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Bioassay assessment of mine pit lake water for aquaculture and biodiversity conservation

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Bioassay assessment of mine pit lake water for aquaculture and biodiversity conservation

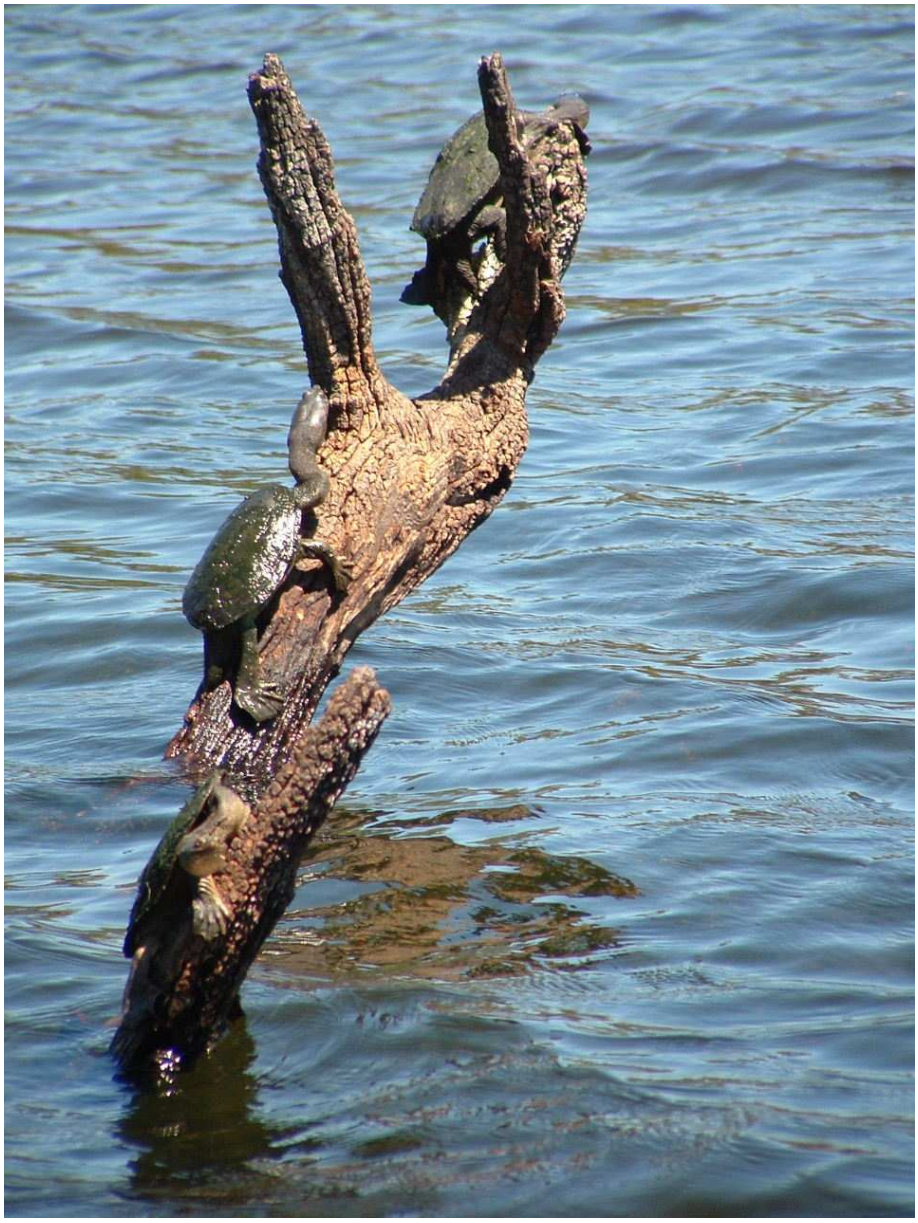

Plate 1. Biodiversity at work

Luke L. Neil

STATEMENT OF SOURCES

Declaration:

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

Signed: Luke Neil Date:

AEC Approval

This study was conducted with the authorisation of the Australian Ethics Committee (AEC) under approval number R16-05. All fish were handled in accordance with the AEC approval.

Acknowledgements

Me, as at one stage in my life I was never going to go to uni because I assumed it would be too difficult. Now I have researched and written a thesis for the award of PhD. Amazing!

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Now I will never be able to have a normal conversation involving anything to do with water with friends and family again!

List of publications and conference abstracts

1) Neil, L., McCullough, C., Tsvetnenko, Y., Evans, L. 2006, Toxicity assessment of limed and phosphorus amended mine pit lake water. Proceedings of the Interact 2006 Conference, Air Water and Earth: Interact in Perth Western Australia (Abstract)

2) Tsvetnenko, Y., Neil, L., Evans, L. 2006, Mine lake water quality assessment using bioassays and chemical analysis. Proceedings of the Interact 2006 Conference, Air Water and Earth: Interact in Perth Western Australia (Abstract)

3) Neil, L.L. McCullough, C.H., Lund, M.A., Evans, L.H., Tsvetnenko, Y. 2007. Bioassay assessment of Lake Kepwari mesocosm treatments. In: Evans, L., Oldham, C., Lund, M., Salmon, U., Tsvetnenko, Y., McCullough, C., and Neil, L. (2007). SWDC Consultancy Report: Review of CSML research on Lake Kepwari and recommendations for water quality management, Final Report, Centre for Sustainable Mine Lakes, Perth.

4) Neil, L.L., McCullough, C.H., Lund, M.A., Tsvetnenko, Y., Evans, L.H. submitted 2007, Bioassay assessment of limed and phosphorus amended mine pit lake water. Ecotoxicology and Environmental Safety

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List of Acronyms

ABARE	Australian Bureau for Agriculture and Resource Economics
ACA	Australian Coal Association
AMD	Acid Mine Drainage
ANOVA	Analysis Of Variance
BLM	Biotic Ligand Model
BRT	Biological Receptor Theory
CCW	Chicken Creek Water
CI	Confidence Interval
CRW	Collie River Water
CV	Coefficient of Variance
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DTA	Direct Toxicity Assessment
EC_x	Effect Concentration. Toxicant concentration affecting x% of the test endpoint
EDTA	EthyleneDinitriloTetraacetic Acid
EMP	Environmental Management Plan
ERA	Ecological Risk Assessment
FAO	Food and Agriculture Organisation
FIAM	Free Ion Activity Model
FLR	Fluidised Limestone Reactor
FLB	Fluidised Limestone Bed
FTD	Final *Tetrahymena thermophila* Density
GFC	Glass Fibre Construction
HHC	High-Hardness Combo
HMGV	Hardness Modified Guideline Value
IC_x	Inhibition Concentration. Toxicant concentration affecting x% of the test endpoint
ICP-AES	Inductively Coupled Plasma Atomic Absorption Spectrophotometry
L	Limestone treatment in mesocosm assessment of Lake Kepwari

L&P	Limestone and Phosphorus treatment in mesocosm assessment of Lake Kepwari
LC_x	Lethal Concentration. Toxicant concentration causing mortality to x% of the test organism
LOEC	Lowest Observable Effect Concentration
LT1	Limestone Treatment one in Chicken Creek assessment
LT2	Limestone Treatment two in Chicken Creek assessment
LT3	Limestone Treatment three in Chicken Creek assessment
MC	Mesocosm Control
N	NaOH treatment
NOEC	No Observable Effect Concentration
NPW	Natural Pond Water
OCM	Open Cast Mine
P	Phosphorus treatment in mesocosm assessment of Lake Kepwari
RCO	Rapid Catalytic Oxidation. Treatment process used on WO5H water
RCO-Cl	Rapid Catalytic Oxidation and pre treatment with Chlorine
RCO-ClO_2	Rapid Catalytic Oxidation and pre treatment with Chlorine dioxide
RMANOVA	Repeated Measures Analysis Of VAriance
SSW	Synthetic Soft Water control for algae bioassays
TOC	Total Organic Carbon
TTC	*Tetrahymena Thermophila* Control. medium for bioassays
UAN	Unionised Ammonia Nitrogen
USEPA	United States of America Environmental Protection Agency
WET	Whole Effluent Testing (US nomenclature for DTA)

EXECUTIVE SUMMARY

For decades, researchers have been working to better understand the effects of acid and metal toxicity to aquatic organisms and to develop ways to effectively mitigate these detrimental effects. Acid mine drainage (AMD) causes environmental pollution that affects many countries having historic or current mining industries. Both surface and underground mining have the potential to create AMD and therefore adversely affect the local and adjacent environments.

Through malignant mining and farming practices, damming rivers and the changing climate, surface freshwater resources are rapidly being reduced in Australia. Mine pit lakes offer a large freshwater resource that if managed correctly may sustain significant environmental, social and economic benefits from selected end uses for the future. However, assessment of mine pit lakes needs to be achieved to validate end use options.

The Collie Basin located in the southwest of Western Australia is a coal mining area with many abandoned open cast mines (OCM). Some of these OCM have filled with water forming mine pit lakes that are affected by AMD. The result is large freshwater bodies with moderate to high concentrations of metals and a low pH. High concentration of metals combined with low pH is of environmental concern to both the aquatic and terrestrial ecosystem. Nevertheless, remediation techniques for AMD are available and applicable to these acidic lakes. However, the lakes in the Collie Basin are low in sulphatic compounds, therefore, restricting the use of the most common remediation treatment of bioremediation with sulphate reducing bacteria.

Three remediation treatments were assessed for there efficacy in toxicity amelioration to three mine pit lakes. The treatments assessed were Limestone addition, phosphorus addition and a Rapid Catalytic Oxidation (RCO) treatment. Toxicity assessment was achieved by means of comparing physico-chemical data to guideline trigger values and biological assessment. Biological assessment was achieved with three commonly used but ecologically and geographically relevant species. The bioassay species were the alga *Chlorella protothecoides,* the crustacean *Ceriodaphnia* cf *dubia* and the protozoa *Tetrahymena thermophila*. Aquaculture is

being trialled adjacent to one of the pit lakes in limestone treated mine pit lake water. Therefore, the aquaculture fish species *Bidyanus bidyanus* early life stages were also used to assess residual toxicity from treated mine pit lake water.

