revise guidelines (DERM, 2010; DNRM 2015). In order to effectively expand our understanding of safe discharge limits for protecting native aquatic animals, the first step is to collate the existing information on the toxicity and environmental harm of coal mining wastewater to help identify existing knowledge gaps.

This review serves to provide 1) an overview of how coal mining activities influence water quality, and 2) a summary of the existing research describing the adverse toxicological effects associated with exposures of aquatic organisms to mine wastewater.

2.1 Characteristics of coal mine wastewater

One of the primary eco-toxicological concerns surrounding coal mining involves substantial volumes of mine wastewater being discharged into the environment. The release of coal mine wastewater (CMW) is often restricted to periods of high rainfall, but uncontrolled release may also occur with extreme rainfall events and flooding. The characteristics of mine water depend largely on the composition of the coal (*e.g.*, pyrite content) (Tiwary 2001). CMW can be saline and acidic, and may often contain high levels of dissolved solids, suspended solids, heavy metals, hydrocarbons and other compounds that can cause surface and ground water contamination (Tiwary 2001; Thiruvenkatachari et al. 2011) and may therefore pose a risk to aquatic wildlife (Figure 1; see Chapter 1 Table 1.1 for typical water quality values, guidelines and release limits). The following sub-sections review mine water characteristics of particular importance and provide an overview of the relevant research surrounding their environmental impacts.

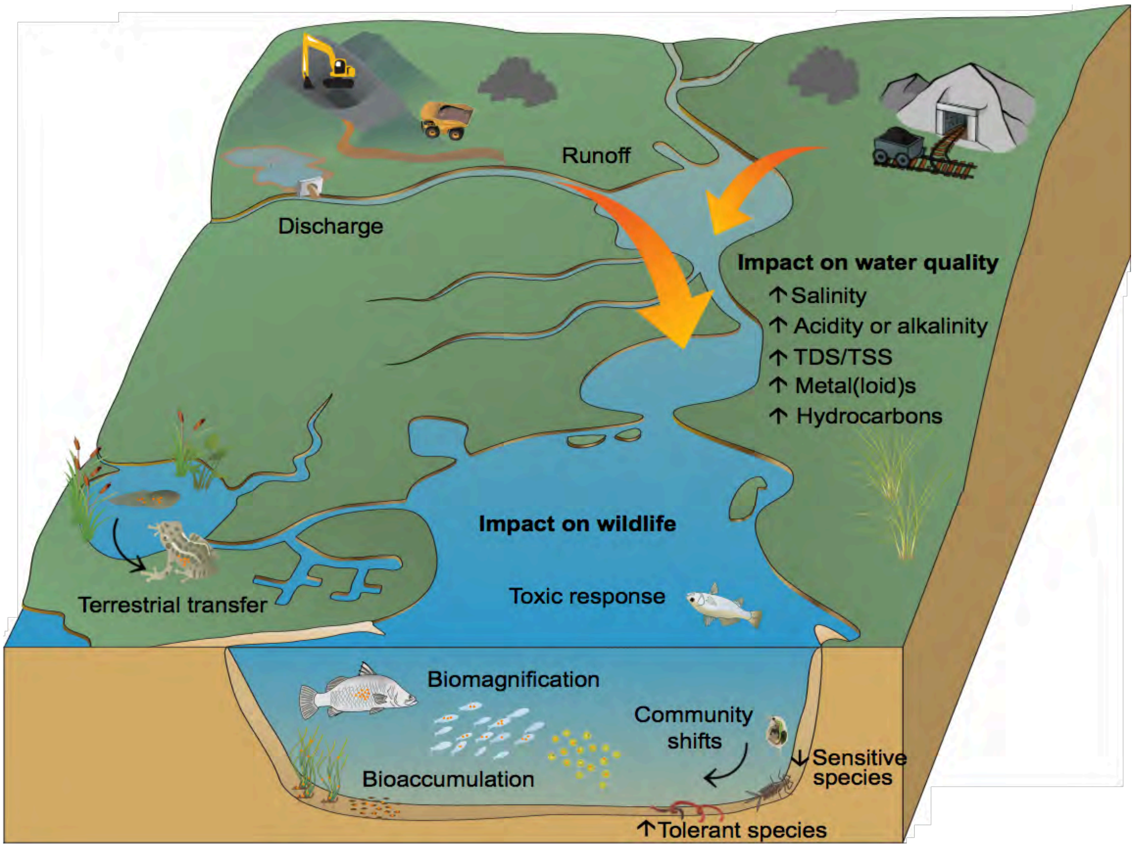

Figure 2-1: Impacts of coal mining on downstream water quality and wildlife. Orange dots represent accumulated or adsorbed toxic elements and compounds from coal mine wastewater. The symbols used are courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

2.1.1 Salinity

Salinity is defined as the total concentration of dissolved salts (including sodium, calcium, magnesium, potassium, sulfate, carbonate, bicarbonate and chloride ions) in water or soil, and is expressed in terms of concentration (mg/L) (Cañedo-Argüelles et al. 2013). Salinity is often estimated by measuring electrical conductivity (EC) due to the practicality of this method. In many parts of the world, freshwater systems are naturally saline but their salinisation has been exacerbated by anthropogenic activities such as irrigation, landscape clearing, de-icing, industrial discharge and mining (Cañedo-Argüelles et al. 2013). Anthropogenic salinisation (also referred to as secondary salinisation) represents a global and growing threat and is an important cause of degrading freshwater quality, declining aquatic biodiversity and shifting ecosystem dynamics (Hart et al. 1991). Salinity is a particularly important environmental concern in Australia (Hart et al. 1990; Land and Water Australia 2007) due to the seasonally hot dry climate in which evaporation and transpiration can lead to increased concentrations of dissolved ions (Vink et al. 2015). Recycled water from Australian coal mining operations where water-reuse programs have been implemented to reduce freshwater consumption is more saline, increasing the risks of secondary salinisation in adjacent ecosystems (Prasad et al. 2012). The impacts of salinisation on freshwater systems have been reviewed extensively (Hart et al. 1991; Nielsen et al. 2003; Dunlop et al. 2005; Cañedo-Argüelles et al. 2013). Generally, these reviews conclude that the impacts of salinity vary between organisms and are dependent on the ionic composition, dose and duration of exposure, as well as the organisms' physiological and morphological characteristics.

Altered salinity can be stressful to freshwater organisms, since they need to actively maintain their internal osmotic pressure relative to their external environment and can only tolerate certain ranges of salinity. Tolerance to changes in salinity can be highly variable amongst organisms. Invertebrates use passive osmoregulatory mechanisms to regulate their internal ionic concentrations and their tolerance is generally dependent on this concentration (Hart et al. 1991). On the other hand, aquatic vertebrates, such as fish and tadpoles, use active ion transport to maintain internal ionic concentrations, which requires high-energy expenditure to maintain optimal internal conditions. Rapid increases in external salinity can lead to osmotic imbalance or failure of osmoregulatory mechanisms, which can result in physiological and behavioural changes as well as loss of cellular function and utilimately can lead to death (Hart et al. 1990).

Species richness and biodiversity are often negatively correlated with salinity levels (García-Criado et al. 1999; Kennedy et al. 2003; Piscart et al. 2005; Pond 2010; Kefford et al. 2011), and salinity is therefore often used as an indicator of pollution for environmental regulation of freshwater rivers and streams. As salinity levels increase, communities dominated by salinity-sensitive taxa are replaced by more tolerant taxa, thus altering the ecosystem structure (reviewed by Hart et al. 2003; Cañedo-Argüelles et al. 2013). Consequently, salinisation above certain thresholds can elicit direct toxicological effects, but also indirect ecological effects on freshwater organisms and ecosystems (Zalizniak et al. 2006; Wood and Welch 2015).

2.1.2 Acidity or alkalinity

Acid mine drainage (AMD) and alkaline mine drainage are common environmental issues related to both active and abandoned mines. AMD is caused by the oxidation of pyrite and sulphides from mine wastes, and can be released when coal with high sulfur content comes in contact with air or water (Gray 1997). Significant contamination can occur from AMD, resulting in serious hazards to aquatic animals in the receiving environment (Salomons 1995; Gray 1997; Sarmiento et al. 2011). Toxicity primarily results from the acidification of natural surface water, and also from increased levels of dissolved and suspended solids and/or the introduction of various heavy metals. Several studies have investigated the environmental impacts of AMD.

Monitoring studies have shown drastic reductions in diversity and abundance of aquatic organisms, as well as increases in tolerant species in sites affected by AMD (Roback and Richardson 1969; Soucek et al. 2000a; 2000b; DeNicola and Stapleton 2002; Tripole et al. 2006). Field and laboratory toxicological studies have revealed acute and chronic lethality of AMD-impacted water to fish (Henry et al. 1999; Barry et al. 2000) and invertebrate species (Soucek et al. 2000b; Cherry et al. 2001; DeNicola and Stapleton 2002; Chapman and Simpson 2005; Lin et al. 2007; Mohti et al. 2012; Holland et al. 2012; Netto et al. 2013). Other studies investigating sub-lethal impacts of AMD have reported genotoxicity (Geremias et al. 2012; Netto et al. 2013) and stress related behavioural effects (Janssens de Bisthoven et al. 2004; Gerhardt et al. 2004; 2005a; 2005b; Mohti et al. 2012; Seo et al. 2012) in various aquatic species. In general, lethal and sublethal toxicity of AMD were found to be largely pH dependent and to increase with decreasing pH. A few studies have therefore concluded that pH is the best predictor of water toxicity (Soucek et al. 2000b; Chapman and Simpson 2005). However, environmental impacts of AMD are complex and are not limited to acidification.

In other instances, coal mining can result in increased alkalinity of downstream environments. Neutral or alkaline mine drainage often occurs in environments with proton buffering capacity, such as elevated hardness and sulfate levels. This is the case in the Fitzroy River catchment, which has a predominately alkaline geology (DERM 2009). Importantly, increases in alkalinity can reduce the solubility of toxic metals in the environment, thereby influencing their bioavailability to aquatic organisms (Riethmuller et al. 2001; Adhikari et al. 2006).

2.1.3 Dissolved and suspended solids

Total dissolved solids (TDS) and total suspended solids (TSS) are often used as indicators of freshwater quality (DEHP 2013). TDS is an integrated measure of all organic and inorganic constituents dissolved in water including minerals, salts, metals, cations and anions. The concentration of TDS in water is often approximated by applying a conversion factor to electrical conductivity (EC) measurements. However, the relationship between TDS and EC is not directly linear, and conversion factors are based on the ionic composition of the water (Walton 1989). TSS, on the other hand, is a measure of all organic or inorganic suspended particulates in water and represents the weight of particulates per volume of water. Turbidity can be used as an indirect measurement of TSS, by measuring the amount of light scattered and absorbed from the water. TDS and TSS may therefore be useful indicators of the potential toxicity of mine water.

Several essential elements and compounds commonly found in aquatic ecosystems can cause adverse effects to aquatic organisms when present at concentrations outside their range of physiological tolerance. For example, most aquatic organisms expend a great deal of metabolic energy to regulate internal water and ion concentrations. Thus, external changes in ion concentration and composition can have negative effects on aquatic organisms by causing osmotic stress and increasing energetic demands (Goodfellow et al. 2000). Elevated TDS has been shown to correlate with stress to aquatic organisms (Kennedy et al. 2003), but toxicological effects relating to dissolved ions are not always easily predictable from TDS alone (Chapman et al. 2000). This can be explained by the fact that different ions may have different mechanisms of action, thus variations in specific combinations and concentrations of ions can influence toxicity.

Mine wastewater typically contains high levels of TDS and TSS (Thiruvenkatachari et al. 2011), which can have adverse effects on water quality when discharged into the receiving environment. A laboratory study exposing benthic macroinvertebrates (*Ceriodaphnia dubia*) and freshwater fish (*Pimephales promelas*) to water impacted by coal-processing found that acute and chronic toxicity effects were correlated to TDS (Kennedy et al. 2003). Similarly, short-term chronic exposure to synthetic effluent representing the ionic composition of mine discharge (Na^+, Ca^{2+}, K^+, Mg^{2+}, SO_4^{2+}, Cl^-) reduced growth and survival of larval chironomids at concentrations above 1,100 mg of TDS/L (Chapman et al. 2000). TSS, on the other hand, can have direct and indirect effects on aquatic life by diminishing light penetration, which impacts photosynthesis and thereby reduces dissolved oxygen levels (Tiwary 2001; Bilotta and Brazier 2008). TSS also reduces visibility, which can impact feeding and reproduction, and can cause smothering by clogging respiratory organs of aquatic organisms (Tiwary 2001; Bilotta and Brazier 2008). The environmental impacts of dissolved and suspended solids related to CMW releases are very complex and depend not only on the concentration of each specific constituents but also the overall composition of the mine discharge and its interactions with the receiving environment.

2.1.4 Heavy metals

Heavy metals occur naturally in the environment and can be beneficial to animals at low concentrations. However, anthropogenic activities such as mining can introduce high loads of toxic metals into the environment, including Al, As, Cd, Cr, Co, Cu, Fe, Hg, Mn, Ni, Se, Pb and Zn, which can lead to detrimental effects on aquatic wildlife. Extensive data exist on the toxicity of heavy metals to aquatic organisms (reviewed by Gerhardt 1993; Golovanova 2008; Langdon et al. 2009), and these data demonstrate that the level of toxicity for a given species depends not only on the chemical nature, dose and exposure duration of the heavy metals, but also on the experimental conditions and biotic and abiotic factors of the local environment. The physicochemical properties of the aquatic environment (*i.e.*, pH, hardness, dissolved organic carbon, temperature) can have a considerable impact on the toxicity and bioavailability of metals (Pascoe et al. 1986; Horne and Dunson 1995; Heijerick et al. 2003).

Mining represents an important source of metal contamination in the environment, and this plays an important role in AMD toxicity (Salomons 1995; Banks et al. 1997). The link between metal toxicity and acidity has been shown in various studies. For example, Mohti et al. (2012) found a decrease in locomotor activity of the fish *Poecilia reticulata* and the prawn *Macrobrachium lanchesteri* with increasing metal concentration in AMD. Trace metals in AMD can also act synergistically to affect aquatic wildlife (Gerhardt 1993). Various mixtures of metals can elicit acute and chronic toxicity as well as other physiological effects in aquatic organisms (reviewed by Atchison et al. 1987; Vijver et al. 2011). For example, Loumbourdis et al. (2007) investigated the effects of a Cr and Cd mixture on adult frogs (*Rana ridibunda*) and found that Cr concentrations in the kidney was doubled when Cd was present and that the concentration of metallothioneins (proteins involved in the detoxification of heavy metals) correlated positively with Cd concentrations in both the liver and the gut of animals exposed to a mixture.

Due to their persistence in the environment, the potential for bioaccumulation and biomagnification of metals is a particular concern. It is possible for aquatic organisms to accumulate heavy metals in various tissues and organs at much higher concentrations than are present in the environment (Loumbourdis and Wray 1998; Rainbow 2002; Roe et al. 2005; Unrine et al. 2007). For example, elevated levels of heavy metals were found in whole bodies of several frog species at different life stages (larvae, recent metamorphs and adults) when exposed to coal combustion waste discharge (Hopkins et al. 2000; Snodgrass et al. 2004; Roe et al. 2005).

Studies investigating bioaccumulation and biomagnification of heavy metals have found that concentrations are dependent on both abiotic factors such as season, pH and temperature, and also biotic factors such as the organism's gender, size, mode of feeding and physiological state (Rainbow 2002; Pourang et al. 2004; Roe et al. 2005; Unrine et al. 2007). In many cases, metal accumulation in tissues is greater than metal elimination, suggesting that once metals have accumulated they are difficult to eliminate from the body (Soegianto et al. 2013). Accumulation of heavy metals in tissues and organs can be severely damaging over time (Soegianto et al. 2013; Zocche et al. 2013). Morphological and physiological-biochemical effects of heavy metals (e.g., Cd, Hg, Zn, Fe, Pb, Cu) in aquatic organisms include (but are not limited to) tissue damage, decreased immunity state, changes in behaviour, growth rate and nutritional state, digestive enzyme activities, efficiency of food assimilation, state of carbohydrate metabolism, teratogenic, mutagenic and gonadotoxic effects, damage to lipid, protein, and peptide metabolism, as well as effects on productivity and life cycles (reviewed by Gerhardt 1993; Golovanova 2008).

2.1.5 Hydrocarbons

Hydrocarbons are organic compounds composed of carbon and hydrogen atoms with a chemical structure containing at least two aromatic rings (Eisler 1987). These compounds are mostly hydrophobic (except naphthalene) and can attach to particulate matter and accumulate in aquatic organisms. Hydrocarbons form fossil fuels, including coal, petroleum and natural gas, and are thus valuable since these represent primary energy sources. Hydrocarbons are also used in the production of plastics, paraffin, waxes, solvents, oil and various other compounds of economic importance.

Polycyclic aromatic hydrocarbons (PAHs) are a class of hydrocarbons and represent a diverse group of organic compounds. PAHs are formed by both the natural and anthropogenic combustion of organic material, such as coal, oil, petrol or wood under oxygen-deficient conditions (Eisler 1987). PAHs are classified as persistent organic pollutants (POPs) (Sánchez-Bayo et al., 2011) and are a particular concern due to their environmental persistence, potential to bioaccumulate, and their known toxicological properties, such as mutagenic, teratogenic or carcinogenic effects (reviewed by Suess 1976; Eisler 1987; Arfsten et al. 1996; Santodonato 1997; Pickering 1999; Xue and Warshawsky 2005; Reynaud and Deschaux 2006). Anthropogenic sources of hydrocarbons, such as mining effluent and coal combustion, can contain various levels and complex mixtures of PAHs, which can cause acute or chronic toxicity in exposed aquatic organisms (Oris and Giesy 1987; Erickson et al. 1999; Lampi et al. 2005; Diamond et al. 2006). For example, several studies have reported changes in copepod feeding, swimming and stress behaviour in response to PAH exposures (Seuront and Leterme 2007; Seuront 2010; Michalec et al. 2013).

2.2 Effects of coal mine wastewater on aquatic biota

As discussed in section 2.1, wastewater generated by coal mining activities contains complex mixtures of assorted contaminants. This raises concerns about possible threats to aquatic biota that may result from surface or ground water contamination, which can occur through both planned and accidental discharge events (Tiwary 2001; Thiruvenkatachari et al. 2011). Despite this known toxicity and risk of exposure, relatively few studies have explored the consequences of exposure to mine wastewater on aquatic organisms. The following sub-sections review the existing literature on the impacts of CMW on aquatic invertebrate, fish and amphibian species.

2.2.1 Invertebrates

Whole effluent toxicity tests are often used to assess the toxicity and effects of complex effluents to a range of aquatic organisms. Several studies have assessed the toxicity of wastewaters from a range of underground, surface and abandoned coal mines to several invertebrate species in acute and chronic laboratory assays, though limited information is available for Australian species (Table 2-1). Tests reveal aquatic invertebrates as generally being sensitive to environmental stress, although large inter-species variations in sensitivity and tolerance are evident. Differences in species sensitivities are generally dependent on differences in osmoregulation, excretion and detoxification capabilities, which enable organisms to cope with changes in water quality differently. Notwithstanding the known differences in sensitivity, studies have generally reported reduced survival in cladocerans (Soucek et al. 2000a; Cherry et al. 2001; Soucek et al. 2001a; Kennedy et al. 2003; Lattuada et al. 2009; Echols et al. 2010; Mitchelmore 2010; Seo et al. 2012; Netto et al. 2013), brine shrimps (Geremias et al. 2003; Defaveri et al. 2009; Netto et al. 2013), freshwater shrimp (Cardno Ecology Lab Pty Ltd 2010) and mayfly nymphs (Diamond et al. 1992; O'Halloran et al. 2008; Echols et al. 2010; Cardno Ecology Lab Pty Ltd 2010). For example, acute lethality values (for electrical conductivity) of CMW ranged between 1962 and > 2980 µS/cm for *Paratya australiensis* and > 1698 µS/cm for *Atalophlebia* sp. (LC_{50}-96 h, Cardno Ecology Lab Pty Ltd 2010) and 6713 – 7142 µS/cm for *Ceriodaphnia dubia* (LC_{50}-48 h, Kennedy et al. 2003; Kennedy and Cherry 2005). *In situ* field studies have found increased mortality of Asian clams *Corbicula fluminea* (Soucek et al. 2000a; Cherry et al. 2001; Soucek et al. 2001b; Hull et al. 2006; Simon et al. 2006) and caddisflies *Hydropsyche* sp. (DeNicola and Stapleton 2002) at sites contaminated by coal mining compared to reference sites. Very few studies have reported no significant differences in survival between control animals and those exposed to coal mine water (Hull et al. 2006; Simon et al. 2006; OEH 2012).

Many studies have also shown that coal mining activities negatively impact freshwater invertebrate communities (Table 2-2). Generally, studies have reported reduced invertebrate family and taxon richness, density and abundance at contaminated sites compared to reference sites. In several cases, shifts in population structure in coal mine-impacted sites were reported, with a decrease in sensitive species (*e.g.*, Ephemeroptera, Plecoptera, Trichoptera (EPT)) and increase in tolerant species (*e.g.*, Chironomidae) (Warner 1971; Letterman and Mitsch 1978; Moon and Lucostic 1979; Scullion and Edwards 1980a; Radwan et al. 1991; Jarvis and Younger 1997; Kennedy et al. 2004; Bruns 2005; Batty et al. 2005; 2007; Ross et al. 2008; Petruck and Stöffler 2011; Bernhardt et al. 2012). Changes in invertebrate community structures can sometimes be correlated with specific water quality parameters. For example, Winterbourn (1998) found that numbers of invertebrate and EPT taxa were negatively correlated with water acidity. Similarly, Timpano et al. (2010) found that EPT richness decreased with elevated TDS levels, and in other studies, decreased taxon richness and abundance was correlated with increased electrical conductivity (Hartman et al. 2005; Proctor and Grigg 2006; Cardno Ecology Lab Pty Ltd 2010).

In many cases, invertebrate population indices and multimetric indices are used to assess ecosystem health. Several studies have identified lower index values in sites contaminated by coal mining compared to reference sites. Specifically, lower scores for Brillouin's H diversity index (Matter and Ney 1981), Shannon-Weaver diversity index (Letterman and Mitsch 1978; Moon and Lucostic 1979; Chadwick and Canton 1983; Simmons et al. 2005; Pond et al. 2008; Ross et al. 2008), Stream Condition Index (Green et al. 2000), Macroinvertebrate Impairment Score (Smucker and Vis 2009), Modified Beck Index (Moon and Lucostic 1979) and Macroinvertebrate Aggregated Index for Streams (Bott et al. 2012) have been observed at polluted sites. The Hilsenhoff Biotic Index, on the other hand, is used to assess the overall tolerance of a community, and was found to be higher in coal-impacted sites, indicating a reduction in sensitive species and increase in tolerant species (Merricks et al. 2007). In Australia, the Australian River Assessment System (AusRivAS) can be used to compare macroinvertebrate communities from impacted sites to predicted reference conditions. Stream assessments in regions impacted by coal mining have generally found impaired macroinvertebrate communities relative to AusRivAS reference conditions (The Ecology Lab Pty Ltd 2008; Bio-Analysis Pty Ltd 2009; Jones et al. 2013; Cardno Ecology Lab Pty Ltd 2013).

Table 2-1: Toxicity information available for Australian freshwater fauna exposed to coal mine wastewater in laboratory studies.

Species	Endpoint	Mine[a]	Treatment effluent	Dilutions (%)	Days	Type[b]	EC_{10} (µS/cm)[c]	EC_{50} (µS/cm)[c]	NOEC % (µS/cm)[d]	Ref[f]
Invertebrates										
Atalophlebia spp. (nymphs)	Immobility	WC	Discharge point (LPD10)	0, 7.5, 15, 30, 60, 100	10	SR	690	2426	15% (503)	[1]
Atalophlebia spp. (nymphs)	Immobility	RW	Dam	0, 1, 2, 5, 10, 20	10	SR	587	1478	1% (575)	[1]
Atalophlebia spp. (nymphs)	Immobility	TM	Weir (Tea Tree Hollow)	0, 7.5, 15, 30, 60, 100	10	SR	310	763	7.5% (377)	[1]
Ceriodaphnia cf. dubia	Reproduction	WC	Discharge point (LPD10)	0, 6, 12, 25, 50, 100	7	SR	537	944	12% (448)	[1]
Ceriodaphnia cf. dubia	Reproduction	RW	Dam	0, 6, 12, 25, 50, 75, 100	7	SR	2366	3829	25% (1582)	[1]
Ceriodaphnia cf. dubia	Reproduction	TM	Weir (Tea Tree Hollow)	0, 6, 12, 25, 50, 100	7	SR	1041	1525	50% (999)	[1]
Ceriodaphnia dubia	Reproduction	WC	Downstream (LPD12)	0, 10, 30, 100	7	SR	-	-	<30-100% (≤1300[e])	[2]
Ceriodaphnia dubia	Reproduction	WC	Discharge point (LPD10)	0, 10, 30, 100	7	SR	-	-	30% (<1960[e])	[2]
Ceriodaphnia dubia	Reproduction	WC	Dam (Brennans Creek)	0, 10, 30, 100	7	SR	-	-	30% (<1580[e])	[2]
Ceriodaphnia dubia	Reproduction	WC	Upstream (LPD11)	0, 10, 30, 100	7	SR	-	-	30-100% (≤249[e])	[2]
Ceriodaphnia dubia	Survival	WC	Clarified process water	50, 100	2	S	-	-	50% (<2750[e])	[3]
Ceriodaphnia dubia	Survival	WC	Downstream (LPD12)	0, 10, 30, 100	2	S	-	-	100% (673-1300)	[2]
Ceriodaphnia dubia	Survival	WC	Discharge point (LPD10)	0, 10, 30, 100	2	S	-	-	100% (1640-1960)	[2]
Ceriodaphnia dubia	Survival	WC	Discharge point (LPD10)	100	2	S	-	-	100% (1860)	[3]
Ceriodaphnia dubia	Survival	WC	Dam (Brennans Creek)	0, 10, 30, 100	2	S	-	-	100% (1550-1720)	[2]
Ceriodaphnia dubia	Survival	WC	Dam (Brennans Creek)	100	2	S	-	-	100% (1643)	[3]
Ceriodaphnia dubia	Survival	WC	Untreated process water	25, 50, 100	2	S	-	-	50% (<3133[e])	[3]
Ceriodaphnia dubia	Survival	WC	Upstream (LPD11)	0, 10, 30, 100	2	S	-	-	100% (161-220)	[2]
Paratya australiensis	Immobility	WC	Discharge point (LPD10)	0, 7.5, 15, 30, 60, 100	10	SR	1406	2932	7.5% (316)	[1]
Paratya australiensis	Immobility	RW	Tailings dam	0, 1, 2, 5, 10, 20	10	SR	635	2717	2% (718)	[1]
Paratya australiensis	Immobility	TM	Weir (Tea Tree Hollow)	0, 6, 12.5, 25, 50, 100	10	SR	1067	1418	25% (841)	[1]
Paratya australiensis	Survival	WC	Clarified process water	50, 100	4	S	-	-	50% (<2750[e])	[3]
Paratya australiensis	Survival	WC	Downstream (LPD12)	0, 10, 30, 100	3	S	-	-	100% (1260)	[2]
Paratya australiensis	Survival	WC	Discharge point (LPD10)	0, 10, 30, 100	3	S	-	-	100% (1780)	[2]
Paratya australiensis	Survival	WC	Discharge point (LPD10)	100	4	S	-	-	< 100% (<1860[e])	[3]
Paratya australiensis	Survival	WC	Discharge point (LPD10)	100	4	SR	-	-	< 100% (<1860[e])	[3]
Paratya australiensis	Survival	WC	Dam (Brennans Creek)	0, 10, 30, 100	3	S	-	-	100% (1620)	[2]
Paratya australiensis	Survival	WC	Dam (Brennans Creek)	100	4	S	-	-	~100% (1643)	[3]
Paratya australiensis	Survival	WC	Untreated process water	25, 50, 100	4	S	-	-	50% (<3133[e])	[3]
Paratya australiensis	Survival	WC	Upstream (LPD11)	0, 10, 30, 100	3	S	-	-	100% (249)	[2]

Species	Endpoint	Mine[a]	Treatment effluent	Dilutions (%)	Days	Type[b]	EC_{10} (µS/cm)[c]	EC_{50} (µS/cm)[c]	NOEC % (µS/cm)[d]	Ref[f]
Fish										
Melanotaenia fluviatilis	Imbalance	WC	Discharge point (LPD10)	0, 7.5, 15, 30, 60, 100	10	SR	-	-	100% (1552)	[1]
Melanotaenia fluviatilis	Imbalance	RW	Dam	0, 7.5, 15, 30, 60, 100	10	SR	-	-	100% (5630)	[1]
Melanotaenia fluviatilis	Imbalance	TM	Weir (Tea Tree Hollow)	0, 7.5, 15, 30, 60, 100	10	SR	-	-	100% (1774)	[1]
Melanotaenia splendida slendida	Imbalance	WC	Discharge point (LPD10)	25, 50, 100	4	S	-	-	< 25% (<1860[e])	[3]
Melanotaenia splendida slendida	Imbalance	WC	Discharge point (LPD10)	100	4	SR	-	-	< 100% (<1860[e])	[3]
Melenotaenia duboulayi	Survival	WC	Downstream (LPD12)	0, 10, 30, 100	2-4	S	-	-	<30-100% (≤1300[e])	[2]
Melenotaenia duboulayi	Survival	WC	Dam (Brennans Creek)	0, 10, 30, 100	2-4	S	-	-	10-100% (≤1720[e])	[2]
Melenotaenia duboulayi	Survival	WC	Upstream (LPD11)	0, 10, 30, 100	2-4	S	-	-	100% (150-249)	[2]
Melenotaenia duboulayi (10-14 d)	Survival	WC	Discharge point (LPD10)	0, 10, 30, 100	2-4	S	-	-	100% (1910-1960)	[2]
Melenotaenia duboulayi (1-6 d)	Survival	WC	Discharge point (LPD10)	0, 10, 30, 100	4	S	-	-	10% (<1750[e])	[2]
Melenotaenia duboulayi (5 d)	Survival	WC	Discharge point (LPD10)	0, 10, 30, 100	3	S	-	-	100% (1780)	[2]

a. Mine names: WC, West Cliff Mine in Appin NSW; RW, Ravensworth Mine in Hunter Valley NSW; TM, Tahmoor Mine in Bargo NSW.
b. Type of exposure: S, static; SR, static renewal.
c. Effective Concentration (EC) values, reported as conductivity units (uS/cm), at which 10 and 50% effects occurred compared to control at the end of the exposure.
d. No Observable Effect Concentration (NOEC) presented as percent effluent and conductivity of effluent at NOEC presented in parentheses.
e. Conductivity value of the dilution treatments is less than the conductivity value of the undiluted effluent.
f. References: [1] Cardno Ecology Lab Pty Ltd 2010, [2] OEH 2012, [3] Short 2012.

Table 2-2: Impacts of coal mining on freshwater invertebrate communities.

Colliery name (type[a])	**Location[b]**	**Result summary[c]**	**Reference**
Appin (U)	NSW, AU	Sites were mostly severely impaired (ranged from significantly impaired to impoverished). Lower family diversity compared to AusRivAS model.	Bio-Analysis Pty Ltd 2009
Appin (U)	NSW, AU	Decreased taxa abundance and richness with increased site conductivity.	Cardno Ecology Lab Pty Ltd 2010
Canyon (AU)	NSW, AU	Decreased total abundance, family richness and EPT family richness. Increased Hydropsychidae abundance.	Wright and Burgin 2009a
Canyon (AU)	NSW, AU	Decreased chironomid species richness. Slightly decreased chironomid abundance (NS). Shifts in chironomid community composition (Increased Orthocladiinae).	Wright and Burgin 2009b
Clarence (U)	NSW, AU	Decreased family richness and abundance. EPT not affected.	Belmer et al. 2014
Neubecks and others (U, AU)	NSW, AU	Decreased taxa richness and density. Chironomedae absent.	Battaglia et al. 2005
Ravensworth (U)	NSW, AU	Decreased taxa richness and abundance with increased conductivity. Shifts in species assemblages (in 5/6 sites).	Cardno Ecology Lab Pty Ltd 2010
Tahmoor (U)	NSW, AU	Decreased taxa richness and abundance with increased conductivity. Shifts in species assemblages (in 5/6 sites).	Cardno Ecology Lab Pty Ltd 2010
West Cliff (U)	NSW, AU	Upstream and downstream conditions similar. Sites are significantly impaired relative to AusRivAS conditions.	The Ecology Lab Pty Ltd 2008
West Cliff (U)	NSW, AU	Sites were mostly severely impaired (ranged from significantly impaired to impoverished). Lower family diversity compared to AusRivAS model.	Bio-Analysis Pty Ltd 2009
West Cliff (U)	NSW, AU	Decreased taxa richness with increased conductivity. Shifts in species assemblages.	Cardno Ecology Lab Pty Ltd 2010
West Cliff (U)	NSW, AU	Macroinvertebrate populations impaired at 3/6 sites relative to the AusRivAS reference condition.	Cardno Ecology Lab Pty Ltd 2013
West Cliff (U)	NSW, AU	Downstream sites (2/4) severely impaired.	Cardno Ecology Lab Pty Ltd 2014
West Cliff (U)	NSW, AU	NS effect in taxa richness and SIGNAL index.	Russell 2014
Blackwater (S)	QLD, AU	Taxa richness and EPT richness generally lower downstream in Taurus and Blackwater Creek compared to upstream.	Jones et al. 2013
Moura (S)	QLD, AU	Decreased total abundance and slightly decreased taxa richness. Taxa richness negatively correlated with conductivity (in December, NS in April).	Proctor and Grigg 2006
Rhenish-Westphalian coalfield (AU)	NRW, DE	Decreased taxa richness and abundance. Increase number to brackish and saltwater taxa.	Petruck and Stöffler 2011
Lublin Basin (S, U)	LU, PL	Decreased diversity and abundance. Increased number of erytopic and euryhaline species.	Radwan et al. 1991
Buller and Reefton coalfields (S, U, A)	SI, NZ	Ephemeroptera, Plecoptera, Trichoptera, Megaloptera, Coleoptera and Diptera were found at various sites, with species of Chironomidae, Scirtidae, Kokiriidae and Oeconesidae below pH 3.	Winterbourn 1998
Buller and Reefton coalfields (S, U, A)	SI, NZ	Decreased number of taxa and EPT taxa in sites with lower pH.	Winterbourn et al. 2000
Stockton Plateau (S)	SI, NZ	Decreased taxa richness and EPT richness. Taxa richness and EPT richness negatively correlated with [Al^{3+}].	Waters and Webster-Brown 2013
West Coast coalfields (S, U, A)	SI, NZ	Decreased density in severely impacted sites.	Gray and Harding 2012
Reefton coalfields (S, U, A)	SI, NZ	Slightly decreased taxa richness and density (NS).	Barnden and Harding 2005
El Bierzo coalfield	León, ES	Decreased family richness and EPT richness, and negatively correlated with sulfate and conductivity. *H'* index negatively correlated with sulfate and conductivity.	García-Criado et al. 1999

Colliery name (type[a])	Location[b]	Result summary[c]	Reference
Dowgang (AU)	CMA, UK	Decreased taxa richness. Shifts in species assemblages.	Armitage and Blackburn 1985
Derbyshire and Yorkshire coalfields (A)	DBY and YKS, UK	Decreased diversity and *H'* index.	Maltby et al. 2002
Morrison Busty, Whittle and Shilbottle (AU)	DUR and NBL, UK	Sites dominated by Chironomidae.	Batty et al. 2005
Durham coalfield	DUR, UK	Decreased total abundance at point of discharge. Predominance of dipterans. No E present. Decreased BMWP score downstream.	Jarvis and Younger 1997
Durham coalfield	DUR, UK	Decreased total abundance at point of discharge. Predominance of dipterans. No E present.	Jarvis and Younger 1997
South Wales coalfield	UK	Shifts in community structure (increased acid-tolerant species). Reduction in faunal abundance.	Scullion and Edwards 1980a
Edna (S)	CO, US	Decreased diversity, density, biomass, and abundance of shredders and predators. Increased abundance of collector-gatherers and collector-filters. *H'* index slightly lower.	Chadwick and Canton 1983
Appalachian coalfield (S)	KY, US	Decreased E richness and abundance.	Pond 2010
Eastern coalfield	KY, US	Decreased MBI with decreasing site quality. Absence of EPT and increased abundance of chironomids and oligochaetes.	Pond and McMurray 2002
Eastern coalfield (S, AS, RS)	KY, US	Decreased taxa richness, EPT index, MBI and clingers and E abundance. Increased HBI and chironomid and oligochaetes abundance.	Howard et al. 2000
McKinley (RS)	NM, US	Diversity and abundance of arthropods variable between years.	Ireland et al. 1994
Western Allegheny Plateau (S, U)	OH, US	Decreased richness and impairment in AMD sites compared to reference and treated sites.	Smucker and Vis 2009
Anthracite region (U)	PA, US	Decreased species diversity, richness, EPT richness and density. Density negatively correlated with conductivity.	MacCausland and McTammany 2007
Anthracite coalfields (A)	PA, US	Decreased total density, EPT density, Diptera and Oligochaeta (NS in remediated site) density, total richness (NS in downstream site), EPT richness and MAIS score in downstream and remediated sites.	Bott et al. 2012
Anthracite coalfields (AU)	PA, US	Decreased richness, EPT richness and abundance, E, Gastropoda, Gammariae and scrapper. Increased Chironomidae.	Bruns 2005
Appalachian coalfield (S, U)	PA, US	Decreased taxa richness, EPT richness and abundance, EPT/chironomid ratio, diversity, *H'* index, scrapper, filterers and herbivore richness and abundance. Increased Diptera and collector-gatherer abundance.	Ross et al. 2008
Appalachian region (AS, AU)	TN, US	Decreased total abundance.	Schorr et al. 2013
Bituminous coalfields (A)	PA, US	Decreased total density, EPT density, total richness, EPT richness and MAIS score. Decreased total density, diptera and oligochaeta density in remediated site.	Bott et al. 2012
Pittsburgh coal seam (S, U, AS, AU)	PA, US	Decreased species diversity and abundance. Diversity positively correlated with pH. Shifts in community structure.	Koryak et al. 1972
Pittsburgh coal seam (S, U, AS, AU)	PA, US	Decreased density (Untreated<Treated).	DeNicola et al. 2012
Redstone Creek Basin (AU, S)	PA, US	Decreased species richness and abundance, modified BI and *H'*. Decreased number of intolerant species. No species found at discharge point.	Moon and Lucostic 1979
Rock Tunnel and Cauffield Mine (AU)	PA, US	Decreased biomass, abundance, *H'* index and intolerant species.	Letterman and Mitsch 1978

Colliery name (type[a])	Location[b]	Result summary[c]	Reference
Central Appalachian coalfield (S, U)	VA, US	Decreased species diversity and abundance. No mussel species present.	Hull et al. 2006
Central Appalachian coalfield (S)	VA, US	EPT and mayfly richness negatively correlated with TDS levels.	Timpano et al. 2010
Southwestern Virginia coalfield (RS, AS)	VA, US	Decreased total density and diversity. Slightly decreased Brillouin's H diversity index (NS). Taxa densities varied between streams.	Matter and Ney 1981
Southwestern Virginia coalfield (RS, AS)	VA, US	Shifts in dominating taxa and functional feeding groups. Increased chironomid in acidic AMD sites.	Soucek et al. 2001b
Southwestern Virginia coalfield (RS, AS)	VA, US	Decreased total abundance, total richness, EPT richness, EPT abundance and E abundance.	Soucek et al. 2000a
Southwestern Virginia coalfield (RS, AS)	VA, US	Decreased total abundance, total richness, E abundance, EPT abundance and richness.	Cherry et al. 2001
Southwestern Virginia coalfield (RS, AS)	VA, US	Decreased taxa richness, EPT abundance and %E in AMD sites compared to other sites and generally lower in remediated sites compared to reference site.	Simon et al. 2006
Southwestern Virginia coalfield (AS)	VA, US	Decreased abundance and diversity. No macroinvertebrates found at 6/20 sites.	Soucek et al. 1998
Appalachian Basin (S)	WV, US	Decreased EPT richness and EPT, E and P abundance. Increased HBI, filterers abundance. NS effect in total density, taxa richness, EPT density, Chironomidae abundance.	Armstead et al. 2004
Appalachian Basin (S)	WV, US	Decreased total taxa richness, EPT taxa richness, Invertebrate IBI. NS effect in HBI index and EPT and Chironomidae abundance.	Fulk et al. 2003
Appalachian Basin (S, RS, AS, U)	WV, US	Decreased taxa richness, EPT richness and mayfly abundance.	Kirk and Maggard 2004
Appalachian Basin (RS)	WV, US	Decreased SCI, EPT richness and abundance, and shredder taxa abundance. SCI and EPT richness negatively correlated with conductivity. No effect in family richness.	Petty et al. 2013
Central Appalachian coalfield (S)	WV, US	Decreased abundance and diversity of intolerant species (mayfly, stonefly, caddisfly, and beetle larvae). Increased tolerant species.	Bernhardt et al. 2012
Central Appalachian coalfield (S, AS)	WV, US	Decreased SCI. Filled/residential sites usually worst SCI.	Green et al. 2000
Central Appalachian coalfield (S, RS)	WV, US	Decreased total richness, EPT richness, species abundance and *H'* index. Shifts in community structure. Decreased total E richness and relative abundance.	Pond et al. 2008
Central Appalachian coalfield (AS, S)	WV, US	Decreased density, taxa richness, *H'* index, %EPT in AMD sites compared to reference and AMD treated sites. Decreased taxa richness, *H'* index and % EPT in treated AMD sites. Decreased abundance in treated AMD sites (*in situ*). Decreased shredders, filterers and scrapers and increased collectors and predators (*in situ*).	Simmons et al. 2005
Coalburg and 5-Block coalfield (S, U)	WV, US	Decreased diversity and evenness. Increased tolerant species, total abundance, Scrapper:Collector/filterer ration and biomass. No effect in total taxa.	REI Consultants et al. 2000
Elkins Coalfield (S, U)	WV, US	Decreased species diversity in AMD sites (pH<3.8). Diversity increased with increased pH. Shifts in community structure (increased acid tolerant species).	Warner 1971
Hobet 21 Coal Mine (S)	WV, US	Decreased Coleoptera, E, Odonata, non-insect, scraper and shredder density. E richness negatively correlated to conductivity. NS effect in total density, EPT density.	Hartman et al. 2005

a. Mine types: A, Abandoned mine; S, active surface mine (including strip, open-cut and mountain top/valley fill mines); AS, abandoned surface mine; RS, reclaimed surface mine; U, active underground mine; AU, abandoned underground mine.
b. State, Country (ISO 3166-1 alpha-2 code).
c. Effect in impacted site(s) compared to reference site(s) (unless indicated otherwise).
AMD, Acid Mine Drainage; AusRivAS, Australian River Assessment System; BI, Beck's Index; BMWP, Biological Monitoring Working Party score; E, Ephemeroptera; EPT, Ephemeroptera, Plecoptera and Trichoptera index; HBI, Hilsenhoff Biotic Index; IBI, Indexes of Biological Integrity; MAIS, Macroinvertebrate Aggregated Index for Streams; MBI, Macroinvertebrate Bioassessment Index; NS, no significant effect; *H'*, Shannon Diversity Index; SCI, Stream Condition Index; TDS, Total Dissolved Solids.