Limestone remediation of pit lake water demonstrated good reduction of dissolved metal concentrations with the exception of Zn and the ability to increase pH to circum-neutral. Biological assessment of limestone treated pit lake water showed that toxicity was removed to the three bioassay species and to the aquaculture species *B. bidyanus* early life stages. A larger field- scale mesocosm experiment with limestone treatment *in situ* is recommended. Assessment of the mesocosm experiment with biological and chemical analysis will confirm the efficacy of this treatment for full scale use.

INTRODUCTION AND OVERVIEW

1 Introduction and overview

Abandoned Open Cast Mines (OCM) have the potential to become a freshwater resource once mining ceases. With the diminishing freshwater resources in Western Australia any large freshwater resource is worthy of investigation and development. Many OCM are located near isolated towns and could therefore be a source of freshwater not only for potable uses but for other end uses as well (Doupé & Lymbery 2005). In many cases when mining ceases these isolated towns with little or no future begin to shrink and finally disappear. Using OCM when flooded with water gives the opportunity for local communities to diversify into other activities such as irrigated horticulture, aquaculture or tourism, all of which require a reliable supply of fresh water.

Water in mining lakes is a significant but under utilised resource in rural Australia. Currently there are more than 1800 abandoned mine pits in Western Australia alone. Pit lake research on end uses has the potential to assist in the diversification of local economies allowing rural communities to become self-sufficient after mining in the area has ceased. Two of the identified possible uses of mine lakes are aquaculture and biodiversity conservation. The viability of aquaculture ventures using water from mining lakes depends on the selection of suitable species, suitable rearing and production systems and good quality water. Preliminary research on pit lakes demonstrated successful poly-culture of marron and silver perch in ameliorated acid mine lake water from a coalmine in Collie (Storer 2005) and trout in America (Viadero Jr & Tierney 2003). Tourism associated with both aquaculture and aquatic ecosystems also has the potential to increase rural town stability.

The long-term viability of projects utilising water from abandoned mining lakes is dependent on the residual toxicity of the water and risk to the desired end use. Preliminary research completed by the Centre of Excellence in Sustainable Mine Lakes (CSML) has demonstrated a range of possible end uses for mine lakes (Evans *et al.* 2006) as well as assessing toxicity in some mine lakes (csml@curtin.edu,au). The current research described in this thesis, aimed at utilising a battery of bioassays for assessment of mine pit lake water, both treated and untreated, entailed

experiments with a macro-invertebrate crustacean, a micro-alga, a protozoan and a fish species. The results from this research will help in determining toxicity and risk from treated and untreated mine pit lake water in Collie WA to aquaculture species and the aquatic ecosystem and have application in other freshwater toxicity assessment throughout Australia and overseas.

1.1 Mining

The exploration and extraction of minerals necessary for the functioning of our technologically thriving world is a ubiquitous endeavour. Mining is found on every continent with almost everyone on Earth being reliant on this activity, either directly or indirectly, for the resources mining provides. Consequently mining and its legacy will be with us far into the future.

Mining is a large revenue earner for Australia, with a total trading profit in 2004-05 of $37.6b. Coal mining contributed $20.3b (~54%) of that total (ABS 2007). There are mines located in every Australian state and territory that impact upon the environment, economy and communities. To a degree, mining has a notorious reputation for environmental tragedy, community break down through mine closure and loss of human life through industrial accidents. Mining's negative attributes can detract from the many positive outcomes of this enormous industry. Mining benefits include; construction of infrastructure; developing employment opportunities; creating communities; and revenue are created for the state and country. In the last few years mining has significantly boosted the economy of Australia with many new and highly paid jobs creating an economic, housing and goods and services boom. Mining has helped reduce the unemployment rate to 4.4% and increased the average Australian wage to (ABS 2007)

Due to advances in technology and increases in commodity prices mining operations around the world are now commonly practiced as open cast mines (OCM) (Martin & Black 1998; Wenderoth & Abraham 2005; McCullough & Lund 2006). Johnson and Wright (2003) have identified that there are approximately 1800 mine voids in WA

alone, ranging from one or two hectares in area and a few meters deep to the increasingly large pits of several square kilometres in area and hundreds of meters deep, found particularly in the gold fields and North of the state. Examples of these OCM can be seen in WA from the coal mines in the south west to the gold and nickel mines in the east and to the iron ore mines in the north. These OCM, however, do not operate forever and must eventually be abandoned.

According to Johnson and Wright (2003) and Doupé and Lymbery (2005), up until recently, abandoned OCM have received little attention. Regulatory bodies governing the actions of mining companies are now becoming proactive in regulating and monitoring activities that can impact on adjacent environments and communities. In Western Australia, mines are subjected to stringent environmental requirements and conditions as part of government legislation. The most important legislation in regard to mining and its possible impact on water resources as the *Mining Act 1978*, the *Rights in Water and Irrigation Act 1914*, and *Environmental Protection Act 1986* (Johnson & Wright 2003). Regardless of government regulation, the adverse effect of mining is inevitably felt by the adjacent environment and communities owing to the nature and magnitude of mining operations. Possibly the only way in which mining can positively contribute to a region's long term sustainability will be achieving a planned landscape of equal or even greater social and environmental value (McCullough *et al.* 2007a).

In a review on Acid Mine Drainage (AMD) remediation Kalin *et al*. (2005) stated that "By definition mining is not sustainable", mine closure is therefore inevitable. Mine closure can have just as serious an impact on communities and the environment as when a mine is active (Otchere *et al.* 2004). A new era of increased corporate and social conscience is emerging and corporations are beginning to assess the environmental and social damage of mining (McCullough & Lund 2006). The growing scale of the mining industry addressing sustainability has placed a strong focus on emphasising the creation of sustainable livelihoods (employment, community development, and infrastructure), optimising resource use, and on the final closure of mining operations in a manner that minimises social and environmental harm while retaining future options for the lease. This change in

attitude will lead to a visual, social and economic change to coal mine abandonment practices. In the near future, mine relinquishment to government and other stakeholders will also eventuate.

1.1.1 Coal mining

Thompson (2000) described the process of coal formation as the accumulation, alteration and compaction of organic remains in anoxic reducing environments commonly found in swamps during the carboniferous period. This ancient resource of energy exists Australia wide in many different grades from Lignite (brown coal) to Anthracite (black coal) (Suggate 1998). Coal grades can influence preliminary environmental constraints, mining approach (e.g. OCM, underground) and final impacts to the surrounding environment and communities through future formation of acid pit lakes (Rolland *et al.* 2001; Denimal *et al.* 2005). Coal has been a major source of energy to the world for many decades but as an unrenewable energy resource is now leaving a legacy of acidic pit lakes of major environmental concern (Frömmichen *et al.* 2003).

Declining and closing coal mine industries world wide have caused the significant problem of abandoned coal mines to become increasingly prominent in recent years and will continue for decades to come (Black & Craw 2001). The need to prevent or mitigate the effects of acid mine problems is becoming of major international concern. Imperfectly formed coal (lignite) with a high sulphur content is mined extensively in Germany (Bachmann *et al.* 2001; Trettin *et al.* 2007), the Appalachian region of America (Viadero Jr & Tierney 2003; Simmons *et al.* 2004) and parts of Australia (Hill *et al.* 1985; Suggate 1998). This mining results in AMD and highly acidic pit lakes. Due to the generally high sulphur content of lignite mines very low pH and high metal concentrations are also a common occurrence in adjacent aquatic environments. Although most coal mined in Australia is lower in sulphur (bituminous or anthracite coal), the consequent effects of this mining are similar to those observed in regions where the coal has a high sulphur level. These effects include ecologically low pH, metal contamination and permanent acidic pit lakes.

As noted by Doupé and Lymbery (2005), the environmental consequences of coal mining issues are now becoming recognised by government, industry and the community alike. Guidelines and regulations are being put in place to prevent or alleviate adverse environmental impacts. The current knowledge of environmental concerns and effects of coal mining, however, are not always completely understood or are deliberately ignored. The Australian Coal Association's position on environmental protection is summed up with this statement from their website "Environmentally, coal mining has two important factors in its favour. It makes only temporary use of the land and produces no toxic chemical wastes" (ACA 2007). If the above statement were to be true, the need for this and other research into coal mine impacts to the environment would not exist. This is clearly not the case. For example DeNicola and Stapleton (2002) noted that AMD from coal mining degrades more than ca. 12000km of streams in the Appalachian region of North-eastern, USA. Johnson and Hallberg (2005a) highlighted that ca.19300km of streams and rivers, and ca. 72000ha of lakes and reservoirs worldwide have been affected. There have been numerous studies on impacts from coal mining practices (Castro & Moore 1997; Davis *et al.* 2006; Triantafyllidis & Skarpelis 2006). Adverse effects have been shown to come mainly from the dumping of overburden adjacent to OCM and from dewatering practices containing waste rock that when oxidised lowers pH and mobilises metals into the surrounding aquatic environment.

Western Australian coal pit lakes formed after OCM are acidic (pH 3–5) but with low concentrations of sulphate and metals when compared to elsewhere around the world (Sappal *et al.* 2000; Whisson & Evans 2003; Lund *et al.* 2006). Knoller *et al.* (2004) showed that low sulphate concentrations prevent or slow natural microbial sulphate reduction by increasing alkalinity and therefore reducing acidity in pit lakes. Nevertheless, stimulation of primary production and associated alkalinity generating processes may provide a cost effective and sustainable solution to the acidity problems (Kopacek *et al.* 2000; Lychie-Solheim *et al.* 2001; Dessouki *et al.* 2005). Evans *et al*. (2006) outlined that once acidity and related problems can be rectified, further end uses of these fresh water bodies can be investigated.

1.1.2 Background for mine pit lakes researched in this thesis

Water from three mine pit lakes in the Collie coal basin in WA were used in this research. They were WO5H (Collie Aquafarm), Chicken Creek Lake and Lake Kepwari. All these lakes are acidic but have very different physio-chemical parameters relating to the in pit water and surrounding surface and groundwater hydrology. All the lakes were previously mined for coal and are now being rehabilitated, and used as study sites for water quality remediation technologies and/or possible future end uses.

1.1.2.1 Chicken Creek Lake

This mine pit lake has been abandoned since the late 1990s. The lake contains approximately 8 million m^3 of water with high acidity (pH of approximately 3). A mine water treatment system was constructed at the Griffin Coal Mining Company's site, to remediate the acidified mine lake water and to make it suitable for aquaculture of plants crustaceans, molluscs or fish.

1.1.2.2 WO5H (Collie Aquafarm)

Mining this OCM was officially ceased in 1996. The lake is approximately 80m deep and has a pH of approximately 3. Storer (2005) utilised water from this lake remediated with a Fluidised Limestone Reactor (FLR) to trial the polyculture of fish and crustaceans within adjacent ponds and cages. Data from these trials displayed successful and commercially realistic growth rates for the two species used. Assessment of the treated pit lake water for aquaculture is an ongoing process at this pit lake.

1.1.2.3 Lake Kepwari

Lake Kepwari was the first Wesfarmers Premier Coal OCM in the Collie basin. The name Kepwari comes from the local Noongar Aboriginal name for the Collie River and means 'playing in water' (SWDC 2007). Lake Kepwari is approximately two kilometres long, one kilometre wide and 70 metres deep, and was formerly known as Western Five. Mining started in 1970, revegetation commenced in 1975 and revegetation was completed in 2004. Of the three acidic pit lakes studied, the water

quality within Lake Kepwari has the least metal and acid contamination, therefore posing the least toxicity/risk to aquatic organisms. This is due to the rapid filling of the lake through diversion of the Collie River over several years. By rapidly filling the lake the acid precipitation from oxidised pyrite occurring with intermittent wetting and drying was reduced significantly. The final pH of 4.8 and dissolved metal concentrations were improved compared to that found in the other two lakes tested in this research. Research into possible end uses of this lake include; tourism, recreational boating, skiing and fishing. The possibility of this lake being a major tourist attraction is becoming increasingly imminent and if successful will be a first for WA.

1.2 Fresh water resources

The fresh water shortage and decline in Western Australia has become all too familiar, especially in the past decade. Not only are water restrictions within the Perth metropolitan area altering the way people plant their gardens, design their homes and take their showers, but also affecting agricultural, horticulture and viticulture farmers, whose livelihoods are threatened by the current 'drying off' effect. Many solutions to the shortage of freshwater supplies are under investigation but until recently mine pit lakes were left out of the equation (Martin *et al.* 2003). Failure to monitor rehabilitate and develop mine pit lakes, can reduce the chance of utilising freshwater from the pit lake and surrounding surface and underground supplies through contamination, salinisation and evapoconcentration (Johnson & Wright 2003; Evans *et al.* 2006; Lund *et al.* 2006). Freshwater is a valuable and finite resource world wide and needs to be conserved and used wisely at all costs.

Many options for fresh water sources in and around the Perth area are being devised including; installation of desalination plants; piping of water from Lake Argyle diversion dam on the Ord River in the Kimberly and bore fields into underground aquifers. Whether any of these approaches will eventuate and address this problem in the short term is unclear. However, as revealed by Johnson and Wright (2003), there are many unused pit lakes in WA that with appropriate remediation techniques could be a feasible source of potable, agricultural or industrial water. Most of these pit

lakes are located in rural or arid areas where diversification into activities such as aquaculture and tourism could be of major benefit to communities as well as the environment. Before such uses can eventuate, however, the suitability of the mine lake water, in particular its toxicity to aquatic life, needs to be determined.

The current research used both biological and chemical toxicity assessment of treated and untreated mine pit lake water. Tests were performed with four aquatic organisms, *Ceriodaphnia* cf *dubia*, *Chlorella protothecoides*, *Tetrahymena thermophila* and *Bidyanus bidyanus,* the latter being an aquaculture species currently being farmed in Western Australia. Three mine pit lake waters were studied, Chicken Creek, WO5H and Lake Kepwari, all from the Collie Basin. Assessments of mine pit lake water toxicity before and after treatment with three different amelioration techniques, limestone addition, phosphorus addition and Rapid Catalytic Oxidation (RCO), were performed. The research entailed many experiments, all of which were conducted *in vitro.*

Acute and chronic bioassay protocols were chosen for the testing of untreated and treated mine pit lake water. The protocols used were; 48 h acute and 8 day three brood chronic *C.* cf *dubia* bioassays; 72 h chronic *C. protothecoides* bioassays (with and without nutrients), 24 h chronic *T. thermophila* bioassays and early life stage toxicity tests on *B. bidyanus*. Chicken creek water was assessed firstly, with only *C. dubia* and *T. thermophila* being used while the three bioassay species were used to assess WO5H and Lake Kepwari water. *Bidyanus bidyanus* was used to assess limestone treated Chicken creek pit lake water.

1.3 Research purpose

1.3.1 Aim

The aim of this research was to use a suitable bioassay battery for assessing toxicity of mine pit lake water and to use this battery to evaluate the efficacy of various water treatment regimes. The bioassays were used in conjunction with chemical analysis before and after water amelioration.

The biological and chemical assessment should show if risk still remains for the end uses of aquaculture and biodiversity conservation. The amelioration techniques assessed have been previously designed for mine pit lake water treatment and were undergoing trials on various pit lakes in the Collie Basin in WA.

1.3.2 Research questions

1) Do any of the pit lake water treatment modalities reduce metal concentrations to below current guideline parameters for aquaculture species and aquatic ecosystems?

2) Do any of the treatment modalities completely eliminate toxicity from mine pit lake water as shown by bioassay results compared to the bioassay control? If so, is one treatment better than the others?

3) Do any of the bioassays consistently confirm toxicity in water samples when metal concentrations exceed guideline trigger values for protection of aquaculture species and aquatic environments? Conversely, do any of the bioassays consistently confirm a lack of toxicity when all metal concentrations are below guideline trigger values?

4) Do bioassays increase the robustness of results obtained from mesocosm assessment of mine pit lake water treatment modalities?

5) Does *B. bidyanus* meet criteria to be a good bioassay candidate for assessing toxicity from limestone treated mine pit lake water for the end use of aquaculture? The selected criteria were; survival in control consistently >80%, small variation (standard error) between replicates of controls or treatments (i.e. similar growth, hatching and survival in replicates), no cannibalism and normal growth and survival in the control.

1.3.3 Thesis layout

The layout of this thesis consists of an exegesis, literature review and main body. A list of acronyms is provided as well as expansion of the acronym for its first occurrence in each chapter. The main body comprises chapters three to seven which are written in journal article format. Therefore, each of the main body chapters has its own abstract, introduction, materials and methods, results, discussion and conclusion. References from each chapter are only presented at the end of the thesis. The use of this structure was chosen to both effectively and fluently show the design and outcome of each set of experiments and to make formatting for publication straightforward. The exegesis is used to explain and tie in the outcomes and effectiveness of using bioassays for assessing the success of the treatment modalities. The research questions are addressed in the general discussion chapter to summarise the findings from the experiments.

LITERATURE REVIEW

2 Literature review

Lakes that develop in open cast mine voids are called mine pit lakes. The purpose of this review is to give a sound foundation of information and reasoning pertaining to the use of *in vitro* bioassays in assessing mine pit lake water and ameliorated mine pit lake water. The focus of the research is the use of a freshwater bioassay battery for direct toxicity and risk assessment to the aquatic environment and aquaculture from remediated mine pit lake water.

2.1 Risk from mine pit lake end uses

Lakes develop from abandoned mine pits that have been mined to below the water table. These mine pit lakes present an element of risk to the surrounding environment via contamination of in lake, natural surface and underground waters (Frömmichen *et al.* 2003). However, there are several end uses available to these mine pit lakes, all of which may need to be individually assessed for risk. Seven beneficial end uses for these mine pit lakes have been identified and a semi-quantitative potential risk has been calculated for each use (Doupé & Lymbery 2005). The lowest risk was assigned to wildlife (biodiversity) conservation. Therefore, biodiversity conservation would be the safest relinquishment option for the ecology of pit lakes and should be given the highest priority for site rehabilitation.

Biodiversity conservation through biologically representative assessment and consideration is the primary goal of ecotoxicology. Conservation of biodiversity is needed due to deforestation, industrialisation and urbanisation which are adversely affecting wetland environments and, as a consequence, the biodiversity of aquatic flora and fauna. Mine pit lakes are a potential resource for biodiversity conservation, providing wetland environments for both terrestrial and aquatic species. .

The end use of aquaculture featured central in potential risk for the seven end uses (Doupé & Lymbery 2005). Aquaculture is already being researched in many mine pit lakes world wide (Axler *et al.* 2000; Viadero Jr *et al.* 2004), including in Collie WA where this research has taken place (Storer *et al.* 2002). Aquaculture in ponds

adjacent to the mine pit lake may aid in the reduction of risk for this end use. Furthermore aquaculture if managed appropriately may be used in conjunction with biodiversity conservation as it has to in other places in Australia providing it adheres with strict regulations requiring the construction and implementation of Ecological Risk Assessments (ERA) and Environmental Management Plans (EMP) and Programs (ANZECC/ARMCANZ. 2000b; DoF 2007).

However, conducting a cost-benefit analysis of the end uses may show a more compatible choice for the community and other stake holders (Doupé & Lymbery 2005). The opportunity for combining end uses in a compatible manner may also be achievable (Evans *et al*. 2006).

In this study, a temperate Australian representative battery of bioassays was employed in conjunction with chemical analysis to evaluate the risk of remediated mine pit lake water to both aquaculture and the conservation of biodiversity.

2.2 Pit lakes

The primary concern at mine closure has been ensuring that any OCM void is geologically stable and safe to the public in accordance to government department guidelines (Johnson & Wright 2003). Currently abandonment for OCM is restricted in WA to three options;

- open voids, where nothing is done (most common);
- waste storage where backfilling of the pit occurs; and
- water storage either from dewatering operations, natural filling or from deliberate filling (Evans *et al*. 2006).