The bulk of research pertaining to the effects of CMW on aquatic invertebrate species has focused on toxicity and community assemblages, with only a small number of studies investigating sub-lethal effects. Decreased growth of *Corbicula fluminea* and *Villosa iris* has been observed at contaminated sites compared to sites not impacted by coal mining in North America (Kennedy et al. 2003; Hull et al. 2006; Simon et al. 2006), whereas no significant differences in *Corbicula fluminea* growth have been reported in other cases (Soucek et al. 2001b; Simon et al. 2006; 2007). Reduced reproduction was found in *Ceriodaphinia dubia* exposed for 7 days to a range of saline effluent dilutions from various coal mines in Australia (Cardno Ecology Lab Pty Ltd 2010; OEH 2012) and the USA (Kennedy et al. 2003; 2004; Kennedy and Cherry 2005; Echols et al. 2010; Kunz et al. 2013). These studies reported NOEC, LOEC and EC_{50} values for conductivity ranging between 2014 – 2910 µS/cm (Kennedy et al. 2004), 2132 – 4250 µS/cm (Kennedy et al. 2003; Kennedy and Cherry 2005; Echols et al. 2010) and 944 – 3829 µS/cm (Cardno Ecology Lab Pty Ltd 2010), respectively. Another study showed that stonefly nymphs, *Acroneuria carolinensis*, exposed to water from an acidic stream contaminated by coal mining (pH = 4.0) lost approximately 50% of their body sodium compared to reference animals (Whipple and Dunson 1993). Decreased feeding rates were also observed in cladocerans *Daphnia magna* and *Moina macrocopa* exposed to acidic effluent (Seo et al. 2012) and freshwater amphipods *Gammarus pulex* exposed downstream of coal mines (Maltby et al. 2002). Finally, invertebrate species collected from contaminated sites were found to contain higher levels of metals, including Al, Fe, Cu, Zn, Se, compared to invertebrates from reference sites (DeNicola and Stapleton 2002; Hull et al. 2006; Arnold et al. 2014).

2.2.2 Fish

While the majority of ecotoxicology studies related to coal mine discharge has focused on invertebrate species, a few studies have described a range of potential impacts in fish, though limited information is available for Australian species (Table 2-1). Observed impacts include effects on survival (Scullion and Edwards 1980a; Mylliemngap and Ramanujam 2012), community structure (Branson and Batch 1971; Daniel et al. 2014), morphometrics (Bharti and Banerjee 2013; 2014), metal uptake (Rudolph et al. 2008; Bharti and Banerjee 2011), genotoxicity (Martin and Black 1998; Benassi et al. 2006; da Silveira et al. 2009), reproduction (Rudolph et al. 2008), metabolism (Miller et al. 2009) and oxidative stress (Benassi et al. 2006). Though most studies are consistent in showing adverse responses, differences in response patterns amongst studies are not uncommon. For example, some laboratory and *in situ* field studies showed severe lethality in fish exposed between 6 h and 1 month to CMW from various active and abandoned coal mines (Scullion and Edwards 1980b; Mylliemngap and Ramanujam 2012;

Bharti and Banerjee 2013; 2014), whereas others reported no significant impacts on fish survival after 4-10 d exposures to effluent from underground coal mines in NSW, Australia (Cardno Ecology Lab Pty Ltd 2010; Short 2012; OEH 2012). Similarly, in some instances, fish collected from sites contaminated by coal mining showed increased body condition (Rudolph et al. 2008; Miller et al. 2009), whereas others had decreased condition (Scullion and Edwards 1980a; Bharti and Banerjee 2013; 2014) or showed no significant differences (Martin and Black 1998) compared to reference fish. Differences in responses between studies can often be attributable to variability in environmental factors, differences in species sensitivity and adaptability, and/or effluent characteristics amongst locations.

Mixtures of toxic metals present in CMW can have detrimental impacts on fish populations due to their high bioaccumulation potential. In fact, high levels of trace metals including Se, Cd, Zn, Mn, Cu and Pb have been detected in various organs of fish exposed to wastewater from different coal mines (Rudolph et al. 2008; Miller et al. 2009; Bharti and Banerjee 2011; Friedrich et al. 2011; Bharti and Banerjee 2013; Arnold et al. 2014). Trace metals present in CMW have been linked to a range of sub-lethal effects in fish. For example, Se has been linked to adverse health effects in fish exposed to mine affected water. *Lepomis cyanellus* collected from a polluted site downstream from Hobet coal mine in West Virginia, USA, exhibited upper jaw deformities characteristic of Se exposure (Arnold et al. 2014). Similarly, Miller et al. (2009) showed that antioxidant responses (*i.e.*, decreased liver glutathione and vitamin levels) were linked to high muscle Se levels in fish downstream of a surface coal mine. Another study linked the reduced reproductive performance and egg viability of *Oncorhynchus clarki lewisi* exposed to polluted water from the Elk Valley coalfield in British Columbia, Canada, to elevated Se concentration (Rudolph et al. 2008).

Studies have also shown a range of morphological and physiological responses and population level effects in fish exposed to CMW. For example, exposure of *Heteropneustes fossilis* to undiluted effluent from Dudhichua coal mine in India for up to 26 d resulted in decreased body condition, hepato-, kidney-, brain- and air breathing organs-somatic index as well as decreased total protein, glycogen, lipid, DNA and RNA in several tissues (Bharti and Banerjee 2013; 2014). Similarly, exposure of *Oreochromis nilotixus* to diluted effluent from the mining area of Siderópolis in Southern Brazil caused increased liver lipid oxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) activity and decreased glutathione (GSH) levels (Benassi et al. 2006). Thyroid hormone levels in *Oncorhychus mykiss* plasma were also found to be lower at polluted sites compared to reference sites (Miller et al. 2009). Additionally, a range of malformations such as skeletal deformities, edemas, tissue lesions (Rudolph et al. 2008), enlarged and fatty livers (Martin and Black 1998) and gill deformation (Mylliemngap and Ramanujam 2012) have been reported in fish exposed to CMW. Importantly, organism level responses can have severe consequences at the population level, and this is supported by field surveys that have reported lower fish species abundance, biomass and diversity at sites contaminated by coal mining compared to reference sites (Branson and Batch 1971; Letterman and Mitsch 1978; Matter and Ney 1981; Stauffer and Ferreri 2002; Fulk et al. 2003; Schorr and Backer 2006; Smucker and Vis 2009; Cravotta et al. 2010; Daniel et al. 2014).

2.2.3 Amphibians

Amphibians are considered to be one of the most threatened organisms on the planet, with close to half of all known species facing extinction (Monastersky 2014; Alroy 2015). Larval amphibians are highly sensitive to the presence of anthropogenic pollution in the aquatic environment, due to their unique physiology and feeding habits (Sparling and Lowe 1996). Many trace elements are known to bioaccumulate in amphibians, and in some cases these can cause severe physiological disruption. For example, a recent study reported higher levels of various metals in liver (Al, Mn, Fe, Cu, Br), kidney (Fe, Cu, Rb) and leg muscles (Fe, Zn, Br) of adult *Hypsiboas faber* collected in proximity to an abandoned open cut coal mine in southern Brazil compared to reference samples (Zocche et al. 2013; 2014). Frogs from this site also had increased DNA damage (Zocche et al. 2013) as well as increased muscle super oxide dismutase (SOD) and glutathione peroxidase (GPx) activities (Zocche et al. 2014) compared to reference animals.

Despite the potential threat of coal mining on amphibians, there have been few attempts to explore effects of CMW on amphibians compared to other organisms. The existing literature on amphibians has primarily explored ecological aspects such as altered community structures (*e.g.*, species richness, abundance) of anurans and salamanders at sites impacted by abandoned and reclaimed coal mines (Ireland et al. 1994; Wood and Williams 2013a; 2013b; Schorr et al. 2013; Petty et al. 2013; Muncy et al. 2014). Studies have found decreased amphibian abundance at several sites polluted by active and abandoned surface coal mines compared to reference sites (REI Consultants and Kirk 2000; Williams and Wood 2004; Schorr et al. 2013). Similarly, a few reclaimed sites have also been found to have decreased amphibian abundance and richness (Wood and Williams 2013a; 2013b; Muncy et al. 2014). Contrarily, in some instances reclaimed sites have proven to be quite successful, showing comparable amphibian richness and diversity to reference locations (Ireland et al. 1994; Petty et al. 2013).

Though research pertaining to CMW and its impacts on amphibians is overall quite scarce, considerable research in the last two decades has been directed at investigating the impacts of coal combustion residues (CCR) from a power plant located in South Carolina, USA (reviewed by Rowe et al. 2001; 2002; Rowe 2014). This body of literature has revealed that exposure to CCR is related to a wide range of detrimental effects on survival, growth, development, morphology, behaviour, physiology and reproduction (Hopkins et al. 2000; Snodgrass et al. 2005; Roe et al. 2006; Ward et al. 2006; Hopkins et al. 2006), and that effects are likely related to increased accumulation of several toxic elements in amphibians (Hopkins et al. 1998; Peterson et al. 2007; Metts et al. 2012). Though coal mine process-affected water may differ from CCR, both contain similar toxic elements such as toxic metals and metalloids (Rowe et al. 2002) that may cause similar responses in aquatic organisms. However, while perhaps not directly relevant to coal mining, this particular example demonstrates the diverse range of acute and sub-lethal impacts that can result from exposures of aquatic animals to contaminants associated with coal mining. The risk may be particularly large for sensitive and threatened organisms like amphibians, highlighting a need for further research.

2.3 References

Adhikari, S., Ghosh, L., and Ayyappan, S. (2006). Combined effects of water pH and alkalinity on the accumulation of lead, cadmium and chromium to *Labeo rohita* (Hamilton). *International Journal of Environmental Science and Technology* **3**, 289–296.

Anon (2007). Coal-mine hollow fill and settling pond influences on headwater streams in southern West Virginia, USA. *Environmental Monitoring and Assessment* **129**, 359–378.

Arfsten, D. P., Schaeffer, D. J., and Mulveny, D. C. (1996). The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: a review. *Ecotoxicology and Environmental Safety* **33**, 1–24.

Armitage, P. D., and Blackburn, J. H. (1985). Chironomidae in a Pennine stream system receiving mine drainage and organic enrichment. *Hydrobiologia* **121**, 165–172.

Armstead, M. Y., Yeager-Seagle, J. L., and Emerson, L. (2004). Benthic macroinvertebrate studies conducted in mountaintop mining/valley fill influenced streams in conjunction with the USEPA environmental impact study. In National Meeting of the American Society of Mining and Reclamation. pp. 87–93

Arnold, M. C., Lindberg, T. T., Liu, Y. T., Porter, K. A., Hsu-Kim, H., Hinton, D. E., and Di Giulio, R. T. (2014). Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. *Ecotoxicology* **23**, 929–938.

Atchison, G. J., Henry, M. G., and Sandheinrich, M. B. (1987). Effects of metals on fish behavior: a review. *Environmental Biology of Fishes* **18**, 11–25

Banks, D., Younger, P. L., Arnesen, R.-T., Iversen, E. R., and Banks, S. B. (1997). Mine-water chemistry: the good, the bad and the ugly. *Environmental Geology* **32**, 157–174.

Barnden, A. R., and Harding, J. S. (2005). Shredders and leaf breakdown in streams polluted by coal mining in the South Island, New Zealand. *New Zealand Natural Sciences* **30**, 35–48.

Barry, K. L., Grout, J. A., Levings, C. D., Nidle, B. H., and Piercey, G. E. (2000). Impacts of acid mine drainage on juvenile salmonids in an estuary near Britannia Beach in Howe Sound, British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences* **57**, 2032–2043.

Battaglia, M., Hose, G. C., Turak, E., and Warden, B. (2005). Depauperate macroinvertebrates in a mine affected stream: Clean water may be the key to recovery. *Environmental Pollution* **138**, 132–141

Batty, L., Atkin, L., and Manning, D. A. C. (2005). Assessment of the ecological potential of mine-water treatment wetlands using a baseline survey of macroinvertebrate communities. *Environmental Pollution* **138**, 413–420.

Belmer, N., Tippler, C., Davies, P. J., and Wright, I. A. (2014). Impact of a coal mine waste discharge on water quality and aquatic ecosystems in the Blue Mountains World Heritage area. In the Australian Stream Management Conference. pp. 1–7. (Townsville, Queensland.)

Benassi, J. C., Laus, R., Geremias, R., Lima, P. L., Menezes, C. T. B., Laranjeira, M. C., Wilhelm-Filho, D., Fávere, V. T., and Pedrosa, R. C. (2006). Evaluation of remediation of coal mining wastewater by chitosan microspheres using biomarkers. *Archives of Environmental Contamination and Toxicology* **51**, 633–640.

Bernhardt, E. S., Lutz, B. D., King, R. S., Fay, J. P., Carter, C. E., Helton, A. M., Campagna, D., and Amos, J. (2012). How many mountains can we mine? Assessing the regional degradation of Central Appalachian rivers by surface coal mining. *Environmental Science & Technology* **46**, 8115–8122.

Bharti, S., and Banerjee, T. K. (2011). Bioaccumulation of metals in the edible catfish *Heteropneustes fossilis* (Bloch) exposed to coal mine effluent generated at Northern Coalfield Limited, Singrauli, India. *Bulletin of environmental contamination and toxicology* **87**, 393–398.

Bharti, S., and Banerjee, T. K. (2013). Bioassay analysis of efficacy of phytoremediation in decontamination of coal mine effluent. *Ecotoxicology and Environmental Safety* **92**, 312–319.

Bharti, S., and Banerjee, T. K. (2014). Toxicity analysis of coal mine effluent on vital organs of *Heteropneustes fossilis* (Bloch) using biometric and biochemical parameters. *Clean - Soil, Air, Water* **42**, 260–266.

Bian, Z., Inyang, H. I., Daniels, J. L., Otto, F., and Struthers, S. (2010). Environmental issues from coal mining and their solutions. *Mining Science and Technology* **20**, 215–223.

Bilotta, G. S., and Brazier, R. E. (2008). Understanding the influence of suspended solids on water quality and aquatic biota. *Water Research* **42**, 2849–2861.

Bio-Analysis Pty Ltd (2009). Illawarra Coal - Bulli seam operations. Appendix D: Aquatic ecology assessment. BHPbilliton.

Bott, T. L., Jackson, J. K., McTammany, M. E., Newbold, J. D., Rier, S. T., Sweeney, B. W., and Battle, J. M. (2012). Abandoned coal mine drainage and its remediation: impacts on stream ecosystem structure and function. *Ecological Applications* **22**, 2144–2163.

Branson, B. A., and Batch, D. L. (1971). Effects of strip mining on small-stream fishes in east-central Kentucky. *Proceedings of The Biological Society of Washington* **84**, 507–517.

Bruns, D. A. (2005). Macroinvertebrate response to land cover, habitat, and water chemistry in a mining-impacted river ecosystem: A GIS watershed analysis. *Aquatic Sciences* **67**, 403–423.

Cañedo-Argüelles, M., Kefford, B. J., Piscart, C., Prat, N., Schäfer, R. B., and Schulz, C.-J. (2013). Salinisation of rivers: an urgent ecological issue. *Environmental Pollution* **173**, 157–167.

Cardno Ecology Lab Pty Ltd (2010). Effects of mine water salinity on freshwater biota investigations of coal minewater discharg e in NSW. Australian Coal Association Research Program.

Cardno Ecology Lab Pty Ltd (2014). West Cliff Longwalls 33-38 Aquatic Ecology Monitoring 2002-2013. BHP Billiton Illawarra Coal.

Cardno Ecology Lab Pty Ltd (2013). West Cliff Longwalls 33-38 Aquatic Ecology Monitoring, May 2002 – December 2012.

Chadwick, J. W., and Canton, S. P. (1983). Coal mine drainage effects on a lotic ecosystem in Northwest Colorado, U.S.A. *Hydrobiologia* **107**, 25–33.

Chapman, H. F., and Simpson, S. L. (2005). Toxicity of acid water from Mt Morgan mine site, Central Queensland, to the freshwater shrimp Caradina indistincta. *Australasian Journal of Ecotoxicology* **11**, 93–99.

Chapman, P. M., Bailey, H. C., and Canaria, E. (2000). Toxicity of total dissolved solids associated with two mine effluents to chironomid larvae and early life stages of rainbow trout. *Environmental Toxicology and Chemistry* **19**, 210–214.

Cherry, D. S., Currie, R. J., Soucek, D. J., Latimer, H. A., and Trent, G. C. (2001). An integrative assessment of a watershed impacted by abandoned mined land discharges. *Environmental Pollution* **111**, 377–388.

Cravotta, C. A. I., Brightbill, R. A., and Langland, M. J. (2010). Abandoned Mine Drainage in the Swatara Creek Basin, Southern Anthracite Coalfield, Pennsylvania, USA: 1. Stream Water Quality Trends Coinciding with the Return of Fish. *Mine Water and the Environment* **29**, 176–199.

da Silveira, F. Z., Defaveri, T. M., Ricken, C., Zocche, J. J., and Pich, C. T. (2009). Toxicity and genotoxicity evaluation of acid mine drainage treatment using *Artemia* sp. and *Geophagus* *brasiliensis* as bioindicators. In (Ed R. I. Barnhisel.) National Meeting of the American Society of Mining and Reclamation. pp. 1725–1742.

Daniel, W. M., Infante, D. M., Hughes, R. M., Tsang, Y.-P., Esselman, P. C., Wieferich, D., Herreman, K., Cooper, A. R., Wang, L., and Taylor, W. W. (2014). Characterizing coal and mineral mines as a regional source of stress to stream fish assemblages. *Ecological Indicators* **50**, 50–61.

Defaveri, T. M., da Silveira, F. Z., Bortolotto, T., Geremias, R., Zocche, J. J., and Pich, C. T. (2009). Evaluation of acid mine drainage treatment using Artemia sp. and Allium cepa as bioindicators of toxicity and genotoxicity. In National Meeting of the American Society of Mining and Reclamation. pp. 283–301.

DEHP (2013). Monitoring and Sampling Manual 2009. Department of Environment and Heritage Protection, State of Queensland.

DeNicola, D. M., and Stapleton, M. G. (2002). Impact of acid mine drainage on benthic communities in streams: the relative roles of substratum vs. aqueous effects. *Environmental Pollution* **119**, 303–315.

DeNicola, D. M., Layton, L., and Czapski, T. R. (2012). Epilithic community metabolism as an indicator of impact and recovery in streams affected by acid mine drainage. *Environmental Management* **50**, 1035–1046.

DERM (2009). A study of the cumulative impacts on water quality of mining activities in the Fitzroy River Basin. Department of Environment and Resource Management.

DERM(2010). Fitzroy Basin draft water resource plan. Environmental assessment–Stage 2 assessment report. Department of Environment and Resource Management.

Diamond, J. M., Winchester, E. L., Mackler, D. G., and Gruber, D. (1992). Use of the mayfly *Stenonema modestum* (Heptageniidae) in subacute toxicity assessments. *Environmental Toxicology and Chemistry* **11**, 415–425.

Diamond, S. A., Mount, D. R., Mattson, V. R., Heinis, L. J., Highland, T. L., Adams, A. D., and Simcik, M. F. (2006). Photoactivated polycyclic aromatic hydrocarbon toxicity in medaka (*Oryzias latipes*) embryos: relevance to environmental risk in contaminated sites. *Environmental Toxicology and Chemistry* **25**, 3015–3023.

DNRM (2015). Fitzroy Basin resource operation plan. Department of Natural Resource and Mines.

Dunlop, J. E., McGregor, G., and Horrigan, N. (2005). Potential impacts of salinity and turbidity in riverine ecosystems. The State of Queensland.

Echols, B. S., Currie, R. J., and Cherry, D. S. (2010). Preliminary results of laboratory toxicity tests with the mayfly, *Isonychia bicolor* (Ephemeroptera: Isonychiidae) for development as a standard test organism for evaluating streams in the Appalachian coalfields of Virginia and West Virginia. *Environmental Monitoring and Assessment* **169**, 487–500.

Eisler, R. (1987). Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service.

Erickson, R. J., Ankley, G. T., DeFoe, D. L., Kosian, P. A., and Makynen, E. A. (1999). Additive toxicity of binary mixtures of phototoxic polycyclic aromatic hydrocarbons to the oligochaete *Lumbriculus variegatus*. *Toxicology and Applied Pharmacology* **154**, 97–105.

Friedrich, L. A., Orr, P. L., Halden, N. M., Yang, P., and Palace, V. P. (2011). Exposure histories derived from selenium in otoliths of three cold-water fish species captured downstream from coal mining activity. *Aquatic Toxicology* **105**, 492–496.

Fulk, F., Autrey, B., Hutchens, J., Gerritsen, J., Burton, J., Cresswell, C., and Jessup, B. (2003). Ecological Assessment of Streams in the Coal Mining Region of West Virginia Using Data Collected by the U.S. EPA and Environmental Consulting Firms. In 'Mountaintop Mining/Valley Fills in Appalachia Draft Programmatic Environmental Impact Statement'. United States Environmental Protection Agency.

García-Criado, F., Tomé, A., Vega, F. J., and Antolín, C. (1999). Performance of some diversity and biotic indices in rivers affected by coal mining in northwestern Spain. *Hydrobiologia* **394**, 209–217.

Geremias, R., Bortolotto, T., Wilhelm-Filho, D., Pedrosa, R. C., and de Fávere, V. T. (2012). Efficacy assessment of acid mine drainage treatment with coal mining waste using *Allium cepa* L. as a bioindicator. *Ecotoxicology and Environmental Safety* **79**, 116–121.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Fávere, V. T., Stolberg, J., Menezes, C. T. B., and Laranjeira, M. C. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental Technology* **24**, 1509–1515.

Gerhardt, A. (1993). Review of impact of heavy metals on stream invertebrates with special emphasis on acid conditions. *Water, Air, and Soil Pollution* **66**, 289–314.

Gerhardt, A., Janssens de Bisthoven, L., and Soares, A. M. (2004). Macroinvertebrate response to acid mine drainage: community metrics and on-line behavioural toxicity bioassay. *Environmental Pollution* **130**, 263–274.

Gerhardt, A., Janssens de Bisthoven, L., and Soares, A. M. (2005a). Evidence for the stepwise stress model: *Gambusia holbrooki* and *Daphnia magna* under acid mine drainage and acidified reference water stress. *Environmental Science & Technology* **39**, 4150–4158.

Gerhardt, A., Janssens de Bisthoven, L., and Soares, A. M. V. M. (2005b). Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). *Archives of Environmental Contamination and Toxicology* **48**, 450–458.

Golovanova, I. L. (2008). Effects of heavy metals on the physiological and biochemical status of fishes and aquatic invertebrates. *Inland Water Biology* **1**, 93–101.

Goodfellow, W. L., Ausley, L. W., Burton, D. T., Denton, D. L., Dorn, P. B., Grothe, D. R., Heber, M. A., Norberg-King, T. J., and Rodgers, J. H. (2000). Major ion toxicity in effluents: A review with permitting recommendations. *Environmental Toxicology and Chemistry* **19**, 175–182.

Gray, D. P., and Harding, J. S. (2012). Acid Mine Drainage Index (AMDI): a benthic invertebrate biotic index for assessing coal mining impacts in New Zealand streams. *New Zealand Journal of Marine and Freshwater Research* **46**, 335–352.

Gray, N. F. (1997). Environmental impact and remediation of acid mine drainage: a management problem. *Environmental Geology* **30**, 62–71.

Green, J., Passmore, M., and Childers, H. (2000). A Survey of the Condition of Streams in the Primary Region of Mountaintop Mining/Valley Fill Coal Mining. US EPA.

Haibin, L., and Zhenling, L. (2010). Recycling utilization patterns of coal mining waste in China. *Resources, Conservation and Recycling* **54**, 1331.

Hart, B. T., Bailey, P., Edwards, R. W., Hortle, K., James, K., McMahon, A., Meredith, C., and Swadling, K. (1990). Effects of salinity on river, stream and wetland ecosystems in Victoria, Australia. *Water Research* **24**, 1103–1117.

Hart, B. T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., Meredith, C., and Swadling, K. (1991). A review of the salt sensitivity of the Australian freshwater biota. *Hydrobiologia* **210**, 105–144.

Hart, B. T., Lake, P. S., Webb, J. A., and Grace, M. (2003). Ecological risk to aquatic systems from salinity increases. *Australian Journal of Botany* **51**, 689–702.

Hartman, K. J., Kaller, M. D., Howell, J. W., and Sweka, J. A. (2005). How much do valley fills influence headwater streams? *Hydrobiologia* **532**, 91–102.

Heijerick, D. G., Janssen, C. R., and De Coen, W. M. (2003). The combined effects of hardness, pH, and dissolved organic carbon on the chronic toxicity of Zn to *D. magna*: development of a surface response model. *Archives of Environmental Contamination and Toxicology* **44**, 210.

Henry, T. B., Irwin, E. R., Grizzle, J. M., Wildhaber, M. L., and Brumbaugh, W. G. (1999). Acute toxicity of an acid mine drainage mixing zone to juvenile bluegill and largemouth bass. *Transactions of the American Fisheries Society* **128**, 919–928.

Holland, A., Duivenvoorden, L. J., and Kinnear, S. H. W. (2012). Humic substances increase survival of freshwater shrimp *Caridina* sp. D to acid mine drainage. *Archives of Environmental Contamination and Toxicology* **64**, 263–272.

Hopkins, W. A., Congdon, J. D., and Ray, J. K. (2000). Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. *Environmental Toxicology and Chemistry* **19**, 862–868.

Hopkins, W. A., DuRant, S. E., Staub, B. P., Rowe, C. L., and Jackson, B. P. (2006). Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. *Environmental Health Perspectives* **114**, 661–666.

Hopkins, W. A., Mendonça, M. T., Rowe, C. L., and Congdon, J. D. (1998). Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. *Archives of Environmental Contamination and Toxicology* **35**, 325–329.

Horne, M. T., and Dunson, W. A. (1995). Effects of low pH, metals, and water hardness on larval amphibians. *Archives of Environmental Contamination and Toxicology* **29**, 500–505.

Howard, H. S., Berrang, B., Flexner, M., Pond, G., and Call, S. (2000). Kentucky Mountaintop Mining Benthic Macroinvertebrate Survey. October 2001. U.S. Environmental Protection Agency, Science and Ecosystem Support Division, Ecological Assessment Branch, Athens, Georgia.

Hull, M. S., Cherry, D. S., and Neves, R. J. (2006). Use of bivalve metrics to quantify influences of coal-related activities in the Clinch River Watershed, Virginia. *Hydrobiologia* **556**, 341–355.

Ireland, T. T., Wolters, G. L., and Schemnitz, S. D. (1994). Recolonization of wildlife on a coal strip-mine in Northwestern New-Mexico. *The Southwestern Naturalist* **39**, 53–57.

Janssens de Bisthoven, L., Gerhardt, A., and Soares, A. M. V. M. (2004). Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitor®. *Environmental Toxicology and Chemistry* **23**, 1123–1128.

Jarvis, A. P., and Younger, P. L. (1997). Dominating chemical factors in mine water induced impoverishment of the invertebrate fauna of two streams in the Durham coalfield, UK. *Chemistry and Ecology* **13**, 249–270.

Jones, C., Sellens, C., Campbell, L., Flint, N., Holland, A., Kerr, J., Stitz, L., Shrestha, K., Tucker, G., Vink, S., and Fabbro, L. D. (2013). Blackwater mine. February and May 2012 aqua-eco health sampling report. BMA Aqua-Eco Health.

Kefford, B. J., Marchant, R., Schäfer, R. B., Metzeling, L., Dunlop, J. E., Choy, S. C., and Goonan, P. (2011). The definition of species richness used by species sensitivity distributions approximates observed effects of salinity on stream macroinvertebrates. *Environmental Pollution* **159**, 302–310.

Kennedy, A. J., and Cherry, D. S. (2005). Evaluation of ionic contribution to the toxicity of a coal-mine effluent using *Ceriodaphnia dubia*. *Archives of Environmental Contamination and Toxicology* **49**, 155–162.

Kennedy, A. J., Cherry, D. S., and Currie, R. J. (2004). Evaluation of ecologically relevant bioassays for a lotic system impacted by a coal-mine effluent, using *Isonychia*. *Environmental Monitoring and Assessment* **95**, 37–55.

Kennedy, A. J., Cherry, D. S., and Currie, R. J. (2003). Field and laboratory assessment of a coal processing effluent in the Leading Creek Watershed, Meigs County, Ohio. *Archives of Environmental Contamination and Toxicology* **44**, 324–331.

Kirk, E. J., and Maggard, R. (2004). Long-term downstream impacts of surface mining and valley fill construction to benthic macroinvertebrates and water quality. In National Meeting of the American Society of Mining and Reclamation. pp. 1095–1120

Koryak, M., Shapiro, M. A., and Sykora, J. L. (1972). Riffle zoobenthos in streams receiving acid mine drainage. *Water Research* **6**, 1239–1247.

Kunz, J. L., Conley, J. M., Buchwalter, D. B., Norberg-King, T. J., Kemble, N. E., Wang, N., and Ingersoll, C. G. (2013). Use of reconstituted waters to evaluate effects of elevated major ions associated with mountaintop coal mining on freshwater invertebrates. *Environmental Toxicology and Chemistry* **32**, 2826–2835.

Lampi, M. A., Gurska, J., McDonald, K. I. C., Xie, F., Huang, X.-D., Dixon, D. G., and Greenberg, B. M. (2005). Photoinduced toxicity of polycyclic aromatic hydrocarbons to Daphnia magna: ultraviolet-mediated effects and the toxicity of polycyclic aromatic hydrocarbon photoproducts. *Environmental Toxicology and Chemistry* **25**, 1079–1087.

Land and Water Australia (2007). Salt, nutrient, sediment and interactions: Findings from the national river contaminants program. Land and Water Australia.

Langdon, K., Warne, M. S. J., and Sunderam, R. I. M. (2009). A compilation of data on the toxicity of chemicals to species in Australasia. Part 4: Metals (2000-2009). *Australasian Journal of Ecotoxicology* **15**, 51–184.

Lattuada, R. M., Menezes, C. T. B., Pavei, P. T., Peralba, M. C. R., and Santos, Dos, J. H. Z. (2009). Determination of metals by total reflection X-ray fluorescence and evaluation of toxicity of a river impacted by coal mining in the south of Brazil. *Journal of Hazardous Materials* **163**, 531–537.

Letterman, R. D., and Mitsch, W. J. (1978). Impact of mine drainage on mountain stream in Pennsylvania. *Environmental Pollution* **17**, 53–73.

Lin, C., Wu, Y., Lu, W., Chen, A., and Liu, Y. (2007). Water chemistry and ecotoxicity of an acid mine drainage-affected stream in subtropical China during a major flood event. *Journal of Hazardous Materials* **142**, 199–207.

Loumbourdis, N. S., and Wray, D. (1998). Heavy-metal concentration in the frog *Rana ridibunda* from a small river of Macedonia, Northern Greece. *Environment international* **24**, 427–431.

Loumbourdis, N. S., Kostaropoulos, I., Theodoropoulou, B., and Kalmanti, D. (2007). Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and cadmium. *Environmental Pollution* **145**, 787–792.

MacCausland, A., and McTammany, M. E. (2007). The impact of episodic coal mine drainage pollution on benthic macroinvertebrates in streams in the Anthracite region of Pennsylvania. *Environmental Pollution* **149**, 216–226. d

Maltby, L., Clayton, S. A., Wood, R. M., and McLoughlin, N. (2002). Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. *Environmental Toxicology and Chemistry* **21**, 361–368.

Martin, L. M., and Black, M. C. (1998). Biomarker assessment of the effects of coal strip-mine contamination on channel catfish. *Ecotoxicology and Environmental Safety* **41**, 307–320.

Matter, W. J., and Ney, J. J. (1981). The impact of surface mine reclamation on headwater streams in Southwest Virginia. *Hydrobiologia* **78**, 63–71.

Metts, B. S., Buhlmann, K. A., Scott, D. E., Tuberville, T. D., and Hopkins, W. A. (2012). Interactive effects of maternal and environmental exposure to coal combustion wastes decrease survival of larval southern toads (*Bufo terrestris*). *Environmental Pollution* **164**, 211–218.

Merricks, T. C., Cherry, D. S., Zipper, C. E., Currie, R. J., Valenti, T. W. (2007). Coal-mine hollow fill and settling pond influences on headwater streams in southern West Virginia, USA. *Environmental Monitoring and Assessment* **129**, 359–378.

Michalec, F.-G., Holzner, M., Menu, D., Hwang, J.-S., and Souissi, S. (2013). Behavioral responses of the estuarine calanoid copepod *Eurytemora affinis* to sub-lethal concentrations of waterborne pollutants. *Aquatic Toxicology* **138-139**, 129–138.

Miller, L. L., Rasmussen, J. B., Palace, V. P., and Hontela, A. (2009). The physiological stress response and oxidative stress biomarkers in rainbow trout and brook trout from selenium-impacted streams in a coal mining region. *Journal of Applied Toxicology* **29**, 681–688.

Mitchelmore, C. L. (2010). Report on the U.S. Environmental Protection Agency's chronic whole effluent toxicity testing at selected sites in the coalfields of Kentucky and West Virginia.

Mohti, A., Shuhaimi-Othman, M., and Gerhardt, A. (2012). Use of the Multispecies Freshwater Biomonitor to assess behavioral changes of *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae) and *Macrobrachium lanchesteri* (Decapoda: Palaemonidae) in response to acid mine drainage: laboratory exposure. *Journal of Environmental Monitoring* **14**, 2505–2511.

Moon, T. C., and Lucostic, C. M. (1979). Effects of acid mine drainage on a Southwestern Pennsylvania stream. *Water, Air, and Soil Pollution* **11**, 377–390.

Muncy, B. L., Price, S. J., Bonner, S. J., and Barton, C. D. (2014). Mountaintop removal mining reduces stream salamander occupancy and richness in southeastern Kentucky (USA). *Biological Conservation* **180**, 115–121

Mylliemngap, B. K., and Ramanujam, S. N. (2012). Morphological changes in the gills of *Heteropneustes fossilis* (Bloch) exposed to coal mining effluent water. *Journal of environmental biology* **33**, 735–739.

Netto, E., Madeira, R. A., Silveira, F. Z., Fiori, M. A., Angioleto, E., Pich, C. T., and Geremias, R. (2013). Evaluation of the toxic and genotoxic potential of acid mine drainage using physicochemical parameters and bioassays. *Environmental Toxicology and Pharmacology* **35**, 511–516.

Nielsen, D. L., Brock, M. A., Rees, G. N., and Baldwin, D. S. (2003). Effects of increasing salinity on freshwater ecosystems in Australia. *Australian Journal of Botany* **51**, 655–665.

O'Halloran, K., Cavanagh, J.-A., and Harding, J. S. (2008). Response of a New Zealand mayfly (*Deleatidium* spp.) to acid mine drainage: implications for mine remediation. *Environmental Toxicology and Chemistry* **27**, 1135–1140.

OEH (2012). Chemical and ecotoxicology assessment of discharge waters from West Cliff Mine.

Oris, J. T., and Giesy, J. P., Jr (1987). The photo-induced toxicity of polycyclic aromatic hydrocarbons to larvae of the fathead minnow (*Pimephales promelas*). *Chemosphere* **16**, 1395–1404.

Pascoe, D., Evans, S. A., and Woodworth, J. (1986). Heavy-Metal Toxicity to Fish and the Influence of Water Hardness. *Archives of Environmental Contamination and Toxicology* **15**, 481–487.

Peterson, J. D., Wood, M. B., Hopkins, W. A., Unrine, J. M., and Mendonça, M. T. (2007). Prevalence of *Batrachochytrium dendrobatidis* in american bullfrog and southern leopard frog larvae from wetlands on the Savannah River site, South Carolina. *Journal of Wildlife Diseases* **43**, 450–460.

Petruck, A., and Stöffler, U. (2011). On the history of chloride concentrations in the River Lippe (Germany) and the impact on the macroinvertebrates. *Limnologica* **41**, 143–150.

Petty, J. T., Gingerich, G., Anderson, J. T., and Ziemkiewicz, P. F. (2013). Ecological function of constructed perennial stream channels on reclaimed surface coal mines. *Hydrobiologia* **720**, 39–53.

Pickering, R. W. (1999). A toxicological review of polycyclic aromatic hydrocarbons. *Cutaneous and Ocular Toxicology* **18**, 101–135.

Piscart, C., Moreteau, J.-C., and Beisel, J.-N. (2005). Biodiversity and structure of macroinvertebrate communities along a small permanent salinity gradient (Meurthe River, France). *Hydrobiologia* **551**, 227–236.

Pond, G. J. (2010). Patterns of ephemeroptera taxa loss in Appalachian headwater streams (Kentucky, USA). *Hydrobiologia* **641**, 185–201.

Pond, G. J., and McMurray, S. E. (2002). A Macroinvertebrate Bioassessment Index for Headwater Streams of the Eastern Coalfield Region, Kentucky. Kentucky Department for Environmental Protection, Frankfort, Kentucky.

Pond, G. J., Passmore, M. E., Borsuk, F. A., Reynolds, L., and Rose, C. J. (2008). Downstream effects of mountaintop coal mining: comparing biological conditions using family- and genus-level macroinvertebrate bioassessment tools. *Journal of the North American Benthological Society* **27**, 717–737.

Pourang, N., Dennis, J. H., and Ghourchian, H. (2004). Tissue distribution and redistribution of trace elements in shrimp species with the emphasis on the roles of metallothionein. *Ecotoxicology* **13**, 519–533.

Prasad, R., Vink, S., Mann, R. M., Nanjappa, V., and Choy, S. C. (2012). Assessing the ecotoxicology of salinity on organisms in seasonally flowing streams in the Fitzroy Catchment. The State of Queensland.

Proctor, H., and Grigg, A. (2006). Aquatic invertebrates in final void water bodies at an open-cut coal mine in central Queensland. *Australian Journal of Entomology* **45**, 107–121.

Radwan, S., Kowalczyk, C. Z., Jarzyne, B., Paleolog, A., Zwolski, W., and Kowalik, W. (1991). Coal mine waters and their influenceon the purity ecological state of riverand the fish production. *Marine Pollution Bulletin* **23**, 733–736.

Rainbow, P. S. (2002). Trace metal concentrations in aquatic invertebrates: why and so what? *Environmental Pollution* **120**, 497–507

REI Consultants, and Kirk, E. J. (2000). Amphibian utilization of sediment control structures compared to a natural vernal pool located on mine permitted areas in southern West Virginia. Beaver, West Virginia.

REI Consultants, Kirk, E. J., Hedrick, J., and Ras, L. (2000). A History of the Benthic Macroinvertebrate and Water Chemistry Studies of two Long-term Monitoring Stations on Trough Fork. Beaver, West Virgina.

Reynaud, S., and Deschaux, P. (2006). The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. *Aquatic Toxicology* **77**, 229–238.

Riethmuller, N., Markich, S. J., van Dam, R. A., and Parry, D. (2001). Effects of water hardness and alkalinity on the toxicity of uranium to a tropical freshwater hydra (*Hydra viridissima*). *Biomarkers* **6**, 45–51.

Roback, S. S., and Richardson, J. W. (1969). The effects of acid mine drainage on aquatic insects. *Proceedings of the Academy of Science* **121**, 81–107.

Roe, J. H., Hopkins, W. A., and Jackson, B. P. (2005). Species- and stage-specific differences in trace element tissue concentrations in amphibians: Implications for the disposal of coal-combustion wastes. *Environmental Pollution* **136**, 353–363.

Roe, J. H., Hopkins, W. A., DuRant, S. E., and Unrine, J. M. (2006). Effects of competition and coal-combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. *Aquatic Toxicology* **79**, 176–184.

Ross, R. M., Long, E. S., and Dropkin, D. S. (2008). Response of macroinvertebrate communities to remediation-simulating conditions in Pennsylvania streams influenced by acid mine drainage. *Environmental Monitoring and Assessment* **145**, 323–338.

Rowe, C. L. (2014). Bioaccumulation and effects of metals and trace elements from aquatic disposal of coal combustion residues: recent advances and recommendations for further study. *Science of The Total Environment* **485-486**, 490–496.

Rowe, C. L., Hopkins, W. A., and Congdon, J. D. (2002). Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: a review. *Environmental Monitoring and Assessment* **80**, 207–276.

Rowe, C. L., Hopkins, W. A., and Congdon, J. D. (2001). Integrating individual-based indices of contaminant effects. How multiple sublethal effects may ultimately reduce amphibian recruitment from a contaminated breeding site. *The Scientific World Journal* **1**, 703–712.

Rudolph, B.-L., Andreller, I., and Kennedy, C. J. (2008). Reproductive success, early life stage development, and survival of Westslope cutthroat trout (*Oncorhynchus clarki lewisi*) exposed to elevated selenium in an area of active coal mining. *Environmental Science & Technology* **42**, 3109–3114.

Russell, M. (2014). Pollution Reduction Program 20-Aquatic Health Monitoring Report 2014. Fish and Macroinvertebrates. Niche Environment and Heritage.

Salomons, W. (1995). Environmental impact of metals derived from mining activities: Processes, predictions, prevention. *Journal of Geochemical Exploration* **52**, 5–23.

Santodonato, J. (1997). Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. *Chemosphere* **34**, 835–848.

Sarmiento, A. M., DelValls, A., Nieto, J. M., Salamanca, M. J., and Caraballo, M. A. (2011). Toxicity and potential risk assessment of a river polluted by acid mine drainage in the Iberian Pyrite Belt (SW Spain). *Science of The Total Environment* **409**, 4763.

Schorr, M. S., and Backer, J. C. (2006). Localized effects of coal mine drainage on fish assesmblages in a Cumberland Plateau stream in Tennessee. *Journal of Freshwater Ecology* **21**, 17–24.

Schorr, M. S., Dyson, M. C., Nelson, C. H., Van Horn, G. S., Collins, D. E., and Richards, S. M. (2013). Effects of stream acidification on lotic salamander assemblages in a coal-mined watershed in the Cumberland Plateau. *Journal of Freshwater Ecology* **28**, 339–353.

Scott, B., Ranjith, P. G., Choi, S. K., and Khandelwal, M. (2010). A review on existing opencast coal mining methods within Australia. *Journal of Mining Science* **46**, 280–297.

Scullion, J., and Edwards, R. W. (1980a). The effects of coal industry pollutants on the macroinvertebrate fauna of a small river in the South Wales coalfield. *Freshwater Biology* **10**, 141–162.

Scullion, J., and Edwards, R. W. (1980b). The effect of pollutants from the coal industry on the fish fauna of a small river in the South Wales coalfield. *Environmental Pollution* **21**, 141–153.

Seo, J., Kang, S.-W., Ji, W., Jo, H.-J., and Jung, J. (2012). Potential risks of effluent from acid mine drainage treatment plants at abandoned coal mines. *Bulletin of environmental contamination and toxicology* **88**, 990–996.

Seuront, L. (2010). Zooplankton avoidance behaviour as a response to point sources of hydrocarbon-contaminated water. *Marine and Freshwater Research* **61**, 263–270.

Seuront, L., and Leterme, S. (2007). Increased zooplankton behavioral stress in response to short-term exposure to hydrocarbon contamination. *The Open Oceanography Journal* **1**, 1–7.

Short, S. (2012). Pollution Reduction Program 11 - West Cliff Mine Water Discharge Ecotoxicity Study. Ecoengineers Pty Ltd.

Simmons, J. A., Lawrence, E. R., and Jones, T. G. (2005). Treated and untreated acid mine drainage effects on stream periphyton biomass, leaf decomposition, and macroinvertebrate diversity. *Journal of Freshwater Ecology* **20**, 413–424.

Simon, M. L., Cherry, D. S., Currie, R. J., and Zipper, C. E. (2006). The ecotoxicological recovery of Ely Creek and tributaries (Lee County, Va) after remediation of acid mine drainage. *Environmental Monitoring and Assessment* **123**, 109–124.

Smucker, N. J., and Vis, M. L. (2009). Use of diatoms to assess agricultural and coal mining impacts on streams and a multiassemblage case study. *Journal of the North American Benthological Society* **28**, 659.