The final outcome of abandoned OCM is determined by environmental social and economical factors. These include:

- Lake area and volume – places significant limitations on possible and desirable end uses
- Water quality – affects economics of remediation and long term liability risk
- Geographical location – climate and proximity to population centres influences choice of end use

- Proximity to rivers or streams – has both negative and positive effects
- Costs of pit lake remediation/development
- Risks during remediation/development and post closure to mining companies and post-closure managers – short and long term
- Stakeholder political pressure – has both positive and negative effects
- Regulatory requirements for rehabilitation and or end use
- Support from stakeholders for end-use options
- Water resource needs at local, regional and national levels

(McCullough *et al*. (2007b)

Pit lakes, a common legacy lingering from OCM across the globe, are very prominent features within Western Australia. After mining and dewatering ceases, OCM constructed below the water table begin to fill with water creating a pit lake, with the water ranging greatly in quality and quantity between individual lakes (Shevenell *et al*. 1999). Within WA alone the range of pit lake water quality can be from hyper-saline and highly alkaline right through to fresh and highly acidic (Doupé & Lymbery 2005). The physio-chemical parameters of pit lakes depend on the geochemistry and hydrology of the pit lake and surrounding area and in most cases will continually evolve with time if not controlled (Blodau 2006). Therefore assessments of the physiochemical parameters of mine pit lakes and their effect to the aquatic environment must be continued over indefinite periods to comprehensively assess potential risk.

There are many end uses available for pit lakes with the simplest and most common end use being to do nothing. This has been the norm for most existing OCM in Australia (Evans *et al*. 2006). McNee *et al*. (2003) found that all final end uses for OCM that become pit lakes are dependant on the physio-chemical parameters, the surrounding limnology as well as current and proven remediation options. Therefore if the pit lake water parameters are not compatible with the proposed end use, remediation of the lake itself or extraction of water from the lake are alternate end use options. Either way such large volumes of fresh water should be viewed as beneficial and not problematic.

2.2.1 Pit lake beneficial end-uses

Beneficial end uses have been defined as goods or services provided by the pit lake, or any element or segment of the pit lake, which provides economic, health, welfare, safety or aesthetic benefits to the community (Johnson & Wright 2003). Seven possible beneficial end uses have been described for mine pit lakes; recreation, biodiversity conservation, stock water, plant irrigation, aquaculture, extraction of industrial chemicals and sources of potable and industrial water (Doupé & Lymbery 2005; McCullough & Lund 2006). Evans *et al.* (2006) indicated that a number of mine pit lake end uses are complementary and if required could be used simultaneously adding greater benefits to the community from both an environmental and economic perspective.

For more than a decade coal (lignite) mining pit lakes have became important water reservoirs worldwide particularly in Eastern Germany (Woelfl *et al.* 2000). However, the expectation is for the same to happen in WA. Priority needs to be given to prior planning and preparation for the possible creation of acidic mine pit lakes when OCM is going to be undertaken (Johnson & Wright 2003; Doupé & Lymbery 2005; Evans *et al.* 2005). Therefore it is important to allow for prior development of end use strategies to allow for straightforwardness of relinquishment to any future stakeholder. Of the previously mentioned end uses biodiversity conservation and aquaculture are focused on within this thesis. The term aquaculture will be used to cover both aquaculture and recreation fishery enhancement (RFE).

2.2.1.1 Aquaculture

Aquaculture is defined by Lawson (2001) as the science and technology of producing aquatic plants and animals or equally well as defined by Otchere *et al.* (2004) as the production of aquatic organisms by the deliberate and controlled manipulation of their rates of growth, mortality, and reproduction, with the ultimate goal of harvesting products of commercial value. However, commercial products are not always the goal, with some enterprises producing fish for conservation purposes, tourism, education or recreation.

Over the past 150 years, human impacts on the oceans have multiplied more than twenty-fold, approximately five-fold due to population growth and approximately 4-fold due to increased consumption and the use of environmentally destructive technologies and sociopolitical arrangements (Woodard 2000). Consequently an estimated seventy five percent of the worlds fish stocks are either fully exploited or over fished (Clover 2004). The many declining and defunct commercial fisheries of the world were once thought to be an unlimited food resource (Woodard 2000; Murphy *et al*. 2001; Clover 2004). However, commercial harvesting of the worlds oceans reached a plateau in the 1990s which was attributed to anthropogenic pressure on global fish populations (FAO 2002; 2004). Therefore, the need for research into aquaculture is becoming essential to fill the gap of seafood left by decades of overfishing.

With the world's ever escalating population and a continually increasing demand for more and healthier seafood products there is no foreseeable way that natural stocks can support this demand. To address this growing seafood shortfall, world aquaculture production has steadily increased over the past decade growing between 9-10% per year and currently employing around 7 million people (FAO 2003). Therefore as shown by aquaculture statistics collected by the FAO (2004), aquaculture of edible products are showing encouraging trends with value increasing by 16% and tonnage increasing by 21% in 2003/04 compared to equivalent data for 2002/03.

"Without water there can be no aquaculture, but water isn't always easy to find, trap or use" (Romanowski 1994). This statement applies greatly to the majority of the Australian mainland and especially to WA. However mine pit lakes offer a large and increasingly valuable freshwater resource, with a 'thirst' to be utilised and aquaculture is a fitting candidate. Species that have been identified as suitable for aquaculture or those that have already been researched and trialed in Australia are presented in Table 1.

Table 1 Species potentially suitable for mine pit lake aquaculture

Common name	Scientific name	Australian Location
Endemic fish		
murray cod	*Macquarri peellii*	NSW, VIC
barramundi	*Lates calcarifer*	WA, NT, QLD
silver perch	*Bidyanus bidyanus*	SA, NSW, VIC
freshwater cobbler	*Tandanas tandanas*	SA, NSW, VIC
freshwater cobbler (WA)	*Tandanas bostoki*	WA
silver cobbler	*Arius miglyii*	WA, NT
black bream	*Hipocanthus butcherii*	WA
Introduced fish		
redfin perch	*Perca fluviatilis*	WA
rainbow trout	*Onchorhynchus mykiss*	WA, SA, VIC, TAS
brown trout	*Salmo trutta L.*	WA, SA, VIC, TAS
Endemic crustaceans		
hairy marron	*Cherax tenuimanus*	WA
smooth marron	*Cherax cainii*	WA
yabby	*Cherax destructor / albidus*	NSW, VIC, SA
redclaw	*Cherax quadricarinatus*	NT, QLD
giant freshwater prawn	*Macrobrachium rosenbergii*	WA, NT, QLD

Many Australian species have been translocated to other Australian states in an attempt to culture them, including within mine pit lakes.

2.2.1.2 Pit Lake Aquaculture

Currently aquaculture utilising mine pit lake water is being researched and trialed around the globe. Trials and research include but are not limited to salmonid farming in Minnesota (Yokom *et al.* 1997); rainbow trout farming in West Virginia (Viadero Jr & Tierney 2003; 2004); aquaculture potential in mine pit lakes (trout and salmon) Otchere (2004) and pit lake poly culture of silver perch and marron in Western Australia (Storer 2005). Consequently with rapidly gained knowledge and experience in aquaculture operations, aquaculture is proving to have the potential to out compete and indirectly preserve wild fisheries (Whisson & Evans 2003).

Aquaculture has led to environmental problems in the past and when used in a confined area such as a pit lake it can cause the build up of nutrients and inturn can affect ground water quality (Axler *et al.* 1996). In contrast, Yokom *et al*. (1997) used the effluent from aquaculture to remediate an acidic pit lake therefore showing positive effects from aquaculture waste. Nevertheless the side effects of any from aquaculture in pit lakes would generally be constrained to the lake itself.

Consequently due to the remoteness of a large portion of pit lakes in Australia, any potentially adverse impacts derived from aquaculture would be localised and easily remediated or managed.

When aquaculture systems are isolated from open waters, environmental impacts (disease, parasites, translocation etc) do not impact on wild fish stocks or other biota (Otchere *et al.* 2004). Therefore isolated pit lakes may be positive for the environment when considering aquaculture as an end use. However adverse effects on the water quality may still have an impact on natural biota within the pit lake or to underground water reservoirs. To circumnavigate this problem, cage culture of fish can reduce nutrient input from uneaten food and fish waste by easy to manage capture and removal devices located underneath the cages (Axler *et al.* 1996).

Treated waters from active and abandoned coal mines are a potentially valuable, though underutilised resource, which may be used to expand the aquaculture industry worldwide (R. C. Viadero & A. E. Tierney 2003). Furthermore in a pilot system Storer (2005) has illustrated that limestone treated coal mining acid pit lake water can be successfully utilised to produce polyculture of marron (*Cherax tenuimanus*) and silver perch (*Bidyanus bidyanus*). The culture of these species in treated pit lake water is a first in Australia giving promising results and a consequent need for further research.

Due to water quality problems many pit lakes require remediation before the water is suitable to be used for aquaculture. This can restrict aquaculture to the outside of the mine pit lake in ponds or tanks. Although the requirement for pumping and out of pit lake remediation processes may then be necessary it is still a possible option. However the advancement of alternative sustainable livelihoods for mining communities through the development of aquaculture in pit lake water could contribute to sustainable development after the closure of mines (Otchere *et al*. 2004; McCullough & Lund 2006). A number of additional benefits to the adjacent communities include biodiversity conservation, economic diversification, increased food security and tourism.

2.2.1.3 Other pit lake uses

Although the following beneficial end uses are beyond the scope of this research they must be pointed out as they will be of future importance for mine pit lakes to WA and the world. Irrigation for horticulture and forestry is necessary in WA due to the low and erratic rainfall experienced, especially in the central and inland areas. Research and teaching through utilising such a valuable large scale water source as a research tool could be invaluable to research institutions. Recreation and tourism at many pit lakes offers a chance for the community and other stakeholders to diversify. Potable water from a pit lake could ease the pressure on the small number of current sources available in WA. Use as an industrial water source for mining industries i.e. dust suppression or for fire fighting. Stock watering sources are crucial in WA as WA is covered in remote cattle and sheep stations and cropping farms. Future chemical extraction when price fluctuations make the prospect of metal extraction from pit lake water a viable option. However, the final use chosen can influence the mining company's decision to relinquish or abandon the OCM to other stakeholders, therefore, restricting rehabilitation or further end uses for the pit lake (Evans *et al.* 2005).

2.3 Mine pit lake water quality issues

2.3.1 Acidic mine drainage and pit lakes

OCM are commonly operated below the water table and consequently at mine closure when dewatering operations cease and surface and ground waters equilibrate, the formation of pit lakes is inevitable (Johnson & Wright 2003). Acidic pit lakes and drainage are universal problems associated with the creation and relinquishment of these mines (Ramey *et al.* 1992; Harries 1997; Rolland *et al.* 2001). Data from a range of mining sites worldwide indicate that on average 90% of acidic water pollution comes from mine voids (mine pit lakes) leaving only 10% to come from waste depositories (Evans *et al.* 2006). In 1989 it was estimated that ca. 19300 km of streams and rivers and ca. 72000 ha of lakes and reservoirs worldwide had been seriously damaged by mine effluents (Johnson & Hallberg 2005a). However, as noted by Poleo *et al.* (1997) estimates of acid affected Norwegian lakes and rivers

alone exceed ca. 8,000,000 ha. Although this acidification is not just the result of mining activities it is nonetheless wrought with many of the same issues as mining related acidification, therefore requiring similar management and remediation approaches.

Commonly OCM expose pyritic rock (FeS_2) to the air causing the rock to oxidise. When the oxidised geologies come into contact with water sulphuric acid is produced. The simplified acid producing process is; iron sulphide oxidation(1), ferrous iron oxidation (2), ferric iron hydrolysis (3), and the enhanced oxidation of ferric sulphide ions (4) (Wildeman *et al.* 1993; Kalin *et al.* 2005)

Equation 1 Acid Mine Drainage generation. (Wildeman *et al.* 1993)

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O \quad (2)$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3(s) + 3H^+ \quad (3)$$

$$FeS_{2+} + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (4)$$

However, depending on the physico-chemistry of the water and rock, metal speciation following this reaction can be very complex. The resultant decreasing pH has a direct adverse effect on aquatic organisms through H^+ ions and an indirect effect through the increased bioavailability of metals as free ions (Lopes *et al.* 1999). As noted by Watten *et al.* (2005), acid generated from the oxidation of pyrite forces the solubilisation of certain base metals, including Al^{3+} and Mn^{2+}, (common in Collie OCM in WA) that contribute to acidity as well as additional solids while undergoing alternate hydrolysis reactions. Pit lake water becomes contaminated with elevated concentrations of heavy metals due to the continual processes of acid mine drainage (AMD) (Maree & du Plessis 1994; McCullough & Lund 2006).

Acid Mine Drainage problems and solutions from mine pit lakes around the world have been investigated by many authors including Harries (1997), Drury (1999), Maree *et al.* (1999) Bachmann *et al.* (2001), (Viadero Jr *et al.* 2004), Denimal *et al.* (2005), Sibrell *et al.*(2006) and McCullough & Lund (2007a). Commonly mine pit lakes affected by AMD have conditions of low pH (pH <3) and high heavy metal

concentrations. Concentrations of trace metals in most natural waters are controlled by the adsorption or coprecipitation processes commonly relating to pH (Lee *et al.* 2002). The low pH allows most metals that would otherwise not be available at higher pH values found in natural systems to become bioavailable in the mine pit lake water. Through the abandoning of OCM with acid generating geologies the inevitable result is acidic pit lakes, reduced biodiversity and limited end uses (Wenderoth & Abraham 2005). Doupé and Lymbery (2005) have highlighted that effort by mining companies into forward planing for the abandonment or relinquishment of final pit lakes, is the key to safe and practicable pit lake developments for the future.

2.3.2 Ground water

Hydrology is often a difficult parameter to understand and model. Mine pit lakes are no exception and require large amounts of time and money to research the full effects to the ground water, often with continual monitoring after abandonment. Wright (1999) noted that hydrological and chemical inputs are qualitatively different from those of natural lakes. Mine pit lake influences on hydrology can be classified in two ways; 1) under a net precipitation regime pit lakes may contribute as sources to local hydrology with either surface or ground water outflow; and 2) pit lakes may function as flowthrough ground water windows (Johnson & Wright 2003; McCullough & Lund 2006). The final water quality is controlled by the amount of oxygen contact and availability to reduced geologies that occurred during mining and continues post mining. Computational modelling is evolving quickly in the area of acid mine drainage and mine pit lakes and some of the complexities in assessing and predicting hydrological effects are being overcome (Prommer *et al*. 2000).

Current models simulate both the production and evolution of the contaminants as they migrate in groundwater, and also to evaluate remediation schemes (Prommer *et al*. 2000). However, biological and chemical processes in somewhat unknown geologies can affect the accuracy of these models (Barr & Turner 2000). Long term validation of models has not been achieved due to the relatively young age of mine

pit lakes under investigation. Nevertheless, modelling quality in pit lakes is becoming well researched (Barr & Turner 2000; Prommer *et al.* 2000; Morin & Hutt 2001; Johnson & Wright 2003; Bozau *et al.* 2007).

2.4 Risk assessment approaches

2.4.1 Chemical toxicological studies

Risk assessment for aquatic environments has traditionally been carried out through toxicological investigations based on chemical analysis (Fochtman *et al.* 2000; Chapman 2002). Chemical analysis is a simple relatively cheap and quick way of assessing the possible risk of a particular activity by inferring toxicity from the measured metal concentrations (Mitchell 2002). Results from chemical studies are used to establish trigger values for use in the management of risk of adverse environmental influences of pollutants released into aquatic environments. Nevertheless empirical methods assessing effects to the aquatic environment can be problematic not only because of bioavailability and susceptibility of organisms, but through measurement technology and detection limits, environmental conditions, and the interrelatedness of the toxicants themselves (Peakall & Burger 2003).

Toxicant trigger values form the basis of the majority of the world's guidelines for the protection of the aquatic environment, as well as for aquaculture, irrigation, stock water and potable water for human consumption (APHA 1998; ANZECC/ARMCANZ. 2000b). However Markich *et al.* (2001) has noted that trigger values are generally over protective, since only a fraction of the total metal concentration in natural waters will be bioavailable due to water quality factors such as dissolved organic carbon (DOC) and calcium hardness (Wang 1987). Although current guidelines are restricted in their application they can be adapted to situations for which they are not specifically designed, such as mine pit lakes. To get accurate data for trigger values, criteria should be researched for individual situations incorporating site specific conditions and aquatic species. While generally being over protective, chemical trigger values provide a sound precautionary approach for

protection of the ecology of the aquatic environment. This approach can make for near impossible targets for industry to achieve. Therefore, understanding the bioavailability and direct effect to aquatic organisms of toxicants is as necessary as measuring concentrations of contaminants.

Metal bioavailability is critically dependant on its physiochemical form, or speciation (Bettermann *et al.* 1996; Markich *et al.* 2001; Anderson *et al.* 2003). One model developed to predict the bioavailability and therefore toxicity from metals is the Free Ion Activity Model FIAM (Campbell & Tessier 1996). However, as noted by Peakall and Burger (2003), some complexes of metals are more readily taken up than the free ion form and complex formation can be affected by other dissolved components in addition to the well understood effects of temperature and pH. Brown and Markich (2000) went further to explain that the FIAM does not account for all toxicity and that the Biological Receptor Theory (BRT) should be incorporated with the FIAM (Extended FIAM). The Extended FIAM allows for incorporation of not only the free metal ion into the model, but also the likely formation of complexes with ligands present in the water. As a consequence, the modelling of concentration-response relationships over a wider range of water chemistry conditions (i.e. varying pH, hardness and dissolved organic matter) can be achieved compared to the original model. Accordingly the Extended FIAM model provides a more useful tool for evaluating metal-organism interactions than the FIAM alone.

Worms *et al.* (2006) have explained that even with the Extended FIAM biological responses, it has become clear that biological, physical and chemical reactions occurring in the immediate proximity of the biological surface also play an important role in controlling trace metal bioavailability through shifts in the limiting of bio-uptake fluxes. Three factors for concern were highlighted: “(i) the organism can employ a number of biological internalization strategies to get around limitations that are imposed on it by the physio-chemistry of the medium; (ii) The use of a single transport site by several metals or the use of several transport sites by a single metal further complicates the prediction of uptake or effects using simple chemical models; and (iii) once inside the micro-organism the cell is able to employ a large number of strategies including complexation, compartmentalization, efflux or the production of

extracellular ligands to minimize or optimize the reactivity of the metal" (Worms *et al.* 2006). The researchers concluded that accurate prediction of metal bioavailability will require multidisciplinary advances in our understanding of the reactions occurring at and near the biological interface.

Another useful model for predicting the interation between toxicants and aquatic organisms is the biotic ligand model (BLM). Although the BLM and the FIAM are mathematically equivalent the main difference between the two models is the ability o take competition into account since determinations of {M-Rs} in the BLM wil take competing ions into account implicitly whearas in the FIAM stability constants for the competing ions must be taken into account explicitly (Slaveykova 2005).

A clear understanding of the speciation of metals within contaminated waters can support the realisation of what is causing toxicity (Hammarstrom *et al.* 2005). PHREEQC (version 2) is a computer modelling program for simulating chemical reactions and transport processes in natural or polluted water (Parkhurst & Appelo 1999). The program is based on equilibrium chemistry of aqueous solutions interacting with minerals, gases, solid solutions, exchangers and sorption surfaces, and also includes the capability to model kinetic reactions with rate equations that are completely user-specified in the form of basic statements (Parkhurst & Appelo 1999). This program, using site specific data, can therefore give calculations of speciation which can then be compared and correlated with biological data.

Aquatic biota can be influenced through antagonistic, synergistic and complexation effects from the chemical cocktails found within the natural environment (Mitchell 2002). These mainly come from or are accelerated by anthropogenic disturbances such as those occurring in Open Cast Mining (OCM). Observations on the inaccuracies in predicting toxicity using empirical data alone has led to an increased use of bioassays, particularly bioassay tests using invertebrates, algae and fish as test organisms. Assessment of aquatic pollution can be achieved via these sensitive biological instruments far more accurately than just chemical assessment or bioavailability models alone (Bettermann *et al.* 1996; Anderson *et al.* 2003). Consequently the use of biological tests (bioassays) is currently still the most accurate means of assessing the risk from whole effluents (Chapman 2000).