Snodgrass, J. W., Hopkins, W. A., Broughton, J., Gwinn, D., Baionno, J. A., and Burger, J. (2004). Species-specific responses of developing anurans to coal combustion wastes. *Aquatic Toxicology* **66**, 171–182.

Snodgrass, J. W., Hopkins, W. A., Jackson, B. P., Baionno, J. A., and Broughton, J. (2005). Influence of larval period on responses of overwintering green frog (*Rana clamitans*) larvae exposed to contaminated sediments. *Environmental Toxicology and Chemistry* **24**, 1508–1514.

Soegianto, A., Winarni, D., Handayani, U. S., and Hartati (2013). Bioaccumulation, elimination, and toxic effect of cadmium on structure of gills and hepatopancreas of freshwater prawn *Macrobrachium sintangese* (De Man, 1898). *Water, Air, and Soil Pollution* **224**, 1575.

Soucek, D. J., Cherry, D. S., and Trent, G. C. (2000). Relative acute toxicity of acid mine drainage water column and sediments to *Daphnia magna* in the Puckett's Creek Watershed, Virginia, USA. *Archives of Environmental Contamination and Toxicology* **38**, 305–310.

Soucek, D. J., Cherry, D. S., and Zipper, C. E. (2001). Aluminum-dominated acute toxicity to the cladoceran *Ceriodaphnia dubia* in neutral waters downstream of an acid mine drainage discharge. *Canadian Journal of Fisheries and Aquatic Sciences* **58**, 2396–2404.

Soucek, D. J., Cherry, D. S., Currie, R. J., Latimer, H. A., and Trent, G. C. (2000). Laboratory to field validation in an integrative assessment of an acid mine drainage–impacted watershed. *Environmental Toxicology and Chemistry* **19**, 1036–1043.

Soucek, D. J., Currie, R. J., Cherry, D. S., Latimer, H. A., and Trent, G. C. (1998). Benthic macroinvertebrate assemblages and sediment toxicity testing in the Ely creek water shed restoration project. In National Meeting of the American Society of Mining and Reclamation. pp. 745–751.

Soucek, D. J., Schmidt, T. S., and Cherry, D. S. (2001). In situ studies with Asian clams (*Corbicula fluminea*) detect acid mine drainage and nutrient inputs in low-order streams. *Canadian Journal of Fisheries and Aquatic Sciences* **58**, 602–608.

Stauffer, J. R., and Ferreri, C. P. (2002). Characterization of stream fish assemblages in selected regions of mountain top removal/valley fill coal mining. In 'Mountaintop Mining/Valley Fills in Appalachia Draft Programmatic Environmental Impact Statement'. United States Environmental Protection Agency.

Suess, M. J. (1976). The environmental load and cycle of polycyclic aromatic hydrocarbons. *Science of The Total Environment* **6**, 239–250.

The Ecology Lab Pty Ltd (2008). West Cliff Colliery Area 5 Longwalls 34-36 Assessment of Mine Subsidence Impacts on Aquatic Habitat and Biota. BHP Billiton Illawarra Coal.

Thiruvenkatachari, R., Younes, M., and Su, S. (2011). Coal mine site investigation of wastewater quality in Australia. *Desalination and Water Treatment* **32**, 357–364.

Timpano, A. J., Schoenholtz, S. H., and Soucek, D. J. (2010). Isolating effects of total dissolved solids on aquatic life in central Appalachian coalfield streams. In National Meeting of the American Society of Mining and Reclamation. pp. 1284–1302

Tiwary, R. K. (2001). Environmental impact of coal mining on water regime and its management. *Water, Air, and Soil Pollution* **132**, 185–199.

Tripole, S., Gonzalez, P., Vallania, A., Garbagnati, M., and Mallea, M. (2006). Evaluation of the impact of acid mine drainage on the chemistry and the macrobenthos in the Carolina stream (San Luis, Argentina). *Environmental Monitoring and Assessment* **114**, 377–389.

Unrine, J. M., Hopkins, W. A., Romanek, C. S., and Jackson, B. P. (2007). Bioaccumulation of trace elements in omnivorous amphibian larvae: implications for amphibian health and contaminant transport. *Environmental Pollution* **149**, 182–192.

Vijver, M. G., Elliott, E. G., Peijnenburg, W. J. G. M., and de Snoo, G. R. (2011). Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. *Environmental Toxicology and Chemistry* **30**, 1482–1487.

Vink, S., and Robbins, S. (2012). Mine water management in variable climate regimes. In International Mine Water Association Symposium. pp. 20A–20I

Vink, S., Kerr, J., Wolhuter, A., Wu, R., Henderson, A., and Beyer-Robson, J. (2015). Guidelines for establishing ecologically sustainable mine water release criteria in seasonally flowing streams. No. C19024. ACARP.

Vink, S., Moran, C. J., Golding, S. D., Baublys, K., and Nanjappa, V. (2009). Understanding mine site water and salt dynamics to support integrated water quality and quantity management. *Mining Technology* **118**, 185–192.

Walton, N. R. G. (1989) Electrical conductivity and total dissolved solids – What is their precise relationship? *Desalination* **72**, 275–292.

Ward, C. K., Appel, A. G., and Mendonça, M. T. (2006). Metabolic measures of male southern toads (*Bufo terrestris*) exposed to coal combustion waste. *Comparative biochemistry and physiology* **143**, 353–360.

Warner, R. W. (1971). Distribution of biota in a stream polluted by acid mine-drainage. *The Ohio Journal of Science* **71**, 202–215.

Waters, A. S., and Webster-Brown, J. G. (2013). Assessing aluminium toxicity in streams affected by acid mine drainage. *Water science and technology* **67**, 1764–1772.

Whipple, A. V., and Dunson, W. A. (1993). Amelioration of the toxicity of H^+ to larval stoneflies by metals found in coal mine effluent. *Archives of Environmental Contamination and Toxicology* **24**, 194–200.

Williams, J. M., and Wood, P. B. (2004). Streamside salamanders in valley fill and reference streams in southern West Virginia. In National Meeting of the American Society of Mining and Reclamation.

Winterbourn, M. J. (1998). Insect faunas of acidic coal mine drainages in Westland, New Zealand. *New Zealand Entomologist* **21**, 65–72.

Winterbourn, M. J., McDiffett, W. F., and Eppley, S. J. (2000). Aluminium and iron burdens of aquatic biota in New Zealand streams contaminated by acid mine drainage: effects of trophic level. *Science of The Total Environment* **254**, 45–54.

Wood, L., and Welch, A. M. (2015). Assessment of interactive effects of elevated salinity and three pesticides on life history and behavior of southern toad (*Anaxyrus terrestris*) tadpoles. *Environmental Toxicology and Chemistry* **34**, 667–676.

Wood, P. B., and Williams, J. M. (2013a). Impact of valley fills on streamside salamanders in Southern West Virginia. *Journal of Herpetology* **47**, 119–125.

Wood, P. B., and Williams, J. M. (2013b). Terrestrial salamander abundance on reclaimed mountaintop removal mines. *Wildlife Society Bulletin* **37**, 815–823.

Wright, I. A., and Burgin, S. (2009a). Comparison of sewage and coal-mine wastes on stream macroinvertebrates within an otherwise clean upland catchment, southeastern Australia. *Water, Air, and Soil Pollution* **204**, 227–241

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

Xue, W., and Warshawsky, D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology* **206**, 73–93.

Zalizniak, L., Kefford, B. J., and Nugegoda, D. (2006). Is all salinity the same? I. The effect of ionic compositions on the salinity tolerance of five species of freshwater invertebrates. *Marine and Freshwater Research* **57**, 75–82.

Zocche, J. J., da Silva, L. A., Damiani, A. P., Mendonça, R. Á., Peres, P. B., Santos, dos, C. E. I., Debastiani, R., Dias, J. F., Moraes de Andrade, V., and Pinho, R. A. (2014). Heavy-metal content and oxidative damage in *Hypsiboas faber*: the impact of coal-mining pollutants on amphibians. *Archives of Environmental Contamination and Toxicology* **66**, 69–77.

Zocche, J. J., Damiani, A. P., Hainzenreder, G., Mendonça, R. Á., Peres, P. B., Santos, C. E. I. D., Debastiani, R., Dias, J. F., and Andrade, V. M. de (2013). Assessment of heavy metal content and DNA damage in *Hypsiboas faber* (anuran amphibian) in coal open-casting mine. *Environmental Toxicology and Pharmacology* **36**, 194–201.

Declaration of co-authorship and contribution

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Lanctôt, C., Wilson, S. P., Fabbro, L., Leusch, F. D. L., Melvin, S. D. (2016). Comparative sensitivity of aquatic invertebrate and vertebrate species to wastewater from an operational coal mine in Central Queensland, Australia. *Ecotoxicology and Environmental Safety* **129**, 1–9. http://dx.doi.org/10.1016/j.ecoenv.2016.03.003

My contribution to the paper involved:

- Initial concept and experimental design.
- Collection and analysis of data.
- Preparation of manuscript.

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Publication of Research Higher Degree Work for Inclusion in the Thesis Procedures

[redacted]

Chantal Lanctôt

Chapter 3

Comparative sensitivity of aquatic invertebrate and vertebrate species to wastewater from an operational coal mine in central Queensland, Australia

Chantal M. Lanctôt [a, b, *], Scott P. Wilson [a], Larelle Fabbro [c], Frederic D.L. Leusch [b], Steven D. Melvin [a, b]

[a] Central Queensland University, School of Medical and Applied Sciences, Gladstone, QLD 4680, Australia

[b] Smart Water Research Centre and Australian Rivers Institute, Griffith School of Environment, Griffith University, Southport, QLD 4215, Australia

[c] Central Queensland University, School of Medical and Applied Sciences, Rockhampton, QLD 4702, Australia

* Corresponding author
e: chantal.lanctot@gmail.com, c.lanctot@cqu.edu.au
Mailing address:
Chantal Lanctôt
Smart Water Research Centre (G51)
Griffith University, Gold Coast Campus
Southport, Queensland, 4215
Australia

3.1 Abstract

Coal excavation and refinement processes generate substantial volumes of contaminated effluent that may be detrimental to aquatic ecosystems. As such, understanding the impacts of coal mine water releases on aquatic animals and ecosystems is essential for effectively managing and protecting neighboring environments. Such information will ultimately be applied towards developing ongoing monitoring strategies that are protective of native wildlife. Despite intensive mining operations in Australia, few studies have documented toxicity associated with coal mine wastewater (CMW) on native species. To address existing knowledge gaps, we investigated acute toxicity (48–96 h) using eight native invertebrate species and sub-chronic effects (2 week) using three vertebrate species following exposure to wastewater from two dams (CMW1 and CMW2) located at an open-cut coal mine licensed to discharge into the Fitzroy catchment (Queensland, Australia). Wastewater from these sites is characterized by elevated conductivity, pH, sulfates as well as relatively high total and dissolved metal(loid)s (including As, Al, B, Cu, Mn, Ni, Se and Zn). Acute exposures revealed cladocerans (*Daphnia carinata*) and planarians (*Dugesia* sp.) to be the most sensitive species, exhibiting significant mortality after 48 and 96 h exposure to CMW2, respectively. Neither wastewater was found to elicit acute toxicity in vertebrates, but a range of sub-lethal morphological effects were observed following the sub-chronic exposures. The overall response pattern was characterized by decreased condition factor and hepatosomatic index in the fish *Hypseleotris compressa* and *Pseudomugil signifier*, and in *Limnodynastes peronii* tadpoles. Tadpoles were generally more sensitive compared to the two fish species. Differences in responses were observed amongst CMW1 and CMW2, which likely relates to differences in physico-chemical properties between sites. Our results have identified several candidate vertebrate and invertebrate species that show promise for ongoing monitoring of water quality and toxicity risk in Central Queensland, Australia.

3.2 Keywords

Coal mine wastewater; Whole effluent toxicity; Tadpole; Fish; Invertebrates; Australia

3.3 Introduction

Coal mining is an important industry globally, and particularly so in Australia, where coal exportation represents a significant part of the country's economy (Höök et al., 2010; IEA, 2012). Black coal is mined predominantly in Queensland, with over 50 mines currently producing approximately 200 million tons of saleable coal annually (DNRM, 2013). However, despite the economic benefits, coal mining presents a number of major environmental issues including landscape alteration, air pollution and aquatic contamination (Bian et al., 2010). One of the foremost environmental concerns related to the daily operations of a coal mine is the production of substantial volumes of process-affected water that may be discharged into adjacent aquatic ecosystems. The extraction and processing of coal require a constant supply of water (Scott et al., 2010; Haibin and Zhenling, 2010; Thiruvenkatachari et al., 2011), and despite regulated releases of mine wastewater, uncontrolled releases are not uncommon during extreme rainfall events and flooding. These discharges can be highly saline and/or acidic, and may often contain high levels of dissolved solids, suspended solids, metal(loid)s (*e.g.*, Al, As, Cd, Cu, Mn, Ni, Fe, Se, Zn), hydrocarbons (*e.g.*, polycyclic aromatic hydrocarbons), and other compounds (Tiwary, 2001; Thiruvenkatachari et al., 2011), which have all been shown to have detrimental impacts on aquatic biota (Birge 1978; Eisler, 1987; Atchison et al., 1987; Nielsen et al., 2003; Muschal, 2006; Langdon et al., 2009; Hogsden and Harding, 2012; Cañedo-Argüelles et al., 2013; DeForest and Meyer, 2015). It is therefore important that both the volume and the quality of coal mine wastewater being discharged into the environment be effectively managed.

Rapid industrialization in Australia over the last century has put pressure on many of its limited freshwater systems, and has subsequently put native species at risk. This holds particularly true for the Fitzroy Catchment in Central Queensland, which coincides with Australia's largest black coal deposit in the Bowen Basin. Environmental research in this region has largely focused on monitoring water quality and have provided extensive data on flows, sediment loads, physical and chemical water quality characteristics, and assemblages of aquatic flora and fauna (*e.g.*, Noble et al., 1996a, 1996b; DEH and DNR, 1999; DERM, 2001; FBA, 2008, 2009; DERM, 2009, 2010, 2011a, 2011b). In recent years, there has been considerable focus directed towards understanding the risks associated with elevated salinity from coal mine water discharge on streams in the Fitzroy Catchment (Vink et al., 2009; Vink and Robbins, 2012). However, despite these research efforts, there have been few studies directed at understanding the potential for organism-level toxicological effects of coal mine discharge on locally relevant aquatic species (Hart et al., 2008). There have been almost no studies investigating chronic toxicological effects related to coal mine discharge on native aquatic vertebrates. A cumulative impact assessment released by the then Queensland Department of Environment and Resource Management recently established that mining discharge into the Fitzroy Basin catchment is currently not protective of the downstream environment, and that data are extremely limited and inadequate for quantifying cumulative impacts (DERM, 2009). Similar conclusions have been raised globally (Bernhardt and Palmer, 2011; US EPA, 2011; Bernhardt et al., 2012; Bharti and Banerjee, 2014), highlighting the need to explore the potential for adverse toxicological outcomes in relevant aquatic invertebrate and vertebrate species exposed to coal mine wastewater.

Whole effluent toxicity tests have been widely applied to assess the potential toxicity of complex effluents to aquatic organisms, and to monitor industrial and municipal water discharge (US EPA, 2000; Chapman, 2000; van Dam and Chapman, 2001). Given the significance of coal mining operations in Australia and the relatively limited whole organism toxicity information that exists for the Fitzroy River Basin, there is a clear need for such testing with a range of native aquatic organisms. This is not only necessary to fill basic knowledge gaps regarding the potential for adverse effects in exposed animals, but to facilitate effective ongoing monitoring of water quality in aquatic systems receiving mine water discharge, through the identification of comparatively sensitive species. We therefore investigated the impacts of wastewater from a Queensland open-cut coal mine on a range of native aquatic organisms. Acute toxicity tests were used to compare the sensitivity of eight native freshwater invertebrates and three aquatic vertebrate species to various dilutions of wastewater. Sub-lethal impacts were considered in tests with vertebrates, including effects on growth, development and hepatic condition of two species of fish and one species of larval amphibian. The study aims to provide basic acute and sub-lethal toxicity information for a range of relevant species, to determine the relative sensitivities of the different organisms and help develop an effective monitoring suite directly relevant for assessing toxicity associated with the release of coal mine wastewater in the Fitzroy Catchment.

3.4 Materials and methods

3.4.1 Animals

Broad sampling was performed at uncontaminated sites in Central Queensland, Australia, to collect various species of aquatic organisms from the local environment (Average water quality parameters at the time of sampling were: temperature: 24 ± 2.3 °C, conductivity: 1.0 ± 0.2 mS cm^{-1}, pH: 8.5 ± 0.4 and dissolved oxygen: 89 ± 19%). Species were selected for their relevance to the coal mining region within the Bowen Basin. For vertebrates, empire gudgeons (*Hypseleotris compressa*) and pacific blue-eyes (*Pseudomugil signifer*) fish (standard length ~2 cm), as well as a fertilized striped marsh frog (*Limnodynastes peronii*) egg mass were collected. A range of invertebrate species was also collected, and sorted by species and size classes in the laboratory. Sufficient numbers were obtained for replicated toxicity testing with eight invertebrate species, including *Paratya australiensis* (Decapoda: Atyidae, 10–14 mm), *Chironomus* sp. larvae (Diptera: Chironomidae, 7–10 mm), Leptophepiid nymphs (Ephemeroptera: Leptophlebiidae, 3–9 mm), Coenagrionid larvae (Odonata: Coenagrionidae, 12–19 mm), *Dugesia* sp. (Tricladida:Dugesiidae), Erpobdellid (Arhynchobdellida: Erpobdellidae), *Sphaerium* sp. (Bivalvia: Sphaeriidae, 2–5 mm) and *Daphnia carinata* (Cladocera: Daphniidae, < 48 h old). *L.*

peronii eggs were hatched in the laboratory in natural pond water, which was slowly replaced by filtered rainwater (5 µm wound sediment removal cartridge filter). All organisms were acclimatized to laboratory conditions in aerated glass aquaria filled with filtered rainwater (Invertebrates > 48 h; Fish and tadpoles > 2 weeks) prior to testing. During this time, fish and tadpoles were fed *ad libitum* daily with flake and pellet foods (Ocean Nutrition™) or Sera Micron® powered food, respectively. Tadpoles of Gosner developmental stage (Gs; Gosner, 1960) 27 were used for experiments. All aspects of experimentation and sampling were approved by the Animal Ethics Committee of Central Queensland University and in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (CQUniversity Approval No. A13/05-301).

3.4.2 Wastewater sampling and water quality

Wastewater was collected from an open-cut coal mine located within the Bowen Basin in Central Queensland, Australia (approximately 250 km west of the coast), in August 2013. At the time of experimentation, the mine had two active release points authorized for discharge of mine-affected water, hereafter referred to as CMW1 and CMW2 dams. *In situ* measurements of temperature, electrical conductivity (EC), pH, dissolved oxygen (DO) and turbidity were taken at the time of sampling using a YSI multiparameter handheld sonde (Xylem Analytics, Hemnant, Australia). Wastewater was collected for experiments in acid washed 5 L plastic containers. Wastewater was transported on ice, stored in the refrigerator at 4 °C, and brought to ambient temperature prior to experimentation (~ 48 h post collection). Wastewater was filtered using small mesh (500 µm) to remove any large particles for invertebrate tests. Water quality parameters (temperature, EC, pH and dissolved oxygen) were measured for each treatment group using YSI EcoSence® probes at start and end of each acute test, and before and after each water renewal in the chronic exposures.

3.4.3 Acute toxicity in invertebrates, fish and tadpoles

For acute toxicity tests, all organisms were exposed to wastewater dilutions from the two holding dams (CMW1 and CMW2), ranging from 0% to 100% for 96 h, with the exception of *Daphnia carinata*, which was exposed for 48 h (based on ASTM (2002) and OECD (2004) guidelines). Specific details of the experimental design for each species are described in Table 3-1. Survival and health observations were recorded daily. At the end of the 96 h exposure, invertebrate species were euthanized in ethanol. *H. compressa*, *P. signifer* and *L. peronii* from the 50% and 75% treatments were also euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) dissolved in water at the end of the 96 h exposure (control and 100% treatments were maintained for sub-chronic testing, see Section 2.4).

Table 3-1: Experimental design of acute and sub-chronic exposures to wastewater dilutions from CMW1 and CMW2 dams.

Species	Treatments	Rep.[a]	*n*[b]	Total *n*	Volume (mL)
Acute					
Pseudomugil signifer	Control; 50, 75%, 100% CMW1 and CMW2	3	5	105	1000
Hypseleotris compressa	Control; 50, 75%, 100% CMW1 and CMW2	3	5	105	1000
Limnodynastes peronii	Control; 50, 75%, 100% CMW1 and CMW2	3	3	63	250
Paratya australiensis	Control; 50, 75%, 100% CMW1 and CMW2	3	4	84	50
Sphaerium sp.	Control; 50, 100% CMW1 and CMW2	3	4	60	50
Erpobdellidae	Control; 50, 100% CMW1 and CMW2	3	1	15	25
Dugesia sp.	Control; 50, 100% CMW1 and CMW2	3	3	45	25
Chironomus sp.	Control; 50, 75%, 100% CMW1 and CMW2	3	3	63	50
Coenagrionidae	Control; 50, 100% CMW1 and CMW2	3	1	15	50
Leptophlebiidae	Control; 50, 100% CMW1 and CMW2	3	3	45	50
Daphnia carinata	Control; 50, 100% CMW1 and CMW2	3	5	75	25
Sub-chronic					
Pseudomugil signifer	Control; 100% CMW1 and CMW2	3	5	45	1000
Hypseleotris compressa	Control; 100% CMW1 and CMW2	3	5	45	1000
Limnodynastes peronii	Control; 100% CMW1 and CMW2	3	3	27	800

a. Number of replicates.

b. Number of animals per replicate.

3.4.4 Sub-chronic toxicity in fish and tadpoles

For sub-chronic toxicity tests, fish and tadpoles from control and 100% treatments were exposed for 13 d (Table 3-1). Exposure aquaria were gently aerated through the sub-chronic exposure, and animals were fed daily (approximately 6.3 mg/fish and 4.2 mg/tadpole). Fish were fed tropical fish crumbles (Wardley®) and tadpoles were fed Sera Micron® powdered fry food (Sera GmbH). Feces and excess food waste were removed daily, and full water changes were performed on days 4 and 9 to renew treatment dilutions.

At the end of the two-week exposure period, all animals were euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) dissolved in water. Morphometric measurements were taken for fish (standard length, total length, weight) and tadpoles (snout-vent length [SVL], total length, weight) at the end of the exposures. Tadpole developmental stage (Gosner, 1960) was also noted, and livers were dissected from each animal and weighed. Measurements were used to calculate the condition factor ($K = 100 \times$ [body weight/standard length or SVL^3]) and hepatosomatic index (HSI = 100 × [liver weight/body weight]).

3.4.5 Statistical analysis

Treatment effects on survival were analyzed using Mantel-Cox log rank tests performed with Graph Pad Prism® v6.0 f for Mac OS X (GraphPad Software, Inc.). Mantel-Cox pairwise comparisons were used to test for differences between each treatment and control, with the level of significance (α) adjusted for the number of comparisons. Treatment effects on morphometric data (lengths, weight, *K*, liver weight, HSI and Gs) were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests performed with SPSS 20.0.0 (SPSS Inc., Chicago, IL). Data were first tested for normality (Kolmogorov–Smirnov) and homogeneity of variance (Levene's test). The significance level was set at $\alpha \leq 0.05$.

3.5 Results

3.5.1 Water quality of wastewater dams

Water quality parameters taken *in situ* at the time of sampling and throughout the experiments are presented in Table 3-2. CMW1 dam had 1.6-times lower EC and 3.6-times higher turbidity compared to CMW2 at the time of sampling. Treatment groups in the laboratory also exhibited higher EC and pH compared to controls. Temperature and DO were generally consistent between treatments throughout the experiment. Results from ongoing chemical analysis at the studied dams (CMW1 and CMW2) are presented in Table 3-3 (Appendix). Wastewater from both dams is characterized by elevated conductivity, salinity, pH, as well as high levels of total and dissolved metal(loid)s (including As, Al, B, Cu, Mn, Ni, Se and Zn).

Table 3-2: Mean (±SD) temperature, dissolved oxygen (DO), electric conductivity (EC), pH and turbidity of CMW1 and CMW2 dams measured *in situ* at the time of sampling and treatment dilutions measured throughout acute and sub-chronic experiments.

	Temperature (°C)	**DO (%)**	**EC (mS cm^{-1})**	**pH**	**Turbidity (NTU)**
CMW1 *in situ*	20.7	43.1	4.51	9.04	28.4
CMW2 *in situ*	20.9	100.1	7.20	8.90	8.0
Acute					
Control	24.0 ± 1.6	86.8 ± 12.4	0.17 ± 0.05	7.77 ± 0.4	-
50% CMW1	23.9 ± 1.6	87.2 ± 8.5	2.58 ± 0.68	8.84 ± 0.1	-
75% CMW1	24.6 ± 1.6	81.4 ± 15.0	4.32 ± 1.31	8.90 ± 0.2	-
100% CMW1	23.9 ± 1.6	81.8 ± 17.9	4.68 ± 0.58	9.02 ± 0.1	-
50% CMW2	24.2 ± 1.7	90.2 ± 10.5	4.13 ± 0.61	8.82 ± 0.1	-
75% CMW2	24.6 ± 1.5	86.8 ± 19.8	5.31 ± 0.95	8.85 ± 0.2	-
100% CMW2	23.9 ± 1.6	88.6 ± 15.4	7.68 ± 0.78	8.98 ± 0.1	-
Sub-chronic					
Control	23.6 ± 0.8	89.9 ± 4.7	0.16 ± 0.01	7.37 ± 0.2	-
100% CMW1	23.1 ± 1.5	74.2 ± 17.3	4.59 ± 0.22	8.70 ± 0.1	-
100% CMW2	23.2 ± 1.5	83.9 ± 18.2	7.33 ± 0.35	8.69 ± 0.1	-

3.5.2 Acute toxicity in invertebrates, fish and tadpoles

Significant treatment effects on survival were observed for *P. australiensis* (Figure 3-1a; X^2 = 16.57, df = 6, p = 0.011), *Dugesia* sp. (Figure 3-1d; X^2 = 40.28, df = 4, p < 0.001), *Sphaerium* sp. (Figure 3-1e; X^2 = 12.72, df = 4, p = 0.013) and *D. carinata* (Figure 3-1f; X^2 = 19.59, df = 4, p = 0.001). Post-hoc analysis revealed that survival of *Dugesia* sp. and *D. carinata* exposed to 100% CMW2 was significantly reduced compared to other treatments. Post-hoc tests for *P. australiensis* and *Sphaerium* sp., on the other hand, was unable to differentiate survival effects between groups. Survival of *Chironomus* sp. and Leptophlebiidae decreased over time in all treatments but survival did not differ significantly between treatment groups and control animals (Figure 3-1b and c; X^2 = 1.05, df = 6, p = 0.984 and X^2 = 109, df = 4, p = 0.896, respectively). No mortalities were observed for Coenagrionid larvae or Erpobdellid in any treatments (data not shown). No mortalities were observed in *P. signifer*, *H. compressa*, and *L. peronii* after 96-h of exposure (data not shown).

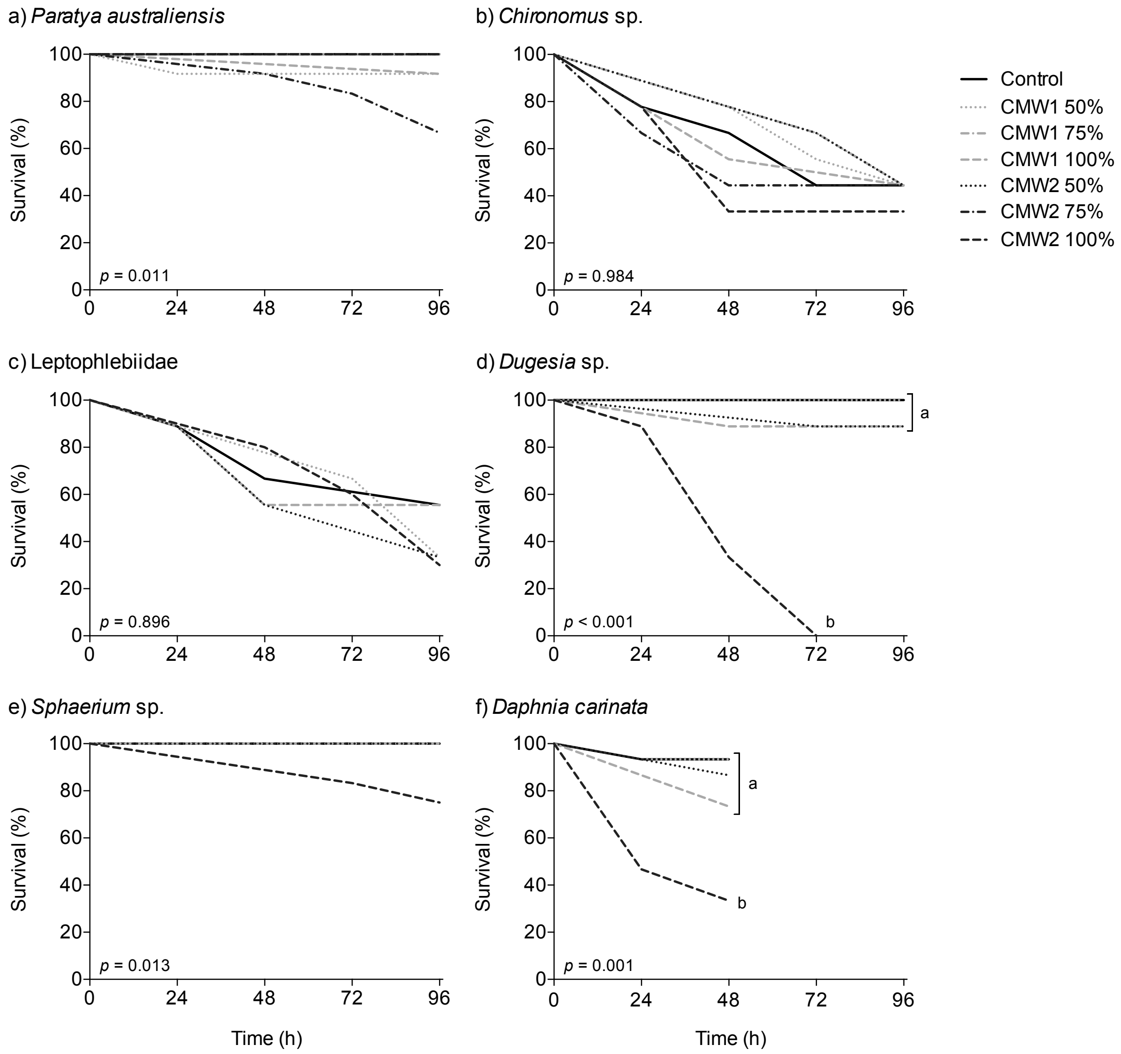

Figure 3-1: Mean survival of (a) *Paratya australiensis*, (b) *Chironomus* sp. larvae, (c) Leptophlebiidae nymphs, (d) *Dugesia* sp., (e) *Sphaerium* sp. and (f) *Daphnia carinata* throughout acute exposures to coal mine wastewater dilutions from CMW1 and CMW2 dams and control. Letters indicate significant differences between treatments.

3.5.3 Sub-chronic toxicity in fish and tadpoles

No significant differences in vertebrate survival were seen after two weeks of exposure to undiluted wastewater from either dam ($p > 0.05$). There were no mortalities observed in controls or 100% CMW1 treatments at the end of the exposure, and only one individual of each species died in the 100% CMW2 treatment. With the stocking densities of our tests this represents 93, 93, and 89% survival of *P. signifer*, *H. compressa* and *L. peronii*, respectively, exposed to 100% CMW2.

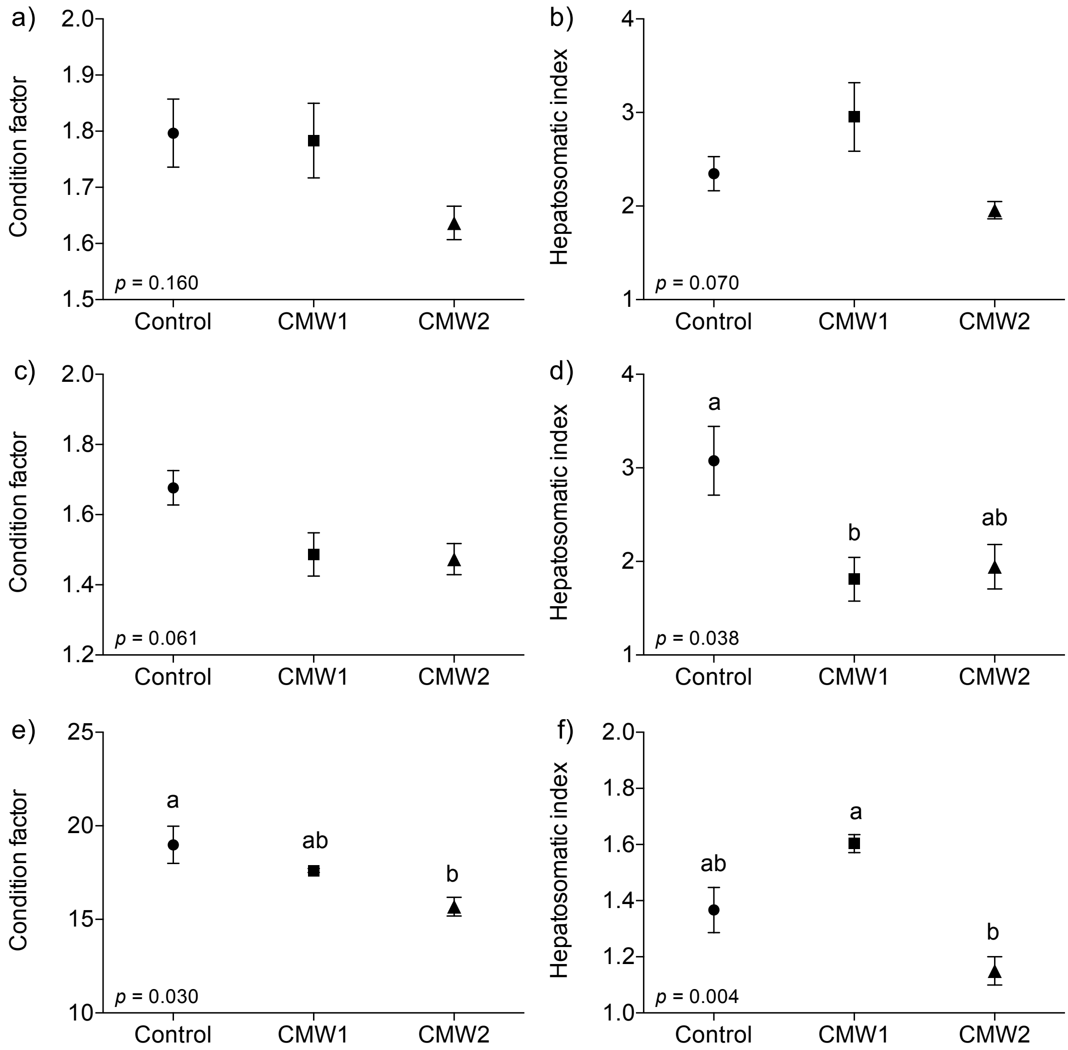

Figure 3-2: Condition factor and hepatosomatic index of *Pseudomugil signifer* (a and b), *Hypseleotris compressa* (c and d) and *Limnodynastes peronii* (e and f) after short-term (13 d) exposure to 100% coal mine wastewater from CMW1 and CMW2 holding dams and control. Symbols indicate the average (±SEM) of 3 replicates of 5 fish (a–d) or 3 tadpoles (e–f). Letters indicate significant differences between treatments. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$.

No significant differences in *P. signifer* standard length ($F_{(2,8)} = 3.29$, $p = 0.109$, data not shown), weight ($F_{(2,8)} = 2.13$, $p = 0.199$, data not shown), K ($F_{(2,8)} = 2.53$, $p = 0.160$, Figure 3-2a) or HSI ($F_{(2,8)} = 4.27$, $p = 0.070$, Figure 3-2b) were observed between treatments at the end of the two-week exposure. *H. compressa* exposed to 100% CMW1 and CMW2 exhibited reduced HSI compared to controls (1.7-and 1.6-times lower, respectively), however differences were only significantly lower in fish exposed to CMW1 ($F_{(2,8)} = 5.93$, $p = 0.038$, Figure 3-2d). No significant differences in *H. compressa* standard length ($F_{(2,8)} = 2.23$, $p = 0.188$, data not shown), weight ($F_{(2,8)} = 2.43$, $p = 0.169$, data not shown) or *K* ($F_{(2,8)} = 4.63$, $p = 0.061$, Figure 3-2c) were observed between treatment groups. Significant decreases in SVL ($F_{(2,8)} = 8.84$, $p = 0.016$, data not shown), total length ($F_{(2,8)} = 69.50$, $p < 0.001$, data not shown), weight ($F_{(2,8)} = 27.18$, $p = 0.001$, data not shown), condition factor ($F_{(2,8)} = 6.65$, $p = 0.030$, Figure 3-2e), and hepatosomatic index ($F_{(2,8)} = 15.65$, $p = 0.004$, Figure 3-2f) were observed in exposed *L. peronii* tadpoles. Specifically, tadpoles exposed to both treatments weighed approximately 1.3–2.0-times less compared to controls (data not shown). Tadpoles exposed to CMW2 also had reduced SVL (data not shown) and *K* (Figure 3-2e) compared to controls (1.2-times lower). Tadpole HSI, on the other hand, was slightly higher in CMW1 and lower in CMW2 compared to control but did not differ significantly (Figure 3-2f). There was an apparent delay in the development of tadpoles exposed to wastewater from both dams, but differences at the end of the experiment were not statistically significant between treatments ($F_{(2,8)} = 4.53$, $p = 0.063$, Figure 3-3).

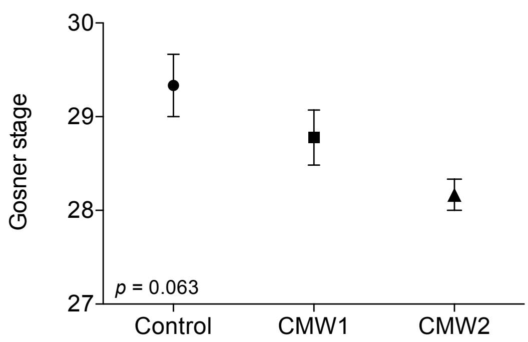

Figure 3-3: Gosner developmental stage of *Limnodynastes peronii* after short-term (13 d) exposure to 100% coal mine wastewater from CMW1 and CMW2 dams and control. Symbols indicate the average (±SEM) of 3 replicates of 3 tadpoles each. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$.

3.6 Discussion

3.6.1 Acute toxicity in invertebrates, fish and tadpoles

We exposed a range of native aquatic invertebrate and vertebrate species to wastewater from two holding dams (CMW1 and CMW2) located at an open-cut coal mine in Central Queensland (Table 3-1). The dams were chosen because they are both authorized for controlled discharge and exhibit very different physico-chemical properties, and thus reflect a range of possible contaminant levels and water quality characteristics that might be associated with mine water releases in the region. Acute toxicity tests revealed *D. carinata* and *Dugesia* sp. to be the most sensitive of the 11 species tested, with low survivability observed following exposure to water from CMW2. Survival of *Sphaerium* sp. and *P. australiensis* was also reduced in animals exposed to 100% and 75% CMW2, respectively. However, differences in survival for these two species were not statistically significant since mortalities were only observed in one replicate whereas all other replicates and treatments exhibited no mortalities. The other invertebrate species did not appear to be suitable for acute toxicity testing of our wastewater due to either an apparent lack of sensitivity (*e.g.*, Coenagrionid and Erpobdellid) or low survivability in control animals (*e.g.*, *Chironomus* sp. and Leptophlebiidea). No mortalities were observed in fish or tadpoles following 96 h exposure. More research is needed to assess the potential sub-lethal and long-term impacts of coal mine discharges on native invertebrate species, particularly *D. carinata* and *Dugesia* sp., in order to establish their suitability for longer term monitoring.

Our results revealed two locally relevant invertebrate species that may be useful for monitoring water quality in the mining region of Central Queensland. Cladocerans are commonly used in standard toxicity bioassays worldwide (ASTM, 2002; US EPA, 2002; OECD, 2004) because of their high sensitivity to various pollutants, short life cycle, and ease of culture and handling. However, numerous researchers have advocated the use of locally relevant species in ecotoxicology, and this has resulted in an increasing number of studies employing other Australian species such as *D. carinata* (van Dam et al., 1998; Kefford et al., 2002; Zalizniak and Nugegoda, 2004; Zalizniak et al., 2006; Cooper et al., 2009; Leung et al., 2011). For the purpose of monitoring water quality in coal mining regions, *D. carinata* makes a good candidate because it is the most abundant and widely distributed daphnid in Australia (Benzie, 1988) and is comparatively more sensitive than other species (Jana and Chakrabarti, 1993). Planarians such as *Dugesia* sp. have been used to a much lesser extent for whole effluent toxicity testing (Kapu and Schaeffer, 1991; Pagán et al., 2009), although they have been extensively studied in regeneration research (Newmark and Sánchez Alvarado, 2002) and more recently in neuropharmacology (Buttarelli et al., 2008). Like cladocerans, planarians commonly respond to lower levels of contamination compared to other species (Kefford et al., 2003; Hughes et al., 2005; Kefford et al., 2012). For example, planarians were previously suggested as good models for monitoring the quality of water from metal polluted areas (Calevro et al., 1999) and as sensitive indicators of ammonia toxicity (Alonso and Camargo, 2011; 2015). Their sensitivity to toxicants and environmental stressors are not surprising given their small size, simple osmoregulatory mechanisms, and lack of specialized detoxification mechanism (Hart et al., 1991; Hughes et al., 2005). Our findings support these earlier studies, and we suggest research investigating the use of sub-lethal behavioral endpoints with this species, since behavioral changes were observed at concentrations roughly 20 times lower than LC50s (Alonso and Camargo, 2011).

3.6.2 Sub-chronic toxicity in fish and tadpoles

Exposure to coal mine wastewater from the two dams was not acutely toxic to fish or tadpoles after 96 h, and there was similarly no significant effect on survival following the full two-week exposure. Slight mortality was observed after two-week exposure to 100% CMW2 wastewater, with only one individual of each species not surviving. However, we observed a range of sub-lethal effects on various morphometric endpoints, with differences in sensitivity apparent amongst the three vertebrate species. The lowest sensitivity was observed for fish, with a minor non-significant decrease in condition and HSI observed in *P. signifer* exposed to 100% CMW2 for two weeks. *H. compressa* was slightly more sensitive, with a significant decrease in HSI observed and an apparent (but non-significant) reduction in condition in both CMW1 and CMW2 treatments. Differences in sensitivity between fish species are not surprizing and have been well documented in the literature (Pickering et al., 1962; Maltby et al., 2005; Dolezelova et al., 2009). In a recent study, *H. compressa* was observed to exhibit sub-lethal morphometric alterations following a similarly short-duration exposure to domestic sewage (Melvin, 2016), supporting the potential for using this species for sub-chronic toxicity testing of stressors in the Australian environment. Nevertheless, amphibian larvae were found to be more sensitive than either fish species, which confirms a recent analysis comparing acute and sub-lethal toxicity of amphibians and fish to a range of contaminants (Weltje et al., 2013). We observed significantly reduced SVL, weight, and condition of tadpoles exposed to CMW2 wastewater, and similar effects with exposure to CMW1, although the latter were not statistically significant. Significant differences in HSI were observed amongst treatment groups, although this was characterized by slightly increased HSI in CMW1 and decreased HSI in CMW2, with neither group being significantly different from controls (Figure 3-2f). Finally, tadpole development was apparently delayed by CMW2 (~ 1 Gs) but differences did not reach statistical significance, which may simply reflect the relatively short duration of the exposure.