2.4.2 Ecotoxicological studies

Traditional approaches to the management of water quality have grown from concerns about human health, and only recently has there been recognition of the need to use toxicity test data to determine water quality requirements for protection of aquatic ecosystems (ANZECC/ARMCANZ. 2000b). Consideration of ecological significance of test organisms has frequently been less important than utilising tests conducted with 'convenient aquatic organisms' that can be easily and readily obtained, cultured and tested. Furthermore most toxicological studies of the aquatic environment are based on the use of single species testing for screening purposes (Fochtman *et al*. 2000; Chapman 2002). Extrapolation from single laboratory species to relevant species in the field, and to whole ecosystem effects may introduce large uncertainties in the estimation of risk (ANZECC/ARMCANZ 2000).

In recent years consideration of the likely ecological effects of chemical pollutants has led to the development of a range of biological test approaches involving the exposure of a suite of aquatic organisms of ecological significance. The use of ecologically representative species and tests is also becoming more common and in doing so, combines environmental with ecological toxicology (ecotoxicology) (Mitchell 2002). Biological methods for assessing toxicity to aquatic environments have the capacity to integrate effects through continuous exposure, and measure directly the level of change at which a particular substance becomes toxic (McCullough 2006). Therefore biological testing produces a more representative ecological based 'eco' toxicity assessment of responses to toxicants, effluents and receiving waters than chemical testing (Chapman 2000; Chapman 2002).

The primary purpose of ecological investigations is to understand and explain natural phenomena and ecological processes (Underwood *et al*. 2000). Understanding of effects of perturbations of natural processes caused by pollutants is achieved through the combination of ecological and toxicological studies. Direct Toxicity Assessment (DTA) or Whole Effluent Toxicity (WET) testing as it is called in the USA, has been developed to identify, characterise and eliminate toxic effects of discharges on

aquatic receiving environments. DTA is capable of assessing the combined toxic effects of all constituents of an effluent, known or unknown (SETAC 2004). In the UK the DTA Demonstration Program Steering Group has recognised that the "simplest practicable form of life should be utilised for ecotoxicity testing" (Mitchell 2002). Therefore the use of 'simple' life forms, such as algae and protozoa as representative species, can provide responses relevant to higher trophic levels due to the inherent reliance of higher organisms on these simple forms of life (Rojickova-Padrtova *et al.* 1998; Chapman 2002).

Ecotoxicity tests using invertebrates or algae are often chosen over acute toxicity tests on fish for logistical, economical and ethical reasons (Sandbacka *et al*. 2000). However, aquatic toxicity studies on certain contaminated waters have shown fish (especially early life stages) to be more sensitive than other invertebrates or algae at differentiating toxicity (Mitchell 2002). The reasons for the sensitivity of fish to different toxicants relates to increased bio-concentration both from the environment and feeding on contaminated food sources and from the different modes of toxicity of toxicants to vertebrates (Sprague 1971; Peakall & Burger 2003). Therefore many fish species are still in use as representative indicators of risk and toxicity to the aquatic environment.

Some authors have shown that sensitivity correlation between related species is possible and from this, the new concept of using a battery of test species has been created (Fogels & Sprague 1977; Manusadzianas *et al.* 2003). The species used in a test battery should be ''representative'' of the whole ecosystem while still sufficiently sensitive to the chemical or effluent to be tested (Fochtman *et al*. 2000). Deanovic *et al.* (1999) noted that the use of the USEPA early life stage three species bioassays has been acknowledged as a cost and time effective means of evaluating the toxicity of toxicants, effluents and AMD. The early life stages approach can be a superior approach to the assessment of biological responses from different taxa and trophic levels to toxicants and effluents (Peakall & Burger 2003). Therefore, using a battery of test species relevant to the biodiversity found in a natural system, will give an improved ecologically representative response to toxicants, whole effluents and AMD (Sherry *et al*. 1997; Rojickova-Padrtova *et al.* 1998; Fochtman *et al*. 2000)

than single species alone. Tests using several species from different taxa and trophic levels were utilised in this research.

2.4.3 Bioassay analysis of mine lakes

The complexities of natural processes found in acidic mine pit lakes make the use of chemical analysis alone a very primitive and undiscerning tool for toxicity evaluation (Deanovic *et al.* 1999). Biological analysis, both *in situ* and *in vitro* has become a very important tool in understanding the complexity of toxicants in naturally and anthropogenically affected aquatic environments such as mine lakes (Handy 1994; Pereira *et al.* 2000; Peakall & Burger 2003). Prior to investing in long term remediation projects efforts should be directed at laboratory investigations of potential toxicants. These relatively quick and cheap investigations can lend support to future field studies where biological recovery will occur much more slowly (Sibrell *et al*. 2006). Remediation attempts on contaminated mine pit lakes can be managed and monitored by Toxicity Reduction Evaluation (TRE). Short term acute and chronic bioassays are commonly used in TRE (SETAC 1998).

Assessing toxicity of acidified systems which are also contaminated with other toxicants is difficult using existing toxicity tests especially when dealing with AMD (Deanovic *et al.* 1999). This is because AMD combines high heavy metal concentrations and low pH as toxicants (Lopes *et al.* 1999). If measured correctly, the response of aquatic organisms to toxicants is the most accurate way of assessing risk to the aquatic environment (Rojickova-Padrtova *et al*. 1998; Fochtman *et al.* 2000). Because responses to environmental stressors are at both biochemical and physiological levels, responses from AMD-sensitive organisms to treated AMD effluents should provide indications of responses likely to occur at all levels of biological organisation (Cole *et al.* 2001).

Studies using bioassay assessment of mine pit lake water have been recently undertaken in the USA using edemic organisms as test species (Deanovic *et al.* 1999). The need to use local species in toxicity evaluations has been emphasised

(Franklin *et al*. 1998; Mitchell 2002). Therefore, four temperate Australian representative species were chosen for the current research.

2.4.4 Species for use in the bioassays

Fresh waters around the world have different individual ionic compositions and chemical characteristics (Bayly & Williams 1973). Fresh waters in southern Western Australia have a chemical composition that is dominated by NaCl (essentially dilute seawater) which is different to fresh waters elsewhere in the world (Morrissy 2000). Elsewhere in the world fresh waters are dominated by salts derived from weathering of rocks and surface areas whereas southern WA has far older rocks that have leached the majority of these salts in the past (Bayly & Williams 1973). Local aquatic species have adapted to the specific chemical environments in which they live and are generally preferred as test species for toxicity tests. Non-endemic species may not be suitable for bioassay assessment due to their lack of adaptation to the local conditions (Chapman 2002). For these reasons it is preferable that Australian species should be utilised in Australian toxicity assessments.

There are no set guidelines for a number of species to be used in a battery of bioassays, but conventionally three of four species are chosen (Rojickova-Padrtova *et al*. 1998; Nalecz-Jawecki *et al*. 2003). The more representative the species and the end points to the test region the higher the precision of risk assessment for the aquatic environment can be achieved. However due to time and cost constraints a limit to the number of species and end points has to be determined. As long as the end points and bioassay designs chosen are relevant to the area and toxicants being tested, smaller numbers of species may prove to be sufficiently sensitive to assess toxicity and risk to the aquatic environment (Mitchell 2002). Four species were chosen for the testing of treatment amelioration effectiveness on mine pit lake water in the current research. All four species were from different taxa and distinctly different trophic levels. The four species used covered vertebrates, invertebrates, protozoa and algae allowing for a comprehensive evaluation of the direct toxicity assessment to the remediated mine pit lake waters.

2.4.4.1 Alga Chlorella protothecoides

Franklin *et al*. (1998) have noted that algae testing is not as highly regarded or utilised as their ecological importance suggests. Unicellular algae are the foundation for most aquatic ecosystem food webs and as such, any adverse impact on algae can therefore have severe effects to higher trophic levels along the food chain (Stauber *et al*. 1994). Hence using algae species in bioassays can give a sensitive indication of toxicity to the aquatic environment. *Chlorella* sp. are a sensitive freshwater species of micro algae, able to differentiate toxic waters (Parent & Campbell 1994; Franklin *et al*. 2000; Sponza 2003). The *Chlorella* sp. algae are found in many tropical and temperate fresh waters Australia wide making it a representative candidate for toxicity testing in Australia.

Unicellular algae have many advantages to toxicity testing as described by Stauber *et al*. (1994) and Franklin *et al*. (1998);

- Measure a sublethal effect (inhibition, cell division or photosynthesis)
- Short term chronic testing
- Can be cultured quickly in the laboratory, providing clones of uniform genetic composition
- Convenient, due to structural simplicity and abundance in nature including Australia
- Highly reproducible results
- Sensitivity to a wide range of organic and inorganic pollutants
- Small samples of effluents required for testing
- Identify waste waters which are biostimulatory which other toxicity tests can not provide.

There are also limitations to these algae tests ;

- Volatile substances such as chlorine may not be detected
- Use of particle counters necessitates filtration of samples possibly removing toxicity. Also they cannot differentiate between live and dead cells. However Tsvetnenko (2003) has shown that the use of a spectrophotometer can remove counting problems and give a more integrated estimate of algal biomass.
- Algae must be maintained axenically and used in the exponential growth phase (Stauber *et al*. 1994).

However, these limitations are minor and overall unicellular algae tests (especially included in a battery of bioassays) have enormous value for ecotoxicity testing.

Stauber *et al*. (1994) developed the test protocol utilised in this research. The method measures the chronic toxicity of whole effluents, specific chemicals and receiving waters to the fresh water green alga *Chlorella protothecoides* during a three day (72 h) static exposure.

2.4.4.2 Tetrahymena thermophila

The ciliated protozoan species *Tetrahymena thermophila* is found in freshwaters worldwide (Plate 2) (Pauli & Berger 2000). Ciliates constitute the largest phylum of protozoa (phylum Ciliophora, class Oligohymenophorea, subclass Hymenostomata). They are the most animal-like of the protozoa and exhibit a high level of organelle development (Ruppert & Barnes, 1991). *Tetrahymena thermophila* are a representative group within the microbial food web, play an important role in the self-purification and matter cycling of natural aquatic ecosystems and in the artificial system of sewage treatment plants (Pauli & Berger 2000).

Tetrahymena sp. ciliates have been used in toxicology for over 40 years and are the best known protozoon in laboratory use. The early use of ciliates in toxicity testing has been reviewed by Persoone & Dive (1978). Toxicity to unicellular organisms is commonly calculated by the impairment of the common end points cell proliferation, respiration or cell viability (Pauli & Berger 2000). Growth assays within this context are performed under axenic conditions, in proteose-based or synthetic media, or in media with bacteria as the sole food source (Pauli & Berger 1997).