Alterations to *K* and HSI are frequently considered as indicators of animal fitness and tertiary stress responses, and may indicate changes in nutritional and energy status resulting from altered allocation of energy reserves (Melvin et al., 2013; Bharti and Banerjee, 2014; Melvin, 2015). Decreased *K* and HSI observed in the present study are consistent with other studies showing similar responses in fish exposed to coal mine wastewater in both laboratory (Bharti and Banerjee, 2014) and field settings (Scullion and Edwards, 1980). Mechanistically, effects on hepatic tissues following exposure of fish to coal mine wastewater may be related to metal accumulation (Bharti and Banerjee, 2011; 2013), tissue damage (Rudolph et al., 2008), DNA damage (Martin and Black, 1998; Benassi et al., 2006; da Silveira et al., 2009; Bharti and Banerjee, 2014) or basic physiological stress responses (Benassi et al., 2006; Miller et al., 2009; Bharti and Banerjee, 2014). Interestingly, while a non-significant decrease in *K* and significant decrease in HSI were observed in the present study, contrasting trends (*i.e.*, increase *K* and HSI) have also been observed in *H. compressa* (Lanctôt et al., 2016) and other fish species (Rudolph et al., 2008; Miller et al., 2009) exposed to coal mine wastewater. Differences in morphometric responses amongst studies may relate to several factors, including differences in water quality and chemistry, species sensitivity and exposure duration. Despite observed differences in responses, results of this and previous studies clearly demonstrate the potential for coal mine wastewater to elicit a broad range of effects in exposed aquatic vertebrates. Moreover, discrepancies highlight the need to establish site-specific monitoring using relevant native species. Overall responses observed in the present study indicated greater toxicity of CMW2 compared to CMW1, following both acute and sub-chronic exposures using a range of species. As discussed, differences in response patterns between the two dams most likely reflect the rather large disparities in water quality and chemistry that were observed. For example, CMW1 had higher turbidity and lower EC compared to CMW2, whereas both sites had similar pH (Table 3-2). Monitoring results from these two dams also revealed CMW1 as generally having higher total and dissolved organic carbon, suspended solids, nutrients (N and P), alkalinity and arsenic levels, whereas CMW2 tends to exhibit higher salinity, hardness and sulfate levels (Appendix: Table 3-3). Both EC and sulfate levels were on average 1.6-times higher in CMW2 compared to CMW1, and salinity levels in both dams exceeded trigger values of the Australian environmental water quality guidelines (ANZECC and ARMCANZ, 2000). Elevated salinity may therefore be an important contributing factor resulting in the increased toxicity observed for CMW2 as several studies have reported that elevated salinity can lead to reduced survival and diversity in Australian species (Christy and Dickman, 2002; Chinathamby et al., 2006; Smith et al., 2007; Kearney et al., 2012; Dunlop et al., 2015). In fact, salinities exceeding 1000 mg/L (approximately 3–5 times lower than our holding dams) have been shown to cause detrimental impacts to Australian freshwater ecosystems at both the organism and population level (Hart et al., 1991; Nielsen et al., 2003; Muschal, 2006). Nevertheless, it is difficult to establish a firm causal relationship from a single study due to the multitude of potentially interacting factors associated with complex wastewater, and the combined pressures resulting from a range of variable water quality characteristics offers the best explanation for disparities in response patterns that sometimes occur in the literature.

3.7 Conclusion

Coal excavation can lead to the introduction of unnaturally high levels of contaminants into surface waters, thereby resulting in the unintentional exposure of aquatic animals to potentially harmful concentrations of these compounds. Our results reveal that coal mine wastewater from two holding dams with different physico-chemical properties elicited a range of toxicological outcomes on native invertebrate and vertebrate species, and that response patterns differed markedly between species and sites. We identified two local invertebrate species, *Dugesia* sp. and *D. carinata*, as showing promise for monitoring water quality and toxicity risks in mine-affected regions throughout Central Queensland, Australia. Acute lethality was not observed in any of three vertebrate species tested, but fish (*H. compressa*) and tadpoles (*L. peronii*) both exhibited morphological effects following relatively short-duration exposure. These species may therefore serve as good models for monitoring sub-lethal responses associated with mine water releases. Combined, this provides a total of four native freshwater species from different trophic levels that exhibit sensitivity to coal mine wastewater.

The present study aimed to fill existing knowledge gaps concerning the basic toxicity of coal mine wastewater to locally relevant species in Queensland's Fitzroy River Basin. Considering the importance of coal mining in Australia and globally, there is a clear need for further research investigating the cumulative impacts of coal mine wastewater on native aquatic organisms. Specifically, relatively little is known on the potential sub-lethal and long-term impacts of coal mine discharges on native species. This information is necessary to continue working towards establishing effective approaches for long-term monitoring. In addition to the basic toxicity information presented herein, we propose further research aimed at identifying sensitive toxicity endpoints and biomarkers of exposure. For example, future studies should explore physiological endpoints related to detoxification and oxidative stress, which may be applicable to field-based monitoring. Behavioral testing provides another option that may be useful for assessing short-term toxicological responses in exposed animals, and may thus be applied towards water quality monitoring during planned release events.

3.8 Acknowledgements

This study was supported by the Central Queensland University Postgraduate Research Award (CL), a Queensland Resource Council Coal Minesite Rehabilitation Trust Fund Scholarship (CL) and in-kind industry support. The funding sources had no involvement in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

3.9 References

Alonso, A., Camargo, J.A., 2011. The freshwater planarian *Polycelis felina* as a sensitive species to assess the long-term toxicity of ammonia. Chemosphere 84, 533–537.

Alonso, A., Camargo, J.A., 2015. Ammonia toxicity to the freshwater planarian *Polycelis felina*: contrasting effects of continuous versus discontinuous exposures. Arch. Environ. Contam. Toxicol. 68, 689–695.

ANZECC and ARMCANZ, 2000. Australian and New Zealand Guidelines for Fresh and Marine Waters. Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand, Canberra.

ASTM. 2002. Standard Guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E 729-96. American Society for Testing and Materials, Philadelphia, PA.

Atchison, G.J., Henry, M.G., Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. Environ. Biol. Fish. 18, 11–25.

Benassi, J.C., Laus, R., Geremias, R., Lima, P.L., Menezes, C.T.B., Laranjeira, M.C., Wilhelm-Filho, D., Fávere, V.T., Pedrosa, R.C., 2006. Evaluation of remediation of coal mining wastewater by chitosan microspheres using biomarkers. Arch. Environ. Contam. Toxicol. 51, 633–640.

Benzie, J., 1988. The systematics of Australian *Daphnia* (Cladocera: Daphniidae). Species descriptions and keys. Hydrobiologia 166, 95–161.

Bernhardt, E.S., Lutz, B.D., King, R.S., Fay, J.P., Carter, C.E., Helton, A.M., Campagna, D., Amos, J., 2012. How many mountains can we mine? Assessing the regional degradation of Central Appalachian rivers by surface coal mining. Environ. Sci. Technol. 46, 8115–8122.

Bernhardt, E.S., Palmer, M.A., 2011. The environmental costs of mountaintop mining valley fill operations for aquatic ecosystems of the Central Appalachians. Ann. N. Y. Acad. Sci. 1223, 39–57.

Bharti, S., Banerjee, T.K., 2011. Bioaccumulation of metals in the edible catfish *Heteropneustes fossilis* (Bloch) exposed to coal mine effluent generated at Northern Coalfield Limited, Singrauli, India. Bull. Environ. Contam. Toxicol. 87, 393–398.

Bharti, S., Banerjee, T.K., 2013. Bioassay analysis of efficacy of phytoremediation in decontamination of coal mine effluent. Ecotoxicol. Environ. Saf. 92, 312–319.

Bharti, S., Banerjee, T.K., 2014. Toxicity analysis of coal mine effluent on vital organs of *Heteropneustes fossilis* (Bloch) using biometric and biochemical parameters. Clean – Soil Air Water 42, 260–266.

Bian, Z., Inyang, H., Daniels, J.L., Otto, F., Struthers, S., 2010. Environmental issues from coal mining and their solutions. Min. Sci. Technol. 20, 0215–1223.

Birge W.J. 1978. Aquatic toxicology of trace elements of coal and fly ash. In: Thorp, J. H., Gibbons, J.W., (Eds.), Energy and Environmental Stress in Aquatic Systems, DOE Symposium Series (CONF-771114). Washington, DC, pp. 219–240.

Buttarelli, F.R., Pellicano, C., Pontieri, F.E., 2008. Neuropharmacology and behavior in planarians: translations to mammals. Comp. Biochem. Physiol. 147, 399–408.

Calevro, F., Campani, S., Filippi, C., Batistoni, R., Deri, P., Bucci, S., Ragghianti, M., Mancino, G., 1999. Bioassays for testing effects of Al, Cr and Cd using development in the amphibian *Pleurodeles waltl* and regeneration in the planarian *Dugesia etrusca*. Aquat. Ecosyst. Health Manag. 2, 281–288.

Cañedo-Argüelles, M., Kefford, B.J., Piscart, C., Prat, N., Schäfer, R.B., Schulz, C.-J., 2013. Salinisation of rivers: An urgent ecological issue. Environ. Pollut. 173, 157–167.

Chapman, P.M., 2000. Whole effluent toxicity testing—usefulness, level of protection, and risk assessment. Environ. Toxicol. Chem. 19, 3–13.

Chinathamby, K., Reina, R.D., Bailey, P.C.E., Lees, B.K., 2006. Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, *Litoria ewingii*. Aust. J. Zool. 54, 97–105.

Christy, M.T., Dickman, C.R., 2002. Effects of salinity on tadpoles of the green and golden bell frog (*Litoria aurea*). Amphibia-Reptilia 23, 1–11.

Cooper, N.L., Bidwell, J.R., Kumar, A., 2009. Toxicity of copper, lead, and zinc mixtures to *Ceriodaphnia dubia* and *Daphnia carinata*. Ecotoxicol. Environ. Saf. 72, 1523–1528.

DeForest, D.K., Meyer, J.S., 2015. Critical review: toxicity of dietborne metals to aquatic organisms. Crit. Rev. Environ. Sci. Technol. 45, 1176–1241.

DEH, DNR, 1999. Testing the Waters. Queensland Government, Brisbane, Australia.

DERM, 2001. Australia-wide Assessment of River Health: Queensland AusRivAS Sampling and Processing Manual (No. 12). Commonwealth of Australia and QLD Department of Natural Resources and Mines, Canberra and Rocklea.

DERM, 2009. A Study of the Cumulative Impacts on Water Quality of Mining Activities in the Fitzroy River Basin. Department of Environment and Resource Management.

DERM, 2010. Fitzroy Basin Draft Water Resource Plan Environmental Assessment-Stage 2 Assessment Report. State of Queensland, DERM, pp. 1–392.

DERM, 2011a. Developing Water Quality Guidelines for the Protection of the Freshwater Aquatic Ecosystems in the Fitzroy Basin (No. 29903). Department of Environment and Resource Management.

DERM, 2011b. Fish Water Quality Guidelines for Fitzroy Basin Freshwaters (No. 29903). Department of Environment and Resource Management.

DNRM, 2013. Queensland Coal Statistics Report for the Financial Year Ended June 2013. Deparment of Natural Resources and Mine, Queensland Government.

Dolezelova, P., Macova, S., Plhalova, L., Pistekova, V., Svobodova, Z., Bedanova, I., Voslarova, E., 2009. Comparison of the sensitivity of different fish species to medical substances. Neuroendocrinol. Lett. 30, 248–252.

Dunlop, J.E., Mann, R.M., Hobbs, D., Smith, R.E.W., Nanjappa, V., Vardy, S., Vink, S., 2015. Assessing the toxicity of saline waters: the importance of accommodating surface water ionic composition at the river basin scale. Austral. Bull. Ecotoxicol. Environ. Chem. 2, 1–15.

Eisler, R., 1987. Polycyclic Aromatic Hydrocarbon Hazards to Fish, Wildlife, and Invertebrates: a Synoptic Review (No. 85(1.11)). U.S. Fish and Wildlife Service.

FAB, 2008. Fitzroy Basin Water Quality Improvement Report. Fitzroy Basin Association Inc.

FAB, 2009. Priority Neighbourhood Catchments Water Quality Monitoring Program; 2005–09. Fitzroy Basin Association Inc.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Haibin, L., Zhenling, L., 2010. Recycling utilization patterns of coal mining waste in China. Resour. Conserv. Recycl. 54, 1331.

Hart, B.T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., Meredith, C., Swadling, K., 1991. A review of the salt sensitivity of the Australian freshwater biota. Hydrobiologia 210, 105–144.

Hart, B.T., Greenfield, P., Pascoe, M., 2008. Review of the Fitzroy River Water Quality Issues. Water Science Pty Ltd. and Water Studies Centre, Monash University.

Hogsden, K.L., Harding, J.S., 2012. Consequences of acid mine drainage for the structure and function of benthic stream communities: a review. Freshw. Sci. 31, 108–120.

Höök, M., Zittel, W., Schindler, J., Aleklett, K., 2010. Global coal production outlooks based on a logistic model. Fuel 89, 3546–3558.

Hughes, R., Reichelt-Brushett, A.J., Newman, L.J., 2005. Identifying suitable invertebrate species from a unique habitat for ecotoxicological testing. Australas. J. Ecotoxicol. 11, 85–92.

IEA, 2012. Coal Information. International Energy Agency.

Jana, B.B., Chakrabarti, R., 1993. Life table responses of zooplankton (*Moina micrura* Kurz and *Daphnia carinata* King) to manure application in a culture system. Aquaculture 117, 273–285.

Kapu, M.M., Schaeffer, D.J., 1991. Planarians in toxicology. Responses of asexual *Dugesia dorotocephala* to selected metals. Bull. Environ. Contam. Toxicol. 47, 302–307.

Kearney, B.D., Byrne, P.G., Reina, R.D., 2012. Larval tolerance to salinity in three species of Australian anuran: an indication of saline specialisation in Litoria aurea. PLoS One 7, e43427.

Kefford, B.J., Hickey, G.L., Gasith, A., Ben-David, E., Dunlop, J.E., Palmer, C.G., Allan, K., Choy, S.C., Piscart, C., 2012. Global scale variation in the salinity sensitivity of riverine macroinvertebrates: Eastern Australia, France, Israel and South Africa. PLoS One 7, e35224.

Kefford, B.J., Papas, P.J., Crowther, D., 2002. Are salts toxicants? Australas. J. Ecotoxicol. 8, 63–68.

Kefford, B.J., Papas, P.J., Nugegoda, D., 2003. Relative salinity tolerance of macro-invertebrates from the Barwon River, Victoria, Australia. Mar Freshw. Res. 54, 755–765.

Lanctôt, C.M., Melvin, S.D., Leusch, F.D.L., Wilson, S., Fabbro, L., 2016. Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. Chemosphere 144, 1560–1566.

Langdon, K., Warne, M.S.J., Sunderam, R.I.M., 2009. A compilation of data on the toxicity of chemicals to species in Australasia. Part 4: metals (2000–2009). Australas J Ecotox. 15, 51–184.

Leung, J., Kumar, M., Glatz, P., Kind, K., 2011. Impacts of un-ionized ammonia in digested piggery effluent on reproductive performance and longevity of *Daphnia carinata* and *Moina australiensis*. Aquaculture 310, 401–406.

Maltby, L., Blake, N., Brock, T., Van Den Brink, P.J., 2005. Insecticide species sensitivity distributions: importance of test species selection and relevance to aquatic ecosystems. Environ. Toxicol. Chem. 24, 379–388.

Martin, L.M., Black, M.C., 1998. Biomarker assessment of the effects of coal strip-mine contamination on channel catfish. Ecotoxicol. Environ. Saf. 41, 307–320.

Melvin, S.D., 2016. Short-term exposure to municipal wastewater influences energy, growth and swimming performance in juvenile Empire Gudgeons (*Hypseleotris compressa*). Aquat. Toxicol. 170, 271–278.

Melvin, S.D., 2015. Oxidative stress, energy storage, and swimming performance of *Limnodynastes peronii* tadpoles exposed to a sub-lethal pharmaceutical mixture throughout development. Chemosphere . http://dx.doi.org/10.1016/j. chemosphere.2015.09.034.

Melvin, S.D., Lanctôt, C.M., Craig, P.M., Moon, T.W., Peru, K.M., Headley, J.V., Trudeau, V.L., 2013. Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. Environ. Pollut. 177, 22–27.

Miller, L.L., Rasmussen, J.B., Palace, V.P., Hontela, A., 2009. The physiological stress response and oxidative stress biomarkers in rainbow trout and brook trout from selenium-impacted streams in a coal mining region. J. Appl. Toxicol. 29, 681–688.

Muschal, M., 2006. Assessment of risk to aquatic biota from elevated salinity—A case study from the Hunter River, Australia. J. Environ. Manag. 79, 266–278.

Newmark, P.A., Sánchez Alvarado, A., 2002. Not your father's planarian: a classic model enters the era of functional genomics. Nat. Rev. Genet. 3, 210–219.

Nielsen, D.L., Brock, M.A., Rees, G.N., Baldwin, D.S., 2003. Effects of increasing salinity on freshwater ecosystems in Australia. Aust. J. Bot. 51, 655–665.

Noble, R.M., Duivenvoorden, L.J., Rummenie, S.K., Long, P.E., Fabbro, L.D., 1996a. Downstream Effects of Land Use in the Fitzroy Catchment. The State of Queensland, Department of Natural Resources.

Noble, R.M., Rummenie, S.K., Long, P.E., Fabbro, L.D., Duivenvoorden, L.J., 1996b. The Fitzroy River catchment-An assessment of the condition of the riverine system. In: Proceedings of the 8th Australian Agronomy Conference. Toowoomba.

OECD, 2004. OECD Guidelines for the Testing of Chemicals: Daphnia sp., Acute Immobilisation Test (No. 202), OECD. Organisation for Economic Co-operation and Development.

Pagán, O.R., Coudron, T., Kaneria, T., 2009. The flatworm planaria as a toxicology and behavioral pharmacology animal model in undergraduate research experiences. J. Undergrad. Neurosci. Educ. 7, A48–A52.

Pickering, Q.H., Henderson, C., Lemke, A.E., 1962. The toxicity of organic phosphorus insecticides to different species of warmwater fishes. Trans. Am. Fish. Soc. 91, 175–184.

Rudolph, B.-L., Andreller, I., Kennedy, C.J., 2008. Reproductive success, early life stage development, and survival of Westslope Cutthroat Trout (*Oncorhynchus clarki lewisi*) exposed to elevated selenium in an area of active coal mining. Environ. Sci. Technol. 42, 3109–3114.

Scott, B., Ranjith, P.G., Choi, S.K., Khandelwal, M., 2010. A review on existing opencast coal mining methods within Australia. J. Min. Sci. 46, 280–297.

Scullion, J., Edwards, R.W., 1980. The effect of pollutants from the coal industry on the fish fauna of a small river in the South Wales coalfield. Environ. Pollut. 21, 141–153.

Smith, M.J., Schreiber, E.S.G., Scroggie, M.P., Kohout, M., Ough, K., Potts, J., Lennie, R., Turnbull, D., Jin, C., Clancy, T., 2007. Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation. Freshw. Biol. 52, 75–84.

Thiruvenkatachari, R., Younes, M., Su, S., 2011. Coal mine site investigation of wastewater quality in Australia. Desalination Water Treat. 32, 357–364.

Tiwary, R.K., 2001. Environmental impact of coal mining on water regime and its management. Water Air Soil Pollut. 132, 185–199.

US EPA, 2000. Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136) (No. EPA 821-B-00-004). US Environmental Protection Agency.

US EPA, 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. US Environmental Protection Agency, USA.

US EPA, 2011. The Effects of Mountaintop Mines and Valley Fills on Aquatic Ecosystems of the Central Appalachian Coalfields. US Environmental Protection Agency, USA.

Vink, S., Moran, C.J., Golding, S.D., Baublys, K., Nanjappa, V., 2009. Understanding mine site water and salt dynamics to support integrated water quality and quantity management. Min. Technol. 118, 185–192.

Vink, S., Robbins, S., 2012. Mine water management in variable climate regimes. In: McCullough, C.D., Lund, M.A., Wyse, L., (Eds.), Presented at the International Mine Water Association Symposium. pp. 20A–20I.

Weltje, L., Simpson, P., Gross, M., Crane, M., Wheeler, J.R., 2013. Comparative acute and chronic sensitivity of fish and amphibians: a critical review of data. Environ. Toxicol. Chem. 32, 984–994.

Zalizniak, L., Kefford, B.J., Nugegoda, D., 2006. Is all salinity the same? I. The effect of ionic compositions on the salinity tolerance of five species of freshwater invertebrates. Mar. Freshw. Res. 57, 75–82.

Zalizniak, L., Nugegoda, D., 2004. Maintenance of *Daphnia carinata* cultures for use in toxicity testing. Australas. J. Ecotoxicol. 10, 65–69.

da Silveira, F.Z., Defaveri, T.M., Ricken, C., Zocche, J.J., Pich, C.T., 2009. Toxicity and genotoxicity evaluation of acid mine drainage treatment using *Artemia* sp. and *Geophagus brasiliensis* as bioindicators. In: Barnhisel, R.I., (Ed.), National Meeting of the American Society of Mining and Reclamation, Billings, MT, Revitalizing the Environment: Proven Solutions and Innovative Approaches; 2009, May 30–June 5. Published by ASMR, 3134 Montavesta Rd., Lexington, KY 40502. pp. 1725–1742.

van Dam, R.A., Barry, M.J., Ahokas, J.T., Holdway, D.A., 1998. Effects of water-borne iron and calcium on the toxicity of diethylenetriamine pentaacetic acid (DTPA) to *Daphnia carinata*. Aquat. Toxicol. 42, 49–66.

van Dam, R.A., Chapman, J.C., 2001. Direct toxicity assessment (DTA) for water quality guidelines in Australia and New Zealand. Australas. J. Ecotoxicol. 7, 175–198.

3.10 Appendix

Table 3-3: Mean and range of water quality and chemistry parameters measured *in situ* throughout on going monitoring of CMW1 and CMW2 dams between 2012 and 2015 (n = 10–34). Water samples were analyzed by the ALS Laboratory Group (Brisbane, Australia).

		CMW1			**CMW2**		
Parameter/Analyte	**LOR[a]**	**Mean[b]**	**Min.**	**Max.**	**Mean[b]**	**Min.**	**Max.**
pH Value	0.01	8.88	8.01	9.51	8.81	7.77	9.28
Electrical Conductivity (mS/cm)	0.001	4.26	0.70	7.89	6.61	0.75	9.38
Suspended Solids (mg/L)	5	67	17	216	42	8	110
Turbidity (NTU)	0.1	67	3	286	35	1.9	230.0
Total hardness as $CaCO_3$ (mg/L)	1	352	215	534	551	494	650
Sulfate as SO_4 (g/L)	0.001	0.60	0.15	1.32	0.99	0.19	1.60
Fluoride (mg/L)	0.1	0.6	< 0.1	0.9	0.4	< 0.1	0.7
Ammonia as N (mg/L)	0.01	0.07	0.03	0.16	0.05	0.02	0.09
Nitrite as N (mg/L)	0.01	0.03	< 0.01	0.30	0.02	< 0.01	0.14
Nitrate as N (mg/L)	0.01	0.20	< 0.01	1.67	0.35	< 0.01	3.81
Nitrite + Nitrate as N (mg/L)	0.01	0.23	< 0.01	1.69	0.37	< 0.01	3.95
Total Kjeldahl Nitrogen as N (mg/L)	0.1	2.4	0.1	10.0	1.1	0.1	3.4
Total Nitrogen as N (mg/L)	0.1	2.5	0.3	10.0	1.4	0.4	5.0
Total Phosphorus as P (mg/L)	0.01	0.54	0.03	1.57	0.07	0.03	0.18
Dissolved Organic Carbon (mg/L)	1	12	7	20	5.8	4	7
Total Organic Carbon (mg/L)	1	14	10	24	5.8	3	10
Alkalinity as $CaCO_3$ (mg/L)							
Hydroxide Alkalinity	1	< 1	< 1	< 1	< 1	< 1	< 1
Carbonate Alkalinity	1	172	69	303	100	21	196
Bicarbonate Alkalinity	1	270	87	463	291	149	431
Total Alkalinity	1	443	299	574	391	170	584
Dissolved Metals and Metalloids (µg/L)							
Aluminium	10	21.9	< 10	240	< 10	< 10	< 10
Arsenic	1	7.9	1	28	4.2	1	11
Cadmium	0.1	< 0.1	< 0.1	0.2	< 0.1	< 0.1	0.2
Chromium	1	1.2	< 1	6	1.0	< 1	4
Copper	1	2.2	1	3	1.7	1	4
Cobalt	1	< 1	< 1	1	< 1	< 1	1
Nickel	1	1.8	1	3	1.8	1	3
Lead	1	<1	< 1	< 1	<1	< 1	< 1
Zinc	5	4.9	< 5	24	4.8	< 5	27
Manganese	1	2.4	< 1	22	1.4	< 1	3
Selenium	10	< 10	< 10	20	< 10	< 10	10
Silver	1	<1	< 1	< 1	<1	< 1	< 1
Uranium	1	2.7	1	5	2.7	1	6
Vanadium	10	< 10	< 10	20	< 10	< 10	30
Boron	50	270	70	470	360	210	610

Parameter/Analyte	LOR[a]	CMW1 Mean[b]	CMW1 Min.	CMW1 Max.	CMW2 Mean[b]	CMW2 Min.	CMW2 Max.
Iron	50	< 50	< 50	180	47.86	< 50	120
Mercury	0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Total Metals and Metalloids (µg/L)							
Aluminium	10	1291	120	4220	545	50	3080
Arsenic	1	9.4	2	28	4.4	2	10
Cadmium	0.1	< 0.1	< 0.1	0.2	< 0.1	< 0.1	0.4
Chromium	1	1.6	< 1	6	< 1	< 1	4
Copper	1	4.3	1	11	2.9	1	7
Cobalt	1	1.3	< 1	4	< 1	< 1	3
Nickel	1	3.8	2	9	2.6	1	7
Lead	1	1.0	< 1	4	< 1	< 1	4
Zinc	5	10.9	< 5	30	9.3	< 5	34
Manganese	1	50	13	147	39	7	107
Selenium	10	< 10	< 10	20	5.4	< 10	10
Silver	1	< 1	< 1	< 1	< 1	< 1	< 1
Uranium	1	3.2	< 1	6	2.7	< 1	4
Vanadium	10	11	< 10	40	< 10	< 10	20
Boron	50	267	60	470	356	200	560
Iron	50	1523	140	6040	798	70	4580
Mercury	0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Total Petroleum Hydrocarbons (µg/L)							
C6 - C9	20	< 20	< 20	< 20	< 20	< 20	< 20
C10 - C14	50	< 50	< 50	< 50	< 50	< 50	< 50
C15 - C28	100	164	< 100	1170.00	63	< 100	200
C29 - C36	50	< 50	< 50	80.00	< 50	< 50	120
C10 - C36 (sum)	50	149	< 50	1250.00	55	< 50	320
Total Recoverable Hydrocarbons (µg/L)							
C6 - C10	20	< 20	< 20	< 20	< 20	< 20	< 20
C6 - C10 minus BTEX (F1)	20	< 20	< 20	< 20	< 20	< 20	< 20
>C10 - C16	100	< 100	< 100	< 100	< 100	< 100	< 100
>C16 - C34	100	422	< 100	1220	64	< 100	120
>C34 - C40	100	< 100	< 100	< 100	< 100	< 100	< 100
>C10 - C40 (sum)	100	422	< 100	1220	64	< 100	120
>C10 - C16 minus Naphthalene (F2)	100	< 100	< 100	< 100	< 100	< 100	< 100
BTEXN							
Benzene	1	< 1	< 1	< 1	< 1	< 1	< 1
Toluene	2	< 2	< 2	< 2	< 2	< 2	< 2
Ethylbenzene	2	< 2	< 2	< 2	< 2	< 2	< 2
meta- and para-Xylene	2	< 2	< 2	< 2	< 2	< 2	< 2
ortho-Xylene	2	< 2	< 2	< 2	< 2	< 2	< 2
Total Xylenes	2	< 2	< 2	< 2	< 2	< 2	< 2
Sum of BTEX	1	< 1	< 1	< 1	< 1	< 1	< 1

		CMW1			CMW2		
Parameter/Analyte	**LOR[a]**	**Mean[b]**	**Min.**	**Max.**	**Mean[b]**	**Min.**	**Max.**
Naphthalene	5	< 5	< 5	< 5	< 5	< 5	< 5
TPH(V)/BTEX Surrogates (%)							
1.2-Dichloroethane-D4	0.1	105	94	130	102	80	128
Toluene-D8	0.1	104	93	124	102	85	124
4-Bromofluorobenzene	0.1	102	93	120	101	86	125

a. LOR, limit of reporting

b. When values < LOR, values of 0.5×(LOR) were used to calculate means (< LOR indicated if mean was below limit of reporting).

Declaration of co-authorship and contribution

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Lanctôt, C. M., Melvin, S. D., Fabbro, L., Leusch, F. D. L., and Wilson, S. P. (2016a). Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (*Hypseleotris compressa*). *Ecotoxicology and Environmental Safety* **127**, 36–42. http://dx.doi.org/10.1016/j.ecoenv.2016.01.010

My contribution to the paper involved:

- Initial concept and experimental design.
- Collection and analysis of data.
- Preparation of manuscript.

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Publication of Research Higher Degree Work for Inclusion in the Thesis Procedures

[redacted]

Chantal Lanctôt

Chapter 4

Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (*Hypseleotris compressa*)

Chantal M. Lanctôt [a, b, *], Steven D. Melvin [a, b], Larelle Fabbro [c], Frederic D.L. Leusch [b], Scott P. Wilson [a]

[a] Central Queensland University, School of Medical and Applied Sciences, Gladstone, QLD 4680, Australia

[b] Smart Water Research Centre and Australian Rivers Institute, Griffith School of Environment, Griffith University, Southport, QLD 4215, Australia

[c] Central Queensland University, School of Medical and Applied Sciences, Rockhampton, QLD 4702, Australia

* Corresponding author
e: chantal.lanctot@gmail.com, c.lanctot@cqu.edu.au
Mailing address:
Chantal Lanctôt
Smart Water Research Centre (G51)
Griffith University, Gold Coast Campus
Southport, Queensland, 4215
Australia

4.1 Abstract

Coal mining represents an important industry in many countries, but concerns exist about the possible adverse effects of minewater releases on aquatic animals and ecosystems. Coal mining generates large volumes of complex wastewater, which often contains high concentrations of dissolved solids, suspended solids, metals, hydrocarbons, salts and other compounds. Traditional toxicological testing has generally involved the assessment of acute toxicity or chronic toxicity with longer-term tests, and while such tests provide useful information, they are poorly suited to ongoing monitoring or rapid assessment following accidental discharge events. As such, there is considerable interest in developing rapid and sensitive approaches to environmental monitoring, and particularly involving the assessment of sub-lethal behavioural responses in locally relevant aquatic species. We therefore investigated behavioural responses of a native Australian fish to coal mine wastewater, to evaluate its potential use for evaluating sub-lethal effects associated with wastewater releases on freshwater ecosystems. Empire gudgeons (*Hypseleotris compressa*) were exposed to wastewater from two dams located at an open cut coal mine in Central Queensland, Australia and activity levels were monitored using the Multispecies Freshwater Biomonitors (LimCo International GmbH). A general decrease in locomotor activity (*i.e.*, low frequency movement) and increase in non-locomotor activity (*i.e*., high frequency movement including ventilation and small fin movement) was observed in exposed fish compared to those in control water. Altered activity levels were observable within the first hour of exposure and persisted throughout the 15-d experiment. Results demonstrate the potential for using behavioural endpoints as tools for monitoring wastewater discharges using native fish species, but more research is necessary to identify responsible compounds and response thresholds, and to understand the relevance of the observed effects for populations in natural receiving environments.

4.2 Keywords

Multispecies Freshwater Biomonitor®; Coal mine wastewater; Behaviour; Locomotor activity; Ventilation; Fish

4.3 Introduction

Coal mining represents a fundamental economic activity in many parts of the world, providing the raw material to produce electricity and heat and thus necessary for many industrial processes. The coal mining industry is extremely prevalent in Australia, with Central Queensland's Bowen Basin containing over 50 active coal mines that produce approximately 200 million tonnes of saleable coal annually (DNRM, 2013). Mining coal requires a constant supply of water, and therefore results in the production of large quantities of wastewater that must be effectively managed (Scott et al., 2010; Haibin and Zhenling, 2010; Thiruvenkatachari et al., 2011). This generally involves holding spent water in large dams and periodically releasing the wastewater into aquatic receiving environments. Releases of mine water are normally restricted to periods of natural flow, but uncontrolled releases are also possible during high rainfall and flooding events. Whether controlled or accidental, concerns exist about the possible consequences of coal mine-affected water (CMW) releases on native Australian species. However, despite the magnitude of the mining industry in Australia and the potential for aquatic wildlife to become exposed to wastewater during discharge events, relatively little information exists about the potential toxicological effects of exposure to CMW on native aquatic organisms.

The quality and toxicity of CMW can be highly variable, as this largely depends on regional geological characteristics that impact the coal composition. Generally, mining effluent contains elevated concentrations of dissolved solids, suspended solids, metals, sulphate, and hydrocarbons and is often highly saline and/or acidic. Any one of these parameters can pose a risk to aquatic wildlife in the event of surface or groundwater contamination (Tiwary, 2001; Thiruvenkatachari et al., 2011). Metals are one of the most harmful components of CMW, due to their persistence in the environment and potential for bioaccumulation and biomagnification (Mishra et al., 2008). Bioaccumulation of trace metals has been observed in various organs of aquatic vertebrates exposed to coal mine wastewater (Bharti and Banerjee 2011; Zocche et al., 2013; Lanctôt et al. 2016). Complex metal mixtures in CMW have been associated with morphological, genotoxic and physiological damage in fish, such as decreased condition factor and decreased total protein, lipid, glycogen and nucleic acid concentrations (Bharti and Banerjee, 2014). However, very little effort has historically been directed at understanding the potential for sub-lethal effects of CMW on aquatic vertebrates (Bharti and Banerjee, 2014), and this holds particularly true for the Australian environment. Research is therefore needed to fill existing knowledge gaps by exploring the potential for sub-lethal effects on relevant indigenous fish species in environments receiving CMW discharge in Australia.

Recent advancements in the field of computer-automated behavioural analysis have provided novel approaches for quickly assessing the potential sub-lethal organism-level effects of environmental contaminants on aquatic wildlife (Bushnell et al., 2010; Maradona et al., 2012; Melvin and Wilson, 2013). Many aquatic organisms, including fish, display sensitive behavioural responses to toxicants at low concentrations, and these may often be predictive of potential higher-level effects related to organism survival and fitness (Atchison et al., 1987; Pestana et al., 2007; Mohti et al., 2012). Examples include altered swimming performance, reduced feeding activities, inability to effectively escape predation, and altered mating and reproductive behaviours (Gerhardt et al., 2005b; Pestana et al., 2007; Macedo-Sousa et al., 2008; Alonso et al., 2009; Melvin 2016). Behavioural alterations tend to be rapidly observable in animals exposed to many contaminants (Melvin and Wilson, 2013), and may therefore provide a useful tool for the monitoring of mining discharges. Industry and regulatory agencies are currently interested in developing these approaches for use as early warning systems to signal potential ecological effects associated with the release of mine water and other complex effluents. Little is known on the implications of coal mine wastewater exposures for influencing behavioural patterns in fish and other aquatic organisms (Lanctôt et al., 2016). Research is therefore needed to explore the sensitivity of locally relevant species to CMW, and to compare rapid behavioural outcomes to those resulting from longer-duration exposures. This is a necessary first step towards developing and optimizing rapid behavioural testing protocols that can be used to monitor water quality in regions characterized by intense mining operations.

The Multispecies Freshwater Biomonitors (MFB; LimCo International GmbH) has previously been used to study the behavioural responses of several macro-invertebrate species (Janssens de Bisthoven et al., 2004; Gerhardt et al., 2004, 2005a, 2005b; Macedo-Sousa et al., 2007; 2008; Mohti et al., 2012) as well as two fish species, *Poecilia reticulata* and *Gambusia holbrooki* (Gerhardt et al., 2005a; Mohti et al., 2012) to acid mine drainage from abandoned mineral mines. The present study explores acute (≤ 3 d) and sub-chronic (15 d) behavioural responses of the Australian fish, *Hypseleotris compressa*, exposed to CMW from an open cut mine in Central Queensland.

4.4 Materials and methods

4.4.1 Animals

Empire gudgeons, *H. compressa*, are a small (maximum standard length ~10 cm) freshwater species that occur throughout northern and eastern Australia and southern New Guinea (Auty, 1978). *H. compressa* was selected for this study because it represents an ecologically relevant species for the coal mine-affected catchments of Central Queensland, Australia. Wild fish (standard length: 2.1 ± 0.2 cm) were collected from Gavial Creek in Central Queensland, Australia (23 °26′47″S, 150 °32′25″E) using D-frame dip-nets. Fish were transported to the laboratory in plastic containers containing water from the collection site, and acclimated to aerated double carbon-filtered tap water prior to experimentation. Fish were housed in aerated glass aquaria containing rock substrate and plastic refuge for over four weeks prior to experiments, and were fed Community Formula Flakes and Pellets (Ocean Nutrition^{TM}) supplemented with frozen Artemia (Ocean Nutrition^{TM}) *ad libitum* twice daily. Sexually immature juvenile males and females that could not be visually distinguished (Allen et al., 2002; Pusey et al., 2004) were used for experimentation. Laboratory conditions were maintained at 25 °C and 12:12 light:dark sequence. The experimental protocol was approved by the Animal Ethics Committee of Central Queensland University and in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (Approval No. A13/05-301).

4.4.2 Water collection and analysis

Wastewater was collected at an open-cut coal mine located in Central Queensland, Australia, in April 2014. At the time of experimentation, the mine had two holding dams authorized for discharge, henceforth referred to as CMW1 and CMW2. Wastewater was collected from the dams in 20 L plastic containers, transported on ice and stored at 4 °C until brought to ambient temperature prior to experimentation. *In situ* measurements of temperature, conductivity (EC), pH, dissolved oxygen (DO), salinity, total dissolved solid (TDS) and turbidity were taken using a YSI 6600 multi-parameter sonde (Xylem Analytics, Hemmant, Australia). Water samples were collected and analysed for a suite of parameters (Appendix: Table 4-2) by the ALS Laboratory Group (Brisbane, Australia). During experiments, water quality parameters (temperature, EC, pH, DO, salinity and TDS) were measured for all treatment groups, prior to and immediately following each water renewal using YSI EcoSenses probes. Nitrogenous waste levels were measured in a subset of replicates throughout the experiments using an ammonia test kit (Aquasonic Pty Ltd, Australia).

4.4.3 Behavioural monitoring of fish activity levels

Behavioural analysis using the MFB is based on quadropole-impedance conversion technology, which provides simultaneous recording of locomotory (*i.e*., swimming) and non-locomotory activities (*i.e*., ventilation) (Gerhardt and Schmidt, 2002). Briefly, the MFB uses cylindrical plexiglass chambers containing two pairs of opposing electrodes; one set of electrodes transmits a high frequency alternating current while the others measure disturbances to the signal resulting from animal movement within the chambers. The associated software package analyzes this data using a stepwise discrete fast Fourier transformation (FFT), providing measurement of the intensity of movement as a percentage of the disturbance to the alternating current at frequencies ranging from 0.5 –8.5Hz. Complete descriptions of the experimental methodology for behavioural analysis using the MFB have been published elsewhere (Gerhardt and Schmidt, 2002).

For experimentation, eight fish were individually transferred to MFB chambers (length = 15 cm, diameter = 5 cm) and randomly assigned to either control or treatment groups (n = 4 per treatment). Four series of experiments were performed, such that fish were exposed to 50% and 100% wastewater from CMW1 and CMW2 holding dams. Baseline activities of all fish were recorded for 20 h prior to testing to ensure that control and treatment groups behaved consistently. Aerated carbon filtered tap water was used as control water and for making treatment dilutions, and each replicate aquarium contained one horizontally submerged MFB chamber. Aquaria measured 30 × 20 × 26 cm and were filled with 4.5 L of control water or the appropriate treatment water. Fish were allowed to acclimatize for 10 min, after which recording was commenced for 250 s on a 10 min interval using the MFB (*i.e.*, 250 s recording followed by a 350 s break). This frequency of recording was carried out for 24 h every second day, with fish released from the test chambers into their respective treatment aquaria for feeding and to allow water to be aerated between recordings. Faeces and uneaten food were removed using a plastic baster 24 h after feeding. Experiments with 100% dilutions were carried out for 15 d, whereas it was only possible to continue the 50% dilutions for 72 h due to logistical constraints limiting the volume of water that could be transported to the laboratory. Full water changes were performed every 3 d. At the end of each experiment, fish were euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) dissolved in water, and measurements of standard length (mm), full length (mm) and weight (mg) were taken for each fish.

4.4.4 Statistical analysis

Data were analysed using SPSS 22.0 (IBM Corp.), with $\alpha = 0.05$. Prior to analyses, data were tested for normality (Kolmogorov– Smirnov) and homogeneity of variance (Levene's test). One-way ANOVAs were used to explore differences in morphometric measurements (*e.g.*, length and weight) between treatment dilutions. Percentage data generated by the MFB were arcsine square root transformed prior to analysis. A t-test was used to ensure that baseline activities of fish in control and treatment groups were consistent prior to commencing the exposures. Differences in behaviours throughout the 3-d exposure (all treatments and controls) and 15-d exposure (100% treatments and controls) were analysed using a full factorial multivariate general linear model, using treatment as a fixed factor and time (daily means) as a covariate. Bonferroni post hoc analyses were used to test for specific differences between treatments. Differences in mean activities between control and treatment groups within the first hour of exposures were analysed using non-parametric Kruskal–Wallis tests followed by Dunn–Bonferroni multiple comparisons.

4.5 Results

4.5.1 Water quality and chemistry

In situ water quality and chemistry data for CMW1 and CMW2 dams are presented in Table 4-2 (Appendix). CMW1 generally had lower conductivity, TDS, hardness and higher ammonia, alkalinity, nutrients (N and P), TOC and DOC compared to CMW2. Dissolved metals detected in the wastewater included arsenic (2–9 μg/L), copper (1–4 μg/L), nickel (1–3 μg/L), manganese (0–3 μg/ L), uranium (3–6 μg/L) and boron (390–470 μg/L). Total and dissolved metal(loid)s were higher in CMW1 with the exception of aluminium, manganese and iron. Petroleum hydrocarbon fractions and BTEXN (benzene, toluene, ethylbenzene, xylene, naphthalene) were below detection limits in both dams. Basic water quality parameters throughout experiments are presented in Table 4-1. Ammonia levels were < 0.01 mg/L in control and CMW2 treatments and ranged between 0–0.1 mg/L in 50% CMW1 and 0.1–0.5 mg/L in 100% CMW1.

Table 4-1: Mean (±SD) temperature, pH, dissolved oxygen, electrical conductivity (EC), salinity and total dissolved solids in controls and 50% and 100% coal mine wastewater from two dams (CMW1 and CMW2) measured post- and pre-water changes throughout exposures.