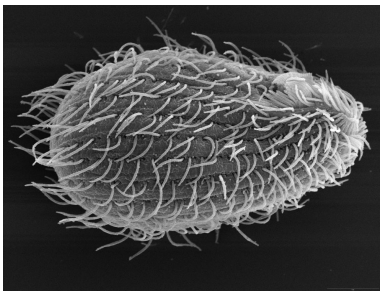


Plate 2. *Tetrahymena thermophila*, (test organism).

Source: (Ondarza 2000)

Tetrahymena thermophila has been used for DTA as an alternative assessment strategy from the use of large animal tests such as acute toxicity tests on rainbow trout (*Salmo gairdneri*) (Swift & Morgan 1983) and fathead minnows (*Pimephales promelas*) (Zischke *et al*. 1983). Ciliates such as *T. thermophila* can be maintained more easily (or dormant) and at less cost than fish cell lines (Dayeh *et al.* 2005a) or whole animals. Due to the relatively long period of time that *T. thermophila* has been used in toxicity testing there is now a large database on effects of toxicants to this species (Gallego *et al*. 2007). Ciliates do not require animal ethics approval which can be a major restricting factor in toxicity assessment species selection (Mitchell 2002). Therefore the use of *T. thermophila* as a representative of the aquatic ecosystem in DTA can be extremely useful (Pauli & Berger 2000).

The protocol for this experiment is based on the PROTOXKIT F™ Freshwater Toxicity Test, Protozoan Standard Operational Procedure (Prototoxkit F™ 1998). The Protoxkit F toxicity test is one of the many new toxkit microbiotests designed to make DTA quicker, easier and cheaper (Pauli & Berger 2000).

2.4.4.3 Ceriodaphnia cf dubia

Ceriodaphnia cf *dubia* have been used regularly in aquatic ecotoxicology in the last few decades. *Ceriodaphnia* cf *dubia* (common name; water flea) is a daphnid which is a member of the phylum Crustacea. Daphnids live exclusively in freshwater, inhabiting streams, ponds, lakes and temporary pools. “Daphnids, frequently dominate the second (herbivore) trophic level in freshwater lakes and in so doing occupy a critical position in the structure of such communities” (Keating 1985). *C.* cf *dubia* reproduce very quickly allowing them to follow their main food sources of algae and bacteria (Gooderham & Tsyrlin 2002). This rapid reproduction allows for chronic testing of this species in a short time when compared to most fishes (Environment Canada 1992).

Shiel & Dickson (1995) and Orr & Foster (1997) noted that in the life cycle of *C.* cf *dubia*, ephipia cysts are produced that are resistant to drying, freezing and digestive juices, allowing them to be distributed throughout a wide range of locations around

the earth. This is an advantage for toxicity testing as *C.* cf *dubia* can be stored as cysts ready for use when needed. Average life spans for *C.* cf *dubia* are 30 days at 25°C and 50 days at 20°C (Orr & Foster 1997). Reproduction occurs after 5 days from when neonates are released from the female.

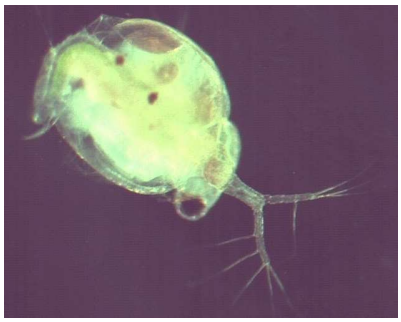


Plate 3. Adult female *Ceriodaphnia* cf *dubia* containing brood.

Ceriodaphnia cf *dubia* is commonly utilised in freshwater ecotoxicology worldwide due to the ease of culturing and handling (ASTM 2002). *Ceriodaphnia* cf *dubia* is recommended for toxicity testing in many international freshwater guidelines (Environment Canada 1992; USEPA 1994; ANZECC/ARMCANZ 2000; ASTM 2002). *Ceriodaphnia* cf *dubia* has been widely researched and now has a large database for comparison of results from similar toxicity tests and reference toxicity tests. *C.* cf *dubia* is currently used in Australia for toxicity testing of receiving waters and toxicants. Most laboratories culture their own animals depending on the requirements of the testing. *C.* cf *dubia* have been provided to the CSIRO to produce the document 'Methods of culturing and performing toxicity tests with the Australian cladoceran *Ceriodaphnia* cf *dubia*' by the Centre for Ecotoxicology (CET) (Orr & Foster 1997). The protocol for testing of *C.* cf *dubia* by Orr & Foster (1997) was used for toxicity assessment in this research due to its Australian origin.

2.4.4.4 Bidyanus bidyanus Mitchel 1838

Bidyanus bidyanus (silver perch) is an Australian native freshwater fish endemic to the Murray-Daring river system. *Bidyanus bidyanus* has been used for aquaculture in ponds since the early 1900s.

Plate 4. Adult silver perch (Bidyanus bidyanus)

The establishment of *B. bidyanus* for a DTA candidate is based on its representativeness as an Australian freshwater aquaculture species. That is, aquaculture species exhibit many characteristics that would be beneficial to toxicity testing and that in the case of mine pit lakes, aquaculture species may represent species that will be grown in the mine pit lake in the future. *Bidyanus bidyanus* demonstrates many traits required for aquaculture and therefore possibly for DTA. Further the species was used in the determining of guidelines for aquaculture species for ANZECC/ARMCANZ (2000b). Traits that make *B. bidyanus* useful for toxicity assessment include;

- established hatchery techniques
- known optimum physical parameters
- can be held in captivity in high densities
- rapid and uniform growth
- amenable to artificial feeds
- Australian native fish
- non-canabalistic
- diseases under hatchery conditions known (Rowland 1994a).

These attributes indicate *B. bidyanus* to be a good candidate for toxicity testing. Although not native to Western Australia, *B. bidyanus* is presently being cultivated in the South West of Western Australia including at a site adjacent to a mine lake in Collie. The limitations for this fish as a toxicity test candidate in Western Australia include;

- non-endemic to WA (possible translocation issues in obtaining test animals)
- limited availability, both for broodstock and larvae (seasonal)

- complicated and time consuming live feed culturing before pelletised feeding occurs
- high maintenance cost for both broodstock and larvae
- chronic testing is lengthy
- large sample quantity for testing required (natural variation)
- possible disease problems

Utilising fish as test species is an accepted way of assessing whole effluent toxicity WET (Sprague 1969 1970 1971; Zischke *et al.* 1983; Sibrell *et al.* 2006). Fish toxicity testing has been incorporated into the USEPA and OECD guidelines. There are many problems with the use of fish for toxicity testing including as noted by Franklin *et al*. (1998) including restrictions imposed by the need to comply with animal ethics regulations. Animal ethics approval is a necessary component of research and further exhibits the need for future tests to not utilise fish. However, fish are representative organisms responding sensitively to environmental toxicants (Tilton *et al*. 2003) and for this reason were included as a test species in this project.

The fathead minnow (*Pimephales promelas*) has been used extensively as a toxicity assessment organism (Zischke *et al.* 1983; Sherry *et al.* 1997; Pyle *et al.* 2002; Grippo & Heath 2003). Vittozzi and De Angelis (1991) highlighted that in nearly all cases fathead minnows were found to be the least sensitive of all fish species used for aquatic ecotoxicology. A lack of sensitivity can lead to an underestimation of the toxicity to more sensitive biota in the aquatic environment. However, there is no doubt that fish do exhibit a representative and functional position in toxicity testing when the right fish and end points are chosen. Furthermore the use of sensitive endpoints such as growth in early life stages of fish including embryo development within the egg, sac-fry and post sac-fry growth stages can give good predictions of risk assessment to the aquatic environment (Clearwater *et al.* 2002; Grippo & Heath 2003; Peakall & Burger 2003).

Recently acidic mine drainage toxicity has being assessed by different fish species including the fathead minnow (Pyle *et al*. 2002), rainbow trout (Viadero Jr & Tierney 2003) and vendance, *Coregonus albula* (Duis & Oberemm 2001). As previously

noted fathead minnows are not necessarily sensitive to all toxicants. Vendance are commonly used in effluent and toxicant testing but may not be representative to Australian conditions. Silver perch may be a possible candidate to fill the gap of a representative toxicology species where the use of freshwater from mine pit lakes could be used for aquaculture.

Furthermore fish are still a part of aquatic toxicity testing and will continue be so until a test that can completely remove the need for their unique responses to contaminants can be substituted or until animal ethics requirements prohibits their use.

2.5 Acidic mine pit lake remediation options

The old mantra 'the solution to pollution is dilution' should not apply in the present day conscience of politicians, communities and mining companies. Nevertheless, it was the main solution for AMD in the past (Kalin 2004). Cole *et al*. (2001) has noted that for decades researchers have been working to better understand the effects of acid and metal toxicity to aquatic ecosystems, and to develop ways to effectively and safely mitigate these effects. An insidious feature of AMD is that its sources (autochthonous and allochthonous) may remain active for decades or even centuries after mine closure (Maree *et al.* 1998; Sheoran & Sheoran 2006). Therefore, many remediation techniques for pit lake water and AMD are in practice as well as being researched (Johnson & Hallberg 2005a). Remediation strategies vary vastly in nature of the cost, duration and effectiveness and will differ depending on specific pit lake needs (Mays & Edwards 2001).

No treatment process is clearly leading in the permanent remediation of AMD (Maree *et al.* 1999; Mays & Edwards 2001). Doupé and Lymbery (2005) noted the best solution to any activity causing adverse affects to the environment is always prevention and this is appropriate to acid mine pit lakes. Seals for tailings dumps and other potentially acid producing areas can prevent acid and heavy metal mobilisation (Deanovic *et al.* 1999). Unfortunately with the thousands of acidic pit lakes already in existence, innovative and cost effective remediation techniques are essentially

required. Johnson and Hallberg (2005a) have identified the types of remediation processes available and divided them into chemical and biological mechanisms, with each including passive and active processes. Passive remediation techniques have been extensively reviewed by Johnson and Hallberg (2005a), Kalin (2004) and Kilborn (1996). Kalin (2004) illustrated that passive remediation techniques require small if any continued input or maintenance, whereas active processes require continual inputs for the remediation mechanism to be successful. Kalin (2004) also noted that "the solution to acid mine drainage will be found only when geomicrobiology has been fully integrated into waste treatment strategies, active/chemical treatment simply does not offer an economically or environmentally acceptable solution". Therefore, the AMD solution should lie in passive or near passive techniques for amelioration.