	Control	50% CMW1	100% CMW1	50% CMW2	100% CMW2
Post-water change (fresh treatments)					
Temperature (°C)	24.8 ± 0.7	24.8	25.2 ± 0.1	24.9	24.2 ± 0.6
pH	7.8 ± 0.2	8.9	9.0 ± 0.02	8.9	8.7 ± 0.03
Dissolved oxygen (%)	96.9 ± 5.1	94.0	100 ± 1.1	85.3	112 ± 4.2
EC (mS/cm)	0.25 ± 0.03	3.16	5.84 ± 0.06	3.97	7.10 ± 0.08
Salinity (ppt)	0.1 ± 0.0	1.6	3.1 ± 0.1	2.1	3.9 ± 0.00
Total dissolved solids (g/L)	0.16 ± 0.02	2.05	3.80 ± 0.04	2.58	4.61 ± 0.05
Pre-water change (aged treatments)					
Temperature (°C)	24.3 ± 0.9	23.8 ± 0.3	25.3 ± 0.06	22.4 ± 0.1	24.3 ± 0.6
pH	7.5 ± 0.3	8.5 ± 0.06	8.8 ± 0.02	8.4 ± 0.03	8.6 ± 0.06
Dissolved oxygen (%)	75.5 ± 10.6	60.8 ± 9.8	56.8 ± 13.1	61.8 ± 1.0	76.0 ± 12.5
EC (mS/cm)	0.26 ± 0.03	3.25 ± 0.03	5.84 ± 0.01	3.97 ± 0.11	7.17 ± 0.08
Salinity (ppt)	0.1 ± 0.0	1.7 ± 0.0	3.2 ± 0.0	2.1 ± 0.0	4.0 ± 0.1
Total dissolved solids (g/L)	0.17 ± 0.02	2.11 ± 0.02	3.48 ± 1.09	2.58 ± 0.07	4.66 ± 0.05

4.5.2 Survival and morphometrics

No mortality occurred in any treatments during the first 2 d of exposure. A single mortality was observed in controls on day 3 as well as in the 100% CMW2 treatment after 13 d. Three mortalities were observed in the 100% CMW1 treatment on day 9, after which the exposure for this treatment was terminated. Size did not vary between control and treatment groups at the end of the exposures ($p > 0.05$). Mean differences were < 1 mm in length and ≤ 0.02 g in weight between the groups.

4.5.3 Behaviour

4.5.3.1 Characterization of behaviours

H. compressa displayed distinctive locomotory behaviour consisting of tail and body movements (*i.e.*, swimming), and non-locomotory behaviour consisting of subtle fin (stabilization/balance) and operculum movements (*i.e.*, ventilation). Locomotory activities resulted in irregular behavioural patterns, detected as low frequencies outputs by the MFB (0.5 Hz, > 1.3 V; Figure 4-1a). Non-locomotory activity generated a more consistent pattern within a higher range of frequencies (2.5–3.5 Hz, < 0.63 V; Figure 4-1b). The observed behavioural patterns were consistent with those described for *G. holbrooki* (Gerhardt et al., 2005a) and *Oryzias latipes* (Gerhardt et al., 2002) in previous studies using this experimental methodology. Baseline locomotory and non-locomotory activities of fish in control and treatment groups were consistent prior to commencing the exposures ($F_{(1,31)} = 0.002$, $p = 0.965$ and $F_{(1,31)} = 0.350$, $p = 0.558$, respectively).

4.5.3.2 Responses to coal mine wastewater exposure

Fish exposed to 50% and 100% dilutions of CMW1 and CMW2 generally spent less time swimming and more time on non-locomotory activities (*i.e.*, ventilation) compared to control fish over the 3-d exposure (Figure 4-2, $F_{(4,57)} = 6.02$, $p < 0.001$ and $F_{(4,57)} = 5.39$, $p = 0.001$, respectively). However, Bonferroni post hoc analysis revealed that only fish exposed to 50% CMW1 spent significantly less time swimming whereas fish exposed to 50% CMW1 and CMW2 spent significantly more time on non-locomotory activities compared to the control. Time spent on locomotory and non-locomotory activities throughout the 3-d exposure was not significantly influenced by exposure time ($F_{(1,57)} = 0.21$, $p = 0.648$ and $F_{(1,57)} = 0.01$, $p = 0.906$, respectively).

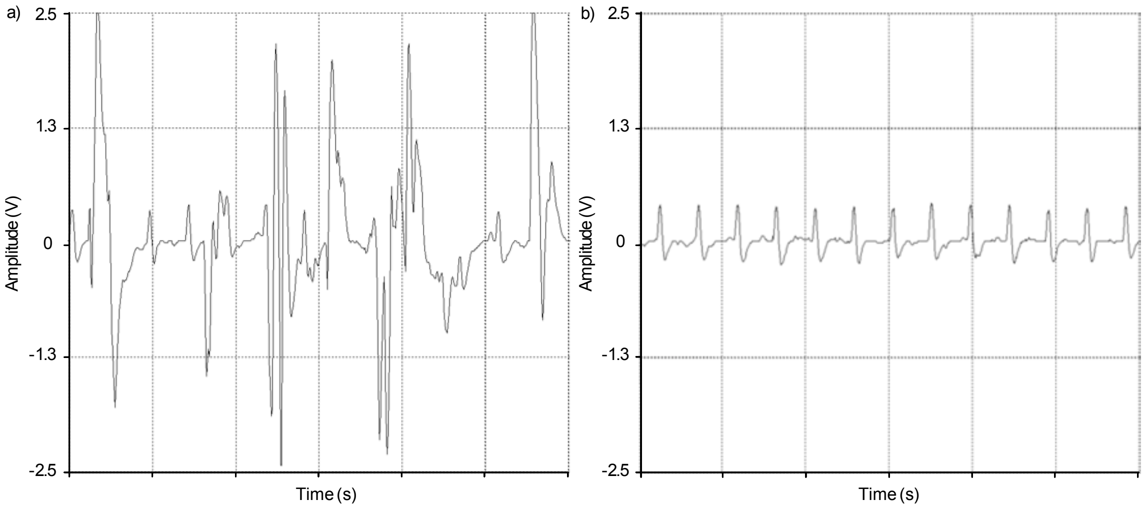

Figure 4-1: Characteristic behavioural pattern of *Hypseleotris compressa* (a) locomotory activity (0–0.5 Hz) and b) non-locomotory activity (2.5–3.5 Hz) as seen in the MFB waveform data output.

In the extended 15-d exposures to raw wastewater, fish exposed to 100% CMW1 and CMW2 spent less time swimming and more time on non-locomotory activities compared to control fish (Figure 4-3, $F_{(2,91)} = 22.79$, $p < 0.001$ and $F_{(2,91)} = 18.18$, $p < 0.001$, respectively). Time spent on locomotory and non-locomotory activities throughout the 15-d exposure was not significantly influenced by exposure time ($F_{(1,91)} = 1.39$, $p = 0.242$ and $F_{(1,91)} = 0.03$, $p = 0.868$, respectively). The trends of decreased swimming and increased non-locomotory activities were observable after 1 h of exposure (Figure 4-4, H = 16.08, df = 4, $p = 0.003$ and H = 14.28, df = 4, $p = 0.006$, respectively), but the decreased time spent swimming was only statistically different from controls in animals exposed to 50% CMW1 and 100% CMW2 treatments, and the increased non-locomotory activities were only statistically different from the control in 50% CMW1 and 50% CMW2 treatments at this time.

4.6 Discussion

Our findings reveal that exposure to wastewater from two dams at an Australian open cut coal mine resulted in reduced swimming activity and increased non-locomotory behaviours (*e.g.*, ventilation) in native empire gudgeons (*H. compressa*) (Figures 4-2 and 4-3). Trends were observed within 1 h of exposure and responses persisted through the full 15 d exposure in the 100% treatments groups. Results demonstrate the rapidity of behavioural responses to sub-lethal exposures of complex wastewaters. Changes in the relative magnitude of activity levels in fish, as observed using the Multispecies Freshwater Biomonitors, may therefore prove useful as an early warning indicator of potential ecological effects associated with CMW releases. More importantly, alterations to normal behaviours warn of possible consequences related to population-level interactions, since behaviour is an important intermediate between physiological and ecological processes (Scott and Sloman, 2004; Kane et al., 2005; Groh et al., 2015). There is evidence to suggest that decreased swimming activities, such as those observed in the present study, can impact upon the ability of exposed fish to feed, avoid predation, migrate and reproduce, leading to various possible higher-level outcomes such as localized population declines and eventual extinctions (Kane et al., 2005). From another perspective, since fish gills are important target sites for the uptake of various toxicants, increased opercular movement (*i.e.*, ventilation) could suggest physical and/or physiological stress related to the exposure (Mohti et al., 2012). Ventilation frequency is a common measure of fish condition and has been shown to be a sensitive indicator of metal and acid exposure (Atchison et al., 1987; Laitinen and Valtonen 1995; Vosyliene et al., 2003; Gerhardt et al., 2005a).

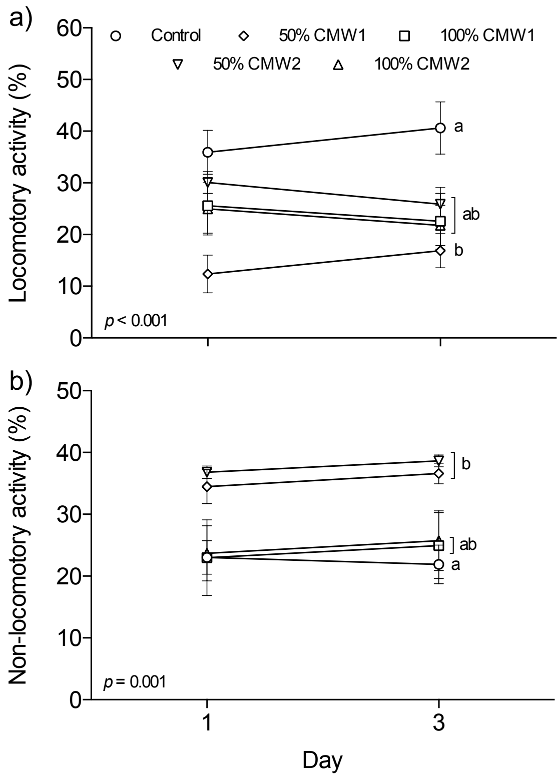

Figure 4-2: Percent time *Hypseleotris compressa* spent on (a) locomotory (0–0.5 Hz) and (b) non-locomotory (2.5–3.5 Hz) activities throughout 3-d exposures to 50% and 100% coal mine wastewater from CMW1 and CMW2 and controls. Activity was recorded for 250 s every 10 min using the Multispecies Freshwater Biomonitors (LimCo International GmbH). Symbols represent

the mean daily activity ± standard error of the mean (SEM) of fish in each treatment. Letters indicate differences between treatments, α = 0.05.

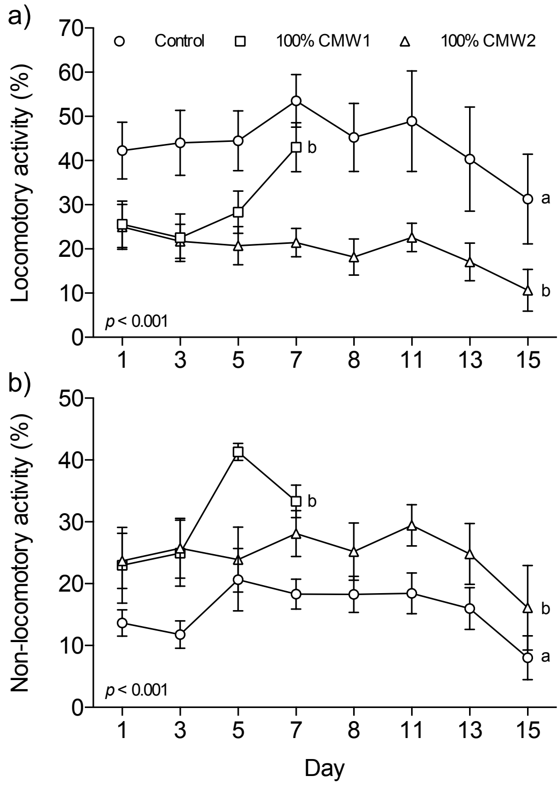

Figure 4-3: Percent time *Hypseleotris compressa* spent on (a) locomotory (0–0.5 Hz) and (b) non-locomotory (2.5–3.5 Hz) activities throughout 15-d exposures to 100% coal mine wastewater from CMW1 and CMW2 and controls. Activity was recorded for 250 s every 10 min using the Multispecies Freshwater Biomonitors (LimCo International GmbH). Symbols represent the mean daily activity ± SEM of fish in each treatment. Letters indicate differences between treatments, α = 0.05.

In previous studies using the MFB, a reduction in time spent swimming was observed in two fish species (*G. holbrooki* and *Poecilia reticulata*) exposed to acid mine drainage (low pH and high metals and salts) from abandoned mineral mines (Gerhardt et al., 2005a; Mohti et al., 2012). In these examples, decreased locomotory activity occurred within the first day of exposure, which is consistent with the rapid response observed in the present study. Increased ventilation was also observed in exposed *G. holbrooki* (Gerhardt et al., 2005a), whereas *P. reticulata* exhibited a slight decrease in ventilation (Mohti et al., 2012). Acid mine drainage has similarly been demonstrated to decrease locomotion in several aquatic invertebrate species (Janssens de Bisthoven et al., 2004;

Gerhardt et al., 2004; 2005b; Mohti et al., 2012). The responses observed in these studies were attributed to low pH and metals, which is not consistent with the alkaline wastewater used in the present study. Craig and Laming (2004), on the other hand, used a similar approach with the MFB and found that ammonia (10 mM NH_4Cl) decreased the swimming activity and increased non-locomotory activities in three-spined sticklebacks, *Gasterosteous aculeatus*. Interestingly, a lower concentration of ammonia (0.1 mM NH_4Cl) caused a greater increase in non-locomotory behaviours compared to the 10 mM treatment (Craig and Laming, 2004). This is again consistent with the non-locomotory responses observed in the present study, in which we observed a greater increase at 50% dilutions compared to 100% CMW after 1 h of exposure. However, ammonia concentrations were not as high in the present study as the nominal concentrations in the aforementioned study, suggesting that observed responses are likely due to a combination of factors associated with the complex wastewater (e.g., pH, metals, salts).

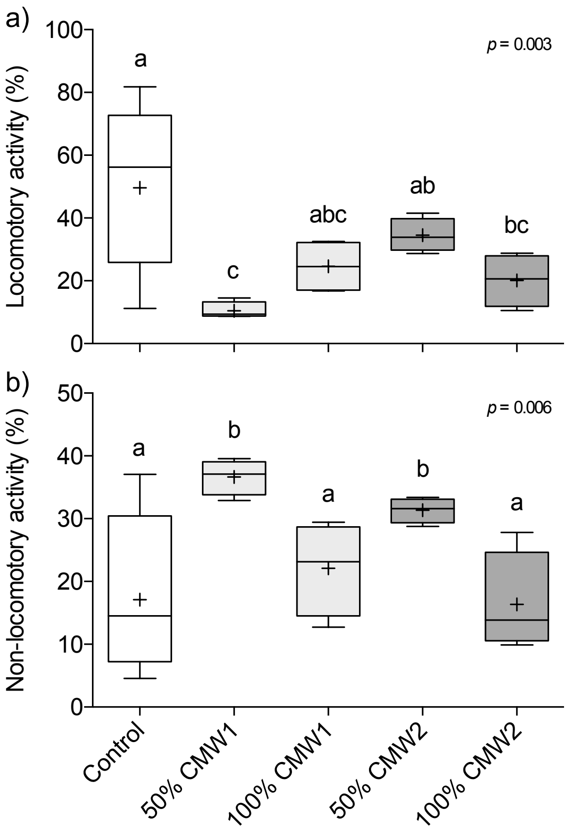

Figure 4-4: Percent time *Hypseleotris compressa* spent on (a) locomotory (0–0.5 Hz) and (b) non-locomotory (2.5–3.5 Hz) activities within 1 h of exposure to 50% and 100% coal mine wastewater from CMW1 and CMW2 and controls. Activity was recorded for 250 s every 10 min using the Multispecies Freshwater Biomonitors (LimCo International GmbH). Boxplots show the interquartile range, median (horizontal line), minimum and maximum values (whiskers) and

average (+) fish activity in each treatment. Letters indicate significant differences between treatments, $\alpha = 0.05$.

Contrary to the behavioural responses observed in the present study, prior exposures in our laboratory documented increased activity in fish (*H. compressa*) and tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater from the CMW1 and CMW2 holding dams (Lanctôt et al., 2016; Lanctôt et al., in review). Differences in responses may relate to seasonal differences in wastewater quality (*e.g.*, physico-chemical properties), which varied slightly between studies. Notably, dissolved As and Se concentration were on average 3 and 10 µg/L higher in CMW1, respectively, in the present study compared to the aforementioned preceding studies (673 µg/L As and 0 µg/L Se), which could help to explain the differential responses between studies. However, similar differences did not exist for CMW2, making it difficult to conclusively identify the cause(s) of the opposing response patterns. It may be important to note that the previous studies investigated behavioural responses using EthoVisions XT video-tracking software, and thus differences between previous studies and the present study with the MFB could be the result of physical differences between the monitoring tools. For example, the MFB measures activity of fish confined in opaque cylindrical chambers whereas the EthoVisions visually quantifies movement in shallow circular dishes (Lanctôt et al., 2016). Test chambers and arena provide different dimensions, resulting in different swimming patterns (*e.g*., linear back and forth *vs* circular movement), which may contribute to differences in activity. To our knowledge, there have been no studies directly comparing and contrasting outputs provided by these two commercial tools, and such research may be important for exploring consistency in behavioural responses amongst studies utilizing different approaches and technologies. Notwithstanding the differences in response patterns that have been noted, rapid behavioural responses were observed using both methods following short-duration exposure to sub-lethal concentrations of CMW, demonstrating the rapid identification of environmental disturbances that is attainable using behavioural analysis techniques.

4.7 Conclusion

Our study demonstrates the potential for using behavioural responses of a native Australian species as tools for evaluating toxicological effects of coal mine wastewater releases on freshwater fishes. The implementation of biological early warning systems towards routine risk assessments could complement existing physico-chemical monitoring regimes in coal mining regions, by measuring rapid and biologically meaningful responses of aquatic biota to environmental pollutants (Kuklina et al., 2013). However, there is still a great deal of research necessary for behavioural tools to be effectively applied towards monitoring efforts, including a need to identify model species, standardize approaches, and to document responses with exposure to various physico-chemical parameters and standard contaminants. Therefore, while our results illustrate the various sub-lethal behavioural effects that may occur in fish exposed to coal mine wastewater, further research is now necessary to explore and understand the biological relevance of behavioural alterations for influencing individual health and fitness, and fundamental population-level interactions. Finally, an important question for the ongoing development of rapid behavioural tests is whether the observed responses are more or less sensitive than outcomes from established bioassays. Therefore, future studies should also involve direct comparison of behavioural and traditional tests with exposures to both complex wastewaters and standard test compounds.

4.8 Acknowledgements

This study was supported by the Central Queensland University Postgraduate Research Award (CL), the Queensland Resource Council Coal Minesite Rehabilitation Trust Fund Scholarship (CL), an Ian Potter Foundation Science Grant (Ref No. 20140140) (SDM) and in-kind industry support. The funding sources had no involvement in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

4.9 References

Allen, G.R., Midgley, S.H., Allen, M., 2002. Field Guide to the Freshwater Fishes of Australia. Western Australian Museum, Perth, Western Australia.

Alonso, A., De Lange, H.J., Peeters, E.T.H.M., 2009. Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the Multispecies Freshwater Biomonitor. Chemosphere 75, 341–346.

Atchison, G.J., Henry, M.G., Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. Environ. Biol. Fish. 18, 11–25.

Auty, E.H., 1978. Reproductive behaviour and early development of the empire fish *Hypseleotris compressus* (Eleotridae). Mar. Freshw. Res. 29, 585–597.

Bharti, S., Banerjee, T.K., 2011. Bioaccumulation of metals in the edible catfish *Heteropneustes fossilis* (Bloch) exposed to coal mine effluent generated at Northern Coalfield Limited, Singrauli, India. Bull. Environ. Contam. Toxicol. 87, 393–398.

Bharti, S., Banerjee, T.K., 2014. Toxicity analysis of coal mine effluent on vital organs of *Heteropneustes fossilis* (Bloch) using biometric and biochemical parameters. Clean Soil. Air Water 42, 260–266.

Bushnell, P.J., Kavlock, R.J., Crofton, K.M., Weiss, B., Rice, D.C., 2010. Behavioral toxicology in the 21st century: challenges and opportunities for behavioral scientists. Summary of a symposium presented at the annual meeting of the Neurobehavioral Teratology Society, June, 2009. Neurotoxicol. Teratol. 32, 313–328.

Craig, S., Laming, P., 2004. Behaviour of the three-spined stickleback, *Gasterosteous aculeatus* (Gasterosteidae, Teleostei) in the multispecies freshwater biomonitor: a validation of automated recordings at three levels of ammonia pollution. Water Res. 38, 2144–2154.

DNRM, 2013. Queensland Coal Statistics Report for the Financial Year Ended June 2013. Deparment of Natural Resources and Mine, Queensland Government.

Gerhardt, A., Janssens de Bisthoven, L., Mo, Z., Wang, C., Yang, M., Wang, Z., 2002. Short-term responses of *Oryzias latipes* (Pisces: Adrianichthyidae) and *Macrobrachium nipponense* (Crustacea: Palaemonidae) to municipal and pharmaceutical waste water in Beijing, China: survival, behaviour, biochemical biomarkers. Chemosphere 47, 35–47.

Gerhardt, A., Janssens de Bisthoven, L., Soares, A.M.V., 2004. Macroinvertebrate response to acid mine drainage: community metrics and on-line behavioural toxicity bioassay. Environ. Pollut. 130, 263–274.

Gerhardt, A., Janssens de Bisthoven, L., Soares, A.M.V., 2005a. Evidence for the stepwise stress model: *Gambusia holbrooki* and *Daphnia magna* under acid mine drainage and acidified reference water stress. Environ. Sci. Technol. 39, 4150–4158.

Gerhardt, A., Janssens de Bisthoven, L., Soares, A.M.V.M., 2005b. Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). Arch. Environ. Contam. Toxicol. 48, 450–458.

Gerhardt, A., Schmidt, S., 2002. The multispecies freshwater biomonitor: a potential new tool for sediment biotests and biomonitoring. J. Soil. Sediment 2, 67–70.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. Chemosphere 120, 778–792.

Haibin, L., Zhenling, L., 2010. Recycling utilization patterns of coal mining waste in China. Resour. Conserv. Recycl. 54, 1331.

Janssens de Bisthoven, L., Gerhardt, A., Soares, A.M.V.M., 2004. Effects of acid mine drainage on larval Chironomus (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitors. Environ. Toxicol. Chem. 23, 1123–1128.

Kane, A.S., Salierno, J.D., Brewer, S.K., 2005. Fish models in behavioral toxicology: automated techniques, updates and perspectives. In: Ostrander, G.K. (Ed.), Methods in Aquatic Toxicology vol. 2. Boca Raton, Florida, pp. 559–590.

Kuklina, I., Kouba, A., Kozak, P., 2013. Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. Environ. Monit. Assess. 185, 5043–5053.

Laitinen, M., Valtonen, T., 1995. Cardiovascular, ventilatory and haematological responses of brown trout (*Salmo trutta* L.), to the combined effects of acidity and aluminium in humic water at winter temperatures. Aquat. Toxicol. 31, 99–112.

Lanctôt, C., Melvin, S.D., Leusch, F.D.L., Wilson, S.P., Fabbro, L., 2016. Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. Chemosphere 144, 1560–1566.

Lanctôt C., Bennett W., Wilson S.P., Fabbro L., Leusch F.D.L., Melvin S.D., Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater. Aquat. Toxicol. In review.

Macedo-Sousa, J.A., Gerhardt, A., Brett, C.M.A., Nogueira, A.J.A., Soares, A.M.V., 2008. Behavioural responses of indigenous benthic invertebrates (*Echinogammarus meridionalis*, *Hydropsyche pellucidula* and *Choroterpes picteti*) to a pulse of acid mine drainage: a laboratorial study. Environ. Pollut. 156, 966–973.

Macedo-Sousa, J.A., Pestana, J.L.T., Gerhardt, A., Nogueira, A.J.A., Soares, A.M.V., 2007. Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. Chemosphere 67, 1663–1670.

Maradona, A., Marshall, G., Mehrvar, M., Pushchak, R., Laursen, A.E., McCarthy, L.H., Bostan, V., Gilbride, K.A., 2012. Utilization of multiple organisms in a proposed early-warning biomonitoring system for real-time detection of contaminants: preliminary results and modeling. J. Hazard Mater. 219–220, 95–102.

Melvin, S.D., 2016. Short-term exposure to municipal wastewater influences energy, growth and swimming performance in juvenile Empire Gudgeons (*Hypseleotris compressa*). Aquat. Toxicol. 170, 271–278. http://dx.doi.org/10.1016/j. aquatox.2015.06.003.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.

Mishra, V.K., Upadhyaya, A.R., Pandey, S.K., Tripathi, B.D., 2008. Heavy metal pollution induced due to coal mining effluent on surrounding aquatic ecosystem and its management through naturally occurring aquatic macrophytes. Bioresour. Technol. 99, 930–936.

Mohti, A., Shuhaimi-Othman, M., Gerhardt, A., 2012. Use of the Multispecies Freshwater Biomonitor to assess behavioral changes of *Poecilia reticulata* (Cyprinodontiformes:

Poeciliidae) and *Macrobrachium lanchesteri* (Decapoda: Palaemonidae) in response to acid mine drainage: laboratory exposure. J. Environ. Monit. 14, 2505–2511.

Pestana, J.L.T., Ré, A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68, 1556–1562.

Pusey, B., Kennard, M., Arthington, A., 2004. Freshwater Fishes of North-Eastern Australia. CSIRO Publishing, Collingwood, Victoria.

Scott, B., Ranjith, P.G., Choi, S.K., Khandelwal, M., 2010. A review on existing opencast coal mining methods within Australia. J. Min. Sci. 46, 280–297.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Thiruvenkatachari, R., Younes, M., Su, S., 2011. Coal mine site investigation of wastewater quality in Australia. Desalination Water Treat. 32, 357–364.

Tiwary, R.K., 2001. Environmental impact of coal mining on water regime and its management. Water Air Soil Pollut. 132, 185–199.

Vosyliene, M.Z., Kazlauskiene, N., Svecevičius, G., 2003. Effect of a heavy metal model mixture on biological parameters of rainbow trout *Oncorhynchus mykiss*. Environ. Sci. Pollut. Res. Int. 10, 103–107.

Zocche, J.J., Damiani, A.P., Hainzenreder, G., Mendonça, R.Á., Peres, P.B., Santos, C.E.I. D., Debastiani, R., Dias, J.F., Andrade, V.M. de, 2013. Assessment of heavy metal content and DNA damage in *Hypsiboas faber* (anuran amphibian) in coal opencasting mine. Environ. Toxicol. Pharm. 36, 194–201.

4.10 Appendix

Table 4-2: *In situ* water quality and chemistry of CMW1 and CMW2 at the time of sampling.

Parameter/Analyte	**LOR**	**CMW1**		**CMW2**	
Temperature (°C)	-	24.4		24.7	
Specific conductivity (mS/cm)	-	5.97		7.02	
Conductivity (mS/cm)	-	5.90		6.98	
Total dissolved solids (g/L)	-	3.88		4.56	
Salinity (ppt)	-	3.24		3.85	
pH	-	9.0		8.8	
Orp (mV)	-	45.2		48.1	
Turbidity (NTU)	-	21.2		17.5	
Suspended Solids (mg/L)	5	38		27	
Total hardness as $CaCO_3$ (mg/L)	1	362		650	
Sulfate as SO_4 (g/L)	1	0.83		1.13	
Fluoride (mg/L)	0.1	0.5		0.4	
Ammonia as N (mg/L)	0.01	0.04		0.02	
Nitrite as N (mg/L)	0.01	<0.01		<0.01	
Nitrate as N (mg/L)	0.01	<0.01		<0.01	
Nitrite + Nitrate as N (mg/L)	0.01	<0.01		<0.01	
Total Kjeldahl Nitrogen as N (mg/L)	0.1	3.2		0.8	
Total Nitrogen as N (mg/L)	0.1	3.2		0.8	
Total Phosphorus as P (mg/L)	0.01	0.56		0.04	
Dissolved Organic Carbon (mg/L)	1	14		7	
Total Organic Carbon (mg/L)	1	14		10	
Alkalinity as $CaCO_3$ (mg/L)					
Hydroxide Alkalinity	1	<1		<1	
Carbonate Alkalinity	1	142		86	
Bicarbonate Alkalinity	1	431		310	
Total Alkalinity	1	574		397	
Dissolved (Dis) and Total (Tot) Metals (µg/L)		**Dis**	**Tot**	**Dis**	**Tot**
Aluminium	10	<10	330	<10	380
Arsenic	1	9	9	3	3
Cadmium	0.1	<0.1	<0.1	<0.1	<0.1
Chromium	1	<1	<1	<1	<1
Copper	1	3	3	1	2
Cobalt	1	<1	<1	<1	<1
Nickel	1	2	3	2	2
Lead	1	<1	<1	<1	<1
Zinc	5	<5	<5	<5	<5

Parameter/Analyte	LOR	CMW1		CMW2	
Manganese	1	1	15	3	16
Selenium	10	10	<10	<10	<10
Silver	1	<1	<1	<1	<1
Uranium	1	3	3	3	3
Vanadium	10	<10	<10	<10	<10
Boron	50	460	470	390	410
Iron	50	<50	330	<50	480
Mercury	0.1	<0.1	<0.1	<0.1	<0.1
Total Petroleum Hydrocarbons (µg/L)					
C6 - C9	20	<20		<20	
C10 - C14	50	<50		<50	
C15 - C28	100	<100		<100	
C29 - C36	50	<50		<50	
C10 - C36 (sum)	50	<50		<50	
Total Recoverable Hydrocarbons (µg/L)					
>C10 - C16	100	<100		<100	
>C16 - C34	100	<100		<100	
>C34 - C40	100	<100		<100	
>C10 - C40 (sum)	100	<100		<100	
>C10 - C16 minus Naphthalene (F2)	100	<100		<100	
TPH(V)/BTEX Surrogates (%)					
1.2-Dichloroethane-D4	0.1	98.9		96.2	
Toluene-D8	0.1	103		106	
4-Bromofluorobenzene	0.1	100		106	
BTEXN					
Benzene	1	<1		<1	
Toluene	2	<2		<2	
Ethylbenzene	2	<2		<2	
meta- and para-Xylene	2	<2		<2	
ortho-Xylene	2	<2		<2	
Total Xylenes	2	<2		<2	
Sum of BTEX	1	<1		<1	
Naphthalene	5	<5		<5	

Declaration of co-authorship and contribution

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Lanctôt, C. M., Melvin, S. D., Leusch, F. D. L., Wilson, S., and Fabbro, L. (2016b). Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. *Chemosphere* **144**, 1560–1566. http://dx.doi.org/10.1016/j.chemosphere.2015.10.026

My contribution to the paper involved:

- Initial concept and experimental design.
- Collection and analysis of data.
- Preparation of manuscript.

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Publication of Research Higher Degree Work for Inclusion in the Thesis Procedures

[redacted]

Chantal Lanctôt

Chapter 5

Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater

Chantal M. Lanctôt [a, b, *], Steven D. Melvin [a, b], Frederic D.L. Leusch [b], Scott P. Wilson [a], Larelle Fabbro [c]

[a] Central Queensland University, School of Medical and Applied Sciences, Gladstone, QLD 4680, Australia

[b] Smart Water Research Centre and Australian Rivers Institute, Griffith School of Environment, Griffith University, Southport, QLD 4215, Australia

[c] Central Queensland University, School of Medical and Applied Sciences, Rockhampton, QLD 4702, Australia

* Corresponding author
e: chantal.lanctot@gmail.com, c.lanctot@cqu.edu.au
Mailing address:
Chantal Lanctôt
Smart Water Research Centre (G51)
Griffith University, Gold Coast Campus
Southport, Queensland, 4215
Australia

5.1 Abstract

Coal mining generates large quantities of complex effluent and may pose a threat to aquatic wildlife. Despite this, few studies have explored the consequences of exposure to mine wastewater on aquatic organisms, and this is particularly true for the Australian environment. We investigated sub-lethal behavioural responses in a native Australian fish exposed to wastewater from two releasing dams (CMW1 and CMW2) located at an open cut coal mine in Central Queensland. Swimming activity and movement of empire gudgeons (*Hypseleotris compressa*) were assessed during a two-week exposure using video-tracking software. Increased activity was observed in exposed fish after 7 and 14 days. Specifically, we found a significant increase in the mean velocity and mobility of fish exposed to CMW1 treatments. Exposed fish also spent on average 23% more time in the peripheral zone compared to controls after 14-d exposures. A similar response pattern was observed in fish exposed to CMW2, but differences between treated and control fish did not generally reach statistical significance. Alterations to normal swimming activity and movement patterns can be indicative of a stress response in fish, and could subsequently lead to negative population-level impacts by increasing the conspicuousness of exposed individuals to predators, or by altering foraging abilities. More research is warranted to explore relationships between behavioural and physiological outcomes, including endocrine disruption, and subsequent population-level outcomes in aquatic organisms at risk of exposure to coal process-affected water.

5.2 Keywords

Video-tracking; EthoVision; Coal mine wastewater; Behaviour; Swimming; Fish

5.3 Introduction

Global human activities are now recognized as the dominant cause of major environmental change (Lewis and Maslin, 2015). Threats to the integrity of freshwater resources represent a foremost concern for future generations, and current generations are therefore obligated to strive to protect aquatic animals and ecosystem health (Weiss, 1990). Perhaps most disconcerting is the widely recognized fact that humans are directly contributing to the accelerated loss of many species (Monastersky, 2014), and that this may be pushing the planet towards a new age of mass extinctions (Ceballos et al., 2015). The exploitation of Earth's natural resources is a prime example of an anthropogenic pursuit that can pose significant threats to the environment (Fransson and Gärling, 1999). However, such activities are necessary for modern society and will foreseeably continue until sustainable alternatives are identified and implemented. It is therefore extremely important that we aim to understand and mitigate potential adverse outcomes on non-target organisms that may be associated with natural resource extraction.

The coal industry has played a significant role in Australia's development for over two centuries and remains embedded in the country's economy. However, rapid expansion of the coal mining industry has in some instances led to the contamination of adjacent freshwater ecosystems, and has subsequently put native species at risk. A primary concern relates to the production of large quantities of complex process-affected water, which can contain high concentrations of metals, hydrocarbons, dissolved and suspended solids, salts and other chemicals that can contaminate aquatic systems through both planned and accidental releases (Tiwary, 2001; Thiruvenkatachari et al., 2011). Laboratory and field studies have revealed that environmental contamination, from both active and abandoned coal mines, can have detrimental impacts on aquatic biota. While the majority of studies have focused on invertebrate species, several studies have described a range of potential sub-lethal impacts in fish, which are related to coal mine discharge. These include effects on survival (Scullion and Edwards, 1980; Mylliemngap and Ramanujam, 2012), community structure (Branson and Batch, 1971; Daniel et al., 2014), morphometrics (Bharti and Banerjee, 2013, 2014), metal uptake (Bharti and Banerjee, 2011; Arnold et al., 2014), genotoxicity (Benassi et al., 2006), reproduction (Rudolph et al., 2008), metabolism (Miller et al., 2009) and oxidative stress (Benassi et al., 2006). However, research pertaining to native Australian fish species has been extremely limited, particularly considering the national importance of the mining industry. Knowledge gaps need to be addressed, and since fish are a vital part of many aquatic ecosystems, the resulting information can serve as a broad indicator of the potential for adverse toxicological outcomes associated with aquatic contaminations resulting from coal mining operations (Adams et al., 1989; Scott and Sloman, 2004).

Behavioural analyses are now commonly applied as sub-lethal response variables in ecotoxicology, and increasing evidence has demonstrated the applicability of these approaches to a range of exposure scenarios (Melvin and Wilson, 2013; Bae and Park, 2014). Many aquatic organisms display sensitive behavioural responses to toxicants at low concentrations, and in many cases such effects may be predictive of potential higher-level outcomes related to organism- or population-level survival and fitness (Plaut, 2001; Groh et al., 2015). For example, swimming performance of fish may impact their ability to feed, escape predation, migrate, disperse, avoid unfavourable conditions, or successfully mate, and these effects have clear associations with survival, growth, metabolism, and reproduction (Beaumont et al., 1995; Kieffer, 2000; Gerhardt et al., 2005; Pestana et al., 2007; Macedo-Sousa et al., 2008; Alonso et al., 2009). Fish provide excellent models for behavioural toxicology, because various ecologically relevant behaviours can be easily observed and quantified in the laboratory (reviewed by Atchison et al., 1987; Kieffer, 2000; Plaut, 2001; Scott and Sloman, 2004). Many fish also respond rapidly to various environmental disturbances (Shedd et al., 2001; Kuklina et al., 2013) including discharges from mineral mining, which was shown to alter locomotor responses of several species (Gerhardt et al., 2005; Mohti et al., 2012). However, there have apparently been no studies investigating behavioural impacts associated with coal mining wastewater on fish, and since discharge events will often have limited duration and result in short-term exposure, rapid responses are highly relevant to explore.

This study describes behavioural impacts on swimming activity and movement patterns of an Australian fish species following short-term exposure to coal mine wastewater from a Queensland mine.

5.4 Materials and methods

5.4.1 Animals

Empire gudgeons, *Hypseleotris compressa* (Eleotridae), are a small-bodied (maximum standard length of ~10 cm) freshwater species, with a broad distribution throughout northern and eastern Australia and southern New Guinea (Auty, 1978). *H. compressa* inhabit a variety of lotic and lentic habitats ranging from fresh to estuarine water and are considered tolerant to a range of environmental variables including temperature, dissolved oxygen, salinity and pH (Allen et al., 2002; Pusey et al., 2004). *H. compressa* was selected for this study because it represents an ecologically relevant species for the coal mine-affected catchments of Central Queensland, Australia. This species was recently shown to exhibit altered swimming performance following short duration exposure to municipal wastewater, and is therefore a good model for behavioural toxicity studies (Melvin, 2015).

Juvenile fish (approximately 60 d of age), bred from adults collected in Central Queensland, were purchased from an aquarium wholesaler (Aquarium Industries Pty Ltd, Australia) and acclimated to laboratory conditions at the Smart Water Research Centre on Griffith University's Gold Coast campus, Queensland, Australia. Fish were housed in aerated glass aquaria containing carbon filtered municipal tap water and rock substrate for over 4 weeks prior to experimentation, and were fed frozen daphnia and a staple flake food (Ocean Nutrition™) ad libitum twice daily during holding. Laboratory conditions were maintained at 22–25 °C and 12:12 light:dark photoperiod. Mean (±SD) standard length, total length, weight, and condition factor of a subset (n = 12) of fish at the start of the experiment were 1.2 ± 0.06 cm, 1.4 ± 0.09 cm, 13.6 ± 5.6 mg and 0.7 ± 0.2, respectively. It was not possible to visually sex fish at this life stage, so (juvenile) males and females were indiscriminately used for experimentation. The experimental protocol was approved by the Animal Ethics Committee of CQUniversity in accordance with guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (Approval No. A13/05-301).

5.4.2 Coal mine wastewater sampling

Wastewater was collected at an open-cut coal mine located in the Bowen Basin of Central Queensland, Australia, in June 2014. At the time of experimentation, this mine had two dams with active release points authorized for the discharge of process-affected water (henceforth referred to as CMW1 and CMW2). Wastewater from these two dams was collected in 20 L plastic containers, transported on ice and stored in the refrigerator at 4 °C. Water was brought to ambient temperature prior to commencement of experimentation.

5.4.3 Water quality and chemistry

In situ measurements of temperature, conductivity (EC), pH, dissolved oxygen (DO), salinity, total dissolved solid (TDS) and turbidity were taken at the time of sampling using a YSI 6600 multi-parameter handheld sonde (Xylem Analytics, Hemnant, Australia). Samples were collected in situ for analysis of a suite of chemical parameters listed in Table 5-2 (Appendix), and were shipped on ice immediately following collection for chemical analysis by the ALS Laboratory Group (Brisbane, Australia). Throughout the experiment, basic water quality parameters (temperature, EC, pH, dissolved oxygen, salinity and TDS) were measured in each treatment group prior to and immediately following water renewals, using YSI EcoSence® probes. Nitrogenous waste was measured in a subset of replicates throughout the experiment using ammonia test kits (Aquasonic Pty Ltd, Australia).

5.4.4 Short-term (14 d) exposure with juvenile fish

Empire gudgeons were exposed to control water and 25, 50 or 100% CMW1 and CMW2 water for 14 days, in 5 L aquaria filled with 2 L of the appropriate treatment. There were four replicates of each treatment, each containing five fish. Fish were fed a controlled food ration throughout the exposure (4% of body weight/day), and uneaten food and faeces was removed daily. Full water renewals were performed twice weekly and survival was monitored daily throughout the experiment. On day 7 and 14, fish were placed individually in 28 petri dishes (D5.5 cm × H1.5

cm) containing 15 mL of the appropriate treatment dilution. Petri dishes were placed on a white LED-backlit acrylic table to maximize contrast. Fish were acclimated for 15 min and video recorded continuously for 45 min using the EthoVision XT 9.0 (Nodulus Information Technology, Netherlands) video-tracking system connected to an acA1300-30gc GigE camera (Basler AG, Germany) positioned above the dishes. This was repeated with three randomly selected fish from each replicate. No personnel were present in the laboratory during video recording. After the final recording, animals were euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS222, Sigma) dissolved in water, and measurements of standard length (mm), total length (mm), and wet weight (mg) were taken for each individual. Morphometric measurements were used to calculate condition factor (k = [weight/standard length^3] × 100) each fish.

5.4.5 Statistical analysis

Differences in survival between treatments were analysed with the Mantel–Cox log rank tests, using Prism® v6.0f (GraphPad Software Inc., USA). All other analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL). Data were first analysed for normality (Kolmogorov–Smirnov) and homogeneity of variance (Levene's test) and transformed ($\log_{10}$) to meet parametric assumptions when the assumptions were violated. One-way ANOVA followed by Tukey's HSD post hoc test was used to analyse differences in morphometric measurements (standard length, total length, weight, k) between treatments. Behavioural data (mean velocity, maximum velocity, time spent in peripheral zone [0.8 cm from border, representing 49% of the arena surface; used as an index of anxiogenic behaviour] and duration of immobility) were analysed using one-way ANOVAs followed by Tukey's HSD post hoc test for each time point. Time spent in peripheral zone data did not satisfy the parametric assumptions, so was analysed using non-parametric Kruskal Wallis test followed by multiple comparisons. Simple linear regression was applied to explore relationships between k and behavioural endpoints at the end of the experiment. The significance level was set at $\alpha \leq 0.05$ for all tests.

5.5 Results

5.5.1 Water quality and chemistry in CMW1 and CMW2 treatments and control

Mean water quality parameters (pH, DO, EC, salinity, and TDS) are presented in Table 5-1. Mean temperature ranged between 22 and 25 °C, coinciding with seasonal temperatures at the time of the exposure. All treatments had higher pH, EC, salinity and TDS compared to controls. Electrical conductivity, salinity and TDS were also marginally higher in CMW2 compared to CMW1. Ammonia concentrations were <0.01 ppm in controls and all of the CMW2 treatments, and ranged between 0 and 0.1 ppm in 25 and 50% CMW1 and 0.1–0.5 ppm in 100% CMW1 treatment.

Table 5-1: Mean (± SD) survival, pH, dissolved oxygen (DO), conductivity (EC), salinity and total dissolved solids (TDS) measured through the exposure.

	Survival (%)	pH	DO (%)	EC (mS)	Salinity (ppt)	TDS (mg L^{-1})
Control	75 ± 19	7.7 ± 0.1	94.8 ± 4.6	0.2 ± 0.01	0.1 ± 0.0	0.1 ± 0.003
25% CMW1	85 ± 19	8.5 ± 0.5	89.7 ± 3.6	2.1 ± 0.03	1.1 ± 0.0	1.3 ± 0.01
50% CMW1	85 ± 19	8.6 ± 0.4	86.9 ± 9.2	4.0 ± 0.02	2.1 ± 0.0	2.6 ± 0.01
100% CMW1	55 ± 10	8.6 ± 0.1	88.7 ± 4.9	7.3 ± 0.04	4.0 ± 0.0	4.7 ± 0.03
25% CMW2	69 ± 19	8.5 ± 0.2	92.9 ± 3.7	2.4 ± 0.03	1.2 ± 0.0	1.5 ± 0.02
50% CMW2	65 ± 19	8.7 ± 0.1	93.5 ± 2.0	4.5 ± 0.06	2.4 ± 0.03	2.9 ± 0.04
100% CMW2	95 ± 10	8.8 ± 0.1	93.0 ± 2.8	8.1 ± 0.06	4.5± 0.04	5.3 ± 0.04

In situ water quality and results from chemical analysis of CMW1 and CMW2 dams at time of sampling are presented in Table 5-2 (Appendix). In general, CMW1 had higher turbidity, TSS, ammonia, fluoride, nutrients, DOC and TOC, but lower conductivity, TDS and salinity compared to CMW2. Total and dissolved metal(loid) concentrations also varied between dams. For example, CMW2 had slightly higher concentrations of dissolved copper, nickel, manganese, uranium, and vanadium compared to CMW1. Total metal concentrations, on the other hand, were higher in CMW1 compared to CMW2 (except for boron). Total and dissolved cadmium, selenium, silver and mercury were below detection limit in both dams. Petroleum hydrocarbons were only detected in CMW1. BTEXN (*i.e.*, benzene, toluene, ethylbenzene, xylenes, naphthalene) were below detection limits in both dams.

5.5.2 Survival and morphometric alterations

Mantel–Cox log rank test revealed no significant differences in survival between treatments (X^2 = 11.07, p = 0.086, Table 5-1). Morphometric endpoints did not vary significantly between treatments at the end of the experiment (p > 0.05), although an apparent trend of increased weight was observed in all treatments relative to controls and corresponded with increased condition in these animals (Figure 5-1). Mean (±SD) standard length, total length, weight, and condition factor of all fish at the end of the exposure was 1.2 ± 0.07 cm, 1.4 ± 0.1 cm, 15.4 ± 6.3 mg and 0.8 ± 0.2, respectively.

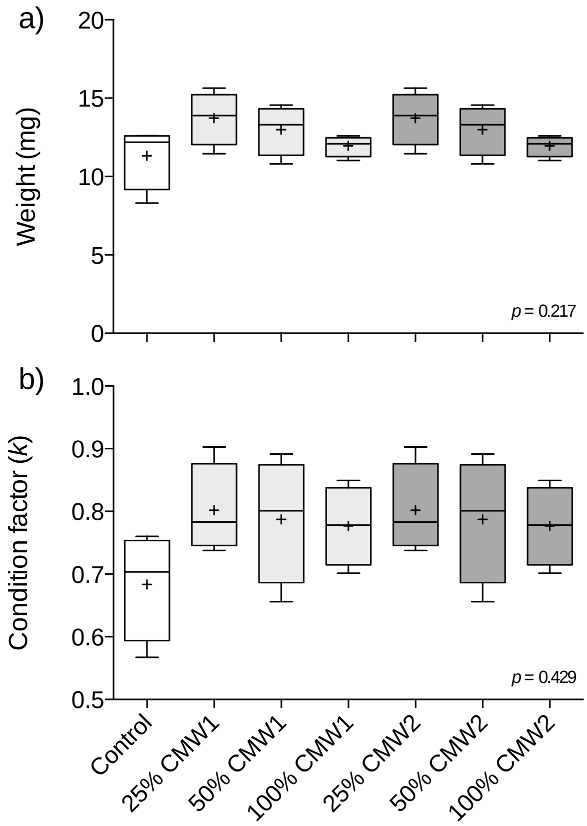

Figure 5-1: Weight (a) and condition factor (b) of *Hypseleotris compressa* after 14 d exposure to 25, 50 and 100% CMW1 and CMW2 and control. Boxplots show the interquartile range, median (horizontal line), minimum and maximum values (whiskers) and average (+) of 5 fish per replicate (n = 4).

5.5.3 Behavioural responses and swimming performance

Significant treatment differences in mean velocity (Figure 5-2a; $F_{(6, 27)} = 5.16$, $p = 0.002$) and immobility (Figure 5-2d; $F_{(6, 27)} = 7.17$, $p < 0.001$) were observed after 7 d of exposure. Mean velocity (Figure 5-2a; $F_{(6, 27)} = 5.78$, $p = 0.001$), time spent in peripheral zone (Figure 5-2c; $F_{(6, 28)} = 15.02$, $p = 0.020$) and immobility (Figure 5-2d; $F_{(6, 27)} = 6.88$, $p < 0.001$) were also significantly different from controls after 14 d exposure. Specifically, 3.3–6.5-fold increases in mean velocity were observed after 7 and 14 d exposure to CMW1 water. Mean velocity between control and CMW2 treatments did not differ significantly. Exposed fish generally spent 2–40% less time stationary compared to controls (measured as immobility state), with significant differences from control animals in 25 and 50% CMW1 after 7 d as well as all CMW1 treatments and 100%

CMW2 after 14 d. There was no effect of treatment on time spent in peripheral zone after 7 d of exposure (Figure 5-2c; $F_{(6, 28)} = 6.68$, $p = 0.351$), however, after 14 d fish exposed to 100% CMW1 spent significantly more time in the peripheral zone compared to controls (26% increase). Maximum velocity was not affected by the exposure after 7 d (Figure 5-2b; $F_{(6, 27)} = 2.20$, $p = 0.084$) or 14 d ($F_{(6, 27)} = 1.87$, $p = 0.135$) of exposure. Linear regression showed a significant positive relationship between *k* and mean velocity, however the linear model only explained 21% of the total variability in the dataset ($R^2 = 0.21$; $p < 0.001$; Appendix Figure 5-3).

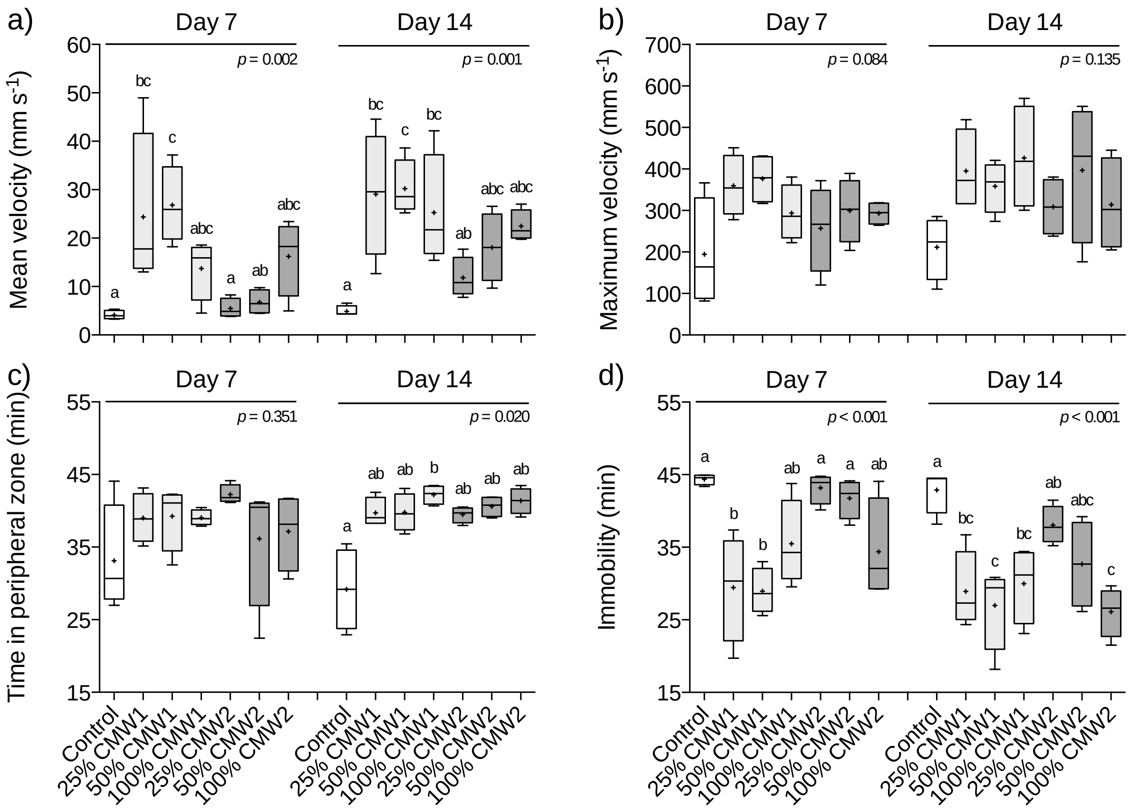

Figure 5-2: Mean velocity (a), maximum velocity (b), time spent in peripheral zone (c) and immobility duration (d) of *Hypseleotris compressa* after 7 and 14 d exposure to 25, 50 and 100% CMW1 and CMW2 and control. Boxplots show the interquartile range (column), median (horizontal line), minimum and maximum values (whiskers) and average (+) of 3 fish per replicate (n = 3 x 4 rep) filmed for 45 min. Letters indicate significant differences between treatments on each day.

5.6 Discussion

Juvenile empire gudgeons were exposed to coal mine wastewater collected from two dams (CMW1 and CMW2) located at an open cut coal mine in Central Queensland's Bowen Basin. After 14 days of exposure, mean mortality was 20% higher in 100% CMW1 exposed fish compared to controls. However, statistical analysis revealed that differences in survival were not significantly influenced by the treatments. Mortalities were higher than expected in control animals, particularly since survival was high (>95%) in the holding tank during the 4-week acclimation period prior to experimentation. During this time fish were held in the same source of filtered water as experimental controls, which suggests that mortalities are not related to laboratory conditions. Decreased survival is therefore more likely related to the sensitivity of juvenile fish to experimental manipulations (*i.e.*, transfer for behavioural monitoring), which is further supported since the majority of mortalities occurred just after the first recording (day 7). Regardless, mortalities were spread amongst replicates, and therefore did not prevent us from obtaining behavioural data during the experiment.

Average morphometric endpoints did not differ statistically between treatments, but a trend of increased weight and condition (*k*) was observed in exposed fish compared to controls after 14 d of exposure. The lack of significance is not unexpected given the relatively short exposure timeframe, however, a recent study observed significantly increased weight and condition in juvenile *H. compressa* from the same population, following 14 d exposure to domestic wastewater (Melvin, 2015). Fish collected from sites contaminated by coal mining and with elevated selenium concentrations have previously been shown to exhibit increased *k* compared to reference fish (Miller et al., 2009). Additionally, a recent study found a similar (non-significant) increase in *k* after 4-weeks exposure of larval amphibians to coal mine wastewater from the same sites as the present study (Lanctôt et al. in review). Increased condition is a characteristic response that has been observed in fish exposed to complex wastewaters from various municipal and industrial sources (Galloway et al., 2003; Knapen et al., 2009; Melvin, 2015). Such responses have generally been attributed to increased food and nutrition resulting from organic matter in the wastewater (Campbell et al., 2003). Contrary to the trend observed in the present study and significant increases in *k* described in other studies, exposure to coal mine wastewater has also resulted in decreased condition in fish in both laboratory (Bharti and Banerjee, 2014) and field settings (Scullion and Edwards, 1980). Differences in morphological responses amongst studies may often be attributable to variability in environmental factors, differences in species sensitivity and adaptability, or effluent characteristics amongst locations. In this case, the fish *H. compressa* is quite tolerant to a range of water conditions including high salt and pH (Pusey et al., 2004), and may therefore represent a conservative estimate of possible toxicological outcomes compared to less tolerant species.

A general trend of increased activity was observed in exposed fish after 7 d compared to controls, and this persisted at 14 days of exposure. Specifically, we observed significant increases in swimming velocity and mobility in the CMW1 treatments after 7 and 14 d. Similar trends were observed in CMW2 treatments but differences were generally not significantly different from the controls. Site differences may relate to differences in physicoechemical parameters, since CMW1 had relatively higher turbidity, suspended solids, ammonia, nutrients, dissolved and total organic carbon and total metal(loid)s compared to CMW2. Several of these parameters are known to elicit toxicological outcomes in aquatic organisms when present outside the range of tolerance (Atchison et al., 1987; Randall and Tsui, 2002; Cañedo-Argüelles et al., 2013), but it is difficult to establish causal relationships from a single study due to the multitude to potentially interacting factors associated with complex wastewater. As indicated, another possible explanation for differences amongst dams, and relatively limited responsiveness overall, could relate to the choice of species. We chose *H. compressa* as our focal species because of its wide distribution and relevance for the specific region where the studied mine is located, but this species is also quite robust and tolerant of changes to salinity and pH (Pusey et al., 2004). More research is therefore warranted, to investigate responses with mixtures of relevant contaminants and water quality characteristics identified through analysis of the studied wastewater, and to explore similar endpoints with other species.

Altered behaviours can result from toxicant damage affecting the nervous and endocrine systems (reviewed by Weis et al., 2001; Scott and Sloman, 2004; Weis and Candelmo, 2012), and this could help to explain the observed responses in the present study. Several organic chemicals and metals can affect neurotransmitters as well as thyroid hormone synthesis, receptor binding, transport and metabolism, which can in turn result in altered behaviour in fish (Morin et al., 1989; Smith et al., 1995; Cooley et al., 2001; Clotfelter et al., 2004). Zhou et al. (1999) demonstrated that metal contaminated water reduces levels of 3,5,30-triiodothyronine (T_3) in fish, suggesting a disruption in the conversion of thyroxine (T_4) to T_3 in peripheral tissues. This is supported by amphibian studies that documented delays in T_3-dependent metamorphosis in tadpoles exposed to arsenic (Davey et al., 2008) and a recent study using wastewater from the same location as the present study (Lanctôt et al. in review). However, reduced T_3 would be expected to be associated with hypothyroidism and general sluggishness, rather than the apparent hyperactivity that was observed (Weis et al., 2001). More research is required to investigate potential endocrine disruption responses of aquatic vertebrates exposed to coal mine wastewater, and its possible links to the observed behavioural outcomes. Furthermore, considering the importance of coal mining in Australia and globally, and with the delayed metamorphosis observed in an associated study (Lanctôt et al. in review) and altered swimming performance in the present study, there is clearly a need for further research investigating the effects of coal mine wastewater on native aquatic organisms.

Consistent with our results, hyperactivity has been previously observed following exposure to sub-lethal doses of metals in various fish species (reviewed by Atchison et al., 1987). Hyperactivity may have important implications for higher-level biological processes, given the major energetic demands and associated high cost of swimming in fishes (Plaut, 2001; Tudorache et al., 2008). Previous studies have demonstrated that effects on behaviours, activity levels, and swimming performance may hold important ramifications for population level interactions. For example, evasive behaviours are a primary survival strategy for many organisms facing the threat of predation (Vamosi and Schluter, 2002; Bradley et al., 2013), and limitations to attainable swimming velocities can influence the ability of fish to traverse barriers during migrations (Castro-Santos, 2005; Starrs et al., 2011). Changes in locomotory activity could also increase the conspicuousness of exposed individuals to predators, or altering foraging abilities (Mesa et al., 1994; Weis et al., 2001). Regardless the outcome, alterations to normal swimming activity and movement patterns are a recognized sign of stress (Garaventa et al., 2010) that are likely related to physical irritation caused by metals or other components of the water (Scarfe et al., 1982; Barry, 2012). Further to this, our study also revealed that exposed fish tended to spend more time in the peripheral zone compared to controls, which can be interpreted as anxiogenic behaviour and indicate that animals are seeking shelter (Baldissarelli et al., 2012). Such behaviour could also conceivably influence higher-level processes, by influencing how fish interact with each other or their habitat. Future work should therefore strive to identify clear linkages, between behavioural responses at the individual level and adverse biological outcomes at the population level.

5.7 Conclusions

Coal mining is an extremely important industry globally, and particularly so in Australia. However, few studies have sought to characterize sub-lethal exposure outcomes in native Australian species exposed to coal mine process-affected waters. Our results address these knowledge gaps, and demonstrate a range of alterations to behaviour and swimming performance in juvenile empire gudgeons exposed to sub-lethal concentrations of minewater from two different holding dams. Hyperactivity, increased average swimming velocity, and more time spent in the peripheral zone of experimental arenas, were observed following relatively short exposure duration (7 and 14 d). These endpoints proved to be more sensitive than developmental or morphological responses, which were unaffected by the exposure. There is growing evidence that effects on behaviour and swimming performance may hold implications for important higher-level outcomes related to survival, growth, and reproduction, but research is needed to further explore such consequences and to investigate responses in different organisms with water collected from different mine operations.

5.8 Acknowledgements

This study was supported by a Central Queensland University Postgraduate Research Award to CL, a Queensland Resource Council Coal Minesite Rehabilitation Trust Fund Scholarship to CL, an Ian Potter Foundation Science Grant (Ref No. 20140140) to SDM, and in-kind industry support. The funding sources had no involvement in the collection, analysis, and interpretation of data, the writing of the report, or the decision to submit the paper for publication.

5.9 References

Adams, S.M., Shepard, K.L., Greeley Jr., M.S., Jimenez, B.D., Ryon, M.G., Shugart, L.R., McCarthy, J.F., Hinton, D.E., 1989. The use of bioindicators for assessing the effects of pollutant stress in fish. Mar. Environ. Res. 28, 459–464.

Allen, G.R., Midgley, S.H., Allen, M., 2002. Field Guide to the Freshwater Fishes of Australia. Western Australian Museum, Perth, Western Australia.

Alonso, A., De Lange, H.J., Peeters, E.T.H.M., 2009. Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the multispecies freshwater biomonitor. Chemosphere 75, 341e346.

Arnold, M.C., Lindberg, T.T., Liu, Y.T., Porter, K.A., Hsu-Kim, H., Hinton, D.E., Di Giulio, R.T., 2014. Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. Ecotoxicology 23, 929–938.

Atchison, G.J., Henry, M.G., Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. Environ. Biol. Fish. 18, 11–25.

Auty, E.H., 1978. Reproductive behaviour and early development of the empire fish *Hypseleotris compressus* (Eleotridae). Aust. J. Mar. Freshw. Res. 29, 585–597.

Bae, M.-J., Park, Y.-S., 2014. Biological early warning system based on the responses of aquatic organisms to disturbances: a review. Sci. Total Environ. 466–467, 635–649.

Baldissarelli, L.A., Capiotti, K.M., Bogo, M.R., Ghisleni, G., Bonan, C.D., 2012. Arsenic alters behavioral parameters and brain ectonucleotidases activities in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C 155, 566–572.

Barry, M.J., 2012. Application of a novel open-source program for measuring the effects of toxicants on the swimming behavior of large groups of unmarked fish. Chemosphere 86, 938–944.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Exposure of brown trout, *Salmo trutta*, to sub-lethal copper concentrations in soft water and its effect upon sustained swimming performance. Aquat. Toxicol. 33, 45–63.

Benassi, J.C., Laus, R., Geremias, R., Lima, P.L., Menezes, C.T.B., Laranjeira, M.C., Wilhelm-Filho, D., Fa vere, V.T., Pedrosa, R.C., 2006. Evaluation of remediation of coal mining wastewater by chitosan microspheres using biomarkers. Arch. Environ. Contam. Toxicol. 51, 633–640.

Bharti, S., Banerjee, T.K., 2011. Bioaccumulation of metals in the edible catfish *Heteropneustes fossilis* (Bloch) exposed to coal mine effluent generated at Northern Coalfield Limited, Singrauli, India. Bull. Environ. Contam. Toxicol. 87, 393–398.

Bharti, S., Banerjee, T.K., 2013. Bioassay analysis of efficacy of phytoremediation in decontamination of coal mine effluent. Ecotox Environ. Safe 92, 312–319.

Bharti, S., Banerjee, T.K., 2014. Toxicity analysis of coal mine effluent on vital organs of *Heteropneustes fossilis* (Bloch) using biometric and biochemical parameters. Clean. Soil Air Water 42, 260–266.

Bradley, C.J., Strickler, J.R., Buskey, E.J., Lenz, P.H., 2013. Swimming and escape behavior in two species of calanoid copepods from nauplius to adult. J. Plankton Res. 35, 49–65.

Branson, B.A., Batch, D.L., 1971. Effects of strip mining on small-stream fishes in east-central Kentucky. Proc. Biol. Soc. Wash 84, 507–517.

Campbell, P.G.C., Hontela, A., Rasmussen, J.B., Gigue re, A., Gravel, A., Kraemer, L., Kovesces, J., Lacroix, A., Levesque, H., Sherwood, G., 2003. Differentiating between direct (physiological) and food-chain mediated (bioenergetic) effects on fish in metal-impacted lakes. Hum. Ecol. Risk Assess. 9, 847–866.

Cañedo-Argüelles, M., Kefford, B.J., Piscart, C., Prat, N., Schäfer, R.B., Schulz, C.-J., 2013. Salinisation of rivers: an urgent ecological issue. Environ. Pollut. 173, 157–167.

Castro-Santos, T., 2005. Optimal swim speeds for traversing velocity barriers: an analysis of volitional high-speed swimming behavior of migratory fishes. J. Exp. Biol. 208, 421–432.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., García, A., Pringle, R.M., Palmer, T.M., 2015. Accelerated modern humaneinduced species losses: entering the sixth mass extinction. Sci. Adv. e1400253.

Clotfelter, E.D., Bell, A.M., Levering, K.R., 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Anim. Behav. 68, 665–676.

Cooley, H.M., Fisk, A.T., Wiens, S.C., Tomy, G.T., Evans, R.E., Muir, D., 2001. Examination of the behavior and liver and thyroid histology of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to high dietary concentrations of C10-, C11-, C12- and C14-polychlorinated n-alkanes. Aquat. Toxicol. 54, 81–99.

Daniel, W.M., Infante, D.M., Hughes, R.M., Tsang, Y.-P., Esselman, P.C., Wieferich, D., Herreman, K., Cooper, A.R., Wang, L., Taylor, W.W., 2014. Characterizing coal and mineral mines as a regional source of stress to stream fish assemblages. Ecol. Indic. 50, 50–61.

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

Fransson, N., Gärling, T., 1999. Environmental concern: conceptual definitions, measurement methods, and research findings. J. Environ. Psychol. 19, 369–382.

Galloway, B.J., Munkittrick, K.R., Currie, S., Gray, M.A., Curry, R.A., Wood, C.S., 2003. Examination of the responses of slimy sculpin (*Cottus cognatus*) and white sucker (*Catostomus commersoni*) collected on the Saint John river (Canada) downstream of pulp mill, paper mill, and sewage discharges. Environ. Toxicol. Chem. 22, 2898–2907.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519.

Gerhardt, A., Janssens de Bisthoven, L., Soares, A.M., 2005. Evidence for the stepwise stress model: *Gambusia holbrooki* and *Daphnia magna* under acid mine drainage and acidified reference water stress. Environ. Sci. Technol. 39, 4150–4158.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. Chemosphere 120, 778–792.

Kieffer, J.D., 2000. Limits to exhaustive exercise in fish. Comp. Biochem. Physiol. 126, 161–179.

Knapen, D., De Wolf, H., Knaepkens, G., Bervoets, L., Eens, M., Blust, R., Verheyen, E., 2009. Historical metal pollution in natural gudgeon populations: inferences from allozyme, microsatellite and condition factor analysis. Aquat. Toxicol. 95, 17–26.

Kuklina, I., Kouba, A., Kozak, P., 2013. Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. Environ. Monit. Assess. 185, 5043–5053.

C. Lanctôt, W. Bennett, S. Wilson, L. Fabbro, F.D.L. Leusch, S.D. Melvin. Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes* *peronii*) exposed to coal mine wastewater. submitted for publication (AQTOX-D-15e00392).

Lewis, S.L., Maslin, M.A., 2015. Defining the anthropocene. Nature 519, 171–180.

Macedo-Sousa, J.A., Gerhardt, A., Brett, C.M.A., Nogueira, A.J.A., Soares, A.M., 2008. Behavioural responses of indigenous benthic invertebrates (*Echinogammarus meridionalis*, *Hydropsyche pellucidula* and *Choroterpes picteti*) to a pulse of acid mine drainage: a laboratorial study. Environ. Pollut. 156, 966–973.

Melvin, S.D., 2015. Short-term exposure to municipal wastewater influences energy, growth and swimming performance in juvenile Empire Gudgeons (*Hypseleotris compressa*). Aquat. Toxicol. http://dx.doi.org/10.1016/j.aquatox.2015.06.003.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.

Mesa, M.G., Poe, T.P., Gadomski, D.M., Petersen, J.H., 1994. Are all prey created equal? A review and synthesis of differential predation on prey in substandard condition. J. Fish. Biol. 45, 81–96.

Miller, L.L., Rasmussen, J.B., Palace, V.P., Hontela, A., 2009. The physiological stress response and oxidative stress biomarkers in rainbow trout and brook trout from selenium-impacted streams in a coal mining region. J. Appl. Toxicol. 29, 681–688.

Mohti, A., Shuhaimi-Othman, M., Gerhardt, A., 2012. Use of the multispecies freshwater biomonitor to assess behavioral changes of *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae) and *Macrobrachium lanchesteri* (Decapoda: Palaemonidae) in response to acid mine drainage: laboratory exposure. J. Environ. Monit. 14, 2505–2511.

Monastersky, R., 2014. Biodiversity: life-a status report. Nature 516, 158e161. Morin, P.-P., Dodson, J.J., Dore , F.Y., 1989. Thyroid activity concomitant with olfactory learning and heart rate changes in Atlantic salmon, *Salmo salar*, during smoltification. Can. J. Fish. Aquat. Sci. 46, 131–136.

Mylliemngap, B.K., Ramanujam, S.N., 2012. Morphological changes in the gills of *Heteropneustes fossilis* (Bloch) exposed to coal mining effluent water. J. Environ. Biol. 33, 735–739.

Pestana, J.L.T., Re , A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68, 1556–1562.

Plaut, I., 2001. Critical swimming speed: its ecological relevance. Comp. Biochem. Physiol. 131, 41–50.

Pusey, B., Kennard, M., Arthington, A., 2004. Freshwater Fishes of North-Eastern Australia. CSIRO Publishing, Collingwood, Victoria.

Randall, D.J., Tsui, T.K.N., 2002. Ammonia toxicity in fish. Mar. Pollut. Bull. 45, 17–23.

Rudolph, B.-L., Andreller, I., Kennedy, C.J., 2008. Reproductive success, early life stage development, and survival of Westslope Cutthroat Trout (*Oncorhynchus clarki lewisi*) exposed to elevated selenium in an area of active coal mining. Environ. Sci. Technol. 42, 3109–3114.

Scarfe, A.D., Jones, K.A., Steele, C.W., Kleerekoper, H., Corbett, M., 1982. Locomotor behavior of four marine teleosts in response to sublethal copper exposure. Aquat. Toxicol. 2, 335–353.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Scullion, J., Edwards, R.W., 1980. The effect of pollutants from the coal industry on the fish fauna of a small river in the South Wales coalfield. Environ. Pollut. 21, 141–153.

Shedd, T.R., van der Schalie, W.H., Widder, M.W., Burton, D.T., Burrows, E.P., 2001. Long-term operation of an automated fish biomonitoring system for continuous effluent acute toxicity surveillance. Bull. Environ. Contam. Toxicol. 66, 392–399.

Smith, G.M., Khan, A.T., Weis, J.S., Weis, P., 1995. Behavior and brain chemistry correlates in mummichogs (*Fundulus heteroclitus*) from polluted and unpolluted environments. Mar. Environ. Res. 39, 329–333.

Starrs, D., Ebner, B.C., Lintermans, M., Fulton, C.J., 2011. Using sprint swimming performance to predict upstream passage of the endangered Macquarie perch in a highly regulated river. Fish. Manag. Ecol. 18, 360–374.

Thiruvenkatachari, R., Younes, M., Su, S., 2011. Coal mine site investigation of wastewater quality in Australia. Desalin Water Treat. 32, 357–364.

Tiwary, R.K., 2001. Environmental impact of coal mining on water regime and its management. Water Air Soil Pollut. 132, 185–199.

Tudorache, C., Viaene, P., Blust, R., Vereecken, H., De Boeck, G., 2008. A comparison of swimming capacity and energy use in seven European freshwater fish species. Ecol. Freshw. Fish 17, 284–291.

Vamosi, S.M., Schluter, D., 2002. Impacts of trout predation on fitness of sympatric sticklebacks and their hybrids. Proc. R. Soc. Lond. B 269, 923–930.

Weis, J.S., Candelmo, A., 2012. Pollutants and fish predator/prey behavior: a review of laboratory and field approaches. Curr. Zool. 58, 9–20.

Weis, J.S., Smith, G.M., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. BioScience 51, 209–217.

Weiss, E.B., 1990. Our rights and obligations to future generations for the environment. Am. J. Int'l L. 84, 198–207.

Zhou, T., John-Alder, H.B., Weis, P., Weis, J.S., 1999. Thyroidal status of mummichogs (*Fundulus heteroclitus*) from a polluted versus a reference habitat. Environ. Toxicol. Chem. 18, 2817–2823.

5.10 Appendix

Table 5-2: Mean (± SD) survival, pH, dissolved oxygen (DO), conductivity (EC), salinity and total dissolved solids (TDS) measured through the exposure.

Parameter/Analyte	**LOR**[a]	**CMW1**		**CMW2**	
Temperature (°C)	-	15.6		16.6	
Specific conductivity (mS cm^{-1})	-	7.15		8.04	
Conductivity (mS cm^{-1})	-	5.86		6.75	
Total dissolved solids (g L^{-1})	-	4.65		5.22	
Salinity (ppt)	-	3.95		4.48	
pH	-	9.51		8.98	
Orp (mV)	-	88.6		87.3	
Turbidity (NTU)	-	261		9.0	
Suspended solids (mg L^{-1})	5	216		8	
Total hardness as $CaCO_3$ (mg L^{-1})	1	314		534	
Sulfate as SO_4 (g L^{-1})	1	0.91		0.93	
Fluoride (mg L^{-1})	0.1	0.7		0.4	
Ammonia as N (mg L^{-1})	0.01	0.11		0.02	
Nitrite as N (mg L^{-1})	0.01	0.03		<0.01	
Nitrate as N (mg L^{-1})	0.01	<0.01		<0.01	
Nitrite + Nitrate as N (mg L^{-1})	0.01	0.03		<0.01	
Total Kjeldahl Nitrogen as N (mg L^{-1})	0.1	10		0.9	
Total Nitrogen as N (mg L^{-1})	0.1	10		0.9	
Total Phosphorus as P (mg L^{-1})	0.01	1.57		0.06	
Dissolved organic carbon (mg L^{-1})	1	20		6	
Total organic carbon (mg L^{-1})	1	24		4	
Alkalinity (mg L^{-1})					
Hydroxide Alkalinity as $CaCO_3$	1	<1		<1	
Carbonate Alkalinity as $CaCO_3$	1	303		196	
Bicarbonate Alkalinity as $CaCO_3$	1	181		388	
Total Alkalinity as $CaCO_3$	1	484		584	
Dissolved (Dis) and Total (Tot) Metals (µg L^{-1})		**Dis**	**Tot**	**Dis**	**Tot**
Aluminium	10	<10	4130	<10	140
Arsenic	1	2	17	2	3
Cadmium	0.1	<0.1	<0.1	<0.1	<0.1
Chromium	1	<1	5	<1	<1
Copper	1	3	11	4	5
Cobalt	1	<1	4	1	<1
Nickel	1	1	8	3	2
Lead	1	<1	2	<1	<1

Parameter/Analyte	LOR[a]	CMW1		CMW2	
Zinc	5	<5	16	<5	<5
Manganese	1	<1	85	2	7
Selenium	10	<10	<10	<10	<10
Silver	1	<1	<1	<1	<1
Uranium	1	3	6	6	3
Vanadium	10	<10	40	30	<10
Boron	50	470	410	410	430
Iron	50	<50	4430	<50	160
Mercury	0.1	<0.1	<0.1	<0.1	<0.1
Total Petroleum Hydrocarbons (µg L^{-1})					
C6 - C9	20	<20		<20	
C10 - C14	50	<50		<50	
C15 - C28	100	1170		<100	
C29 - C36	50	80		<50	
C10 - C36 (sum)	50	1250		<50	
Total Recoverable Hydrocarbons (µg L^{-1})					
>C10 - C16	100	<100		<100	
>C16 - C34	100	1220		<100	
>C34 - C40	100	<100		<100	
>C10 - C40 (sum)	100	1220		<100	
>C10 - C16 minus Naphthalene (F2)	100	<100		<100	
TPH(V)/BTEX Surrogates (%)					
1.2-Dichloroethane-D4	0.1	98.6		100	
Toluene-D8	0.1	95.2		100	
4-Bromofluorobenzene	0.1	98.2		90	
BTEXN					
Benzene	1	<1		<1	
Toluene	2	<2		<2	
Ethylbenzene	2	<2		<2	
meta- and para-Xylene	2	<2		<2	
ortho-Xylene	2	<2		<2	
Total Xylenes	2	<2		<2	
Sum of BTEX	1	<1		<1	
Naphthalene	5	<5		<5	

a. LOR, Limit of reporting.

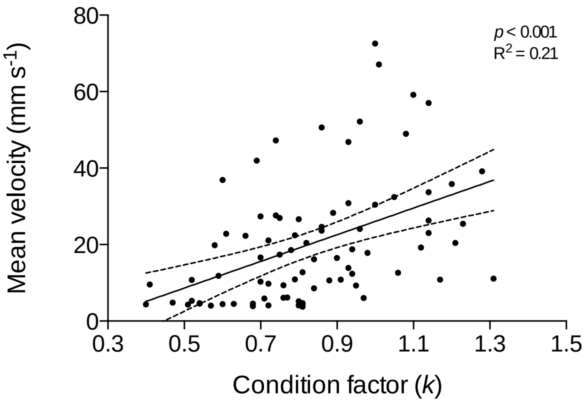

Figure 5-3: Correlation between condition factor and mean swimming velocity in *Hypseleotris compressa* exposed to 25, 50 and 100% CMW1 and CMW2 and control after 14 d (± 95% confidence band).

Declaration of co-authorship and contribution

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Lanctôt, C., Bennett, W., Wilson, S. P., Fabbro, L., Leusch, F. D. L., Melvin, S. D. (2016c). Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater. *Aquatic Toxicology* **173**, 218–227. doi:10.1016/j.aquatox.2016.01.014

My contribution to the paper involved:

- Initial concept and experimental design.
- Collection and analysis of data (except metal quantification by ICP-MS).
- Preparation of manuscript.

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Publication of Research Higher Degree Work for Inclusion in the Thesis Procedures

[redacted]

Chantal Lanctôt

Chapter 6

Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater

Chantal M. Lanctôt [a, b, *], William Bennett [c], Scott P. Wilson [a], Larelle Fabbro [d], Frederic D. L. Leusch [b], Steven D. Melvin [a, b]

[a] Central Queensland University, School of Medical and Applied Sciences, Gladstone, QLD 4680, Australia

[b] Smart Water Research Centre and Australian Rivers Institute, Griffith School of Environment, Griffith University, Southport, QLD 4215, Australia

[c] Environmental Futures Research Institute and Griffith School of Environment, Griffith University, Southport, QLD 4222, Australia

[c] Central Queensland University, School of Medical and Applied Sciences, Rockhampton, QLD 4702, Australia

* Corresponding author
e: chantal.lanctot@gmail.com, c.lanctot@cqu.edu.au
Mailing address:
Chantal Lanctôt
Smart Water Research Centre (G51)
Griffith University, Gold Coast Campus
Southport, Queensland, 4215
Australia

6.1 Abstract

Coal mining generates large quantities of complex effluent, and this often contains high levels of dissolved solids, suspended solids, metals, hydrocarbons, salts and other compounds. Substantial volumes of mine wastewater are periodically discharged into the environment, through both planned and accidental releases, and this raises concerns about the potential for adverse impacts on aquatic wildlife. There have been few attempts to explore sub-lethal effects of coal mine wastewater on amphibians compared to other organisms, and this is particularly true for Australian species. To address existing knowledge gaps, we exposed striped marsh frog (*Limnodynastes peronii*) tadpoles to 25, 50 and 100% coal mine wastewater collected from two holding dams (CMW1 and CMW2) located at an open cut mine in Central Queensland, Australia. The exposure lasted for four weeks, after which survival, growth and development, swimming behaviour, and concentrations of metals and metalloids in tail and liver tissues were assessed. Physico-chemical parameters varied considerably between sites, with higher turbidity, nutrients, total and dissolved organic carbon, alkalinity and arsenic (As) concentrations at CMW1, and higher conductivity, salinity, dissolved solids, hardness and sulfate levels at CMW2. There was no mortality in controls and less than 5% mortality in CMW1 treatments, whereas survival was significantly decreased in tadpoles exposed to CMW2 with 40 and 55% mortality in the 50 and 100% treatments, respectively. Development was significantly delayed in 100% CMW1 wastewater, but tadpole size (growth) was not influenced by the exposure. Hepatosomatic indices were significantly increased in tadpoles exposed to 25 and 50% CMW1 but not the 100% treatment group. Exposed tadpoles (predominantly those exposed to CMW1) exhibited increased activity after very short-term exposure (24 h), but this did not persist as animals approached metamorphic climax. At the end of the experiment, tadpoles exposed to both wastewaters had elevated levels of selenium (Se), cobalt (Co) and As in tail and liver tissue compared to controls ($p \leq 0.001$). Manganese (Mn) levels were also elevated in livers and tails of CMW2 exposed tadpoles. Hepatic tissue accumulated 8–9 times higher concentrations of Co, Mn and Se compared to tail tissue, irrespective of treatments. Future research is warranted to explore possible relationships between metal bioaccumulation, morpho-physiological effects during development, and subsequent higher-level outcomes related to individual performance and population fitness.

6.2 Keywords

Coal mine wastewater; Video-tracking; Swimming velocity; Behaviour; Metal bioconcentration; Amphibian

6.3 Introduction

Wastewater generated by coal mining activities contain complex mixtures of assorted contaminants (*e.g.* salts, metals, hydrocarbons), raising concerns about possible threats to aquatic biota that may result from surface or ground water contamination (Tiwary 2001; Thiruvenkatachari et al., 2011). Australia represents one of the world's top coal producers, and the Bowen Basin in Central Queensland holds the country's largest black coal deposit with over 50 active mines in a 75,000 km^2 area (DNRM, 2013). Due to the extremely variable sub-tropical to tropical Australian environment, which is dominated by climatic extremes including periods of flood and drought, regulatory discharge guidelines limit the release of coal mine wastewater to periods of natural flow which generally coincides with high rainfall (DERM, 2009). However, extreme rainfall events and flooding may result in uncontrolled discharge of large volumes of mine affected wastewater into the environment. Aquatic animals may also choose to exploit wetland habitat created by wastewater holding dams, presenting an additional possible exposure scenario. Despite the intense coal mining activities in Australia, and the various potential exposure pathways that exist, efforts to understand chronic sub-lethal effects of mining discharge on native Australian organisms have been limited (Hart et al., 2008). Exploring consequences of exposure in sensitive aquatic organisms is an important step in maintaining the health and integrity of our freshwater resources.

Amphibians are now considered to be the most threatened organisms on the planet, with close to half of all known species facing extinction (Monastersky, 2014; Alroy, 2015). Larval amphibians are highly sensitive to the presence of anthropogenic pollution in the aquatic environment, due to their unique physiology and feeding habits, which introduce both diet and absorption through their highly permeable and vascularised skin as potential modes of exposure (Sparling and Lowe, 1996). Many trace elements are known to bioaccumulate in amphibians, and in some cases these can cause severe physiological disruption. For example, metal and metalloid uptake has been linked to oxidative stress, immunosuppression, histological damage, DNA damage, endocrine disruption as well as mortality in amphibians (Levesque et al., 2003; Unrine et al., 2007; Davey et al., 2008; Zocche et al., 2013). From another perspective, uptake of toxic elements in amphibian larvae may present an additional ecological concern since the process of metamorphosis can present a source of contamination transference between aquatic and terrestrial habitats (reviewed by Rowe, 2014). Exposure of amphibian larvae to coal mine wastewater is therefore of interest from multiple perspectives, highlighting the need to address existing knowledge gaps in this area.

Metal pollution resulting from anthropogenic activities, including coal mining, can have damaging impacts on aquatic ecosystems, due to the persistence, toxicity and bioaccumulation potential of metals in the environment. Bioaccumulation is therefore frequently used as an indicator of metal exposure in polluted systems (Luoma et al., 2010; Adams et al., 2011), with many studies investigating metal toxicity in fish and invertebrate species and far fewer examining effects on amphibians (reviewed by Atchison et al., 1987; Rainbow, 2007; Wang, 2013; DeForest and Meyer, 2015). Furthermore, the main body of research evaluating the impacts of coal related industries (*i.e.* mining, processing, combustion) on larval amphibians has focused on coal combustion waste from a single location in South-Carolina, USA (reviewed by Rowe et al., 2001, 2002; Rowe, 2014). The few other available studies have primarily explored ecological aspects such as altered community structures (*e.g.* species richness, abundance) of anurans and salamanders at sites impacted by abandoned and reclaimed coal mines (Ireland et al., 1994; Wood and Williams, 2013a,b; Schorr et al., 2013; Petty et al., 2013; Muncy et al., 2014), but not direct organism-level effects in exposed animals. The paucity of research examining lethal and sub-lethal impacts of coal mining on amphibians is particularly relevant for Australian species, because many inhabit and breed in geographical regions characterized by intensive mining operations.

Behavioural alterations have been emerging as important sub-lethal endpoints in toxicity studies because they are rapid and appear sensitive to a broad range of contaminants (Melvin and Wilson, 2013), and since they often have implications for higher-level biological processes (Groh et al., 2015). For example, many aquatic organisms have been shown to display behavioural responses to toxicants at low concentrations, and such responses can be predictive of potential population-level outcomes including altered survival and fitness (Atchison et al., 1987; Pestana et al., 2007; Mohti et al., 2012). Reduced feeding behaviour, inability to effectively escape predation, and altered mating and reproductive behaviours are all examples of toxicity-induced behavioural alterations with broad ecological relevance (Gerhardt et al., 2005, 2006; Pestana et al., 2007; Macedo-Sousa et al., 2008; Alonso et al., 2009). Discharge from several tin and cupriferous pyrite mines have been shown to influence behavioural responses of macroinvertebrates and fish (Janssens de Bisthoven et al., 2004; Gerhardt et al., 2005; Macedo-Sousa et al., 2008; Mohti et al., 2012), but little is known about the implications of exposure to coal mining discharge for influencing behavioural patterns in aquatic animals (Butler et al., 1973; Scullion and Edwards, 1979; Lanctôt et al., 2015; Lanctôt et al., 2016a,b). Based on our review of the literature, there have apparently been no prior studies exploring subtle behavioural responses in amphibians exposed to coal mine wastewater.

The present study describes the sub-lethal impacts of coal mine wastewater from a Queensland mine, on tadpoles of an Australian frog species (*Limnodynastes peronii*). This species was chosen because of its widespread distribution across eastern Australia and its relevance to coal mining regions of Central Queensland. The objectives of the study were to assess survival, growth, development and behavioural effects in larval *L. peronii* after chronic exposure to coal mine wastewater, and to further investigate associations between toxicological outcomes and metal(loid) bio-concentration in exposed individuals.

6.4 Materials and methods

6.4.1 Coal mine wastewater sampling

Wastewater was collected at an open-cut coal mine located in Central Queensland, Australia, on January 23rd and February 10th, 2015. At the time of experimentation, the mine had two active release points authorised for discharge of mine-affected water; CMW1 dam and CMW2 dam. Wastewater was collected for experiments in 20L plastic containers (acid washed). Collected water was transported on ice, stored in the refrigerator at 4°C, and was brought to ambient temperature prior to experimentation (~24 h post collection).

6.4.2 Water quality and chemistry

In situ measurements of temperature, conductivity (EC), pH, dissolved oxygen (DO), salinity, total dissolved solid (TDS) and turbidity were taken at the time of sampling using a YSI multi-parameter handheld sonde (Xylem Analytics, Hemnant, Australia). Water samples were collected twice in situ at the time of wastewater sampling, and from each treatment dilution in the laboratory at the start, middle and end of the experiment for analysis of a suite of parameters (listed in Appendix Table 6-4). Samples were refrigerated at 4°C prior to analysis by the ALS Laboratory Group (Brisbane, Australia). Basic water quality parameters (temperature, EC, pH, DO, salinity and TDS) were measured before and after each water renewal using YSI EcoSence® probes. Nitrogenous waste was monitored through the experiment using an ammonia test kit (Aquasonic Pty Ltd, Australia).

6.4.3 Animals

A single fertilized *L. peronii* egg mass was collected locally and hatched in the laboratory in natural pond water. After hatching, water levels were slowly increased and replaced with moderately hard reconstituted water made according to standard test guidelines (US EPA, 2002). Tadpoles were fed a combination of finely crushed dehydrated kale and Sera Micron® commercial powder (1:1) ad libitum twice daily. Laboratory conditions were maintained similar to ambient conditions for the time of year (*i.e.* 27°C and 12:12 light:dark sequence). All aspects of experimentation and sampling were approved by the Animal Ethics Committee of Central Queensland University, in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (CQUniversity Approval No. A13/05-301).

6.4.4 Experimental procedure

Upon reaching Gosner developmental stage 25 (Gs; Gosner, 1960), 140 tadpoles were randomly allocated to thirty-five glass aquaria divided into seven treatment groups (5 replicates per treatment) and containing 4 L of aerated control or treatment water (25, 50, 100% CMW1 and CWM2). Each replicate contained 4 tadpoles. Moderately hard reconstituted water (US EPA, 2002) was used as diluent and control. Tadpoles were exposed for 4 weeks and water was completely renewed twice weekly. Water was gently aerated throughout the exposure. Animals were fed equal portions twice daily as described above (approximately 10% of the average body mass per day). Food rations were increased throughout the experiment and adjusted based on the density of tadpoles in each replicate. Faeces and excess food waste were removed daily.

6.4.4.1 Survival, growth, development and sex determination

Tadpole health and survival was monitored daily throughout the exposure period. At the start of the experiment, all tadpoles were staged using the Gosner staging system (Gs; Gosner, 1960), weighed and photographed on a 10 mm × 10 mm grid for measurement (snout-vent length [SVL] and tail length) using ImageJ image analysis software (National Institutes of Health, USA). After 15 days, all tadpoles were again staged and photographed for length measurements. At the end of the four-week exposure, animals were euthanized by immersion in buffered 3-aminobenzoic acid ethyl ester (MS222; Sigma–Aldrich) dissolved in water. Subsequently, tadpoles were staged and measurements of SVL (mm), tail length (mm) and weight (mg) were taken. Animals were sexed based on visual inspection of gross gonadal morphology, and livers and tails were weighed and stored at −20°C until further analysis. Morphometric measurements were used to calculate condition factor ($K = [\text{weight}/\text{SVL}^3] \times 100$) and hepatosomatic index ($HSI = [\text{liver weight}/\text{body weight}] \times 100$) for each tadpole.

6.4.4.2 Behavioural monitoring using EthoVision® software

Swimming activity and movement patterns of one randomly selected tadpole per replicate (n = 35) were recorded simultaneously on day 1, 4, 7, 14 and 21, and all tadpoles from each replicate were recorded one day prior to the termination of the experiment (day 27). Tadpoles were randomly selected and placed individually in glass petri dishes (D14.5 cm × H1.9 cm) containing 200 mL of the appropriate treatment water. After a 30-min acclimation period, tadpoles were filmed continuously for 30 min using a GoPro Hero4 camera (GoPro Inc., USA). No personnel were present in the laboratory room during video recording. After filming, each tadpole was returned to its respective exposure aquaria. EthoVision XT 9.0 (Noldus Information Technology, Netherlands) video-tracking software was used to analyse recorded swimming activity and movement. The software analyses each frame in order to distinguish the tracked subject from the background and converts movement patterns into x, y coordinate data for the centre point of each. Data are subsequently used for automatic calculation of the relevant parameters (*e.g.* distance moved, velocity, space utilization). Recent studies have demonstrated the value of this technology for assessing contaminant impacts on larval amphibians (Denoël et al., 2013; Melvin, 2015).

6.4.4.3 Metal and metalloid concentration in hepatic and tail tissues

Whole liver and tail tissue samples were placed into TFM PTFE digestion vessels (EasyPrep Plus; CEM) with 4 mL Milli-Q water, 4 mL concentrated sub-boiling distilled nitric acid and 1 mL hydrogen peroxide (30%, w/w) and digested using a MARS 6 microwave reaction system (CEM Corporation) following the EPA3052 protocol (EPA, 1996). A minimum of one reagent blank was added to every set of twelve digestions (total = 14 blanks per tissue type). Digests (9 mL) were transferred to polypropylene tubes and diluted 1:20 with Milli-Q water.

Concentrations of aluminium (Al), antimony (Sb), arsenic (As), boron (B), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), uranium (U), vanadium (V) and zinc (Zn) were determined by ICP-MS (Agilent 7900 ICP-MS). Interference removal was achieved using the collision-reaction cell mode, with helium as the cell gas. Scandium (Sc), yttrium (Y), indium (In) and terbium (Tb) were added online to the sample flow as internal standards, and independent quality control standards were analysed frequently throughout the analytical run.

6.4.5 Statistical analysis

Mantel–Cox log rank test was used to test for differences in survival between treatments. Mantel-Cox pairwise comparisons were used to test for differences between each treatment and control, with the level of significance (α) adjusted for the number of comparisons. Survival analyses were performed using Graph Pad Prism® v6.0f. All other analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL). Normality and homogeneity of variance of the data were verified by visual inspection of the residual plots and Levene's test, respectively. Data were transformed (log10) to meet parametric assumptions when necessary, and non-parametric tests were used when assumptions were violated. Differences in water quality parameters measured in the laboratory were analysed using the non-parametric Kruskal–Wallis test followed by multiple comparisons. Repeated-measures ANOVA was used to analyse for interactive and main effects of treatments over time, for influencing tadpole developmental stage. The additional assumption of sphericity was therefore tested with Mauchley's test, and a Greenhouse–Geisser correction was applied since this assumption was violated. Tukey HSD post hoc comparisons were used to determine specific differences between treatment groups. Alterations in sex ratios between treatment and control groups, and relative to an equal proportion of males and females were analysed using the Chi-Square test. Differences in morphometric measurements (SVL, weight, condition factor, HSI) between treatments at the end of the exposure and behavioural data (mean velocity [mm/s], maximum velocity [mm/s], time spent in peripheral zone [min, 2 cm from border, representing 48% of the arena surface] and frequency of immobility [min]) at day 1 and 27 were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. The immobility threshold (*i.e.*, percent change in area below which the subject is considered to be immobile) was set to <20%. Changes in hepatic and tail concentrations of trace elements were analysed using non-parametric Kruskal–Wallis test followed by multiple comparisons. For all analysis, aquaria were used as the experimental unit (n = 5 per treatment) and the significance level was set at $\alpha \leq 0.05$ for all tests.

6.5 Results

6.5.1 Water quality and chemistry

Average pH, conductivity, salinity and TDS levels measured before and after water changes differed significantly between treatments (Table 6-1, Kruskal–Wallis, $p < 0.001$). Specifically, both CMW1 and CMW2 had higher pH, conductivity, salinity and TDS compared to control aquaria. CMW2 also had higher conductivity, salinity and TDS compared to CMW1. Average temperature and DO were 26.1 ± 0.7°C and 86.5 ± 8.9%, respectively, and these parameters did not differ amongst treatments throughout the experiment ($p > 0.05$, data not shown).

Table 6-1: Average (SD) pH, electrical conductivity (EC), salinity and total dissolved solids (TDS) in control and treatments (n = 17).

		CMW1			CMW2		
Parameter	**Control**	**25%**	**50%**	**100%**	**25%**	**50%**	**100%**
pH	7.6 (0.2)	8.5 (0.5)	8.7 (0.5)	8.8 (0.5)	8.1 (0.3)	8.3 (0.3)	8.4 (0.3)
EC (mS)	0.3 (0.02)	1.3 (0.1)	2.2 (0.2)	4.0 (0.5)	1.8 (0.4)	3.2 (0.9)	5.8 (1.5)
Salinity (ppt)	0.1 (0)	0.7 (0.1)	1.1 (0.1)	2.1 (0.3)	0.9 (0.2)	1.7 (0.5)	3.2 (0.9)
TDS (g/L)	0.20 (0.01)	0.84 (0.08)	1.46 (0.15)	2.62 (0.31)	1.19 (0.29)	2.11 (0.56)	3.79 (1.00)

Total and dissolved metals measured in each of the treatments are presented in Table 6-2. All other analytes measured in situ are presented in Table 6-4 (Appendix). Measured analytes were generally lower in control water compared to other treatments, except for dissolved Al, Cd and Mn levels, which were higher in control water. Analyte levels in the treatments were also generally lower with increasing dilution factor, with the exception of ammonia, total and dissolved organic carbon (TOC and DOC), dissolved Al, Cu and Mn, total Cu and C_{15}–C_{28} hydrocarbon fraction. Marked differences were observed between CMW1 and CMW2 waters. Specifically, CMW1 had higher turbidity, nutrients, TOC, DOC and total alkalinity, and had lower salinity, TDS, DO, hardness and sulfate levels compared to CMW2. Dissolved and total As and total Fe were higher in CMW1 compared to CMW2, whereas dissolved Ni and Zn and total Mn were lower in CMW1. Dissolved and total Co, U and B as well as total Ni and Zn were similar in both sites. Total petroleum hydrocarbons (fraction $C_{15–28}$ and $C_{29–36}$) and total recoverable hydrocarbons (fraction $C_{16–34}$) were higher in CMW1 than CMW2. All other fractions were below limits of reporting (LORs). BTEXN (benzene, toluene, ethylbenzene, xylenes, naphthalene) were below LORs in both dams.

Table 6-2: Dissolved and total metal and metalloids concentration (µg/L) in control and treatments (25, 50, 100% CMW1 and CMW2) measured after the first (1) and second (2) field collection.

		Control		**CMW1 25%**		**CMW1 50%**		**CMW1 100%**		**CMW2 25%**		**CMW2 50%**		**CMW2 100%**	
	LOR	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Dissolved concentration															
Al	10	<10	**30**	<10	<10	<10	<10	<10	<10	<10	<10	**10**	<10	<10	<10
As	1	<1	<1	**2**	**2**	**4**	**3**	**7**	**6**	<1	<1	**2**	<1	**4**	**2**
Cd	0.1	<0.1	**0.1**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Co	1	<1	<1	<1	<1	<1	<1	**1**	<1	<1	<1	<1	<1	**1**	<1
Cu	1	**1**	<1	**8**	<1	**2**	**1**	**2**	**2**	**2**	<1	**3**	<1	**5**	**2**
Fe	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Pb	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Mn	1	**2**	**3**	**1**	**2**	<1	<1	<1	<1	**1**	**2**	<1	<1	<1	<1
Se	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Zn	5	<5	<5	**6**	<5	<5	<5	<5	<5	<5	<5	**6**	<5	**7**	**10**
Cr	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ni	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	**2**
Ag	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
U	1	<1	<1	<1	<1	<1	<1	**2**	**1**	<1	<1	**1**	<1	**2**	**1**
V	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
B	50	<50	<50	**80**	**60**	**140**	**130**	**350**	**260**	**100**	<50	**170**	**100**	**340**	**190**
Hg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total concentration															
Al	10	<10	**40**	**70**	**140**	**70**	**300**	**260**	**740**	**40**	**120**	**90**	**380**	**280**	**290**
As	1	<1	<1	**2**	**2**	**4**	**4**	**9**	**8**	<1	<1	**2**	**1**	**4**	**2**
Cd	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Co	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Cu	1	**2**	<1	**12**	**1**	**3**	**3**	**3**	**5**	**2**	<1	**3**	**2**	**7**	**2**
Fe	50	<50	<50	**50**	**90**	**60**	**240**	**250**	**640**	<50	<50	**80**	**300**	**300**	**270**
Pb	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Mn	1	**3**	**3**	**10**	**7**	**14**	**15**	**31**	**32**	**6**	**31**	**9**	**72**	**18**	**124**
Se	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Zn	5	<5	<5	<5	<5	<5	<5	**21**	**16**	<5	<5	<5	**10**	<5	**21**
Cr	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ni	1	<1	<1	<1	<1	<1	**1**	**2**	**2**	<1	<1	**1**	**1**	**2**	**2**
Ag	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
U	1	<1	<1	<1	<1	<1	<1	**2**	**2**	<1	<1	**1**	<1	**2**	**1**
V	10	<10	<10	<10	<10	<10	<10	<10	**10**	<10	<10	<10	<10	<10	<10
B	50	<50	<50	**80**	**70**	**160**	**160**	**310**	**300**	**90**	**70**	**170**	**120**	**330**	**220**
Hg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Bold indicates values above limits of reporting (LORs).

6.5.2 Survival, growth and development, and sex ratios

Mantel–Cox log rank test revealed significant differences in survival between treatments (Figure 6-1a, $X^2 = 49.4$, $p < 0.0001$). No significant differences were observed between control and CMW1 exposed animals, which had ≥ 95% survival (Mantel–Cox log rank, $p > 0.05$). Contrarily, survival was reduced by 25 and 50% compared to control within one week of exposure in the 50% and 100% CMW2 treatments, and further decreased to 40 and 55% survival, respectively, after four weeks of exposure (Mantel–Cox log rank, $p < 0.0083$). Repeated-measures ANOVA identified a significant time × treatment interaction (Figure 6-1b; $F_{(12,54)} = 7.53$, $p < 0.001$) and a significant main effect of treatment (Figure 6-1b; $F_{(6,27)} = 7.52$, $p < 0.001$) on tadpole development. Post hoc analysis revealed that tadpole development was reduced by seven Gosner stages on average in animals exposed to 100% CMW1 wastewater compared to controls ($p < 0.001$). No significant differences in development were observed between control and the other treatment groups ($p > 0.05$).

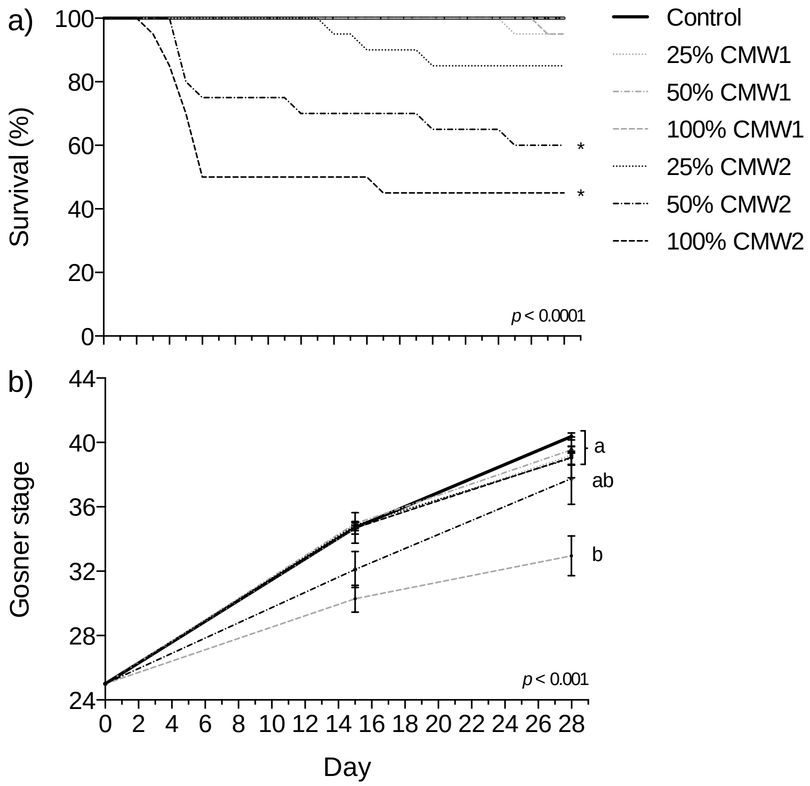

Figure 6-1: (a) Average survival (%) and (b) developmental Gosner stage (± SEM) of *L. peronii* tadpoles throughout 4-week exposure to coal mine wastewater from two dams (25, 50, 100% CMW1 and CMW2) and control. Asterisks indicate differences between treatment and control group. Letters indicate significant differences between all treatments. Data were analysed using (a) Mantel–Cox log rank test and (b) repeated-measures ANOVA followed by Tukey *post hoc* test, $p < 0.05$.

One-way ANOVA revealed significant differences in SVL ($F_{(6,27)} = 3.00$, $p = 0.022$), weight ($F_{(6,27)} = 2.55$, $p = 0.044$) and HSI ($F_{(6,27)} = 8.87$, $p < 0.001$) between treatments (Figure 6-2). Post-hoc comparisons indicated that the differences in SVL and weight did not reach statistical significance between control and CMW treatment groups ($p > 0.05$). Hepatosomatic index, on the other hand, exhibited a 1.2-fold increase in tadpoles exposed to the 25% and 50% CMW1 treatments relative to control animals (Tukey HSD post hoc test, $p < 0.05$). Although a similar (1.2-fold) increase in K was observed in tadpoles exposed to 100% CMW1 compared to controls, differences did not reach statistical significance (Figure 6-2c; $F_{(6,27)} = 2.31$, $p = 0.063$). Sex ratios in the control population were 1:1 and represent the expected male to female ratio. Although all treatments had slightly more females compared to control, differences were only significant in 50% CMW2 (Figure 6-3, $X^2 = 5.3$, $p = 0.021$). Undifferentiated tadpoles were also observed in animals exposed to 100% CMW1, corresponding to the delays in development.

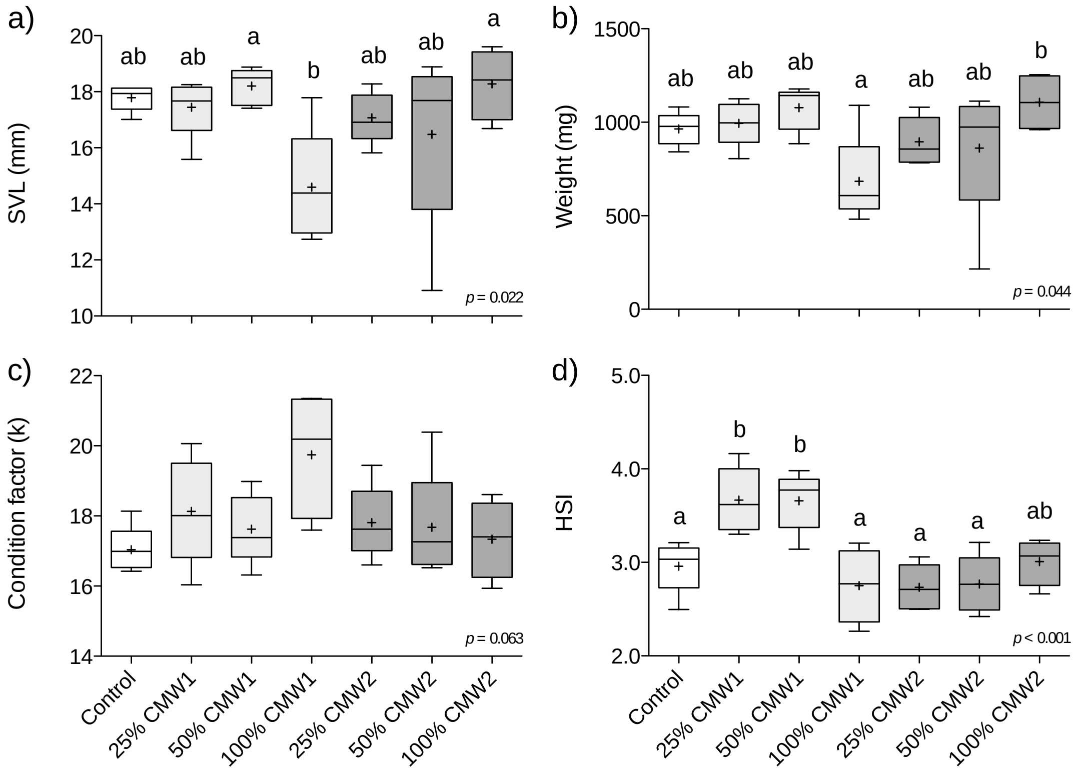

Figure 6-2: (a) Snout-vent length, (b) weight, (c) condition factor, and (d) hepatosomatic index of *L. peronii* tadpoles after 4-week exposure to coal mine wastewater from two dams (25, 50, 100% CMW1 and CMW2) and control. Boxplots show the interquartile range (column), median (horizontal line), minimum and maximum values (whiskers) and average (+) of 5 replicates. Letters indicate significant differences between all treatments. Data were analysed using one-way ANOVA followed by Tukey's *post hoc* test, $p < 0.05$.

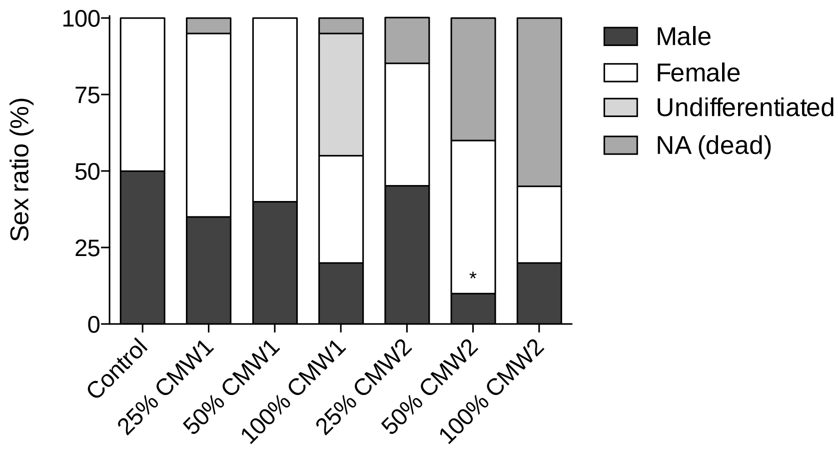

Figure 6-3: Sex ratio (%) of *L. peronii* tadpoles after 4-week exposure to coal mine wastewater from two dams (25, 50, 100% CMW1 and CMW2) and control. Bars represent the average of 5 replicates. Asterisks indicate significant differences from control population ratio (1:1).

6.5.3 Behavioural responses to coal mine wastewater

A significant treatment effect was observed after only one day of exposure for mean velocity (Figure 6-4a; $F_{(6,28)} = 3.36$, $p = 0.013$), maximum velocity (Figure 6-4b; $F_{(6,28)} = 4.50$, $p = 0.003$) and time spent in the peripheral zone (Figure 6-4c; $F_{(6,28)} = 4.04$, $p = 0.005$). Specifically, we observed significantly increased average swimming velocity in animals exposed to 25 and 50% CMW1 and 50% CMW2, and a significant increase in maximum velocity in all CMW1 treatments relative to controls. Time spent in the peripheral zone was significantly reduced in 100% CMW1 relative to all other treatments. Immobility was not significantly affected by treatments (Figure 6-4d; $F_{(6,28)} = 1.60$, $p = 0.183$). After 27 days of exposure, significant differences in maximum velocity ($F_{(6,27)} = 3.84$, $p = 0.007$) persisted, whereas no differences were observed for mean velocity ($F_{(6,27)} = 0.93$, $p = 0.487$), time spent in the peripheral zone ($F_{(6,27)} = 1.47$, $p = 0.227$) or immobility frequency ($F_{(6,27)} = 0.83$, $p = 0.557$). Tukey *post hoc* analysis for maximum velocity did not however distinguish differences between specific treatment groups at this time ($p > 0.05$).

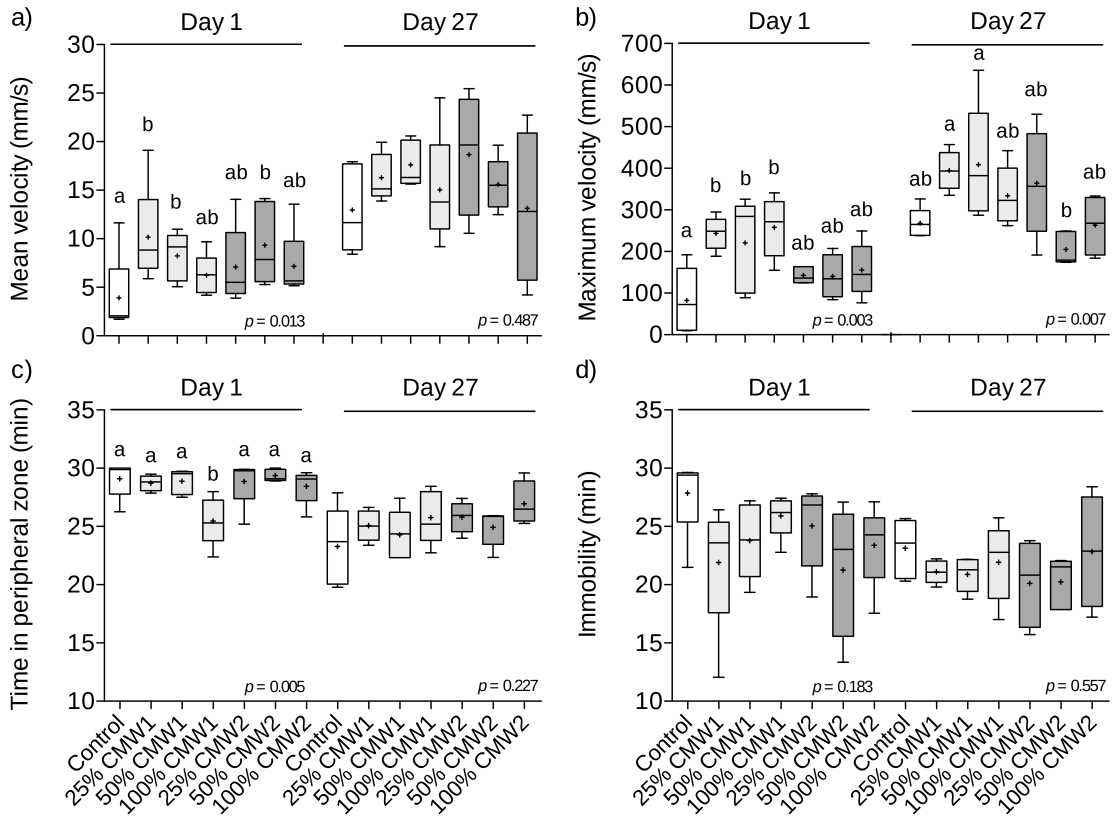

Figure 6-4: (a) Mean velocity (mm/s), (b) maximum velocity (mm/s), (c) time spent in peripheral zone (min) and (d) immobility frequency (min) of *L. peronii* tadpoles filmed for 30 min after 1 and 27 days of exposure to 25, 50 and 100% CMW1 and CMW2 and control. Boxplots show the interquartile range (column), median (horizontal line), minimum and maximum values (whiskers) and average (+) of 5 replicates per treatment (day 1 n = 1 tadpole/replicate, total = 5 tadpole/treatment; day 27 n = 0–4 tadpoles/replicate, total = 9–20 tadpoles/treatment). Letters indicate significant differences between treatments on each day. Data were analysed using one-way ANOVA followed by Tukey's *post hoc* test, $p < 0.05$.

6.5.4 Bioconcentration of metals and metalloids in hepatic and tail tissues

Concentrations of Al, As, Cd, Co, Cu, Fe, Pb, Mn, Se, Zn measured in liver and tail tissues are presented in Table 6-3. Non-parametric Kruskal–Wallis test revealed significant differences in As, Co, Mn, Se and Zn in livers and tails, as well as differences in Fe and Pb in livers between treatments ($p < 0.05$, see Table 6-3 for analysis details). Tadpoles exposed to 100% CMW1 had 4–9 times higher levels of As, Co, Se, levels in their livers as well as 2–8 times higher levels of As, Co, Se, Zn in the tail compared to control, respectively. Levels of Se in the tail and liver, and As in the tail, were also elevated in the 25 and 50% CMW1 treatment dilutions. Tadpoles exposed to 25 and 50% CMW2, on the other hand, had 4–5 times higher levels of Mn compared to the controls ($p < 0.05$) but the increase was not statistically different from control in the 100% CMW2 treatment ($p > 0.05$). Concentrations of Co in livers and tails were also on average 4 and 9 times higher, respectively, in the 50 and 100% CMW2 treatments compared to controls ($p > 0.05$). Levels of Fe and Pb in livers of exposed tadpoles did not differ significantly from controls ($p > 0.05$). Irrespective of treatments, liver tissue generally had 2–311 times higher levels of Cd, Co, Cu, Fe, Mn, Se and Zn compared to tail tissue. Levels of Sb, B, Cr, Mo, Ni, U, V were below the limit of detection in both tissues. Bioconcentration factors (BCF) are presented in Table 6-5 (Appendix). BCFs were generally greater in livers compared to tails, with the exception of As in treated animals. Significant treatment effects were detected for all metals, except Al and As in the liver ($p > 0.05$).

6.6 Discussion

6.6.1 Survival, growth and development, and sex ratios

Tadpoles of the native Australian frog *L. peronii* were exposed to coal mine wastewater collected from two holding dams (CMW1 and CMW2) located at an open cut mine in Central Queensland's Bowen Basin throughout the larval developmental period. Survival was 100% in control animals and ≥ 95% for all CMW1 treatments, but was reduced after only 5 days in tadpoles exposed to 50 and 100% CMW2. Additional mortality occurred in these treatment groups throughout the duration of the experiment, such that 60 and 45% of tadpoles survived in 50 and 100% CMW2, respectively, after 4 weeks of exposure. Contrary to survival, morphological effects were only observed in the 100% CMW1 treatment group, characterised by significantly delayed development in these animals relative to the controls. Delayed tadpoles consequently had slightly smaller body size (SVL and weight) and increased condition factor compared to the other treatments, however treatments did not differ statistically from controls for these endpoints. The lack of a significant growth effect may relate to marginally increased heterogeneity in tadpole size in the delayed group. Our results may indicate that exposure to 100% CMW1 resulted in either direct or indirect endocrine disrupting effect on the thyroid axis, since tadpole development is directly mediated by thyroid hormones (reviewed by Brown and Cai, 2007). While no studies to our knowledge have specifically investigated the potential endocrine disrupting effects of coal mine wastewater, several studies have examined the impacts of its key constituents such as metals and metalloids (reviewed by Iavicoli et al., 2009). In one example, Davey et al. (2008) demonstrated that As (>1 M) suppresses RAR- and TR-mediated gene expression and decreases T_3-depedent tail regression in *Xenopus laevis* tadpoles, and that this occurs in a dose dependent manner. Although the specific mechanism of action underlying the effects of As or other metals remains somewhat unclear, results of this prior study are consistent with the developmental effects observed in our 100% CMW1 treatment dilution, which contained the highest dissolved As concentrations (6–7 μg/L, ~0.1 M).

Table 6-3: Metal and metalloids concentration (μg/g wet weight) in liver and tail tissues of tadpoles exposed to control and to coal mine wastewater from two dams (25, 50, 100% CMW1 and CMW2). Letters indicate significant differences between treatments.

	Control		**CMW1 25%**		**CMW1 50%**		**CMW1 100%**		**CMW2 25%**		**CMW2 50%**		**CMW2 100%**		***p*-value**	**Test statistic (6,34)**
	Average	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**		
Liver																
Al	3.31	6.34	0.15	0.25	0.70	0.60	2.14	1.65	0.36	0.54	0.78	1.29	0.21	0.42	0.075	11.5
As	0.03^{a}	0.06	0.02^{a}	0.01	0.05^{a}	0.02	0.11^{b}	0.05	0.00^{ab}	0.01	0.01^{ab}	0.01	0.02^{ab}	0.00	0.001	22.2
Cd	0.02	0.04	BDL		0.01	0.00	BDL		0.01	0.01	0.02	0.01	BDL		0.078	11.4
Co	0.05^{a}	0.01	0.07^{ab}	0.01	0.08^{abc}	0.01	0.20^{c}	0.05	0.12^{abc}	0.01	0.21^{bc}	0.14	0.20^{c}	0.05	<0.001	28.6
Cu	62.2	12.6	44.0	17.6	51.6	8.99	71.2	16.8	62.3	23.3	84.8	72.4	83.1	24.1	0.117	10.2
Fe	73.5^{ab}	24.0	55.7^{a}	22.8	61.1^{ab}	10.4	111.1^{b}	13.4	57.3^{a}	21.1	78.4^{ab}	28.3	67.5^{ab}	5.85	0.025	14.5
Pb	0.08	0.17	BDL		BDL		BDL		BDL		BDL		BDL		-	-
Mn	1.33^{a}	1.23	1.61^{ab}	0.61	2.17^{ab}	1.87	1.14^{ab}	0.33	4.70^{ab}	2.43	5.47^{b}	3.53	2.78^{ab}	1.75	0.015	15.8
Se	0.52^{a}	0.15	2.92^{abc}	0.26	3.78^{bc}	0.46	4.60^{c}	0.63	1.03^{ab}	0.34	1.28^{ab}	0.67	3.25^{abc}	1.44	<0.001	28.7
Zn	17.6^{a}	1.57	16.0^{ab}	6.36	13.1^{b}	1.10	18.0^{ab}	2.28	15.9^{ab}	1.72	19.3^{ab}	8.76	15.8^{ab}	1.28	0.025	15.4
Tail																
Al	BDL		BDL		BDL		BDL		BDL		BDL		BDL		-	-
As	0.01^{a}	0.00	0.02^{ab}	0.01	0.03^{b}	0.01	0.03^{b}	0.01	0.01^{ab}	0.00	0.02^{ab}	0.01	0.03^{ab}	0.01	0.001	23.6
Cd	BDL		BDL		BDL		BDL		BDL		BDL		BDL		-	-
Co	0.00^{a}	0.00	0.01^{ab}	0.00	0.01^{ab}	0.00	0.02^{b}	0.00	0.02^{b}	0.00	0.03^{b}	0.02	0.03^{b}	0.01	<0.001	25.2
Cu	0.20	0.03	0.19	0.02	0.25	0.14	0.25	0.22	0.36	0.29	0.46	0.46	0.27	0.17	0.493	5.4
Fe	2.29	0.30	3.05	1.14	2.48	0.30	2.77	0.71	2.48	0.45	2.89	1.52	2.46	0.54	0.872	2.5
Pb	BDL		BDL		0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.648	4.2
Mn	0.13^{a}	0.07	0.19^{ab}	0.03	0.19^{ab}	0.07	0.17^{ab}	0.03	0.49^{b}	0.21	0.69^{b}	0.55	0.38^{ab}	0.22	0.004	19.0
Se	0.05^{a}	0.02	0.34^{b}	0.02	0.41^{c}	0.05	0.37^{b}	0.08	0.16^{abc}	0.02	0.18^{abc}	0.03	0.34^{abc}	0.11	<0.001	27.9
Zn	4.33^{a}	0.47	4.83^{ab}	1.01	4.81^{ab}	0.40	8.34^{b}	0.82	7.29^{ab}	5.69	7.62^{ab}	3.84	5.33^{ab}	1.09	0.007	17.8

BDL–below detection limit.

Hepatosomatic index (HSI) was significantly increased in tadpoles exposed to 25 and 50% CMW1 dilutions, but not 100% wastewater. The lack of effect in the highest dilution could be an artefact of the decreased developmental rates that were observed in tadpoles in the 100% CMW1 treatment group. However, there is no reason to expect a major shift in the relative size of the liver, and thus HSI would be expected to remain relatively consistent between less and more developed individuals. This type of U-shaped response pattern is, however, consistent with the phenomenon hormesis (reviewed by Calabrese, 2008), and could thus simply reflect a change in responsiveness at increasing concentrations due to differences in the toxicity of the wastewater. A possible explanation for the observed response pattern could therefore be that, at higher dilutions (lower concentrations) detoxification processes lead to compensatory hyperplasia of hepatic tissue, whereas pure raw wastewater (100% CMW1) overloads these processes and leads to liver damage. This is somewhat speculative and requires further investigation, but differences in response patterns between the various treatments are certainly an interesting outcome. It is also noteworthy that no effects on HSI were observed in animals exposed to CMW2 at any concentrations. Site differences could relate to differences in water chemistry (discussed in Section 6.6.4), or perhaps be a reflection of the increased mortality observed in animals exposed to high CMW2 treatments.

All treatment groups tended to exhibit slight female biases compared to the 1:1 ratio that was observed in control animals (and expected in unexposed populations). However, differences were only significant in the 50% CMW2 treatment, in which less than one third of the 60% surviving tadpoles were males. Considering that 40% of the total tadpole population in this group died and that 50% of the survivors were females we can speculate that either 1) the exposure had a feminizing effect, or 2) mortality was higher in males. Several studies have demonstrated that male tadpoles develop slower than females in laboratory and mesocosm experiments (Dare and Forbes, 2008; Navarro-Martín et al., 2014; Lanctôt et al., 2014), and this could result in males being more sensitive than females during the developmental period. It is also possible that the wastewater contained unidentified endocrine disrupting compounds, and that these had a feminizing effect on the tadpoles. Previous studies have demonstrated that various metals and metalloids can cause estrogenic effects (Garcia-Morales et al., 1994; Jana et al., 2006) and impair fish reproduction (Levesque et al., 2003; Alquezar et al., 2006; Boyle et al., 2008). Trace elements present in our wastewater could therefore be one of many possible explanations for the increased proportion of females in our treatment groups, but the possibility that other organic and inorganic compounds influenced the observed sex ratios cannot be excluded at this time. More research is necessary to form better conclusions about potential compounds responsible for the observed effects on sex ratios.

6.6.2 Tadpole behaviour and swimming performance

Behavioural alterations have previously been shown to be sensitive indicators of sub-lethal metal exposure in fish (reviewed by Atchison et al., 1987), however few studies have focused on amphibian responses to coal-related contaminants, including salinity, metals and mixtures (Jung and Jagoe, 1995; Raimondo et al., 1998; Lefcort et al., 1998; Hopkins et al., 2000; Chen et al., 2009; Wood and Welch, 2015). In the present study, swimming performance was significantly influenced in exposed tadpoles (predominantly those exposed to CMW1). The observed responses included a rapid increase in maximum velocity following 24 h exposure to all CMW1 treatments, and significantly less time spent in the peripheral zone in animals exposed to the highest dilution of CMW1 compared to controls and the other treatment groups. Although similar trends in maximum velocity were apparent in the data, significant differences did not persist on day 27 of the exposure, and this may be due to increased population heterogeneity as animals developed. As expected, regression analysis identified a positive relationship between tadpole size and swimming performance (*e.g.* mean velocity *vs* total length $R^2 = 0.36$, $p = 0.0053$), and this is consistent with previous findings in the literature (Huey, 1980; Jung and Jagoe, 1995). Thus, while early stage tadpoles provided a good measure of responses related to the exposure, this appears to have been confounded by size and stage variances within experimental replicates as the experiment progressed (Smith and Van Buskirk, 1995). Despite effects on swimming and activity not persisting as the animals developed, exposed tadpoles exhibited an extremely rapid behavioural response to coal mine wastewater. This may indicate a potential for using behavioural analysis for ecological monitoring of water quality in mine affected regions, but the practicality of doing so in the field is somewhat questionable. Furthermore, although hyperactivity was observed after a single day of exposure to CMW1 this was not useful for predicting subsequent mortality, which was only observed in the highest CMW2 treatments (50 and 100%). Nevertheless, responses observed in early stage tadpoles may still foreshadow possible physiological effects, since increased activity and burst swimming may be linked to increased stress levels in exposed animals (Garaventa et al., 2010).

6.6.3 Bioconcentration of metals and metalloids in hepatic and tail tissues

Amphibians are at high risk of metal pollution because of their ability to bioaccumulate toxic ions through their semipermeable skin and gills, and via their diet (Zocche et al., 2013). At the end of the 4-week exposure, tadpoles exposed to CMW1 and CMW2 had elevated levels of Se, Co and As in the tail and liver compared to controls. Mn levels, on the other hand, were only elevated in livers and tails of CMW2 exposed tadpoles. Interestingly, Co, Mn and Se concentrations in treatments were 8–9 times higher in livers compared to tails. This is supported by several other studies showing that hepatic tissue accumulates higher levels of metals compared to other tissues (Bharti and Banerjee, 2013; Zocche et al., 2013; Bharti and Banerjee, 2014; Arnold et al., 2014). Contrary to our results, with the exception of Mn, Zocche et al. (2013) observed higher levels of Al, Mn, Fe, Cu, Zn and Br in liver and leg muscles of adult *Hypsiboas faber* collected in proximity to an abandoned open cut coal mine in southern Brazil (compared to reference samples). However, such discrepancies are not surprising since geochemical and physico-chemical (*e.g.* pH, hardness, salinity) characteristics amongst sites can influence the proportions and chemical state of metals and metalloids, thereby impacting their bioavailability (Deb and Fukushima, 1999; Luoma and Rainbow, 2005; Kang et al., 2011). Metal bioaccumulation in frogs may also vary between species (Sparling and Lowe, 1996), developmental stage (Roe et al., 2005) and sex (Dobrovoljc et al., 2012), adding to the potential variability between studies performed at different locations. The results of the present study are therefore important because they help to fill knowledge gaps in the literature, which has thus far focussed on a relatively small number of sites that poorly reflect differences in the composition of coal mine wastewater on a global scale. Several studies have linked metal and metalloid uptake to severe physiological consequences including oxidative stress, immunosuppression, histological damage, DNA damage, endocrine disruption as well as mortality in amphibians (Levesque et al., 2003; Unrine et al., 2007; Davey et al., 2008; Zocche et al., 2013). Thus, while it is difficult to relate tissue uptake to the observed toxicological outcomes, our study indicates the potential for adverse higher-level consequences that might be associated with exposures of larval amphibians to mine water discharge.

6.6.4 Water quality and chemistry

Differences in overall response patterns between the two study sites may relate to the relatively large disparities in water quality and chemistry that were observed. For example, CMW1 had higher turbidity, nutrients (N and P), TOC, DOC, total alkalinity and As levels compared to CMW2, whereas CMW2 had higher conductivity, salinity, dissolved solids, hardness and sulfate levels. High salinity (3.2 ppt) in the 100% CMW2 treatment could help to explain the low survival rates of exposed tadpoles. Several studies have reported that high salinity (>3.5 ppt) reduced survival and diversity in other Australian tadpole species (Christy and Dickman, 2002; Chinathamby et al., 2006; Smith et al., 2007; Kearney et al., 2012). It has also been demonstrated that increased salinity can delay metamorphosis in a range of tadpole species (Christy and Dickman, 2002; Gomez-Mestre and Tejedo, 2003; Chinathamby et al., 2006; Kearney et al., 2012). However, Kearney et al. (2012) observed that when salinity was above the tolerance threshold of 3.5 ppt, development of *Limnodynastes tasmaniensis* tadpole was accelerated in order to escape unfavourable conditions. This might help to explain why development of surviving tadpoles exposed to 100% CMW2 in our study was not delayed, but this requires further research.

In addition to site-specific differences, temporal fluctuations in water quality and chemistry were observed in the wastewater dams (data not shown), which are not uncommon and can depend on the intensity of coal mining activities and climatic conditions at a given site. In an effort to match with relevant timeframes of potential discharge and developmental stages of amphibian for the studied region, wastewater samples and egg masses were collected in the wet season (January–February) immediately following heavy rainfall events. Although this was the most appropriate sampling regime in terms of environmental and ecological relevance, it is important to consider that heavy rainfalls are likely to influence contaminant levels in holding dams. For example, rainfall often initially increases levels of contamination due to runoff and mixing, whereas extended periods of rain can decrease levels of contaminants as a result of dilution of the wastewater. Chemical analysis identified all analytes at levels below maximum environmental concentrations (MEC) that have previously been observed through ongoing monitoring of the studied dams (Appendix: Table 6-4), indicating that all treatments were within the range of environmentally realistic exposure concentrations. More importantly, this also demonstrates that greater concentrations of some of the identified contaminants occasionally occur at these sites, suggesting that the observed responses offer a conservative estimate of the possible toxicological outcomes that might be expected from these locations.

6.7 Conclusion

Our results reveal that wastewater from two coal mine dams with different physico-chemical properties and metal concentrations elicited a range of toxicological outcomes on native *L. peronii* tadpoles, and that response patterns differed markedly between sites. While overt toxicity was higher in CMW2, tadpoles exposed to CMW1 effluent suffered delayed development in the highest concentration, and had increased HSI at intermediate dilutions. Tadpoles from both treatment groups exhibited increased activity and swimming performance, and had significantly higher levels of Se, Co and As in hepatic tissue and tails compared to controls. Although whole effluent studies offer a much closer representation of natural exposure scenarios than studies investigating single contaminants (Eggen et al., 2004), interpretation can be extremely challenging as a result of the multitude of possible interactions. Nevertheless, the study helps to address important gaps in the literature by expanding on the limited number of studies exploring sub-lethal impacts of coal mining on amphibians. More research is needed to explore the significance of the observed effects for influencing amphibian survival and development in natural environments impacted by coal mining activities, and to continue characterising responses across a range of sites with different physico-chemical properties and chemical constituents.

6.8 Acknowledgements

This study was supported by the Central Queensland University Postgraduate Research Award (CL), the Queensland Resource Council Coal Minesite Rehabilitation Trust Fund Scholarship (CL), the Ian Potter Foundation (SDM) and in-kind industry support. The funding sources had no involvement in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication. The authors thank Dr. Michael Arthur for statistical advice.

6.9 References

Adams, W.J., Blust, R., Borgmann, U., Brix, K.V., DeForest, D.K., Green, A.S., Meyer, J.S., McGeer, J.C., Paquin, P.R., Rainbow, P.S., Wood, C.M., 2011. Utility of tissue residues for predicting effects of metals on aquatic organisms. Integr. Environ. Assess. Manag. 7, 75–98.

Alonso, A., De Lange, H.J., Peeters, E.T.H.M., 2009. Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the Multispecies Freshwater Biomonitor. Chemosphere 75, 341–346.

Alquezar, R., Markich, S.J., Booth, D.J., 2006. Effects of metals on condition and reproductive output of the smooth toadfish in Sydney estuaries: south-eastern Australia. Environ. Pollut. 142, 116–122.

Alroy, J., 2015. Current extinction rates of reptiles and amphibians. PNAS, http://dx.doi.org/10.1073/pnas.1508681112.

Arnold, M.C., Lindberg, T.T., Liu, Y.T., Porter, K.A., Hsu-Kim, H., Hinton, D.E., Di Giulio, R.T., 2014. Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. Ecotoxicology 23, 929–938.

Atchison, G.J., Henry, M.G., Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. Environ. Biol. Fish 18, 11–25.

Bharti, S., Banerjee, T.K., 2013. Bioassay analysis of efficacy of phytoremediation in decontamination of coal mine effluent. Ecotoxicol. Environ. Saf. 92, 312–319.

Bharti, S., Banerjee, T.K., 2014. Toxicity analysis of coal mine effluent on vital organs of *Heteropneustes fossilis* (Bloch) using biometric and biochemical parameters. Clean Soil Air Water 42, 260–266.

Boyle, D., Brix, K.V., Amlund, H., Lundebye, A.-K., Hogstrand, C., Bury, N.R., 2008. Natural arsenic contaminated diets perturb reproduction in fish. Environ. Sci. Technol. 42, 5354–5360.

Brown, D.D., Cai, L., 2007. Amphibian metamorphosis. Dev Biol. 306, 20–33. Butler R.L., Cooper E.L., Crawford J.K., Hales D.C., Kimmel W.G., Wagner C.C., 1973. Fish and food organisms in acid mine waters of Pennsylvania, US EPA-R3-73-032, p. 158.

Calabrese, E.J., 2008. Hormesis: Why it is important to toxicology and toxicologists. Environ. Toxicol. Chem. 27, 1451–1474. Chen, T.-H., Gross, J.A., Karasov, W.H., 2009. Chronic exposure to pentavalent arsenic of larval leopard frogs (*Rana pipiens*): bioaccumulation and reduced swimming performance. Ecotoxicology 18, 587–593.

Chinathamby, K., Reina, R.D., Bailey, P.C.E., Lees, B.K., 2006. Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, *Litoria ewingii*. Aust. J. Zool. 54, 97–105.

Christy, M.T., Dickman, C.R., 2002. Effects of salinity on tadpoles of the green and golden bell frog (*Litoria aurea*). Amphib. Reptilia 23, 1–11. Dare, O.K., Forbes, M.R., 2008. Rates of development in male and female Wood Frogs and patterns of parasitism by lung nematodes. Parasitology 135, 385–393.

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

Deb, S.C., Fukushima, T., 1999. Metals in aquatic ecosystems: mechanisms of uptake: accumulation and release-ecotoxicological perspectives. Int. J. Environ. Stud. 56, 385–417.

DeForest, D.K., Meyer, J.S., 2015. Critical review: toxicity of dietborne metals to aquatic organisms. Crit. Rev. Environ. Sci. Technol 45, 1176–1241.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.-F., De Pauw, E., 2013. Effects of a sublethal pesticide exposure on locomotor behavior: a video-tracking analysis in larval amphibians. Chemosphere 90, 945–951.

DERM, 2009. A Study of the Cumulative Impacts on Water Quality of Mining Activities in the Fitzroy River Basin. Department of Environment and Resource Management, Queensland Government.

DNRM, 2013. Queensland Coal Statistics Report for the Financial Year Ended June 2013. Department of Natural Resources and Mines, Queensland Government.

Dobrovoljc, K., Falnoga, I., Žnidarič, M.T., Mazej, D., Ščančar, J., Bulog, B., 2012. Cd, Cu, Zn, Se, and metallothioneins in two amphibians *Necturus maculosus* (Amphibia, Caudata) and *Bufo bufo* (Amphibia, Anura). Biol. Trace Elem. Res. 150, 178–194.

Eggen, R.I.L., Behra, R., Burkhardt-Holm, P., Escher, B.I., Schweigert, N., 2004. Challenges in ecotoxicology. Environ. Sci. Technol., 58A–64A.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519.

Garcia-Morales, P., Saceda, M., Kenney, N., Kim, N., Salomon, D.S., Gottardis, M.M., Solomon, H.B., Sholler, P.F., Jordan, V.C., Martin, M.B., 1994. Effect of cadmium on estrogen-receptor levels and estrogen-induced responses in human breast-cancer cells. J. Biol. Chem. 269, 16896–16901.

Gerhardt, A., Ingram, M.K., Kang, I.J., Ulitzur, S., 2006. In situ on-line toxicity biomonitoring in water: Recent developments. Environ. Toxicol. Chem. 25, 2263–2271.

Gerhardt, A., Janssens de Bisthoven, L., Soares, A.M.V.M., 2005. Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). Arch. Environ. Contam. Toxicol. 48, 450–458.

Gomez-Mestre, I., Tejedo, M., 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. Evolution 57, 1889–1899.

Gosner, K.L., 1960. A simplified Table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. Chemosphere 120, 778–792.

Hart, B.T., Greenfield, P., Pascoe, M., 2008. Review of the Fitzroy River Water Quality Issues. Report to Queensland Premier. Water Science Pty Ltd. and Water Studies Centre, Monash University.

Hopkins, W.A., Congdon, J.D., Ray, J.K., 2000. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. Environ. Toxicol. Chem. 19, 862–868.

Huey, R.B., 1980. Sprint velocity of tadpoles (*Bufo boreas*) through metamorphosis. Copeia 1980 (3), 537–540.

Iavicoli, I., Fontana, L., Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. J. Toxicol. Environ. Health B 12, 206–223.

Ireland, T.T., Wolters, G.L., Schemnitz, S.D., 1994. Recolonization of wildlife on a coal strip-mine in Northwestern New-Mexico. Southwest. Nat. 39, 53–57.

Jana, K., Jana, S., Samanta, P.K., 2006. Effects of chronic exposure to sodium arsenite on hypothalamo–pituitary–testicular activities in adult rats: possible an estrogenic mode of action. Reprod. Biol. Endocrinol. 4, 9.

Janssens de Bisthoven, L., Gerhardt, A., Soares, A.M.V.M., 2004. Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the multispecies freshwater Biomonitor® . Environ. Toxicol. Chem. 23, 1123–1128.

Jung, R.E., Jagoe, C.H., 1995. Effects of low pH and aluminum on body size swimming performance, and susceptibility to predation of green tree frog (*Hyla cinerea*) tadpoles. Can. J. Zool. 73, 2171–2183.

Kang, Y., Liu, G., Chou, C.-L., Wong, M.H., Zheng, L., Ding, R., 2011. Arsenic in Chinese coals: distribution, modes of occurrence, and environmental effects. Sci. Total Environ. 412–413, 1–13.

Kearney, B.D., Byrne, P.G., Reina, R.D., 2012. Larval tolerance to salinity in three species of Australian anuran: an indication of saline specialisation in *Litoria aurea*. PLoS One 7 (8), e43427.

Lanctôt, C.M., Melvin, S.D., Leusch, F.D.L., Wilson, S.P., Fabbro, L., 2016a. Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. Chemosphere 144, 1560–1566, http://dx.doi.org/10.1016/j.chemosphere.2015.10.026.

Lanctôt, C.M., Melvin, S.D., Fabbro, L., Leusch, F.D.L., Wilson, S.P., 2016b. Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (*Hypseleotris compressa*). Ecotox. Environ. Safe. 127, 37–42.

Lanctôt, C.M., Navarro-Martín, L., Robertson, C., Park, B., Jackman, P., Pauli, B.D., Trudeau, V.L., 2014. Effects of glyphosate-based herbicides on survival development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions. Aquat. Toxicol. 154, 291–303.

Lefcort, H., Meguire, R.A., Wilson, L.H., Ettinger, W.F., 1998. Heavy metals alter the survival growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. Arch. Environ. Contam. Toxicol. 35, 447–456.

Levesque, H.M., Dorval, J., Hontela, A., Van Der Kraak, G.J., Campbell, P., 2003. Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. J. Toxicol. Environ. Health A 66, 657–676.

Luoma, S.N., Cain, D.J., Rainbow, P.S., 2010. Calibrating biomonitors to ecological disturbance: a new technique for explaining metal effects in natural waters. Integr. Environ. Assess. Manag. 6, 199–209.

Luoma, S.N., Rainbow, P.S., 2005. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environ. Sci. Technol. 39, 1921–1931.

Macedo-Sousa, J.A., Gerhardt, A., Brett, C.M.A., Nogueira, A.J.A., Soares, A.M., 2008. Behavioural responses of indigenous benthic invertebrates (*Echinogammarus meridionalis*, *Hydropsyche pellucidula* and *Choroterpes picteti*) to a pulse of Acid Mine Drainage: a laboratorial study. Environ. Pollut. 156, 966–973.

Melvin, S.D., 2015. Oxidative stress, energy storage, and swimming performance of *Limnodynastes peronii* tadpoles exposed to a sub-lethal pharmaceutical mixture throughout development. Chemosphere, http://dx.doi.org/10.1016/j.chemosphere.2015.09.034.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.

Mohti, A., Shuhaimi-Othman, M., Gerhardt, A., 2012. Use of the Multispecies Freshwater Biomonitor to assess behavioral changes of *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae) and *Macrobrachium lanchesteri* (Decapoda: Palaemonidae) in response to acid mine drainage: laboratory exposure. J. Environ. Monit. 14, 2505–2511.

Monastersky, R., 2014. Biodiversity: life-a status report. Nature 516, 158–161. Muncy, B.L., Price, S.J., Bonner, S.J., Barton, C.D., 2014. Mountaintop removal mining reduces stream salamander occupancy and richness in southeastern Kentucky (USA). Biol. Conserv. 180, 115–121.

Navarro-Martín, L., Lanctôt, C.M., Jackman, P., Park, B.J., Doe, K., Pauli, B.D., Trudeau, V.L., 2014. Effects of glyphosate-based herbicides on survival development, growth and sex ratios of wood frogs (*Lithobates sylvaticus*) tadpoles. I: chronic laboratory exposures to VisionMax® . Aquat. Toxicol. 154, 278–290.

Pestana, J.L.T., Ré, A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68, 1556–1562.

Petty, J.T., Gingerich, G., Anderson, J.T., Ziemkiewicz, P.F., 2013. Ecological function of constructed perennial stream channels on reclaimed surface coal mines. Hydrobiologia 720, 39–53.

Raimondo, S.M., Rowe, C.L., Congdon, J.D., 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (*Rana catesbeiana*). J. Herpetol. 32, 289–292.

Rainbow, P.S., 2007. Trace metal bioaccumulation: models: metabolic availability and toxicity. Environ. Int. 33, 576–582.

Roe, J.H., Hopkins, W.A., Jackson, B.P., 2005. Species- and stage-specific differences in trace element tissue concentrations in amphibians: implications for the disposal of coal-combustion wastes. Environ. Pollut. 136, 353–363.

Rowe, C.L., 2014. Bioaccumulation and effects of metals and trace elements from aquatic disposal of coal combustion residues: recent advances and recommendations for further study. Sci. Total Environ. 485–486, 490–496.

Rowe, C.L., Hopkins, W.A., Congdon, J.D., 2001. Integrating individual-based indices of contaminant effects. How multiple sublethal effects may ultimately reduce amphibian recruitment from a contaminated breeding site. Sci. World J. 1, 703–712.

Rowe, C.L., Hopkins, W.A., Congdon, J.D., 2002. Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: a review. Environ. Monit. Assess. 80, 207–276.

Schorr, M.S., Dyson, M.C., Nelson, C.H., Van Horn, G.S., Collins, D.E., Richards, S.M., 2013. Effects of stream acidification on lotic salamander assemblages in a coal-mined watershed in the Cumberland Plateau. J. Freshw. Ecol. 28, 339–353.

Scullion, J., Edwards, R.W., 1979. Movement of hatchery-reared rainbow and brown trout in a polluted river in the South-Wales coalfield. J. Fish Biol. 14, 173–178.

Smith, D.C., Van Buskirk, J., 1995. Phenotypic design plasticity, and ecological performance in two tadpole species. Am. Nat. 145, 211–233.

Smith, M.J., Schreiber, E.S.G., Scroggie, M.P., Kohout, M., Ough, K., Potts, J., Lennie, R., Turnbull, D., Jin, C., Clancy, T., 2007. Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation. Freshw. Biol. 52, 75–84.

Sparling, D.W., Lowe, T.P., 1996. Metal concentrations of tadpoles in experimental ponds. Environ. Pollut. 91, 149–159.

Thiruvenkatachari, R., Younes, M., Su, S., 2011. Coal mine site investigation of wastewater quality in Australia. Desalin. Water Treat. 32, 357–364.

Tiwary, R.K., 2001. Environmental impact of coal mining on water regime and its management. Water Air Soil Pollut. 132, 185–199.

Unrine, J.M., Hopkins, W.A., Romanek, C.S., Jackson, B.P., 2007. Bioaccumulation of trace elements in omnivorous amphibian larvae: implications for amphibian health and contaminant transport. Environ. Pollut. 149, 182–192.

US EPA, 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. US Environmental Protection Agency, Washington DC, EPA-821-R-02-013.

Wang, W.-X., 2013. Dietary toxicity of metals in aquatic animals: recent studies and perspectives. Chin. Sci. Bull. 58, 203–213.

Wood, L., Welch, A.M., 2015. Assessment of interactive effects of elevated salinity and three pesticides on life history and behavior of southern toad (*Anaxyrus terrestris*) tadpoles. Environ. Toxicol. Chem. 34, 667–676.

Wood, P.B., Williams, J.M., 2013a. Impact of valley fills on streamside salamanders in Southern West Virginia. J. Herpetol. 47, 119–125.

Wood, P.B., Williams, J.M., 2013b. Terrestrial salamander abundance on reclaimed mountaintop removal mines. Wildl. Soc. Bull. 37, 815–823.

Zocche, J.J., Damiani, A.P., Hainzenreder, G., Mendonc¸a, R.Á., Peres, P.B., dos Santos, C.E.I., Debastiani, R., Dias, J.F., de Andrade, V.M., 2013. Assessment of heavy metal content and DNA damage in *Hypsiboas faber* (Anuran amphibian) in coal open-casting mine. Environ. Toxicol. Pharm. 36, 194–201.

6.10 Appendix

Table 6-4: *In situ* water quality and chemistry of CMW1 and CMW2 dams at time of sampling and maximum environmental concentrations (MEC) of the two dams.

	CMW1		CMW2		MEC[b]
Parameter/Analyte	**23/01/15**	**10/02/15**	**23/01/15**	**10/02/15**	
Temperature (°C)[a]	25.5	-	27.5	-	-
SpCond (mS/cm)[a]	4.56	-	7.44	-	-
Cond (mS/cm)[a]	4.60	-	7.80	-	-
TDS (g/L)[a]	2.96	-	4.83	-	-
Sal (ppt)[a]	2.42	-	4.06	-	-
pH[a]	9.22	-	8.67	-	-
Orp (mV)[a]	48	-	86	-	-
Turbidity (NTU)[a]	76.4	-	23.3	-	-
ODO (sat) % [a]	88	-	128	-	-
ODO (ppm)[a]	7.1	-	9.9	-	-
pH Value	9.20	9.48	8.79	8.44	9.51
Electrical Conductivity @ 25°C (mS/cm)	4.46	3.41	7.36	3.96	9.38
Suspended Solids (mg/L)	29	68	23	10	216
Turbidity (NTU)	55.3	70.1	21.2	12.9	286
Total Hardness as CaCO3 (mg/L)	260	215	584	506	650
Sulfate as SO4 - Turbidimetric (g/L)	0.43	0.33	1.13	0.63	1.6
Fluoride (mg/L)	0.5	<0.1	0.5	<0.1	0.9
Ammonia as N (mg/L)	0.06	0.07	0.03	0.05	0.16
Nitrite as N (mg/L)	<0.01	<0.01	0.02	<0.01	0.30
Nitrate as N (mg/L)	<0.01	<0.01	0.08	<0.01	3.81
Nitrite + Nitrate as N (mg/L)	<0.01	<0.01	0.10	<0.01	3.95
Total Kjeldahl Nitrogen as N (mg/L)	5.6	5.4	1.4	0.8	10
Total Nitrogen (TKN + NOx) as N (mg/L)	5.6	5.4	1.5	0.8	10
Total Phosphorus as P (mg/L)	0.36	0.41	0.08	0.05	1.57
Dissolved Organic Carbon (mg/L)	10	7	6	6	20
Total Organic Carbon (mg/L)	12	10	6	6	24
Alkalinity					
Hydroxide Alkalinity as CaCO3 (mg/L)	<1	<1	<1	<1	<1
Carbonate Alkalinity as CaCO3 (mg/L)	134	212	55	21	303
Bicarbonate Alkalinity as CaCO3 (mg/L)	190	87	176	149	463
Total Alkalinity as CaCO3 (mg/L)	324	299	231	170	584
Dissolved Metals and Metalloids (µg/L)					
Aluminium	<10	<10	<10	<10	240
Arsenic	8	7	3	2	28

Parameter/Analyte	CMW1 23/01/15	CMW1 10/02/15	CMW2 23/01/15	CMW2 10/02/15	MEC[b]
Cadmium	<0.1	<0.1	<0.1	<0.1	<0.1
Chromium	<1	<1	<1	<1	6
Copper	2	2	2	2	4
Cobalt	<1	<1	<1	<1	1
Nickel	<1	<1	1	2	3
Lead	<1	<1	<1	<1	<1
Zinc	<5	<5	<5	<5	27
Manganese	<1	<1	1	2	22
Selenium	<10	<10	<10	<10	20
Silver	<1	<1	<1	<1	<1
Uranium	2	1	2	1	6
Vanadium	<10	<10	<10	<10	30
Boron	310	270	320	210	610
Iron	<50	<50	120	<50	180
Total Metals and Metalloids (µg/L)					
Aluminium	230	510	270	300	4220
Arsenic	9	7	4	2	28
Cadmium	<0.1	<0.1	<0.1	<0.1	<0.1
Chromium	<1	<1	<1	<1	6
Copper	2	3	2	1	11
Cobalt	<1	<1	<1	<1	4
Nickel	2	2	2	2	9
Lead	<1	<1	<1	<1	4
Zinc	8	<5	10	<5	34
Manganese	29	20	19	106	147
Selenium	<10	<10	<10	<10	20
Silver	<1	<1	<1	<1	<1
Uranium	2	2	2	1	6
Vanadium	<10	10	<10	<10	40
Boron	300	270	370	200	560
Iron	240	470	320	280	6040
Total Petroleum Hydrocarbons (µg/L)					
C6 - C9 Fraction	<20	<20	<20	<20	<20
C10 - C14 Fraction	<50	<50	<50	<50	<50
C15 - C28 Fraction	290	520	110	<100	1170
C29 - C36 Fraction	<50	<50	<50	<50	80
C10 - C36 Fraction (sum)	290	520	110	<50	1250

	CMW1		CMW2		MEC[b]
Parameter/Analyte	**23/01/15**	**10/02/15**	**23/01/15**	**10/02/15**	
Total Recoverable Hydrocarbons (µg/L)					
C6 - C10 Fraction	<20	<20	<20	<20	<20
C6 - C10 Fraction minus BTEX (F1)	<20	<20	<20	<20	<20
>C10 - C16 Fraction	<100	<100	<100	<100	<100
>C16 - C34 Fraction	300	490	120	<100	1220
>C34 - C40 Fraction	<100	<100	<100	<100	<100
>C10 - C40 Fraction (sum)	300	490	120	<100	1220
>C10 - C16 Fraction minus Naphthalene (F2)	<100	<100	<100	<100	<100
BTEXN					
Benzene	<1	<1	<1	<1	<1
Toluene	<2	<2	<2	<2	<2
Ethylbenzene	<2	<2	<2	<2	<2
meta- & para-Xylene	<2	<2	<2	<2	<2
ortho-Xylene	<2	<2	<2	<2	<2
Total Xylenes	<2	<2	<2	<2	<2
Sum of BTEX	<1	<1	<1	<1	<1
Naphthalene	<5	<5	<5	<5	<5
TPH(V)/BTEX Surrogates (%)					
1.2-Dichloroethane-D4	95.3	113	92.9	113	130
Toluene-D8	101	105	97.1	109	124
4-Bromofluorobenzene	93.0	102	86.7	103	125

a. Parameters measured in the field using YSI probe.

b. MEC = maximum environmental concentration measured from CMW1 and CMW2 between 2012 and 2015 (n = 10-34).

Table 6-5: Bioconcentration factor (BCF) in liver and tail tissues of tadpoles exposed to control and to coal mine wastewater from two dams (25, 50, 100% CMW1 and CMW2). BCF = tissue concentration (µg/g) / water concentration µg/mL.

	Control		CMW1 25%		CMW1 50%		CMW1 100%		CMW2 25%		CMW2 50%		CMW2 100%		*p*-value	Test statistic (df = 6)
	Average	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**		
Liver																
Al	165.3	317.1	1.4	2.4	3.8	3.2	4.3	3.3	4.5	6.8	3.3	5.5	0.7	1.48	0.131	9.84
As	50.3	112.6	8.0	6.2	12.8	5.9	12.6	5.9	5.9	10.8	5.9	3.9	7.9	1.33	0.112	10.31
Cd	477.9	772.9	-		105.6	66.4	-		166.7	102.9	313.5	243.2	-		-	-
Co	103.8	28.9	132.8	20.4	153.1	12.3	400.2	93.9	218.3	21.9	421.7	287.4	379.9	94.65	< 0.001	28.58
Cu	62179.4	12547.2	6767.3	2703.8	17191.0	2996.8	17796.3	4190.1	62265.0	23293.7	33906.1	28955.6	18458.9	5354.06	< 0.001	25.14
Fe	2938.9	961.2	795.8	325.4	407.6	69.3	249.6	30.2	2291.4	843.9	412.8	149.1	236.8	20.52	< 0.001	30.60
Pb	168.9	335.5	-		-		-		-		-		-		-	-
Mn	442.4	410.9	189.3	71.2	149.7	128.9	36.1	10.4	253.8	131.4	135.0	87.2	39.2	24.70	0.001	22.00
Se	103.8	29.1	583.2	52.9	755.3	91.6	919.6	126.8	206.2	67.0	256.2	134.0	649.0	287.01	< 0.001	28.67
Zn	7041.8	628.4	6415.2	2542.5	5237.0	441.8	973.1	123.4	6361.7	686.0	3867.4	1751.3	1502.6	121.50	< 0.001	26.76
Tail																
Al	-		-		-		-		-		-		-		-	-
As	12.3	5.2	10.5	2.5	7.2	2.2	3.6	1.2	20.9	7.1	10.1	5.8	8.9	2.3	0.003	19.80
Cd	-		-		-		-		-		-		-		-	-
Co	6.6	2.0	21.1	2.6	18.8	3.2	41.8	8.2	41.9	8.3	57.3	38.5	65.3	19.1	< 0.001	25.21
Cu	200.0	34.8	29.5	2.3	82.0	46.2	63.0	54.8	355.8	289.3	183.4	183.7	60.3	38.8	< 0.001	26.04
Fe	91.7	11.9	43.6	16.3	16.5	2.0	6.2	1.6	99.1	17.8	15.2	8.0	8.6	1.9	< 0.001	30.77
Pb	-		-		12.9	26.2	16.1	36.0	25.3	47.3	22.4	32.9	10.1	20.0	-	-
Mn	44.5	24.3	22.2	3.8	13.0	4.7	5.2	0.9	26.3	11.1	17.1	13.5	5.3	3.1	< 0.001	24.61
Se	8.9	4.3	68.1	3.6	82.4	10.4	74.2	16.5	32.3	3.4	36.9	5.3	68.9	23.0	< 0.001	27.94
Zn	1730.7	186.0	1930.8	405.5	1922.5	159.7	450.7	44.2	2915.4	2276.6	1524.5	768.6	508.0	103.4	0.001	23.13

Chapter 7
General discussion

7.1 Critical review of experimental outcomes

The research presented in this thesis aimed to fill important knowledge gaps concerning the potential toxicity associated with coal mine wastewater, and specifically sub-lethal effects that might manifest in locally relevant species found in Queensland's Fitzroy River catchment. The research was primarily focused on evaluating the use of behavioural responses as a toxicity endpoint in native aquatic vertebrates, with the broad goal of assessing the potential application of behavioural tests as tools for monitoring sub-lethal effects of wastewater releases on these organisms. The research was performed as a series of laboratory experiments, in which native species were exposed to coal mine wastewater (CMW) collected from two major holding dams located at an operational open-cut mine in Central Queensland. Initial experiments described in Chapter 3 were carried out to compare the sensitivity of a range of invertebrate and vertebrate species to CMW. Two vertebrate species were identified as the most suitable based on their high comparative sensitivity to the wastewater (*Hypseleotris compressa* and *Limnodynastes peronii* tadpoles), and these species were subsequently used to further investigate sub-lethal toxicity of CMW, including consideration of a range of behavioural endpoints (Chapters 4-6).

Though some of the invertebrate species tested in Chapter 3 exhibited sensitivity to coal mine wastewater (*Dugesia* sp. and *Daphnia carinata*), preliminary behavioural data with invertebrates showed much greater variability amongst replicates. Consequently, due to the anticipated time required for optimising behavioural protocols with these species and limited timeframe for the project, further investigation was not pursued with invertebrates.

The main experimental works for the thesis are presented in Chapters 4 to 6. The overall outcomes demonstrate a range of alterations to behaviour and swimming performance in juvenile *H. compressa* and *L. peronii* tadpoles exposed to sub-lethal concentrations of CMW. One of the key observations from this research is that behavioural responses were generally more rapidly detectable than other parameters. Behavioural endpoints also tended to be more sensitive compared to other measured endpoints such as survival, development and morphometrics. This is consistent with a recent meta-analysis on the topic, which indicated greater sensitivity, rapidity and statistical power from behavioural studies compared to traditional toxicity endpoints (Melvin and Wilson 2013). The research presented in this thesis effectively demonstrates the potential benefits of incorporating behavioural responses as tools for evaluating the toxicological effects of

CMW releases on two locally relevant freshwater species. It has been suggested that the implementation of biological early warning systems (BEWS) towards routine risk assessments could complement existing physico-chemical monitoring regimes in coal mining regions, by measuring rapid and biologically meaningful behavioural responses in aquatic biota exposed to environmental pollutants (Kuklina et al. 2013). Despite the progress made through this thesis, a considerable amount of research is still necessary for behavioural endpoints to be effectively applied as monitoring tools. This includes: 1) a need to identify the most relevant model species, 2) further basic experimentation to standardise approaches, and 3) evaluation of responses with exposure to a wide range of physico-chemical parameters and chemical standards.

Experiments were carried out using two commercially available tools for analysing behaviour in aquatic animals, one based on visual and the other non-visual technologies. Although both methods were found to be well suited to assess sub-lethal behavioural endpoints, the different choices of equipment offer distinct advantages and disadvantages for environmental monitoring (Table 7-1). Visual monitoring using EthoVision® video-analysis software allows the tracking of aquatic organisms in real-time and assessment of a range of behavioural and swimming performance endpoints, including velocity, movement, space utilisation and social interactions. Experiments using this approach were however limited to daytime activity, as sufficient lighting was required to maintain adequate contrast for motion tracking. On the other hand, the Multispecies Freshwater Biomonitor® (MFB®), a non-visual monitoring tool, permits constant real-time monitoring of animals during both day and night. This tool can also be applied to various matrices, including water with a range of turbidities, and sediment and soil, which presents added benefits. Furthermore, this tool shows potential applicability for *in situ* field monitoring of real-time pollution inputs, which merits further investigation. Nevertheless, the MFB® also presented some disadvantages, primarily relating to replication, which is limited to the number of channels and chambers available (*i.e.*, 8 in our case). Experiments using both methods led to the hypothesis that physical differences between the monitoring tools may lead to variable responses (Chapters 3 and 4 (Lanctôt et al. 2016a; 2016b)). For example, the MFB® quantifies activities of animals confined in opaque cylindrical chambers whereas EthoVision® visually quantifies movement in shallow vessels. Test chambers for the MFB® and arenas for EthoVision® provide different dimensions, resulting in dissimilar swimming patterns (*e.g.*, linear back and forth *vs* circular movement), which may contribute to measureable differences in experimental outcomes. To our knowledge, there have been no studies directly comparing and contrasting outputs provided by these two commercial tools, and such research may be important for exploring consistency in behavioural responses amongst studies utilising different approaches and technologies. Notwithstanding the differences in response patterns that have been noted, rapid behavioural responses were observed using both methods following short-duration exposure to sub-lethal concentrations of CMW, demonstrating the rapid identification of environmental disturbances attainable using behavioural analysis.

Table 7-1: Advantages and disadvantages of visual (EthoVision®) and non-visual (Multispecies Freshwater Biomonitor®) behavioural tools for aquatic monitoring.

	Advantages	Disadvantages
EthoVision®	• Real-time monitoring • Multiple endpoints (velocity, movement, space utilisation, interactions)	• Limited to day time measurements (unless using night vision camera) • Tracking ability limited by contrast with background • Not well suited for *in situ* monitoring (though may have some applications)
MFB®	• Real-time monitoring • Circadian monitoring (no light needed) • Suitable for water, sediment and soil • Potential for *in situ* monitoring	• Limited endpoints (locomotory, non-locomotory behaviours) • Target species limited by chamber dimensions and organism's viability • Replication limited by number of channels and chambers available

7.2 General discussion

The bioaccumulation potential of a range of metal(loid)s in *L. peronii* tadpoles exposed to coal mine wastewater was investigated in Chapter 6 (Lanctôt et al. 2016c), to complement the behavioural data. Results indicated the bioconcentration of As, Co, Se, Mn, and Zn in hepatic and/or tail tissue of tadpoles exposed to CMW for 4 weeks. Amphibians are very susceptible to the bioaccumulation of metals because of their larval aquatic life stage and their ability to uptake toxic ions through their skin, gills and diet. Previous studies have linked the uptake of several metals and metalloids to severe physiological consequences including oxidative stress, immunosuppression, histological damage, DNA damage, endocrine disruption, as well as mortality in amphibians (Levesque et al. 2003; Unrine et al. 2007; Davey et al. 2008; Zocche et al. 2013). However, hepatic and tail metal burdens presented in Chapter 6 were consistently lower compared to the concentrations associated with toxicological effects reported in the few studies assessing single metal uptake in amphibians, though exposure concentrations were generally much higher in these previous studies (Falfushynska et al 2015; Koch et al 2015; Loumbourdis 2006; Papadimitriou and Loumbourdis 2003; Mouchet et al 2015). Although it was difficult to relate tissue uptake to the observed toxicological outcomes due to the complexity of the studied wastewater, the results indicate the potential for adverse higher-level consequences that might be associated with exposures of larval amphibians to metal(loid)s from mine water discharge. Future research related to bioaccumulation of metals should further explore tissue specific uptake, and investigate linkages between tissue concentrations, physiological status (*e.g.*, energy reserves, neurological functioning) and subsequent behavioural outcomes.

Importantly, the results from the different experiments reveal that toxicological response patterns can differ significantly amongst sites (*i.e.*, CMW1 and CMW2) and between studies. In both cases, the likely explanation relates to differences in water quality and chemistry, which were observed throughout the research (Tables 3-2, 3-3, 4-1, 4-2, 5-1, 5-2, 6-1, 6-2, 6-4). Water chemistry from the two studied dams frequently differed, and this was generally characterised by higher turbidity, suspended solids, total and dissolved organic carbon, nutrients, alkalinity, total metals and As levels at CMW1, but lower conductivity, salinity, hardness and sulfate levels compared to CMW2. Water quality results also showed that the wastewater from both dams had relatively high pH, conductivity, salinity, total dissolved solids and dissolved metals compared to reference water (control). Dissolved metal(loid)s detected in the wastewater from both sites included As (2-10 μg/L), Cu (1-4 μg/L), Co (<1-1 μg/L), Ni (<1-3 μg/L), Fe (<50-120 μg/L), Mn (<1-3 μg/L), Se (<10-10 μg/L), U (1-6 μg/L), V (<10-30 μg/L) and B (210-470 μg/L). As, Se (CMW1 only) and B levels in undiluted effluent were found to exceed trigger values of the Australian environmental water quality guidelines established for the protection of 99% of freshwater species (ANZECC and ARMCANZ 2000). U, V and B concentrations also sometimes exceeded release water trigger values set out by the Australian Environmental Authority (Department of Environment and Heritage Protection 2015 permit). Conductivity levels in both dams exceeded ANZECC trigger values (ANZECC and ARMCANZ 2000), but were below the discharge license limit for this site (10000 μS/cm).

In addition to site-specific differences, temporal fluctuations in water quality and chemistry were observed in the wastewater dams. These fluctuations are not uncommon and can depend on the intensity of coal mining activities and climatic conditions at a given site. For example, rainfall often initially increases contaminant concentrations due to runoff and mixing, whereas extended periods of rain can decrease contaminant concentrations as a result of dilution of the wastewater. Unfortunately, due to the time, logistical and financial limitations of the project, wastewater could not be collected at the same time for all experiments. Therefore, though the wastewater was collected from the same holding dams, there is variability in water chemistry of the CMW collected for each study, which could partly explain discrepancies in responses between studies. This highlights a need for future research aimed at characterising behavioural responses in animals exposed to a range of water quality characteristics, as this could prove useful when interpreting outcomes following exposure to highly complex wastewaters.

It is important to consider the resulting dilution factor of coal mine wastewater that will normally be achieved in situations of controlled or accidental releases. Concentrations of contaminants are generally much lower in the receiving environment compared to the holding dams, although some trace elements are known to be persistent and can accumulate significantly in water, sediment and wildlife. When releasing CMW, mining operations are required to adhere to the site-specific guidelines set by the Environmental Authority. Mine operators carefully manage releases to ensure adherence to those guidelines, which are set to avoid adverse outcomes on local species. Guidelines are based on several factors, including flow and water quality, in order to provide sufficient dilution to minimise risks of cumulative impacts. Appropriate flow of the receiving system creates a contamination gradient, such that potential impacts of discharged CMW will decrease as it moves further downstream from the release point. Though this is important to keep in mind, it is also important to consider worst-case scenarios in the events of uncontrolled releases that may lead to large volumes of wastewater to be released in the environment when flow does not allow appropriate dilutions. Experiments presented in this thesis were carried out using dilution factors that are representative of these worst-case scenario events (> 25%). Moreover, since the main objective was to assess the use of novel behavioural techniques and the wastewater exhibited relatively low toxicity at these concentrations, environmental relevance was not a primary objective for the research. Logistical and financial contraints surrounding the collection of water for the project also precluded our ability to use water from the receiving environment, and thus laboratory water was used for controls. While this represents the standard acceptable approach according to established laboratory toxicity protocols (EPA, ATSM, OECD), it is important to acknowledge that control water may not be representative of the receiving waters of the studied minesite. Future research is needed to further explore behavioural responses following pulse exposures to environmentally relevant wastewater concentrations (*i.e.*, 1-10%) and using reference water that is representative of the receiving environment. This would ideally be paired with *in situ* experimentation to compare responses between lab and field settings and evaluate the approach for ongoing monitoring of water quality in coal mine impacted areas.

7.3 Main conclusions

- Coal mine wastewater from two holding dams with different physico-chemical properties elicited a range of toxicological outcomes on native invertebrate and vertebrate species, and response patterns differed markedly between species and sites.

- Two local invertebrate species, *Dugesia* sp. and *Daphnia carinata*, and two vertebrate species, *Hypseleotris compressa* and tadpoles *Limnodynastes peronii*, showed promise for monitoring water quality and toxicity risks in mine-affected regions.

- Results confirmed the potential for using behavioural endpoints as tools for monitoring wastewater discharges using native fish and amphibian species.

- Behaviour and swimming performance endpoints proved to be more sensitive than developmental or morphological responses in empire gudgeons, *H. compressa*.

- Exposure of striped marsh frog *L. peronii* tadpoles to coal mine wastewater caused a range of toxicological effects including increased activity and swimming performance, and led to bioconcentration of Se, Co and As in hepatic and tail tissue.

7.4 Recommendations

Considering the importance of the coal mining industry in Australia and globally, there is a clear need for further research investigating the cumulative impacts of coal mine wastewater on native aquatic organisms. Despite our research efforts, relatively little remains known about the potential sub-lethal and long-term impacts of coal mine discharges on aquatic ecosystems. This information is crucial in working towards establishing effective approaches for long-term monitoring and management. Mounting evidence suggests that effects on behaviour and swimming performance may hold implications for important higher-level outcomes related to survival, growth, and reproduction (Groh et al. 2015). While the results of this thesis demonstrate various sub-lethal behavioural effects that may occur in fish and amphibians exposed to coal mine wastewater, further research is now necessary to explore and understand the biological relevance of behavioural alterations to influence individual health and fitness and fundamental population-level interactions in impacted environments. Lastly, an important question for the ongoing development of rapid behavioural tests is whether the observed responses are more or less sensitive than outcomes from established bioassays. Future research should therefore also investigate direct comparisons of behavioural and traditional tests, with exposures to both standard test compounds and complex wastewaters from a range of sites with different physico-chemical properties.

7.5 References

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

Falfushynska, H., Gnatyshyna, L., Fedoruk, O., Mitina, N., Zaichenko, A., Stoliar, O., Stoika, R., (2015). Hepatic metallothioneins in molecular responses to cobalt, zinc, and their nanoscale polymeric composites in frog *Rana ridibunda*. *Comparative Biochemistry and Physiology* **172-173**, 45–56.

Groh, K. J., Carvalho, R. N., Chipman, J. K., Denslow, N. D., Halder, M., Murphy, C. A., Roelofs, D., Rolaki, A., Schirmer, K., and Watanabe, K. H. (2015). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. *Chemosphere* **120**, 778–792.

Koch, I., Zhang, J., Button, M., Gibson, L.A., Caumette, G., Langlois, V.S., Reimer, K.J., Cullen, W.R. (2015). Arsenic(+3) and DNA methyltransferases, and arsenic speciation in tadpole and frog life stages of western clawed frogs (*Silurana tropicalis*) exposed to arsenate. *Metallomics* **7**, 1274–1284.

Kuklina, I., Kouba, A., and Kozak, P. (2013). Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. *Environmental Monitoring and Assessment* **185**, 5043–5053.

Lanctôt, C. M., Melvin, S. D., Fabbro, L., Leusch, F. D. L., and Wilson, S. P. (2016a). Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (*Hypseleotris compressa*). *Ecotoxicology and Environmental Safety* **127**, 36–42.

Lanctôt, C. M., Melvin, S. D., Leusch, F. D. L., Wilson, S., and Fabbro, L. (2016b). Locomotor and behavioural responses of empire gudgeons (*Hypseleotris compressa*) exposed to coal mine wastewater. *Chemosphere* **144**, 1560–1566.

Lanctôt, C., Bennett, W., Wilson, S. P., Fabbro, L., Leusch, F. D. L., Melvin, S. D. (2016c). Behaviour, development and metal accumulation in striped marsh frog tadpoles (*Limnodynastes peronii*) exposed to coal mine wastewater. *Aquatic Toxicology* **173**, 218–227.

Levesque, H. M., Dorval, J., Hontela, A., Van Der Kraak, G. J., and Campbell, P. G. C. (2003). Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. *Journal of toxicology and environmental health. Part A* **66**, 657–676.

Loumbourdis, N. S. (2006). Copper kinetics and hepatic glutathione levels in the copper exposed frog *Rana ridibunda* after tetrathiomolybdate treatment. *Bulletin of Environmental Contamination and Toxicology* **76**, 522–528.

Melvin, S. D., and Wilson, S. P. (2013). The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. *Chemosphere* **93**, 2217–2223.

Mouchet, F., Teaniniuraitemoana, V., Baudrimont, M., Daffe, G., Gauthier, L., Gonzalez, P. (2015). Recovery capabilities of *Xenopus laevis* after exposure to cadmium and zinc. *Chemosphere* **139**, 117–125.

Papadimitriou, E.A., Loumbourdis, N.S. (2003). Copper kinetics and hepatic metallothionein levels in the frog *Rana ridibunda*, after exposure to $CuCl_2$. *Biometals* **16**, 271–277.

Unrine, J. M., Hopkins, W. A., Romanek, C. S., and Jackson, B. P. (2007). Bioaccumulation of trace elements in omnivorous amphibian larvae: implications for amphibian health and contaminant transport. *Environmental Pollution* **149**, 182–192.

Zocche, J. J., Damiani, A. P., Hainzenreder, G., Mendonça, R. Á., Peres, P. B., Santos, C. E. I. D., Debastiani, R., Dias, J. F., and Andrade, V. M. de (2013). Assessment of heavy metal content and DNA damage in *Hypsiboas faber* (anuran amphibian) in coal open-casting mine. *Environmental Toxicology and Pharmacology* **36**, 194–201.