2.5.1 Biological treatments

The basis for bioremediation of AMD is derived from the ability of micro-organisms to generate alkalinity and immobilise metals, thereby essentially reversing the reactions responsible for the generation of AMD (Tostche *et al.* 2003). The alkalinity generating processes noted by Johnson and Hallberg (2005a) are mostly reductive processes that include denitrification, methanogenesis, and sulphate, iron and manganese reduction. Lund *et al*. (2006) have highlighted that sulphate reduction requires large quantities of labile organic material. Hence prior testing to determine efficacy and cost of preferred organic substrates is essentially required.

2.5.1.1 Wetlands

In 1984 it was noted that AMD flowing through a bog dramatically improved the quality of that water (Wildeman *et al.* 1993). In 1987, a pilot constructed wetland was built at the Big Five Tunnel in Idaho Springs, Colorado. In their handbook for wetland construction Wildeman *et al*. (1993) highlighted that this was among the first pilot systems to receive AMD. Since then there has been a great deal of interest and research into the use of constructed wetlands, both alone and in conjunction with other AMD remediation techniques (Sheoran & Sheoran 2006).

Johnson and Hallberg (2005a) indicated that aerobic wetlands are generally constructed to treat mine waters that are n*et al*kaline, due to the main remediative reaction being net acidic from the oxidation of ferrous iron and subsequent hydrolysis of the ferric iron. Research by Kalin *et al*. (2005) showed that by combining an anaerobic limestone drain (ALD) into a wetland sufficient alkalinity can be produced to counteract the iron oxidation reaction. Arsenic, a metal which is difficult to remove at any pH, as reviewed by Johnson and Hallberg (2005a) can also be removed by aerobic wetlands mainly by the adsorption onto positively charged ferric iron colloids and by the formation of scorodite ($FeAsO_4$).

Mays and Edwards (2001) and Tang (1993) noted that since wetlands act as sinks for toxic metals found in AMD, accumulation of these toxicants to levels that would adversely affect the food web and consequently the wetland performance is of growing concern. Kalin (2004) insisted that to ensure that a wetland treatment system is self sustaining and long lasting it must be protected from the metals and or inorganic pollutants contained in the water. Kalin (2004) further explained that this can naturally occur by firstly; the metals being adsorbed onto particulates either inorganic or organic forming organic metal complexes or colloids that settle to the sediment where microbial mediated biomineralisation occurs and secondly; within deeper areas of the sediment, the organically bound metals are mineralised into stable compounds.

2.5.1.2 Organic matter / compost reactors

Compost bioreactors often called anaerobic wetlands, (though most do not actually require the use of macrophytes, which can in fact introduce oxygen into the system via roots) microbially generate net alkalinity and biogenic sulphide (Johnson & Hallberg 2005a). These reactors are suitable to remediate net acidic and metal rich AMD such as that found in the Collie Coal Basin of WA.

Organic matter has been used worldwide as a carbon source for the bioremediation of many toxic effluents (Thompson 2000; Frömmichen *et al.* 2003; Fauville *et al.* 2004; Fyson *et al.* 2006). Zagury *et al*. (2006) characterised six natural sources of organic matter (carbon sources) and their suitability for treating AMD. Zagury *et al*. (2006)

found that single natural organic substrates alone were not as effective as mixtures of these substrates and that chemical characterization could not identify the source that would be most effective at metal removal.

The addition of organic matter to treat an acidic mine lake has been trialed at Ewington Lake in the Collie Coal Basin, WA by Lund *et al*. (2006) in a field-scale experiment involving treatment with *in-situ* macrocosms. A combination of municipal mulch and phosphorus additions was utilised to enhance primary production. Lund *et al*. (2006) found that the addition of carbon and phosphorus was not enough to increase pH and that limestone addition should be included into the treatment regimen. This experiment exposed the fact that a single solution to AMD is unlikely and therefore combinations of remediation technology and treatments will ultimately be the answer.

2.5.1.3 Permeable reactive barriers

These barriers are increasingly being applied to treat a wide range of polluted ground waters including AMD and use the same principles and materials of the compost reactors, such as limestone and manures. Zagury *et al*. (2006) noted that passing water to be treated through limestone (neutralizing agent), gravel (porous support) and organic matter (carbon source) without restricting the flow of water, similar reactions to that of compost bioreactors take place. Permeable Reactive Barriers (PCR) are installed in the path of the contaminated groundwater plume, and are designed to have physical characteristics similar to those in the aquifer. PCR are effective in removing metals and increasing pH of AMD but can only be used for point source effluents or influents and therefore are not suitable for most acid pit lakes.

2.5.1.4 Enhancement of primary production (nutrients)

Primary production is a small (passive) process contributing to alkalinity and heavy metal removal within pit lakes. Alkalinity can be produced when nitrate (often high in pit lakes as a result of blasting activities) is biologically assimilated followed by organic matter being produced and latter anaerobically decomposed. Phosphorus is generally a limiting factor in freshwater due to reactions with Fe and Al forming

(hydroxy) oxides (Wallihan 1948), removing phosphorus from availability which is increased within acidic pit lakes (Kopacek *et al.* 2000; Lychie-Solheim *et al.* 2001). Consequently, assimilative alkalinity-generating processes are generally more important in the early stages of nutrient amendments when sediment conditions are oxic, with dissimilative processes in later stages of nutrient amendment when sediments become anoxic (Lund *et al.* 2006). Active assimilation of heavy metals may also occur within plant cells; either directly through metabolic uptake, or incidentally through the uptake of non-essential cations as analogue ions (Lund *et al.* 2006). However, removal by biotic substrates such as algae will require conditions conducive to their growth and survival whereas inert materials do not require such optimal conditions (McCullough & Lund 2006).

2.5.2 Chemical treatments

2.5.2.1 Alkaline substances

The most widespread method used to mitigate acidic effluents was shown to be active treatment processes which involve the addition of chemical-neutralizing agents (Maree & du Plessis 1994; Johnson & Hallberg 2005a). These alkaline neutralising agents include lime (CaO), slaked lime, limestone ($CaCO_3$), sodium carbonate, sodium hydroxide, magnesium oxide and magnesium hydroxide (Maree *et al.* 1998; Cravotta III & Trahan 1999; LeFevre & Sharpe 2002; Watten *et al.* 2005). The addition of neutralising material increases pH, reduces acidity and precipitates many metals, resulting in a metal rich sludge that needs to be removed and disposed. Johnson and Hallberg (2005a) highlighted that due to high associated expense, liming of an entire pit lake has only occurred infrequently, with many of these treatments failing to achieve lasting remediation. However, lakes with low sulphur levels such as those found in the Collie Coal Basin may only need relatively small amounts of limestone for remediation to occur. Canty & Everett (2006) injected fluidised bed combustion ash directly into an acidic pit lake. This reduced metal concentrations and increased alkalinity and pH in accordance with USEPA guideline values.

The use of active fluidised limestone bed (FLB) technology has provided promising results and shown that remediation is possible with this technology (Tsvetnenko 2003; Evans *et al*. 2006). Water is pumped from the pit lake and under pressure forced to the bottom of a cone containing limestone, where the acidic water reacts with the limestone increasing pH, alkalinity and precipitating metals. The force of the water moving the limestone particles helps to reduce the armouring encountered when metals precipitate. Problems encountered included loss of limestone, a resultant sludge containing metal contamination, the cost of pumping, limestone replacement and maintenance. However, the technology was shown to be superior to oxic or anoxic limestone beds but has yet to be commercialised.

The restoration of normal biological functioning to streams polluted by AMD may require years of continuous treatment with alkaline agents, and demonstration of biological recovery requires long term monitoring of population and community level changes (2001). To decrease treatment time and to increase effectiveness, Cole et. al. (2001) designed a calcium carbonate based pulsed fluidised column treatment with a pre treatment of CO_2 to increase acidity and consequently increase the remediation rate. Intermittent pulsing fluidises limestone granules, reducing the armouring effect from Fe and Al precipitation. In the study by Cole et. al. (2001) treated effluent was routed into 568 L tanks for CO2 removal and settling of metal precipitates. The treatment system with and with out pre-treatment provided water that was non-toxic to the test animals though some parameters were outside guideline values. Sibrell *et al*. (2006) completed a similar study with a limestone based fluidised bed system. The process consistently increased alkalinity and pH above that of the source water.

Johnson and Hallberg (2005a) outlined an alternative (passive) approach for addition of alkalinity to acid waters, the use of anoxic limestone drains (ALD). However a natural flow must be present to be effective. By keeping the drain anoxic it reduces the occurrence of ferrous iron precipitating on to the limestone and causing an armouring effect. Within the drain the partial pressure of carbon dioxide is increased, accelerating the rate of limestone dissolution and consequently increasing the concentration of alkalinity, which may reach up to 275mg/L compared to an open system which in equilibrium would produce only 50-60 mg alkalinity/L (Johnson &

Hallberg 2005b). ALD also increase the efficiency of compost wetlands receiving AMD by increasing pH and removing some metals before the plants are exposed.

2.5.2.2 Phosphorus / phosphate

Phosphorus covering pyritic sediments can prevent further acid forming reactions. Phosphorus also provides nutrients for microbial remediation processes that can increase alkalinity (Lychie-Solheim *et al.* 2001). Evangelou (1998) has developed several techniques for remediation using phosphate. For example when sealing tailings with clay and other materials a layer of solid-phase phosphates (such as apatite) is added to pyritic mine waste in order to precipitate iron (III) as ferric phosphate, thereby reducing its potential to act as an oxidant of sulphide minerals. Evangelou 1998 showed that the application of soluble phosphate (together with hydrogen peroxide) oxidises pyrite, producing ferric iron, which reacts with the phosphate to produce surface protective coating of ferric phosphate. This approach prevents the future production of acidic leachates.

Davison *et al*. (1995) used the addition of phosphate to an acidified lake to increase phytoplankton production and consequently alkalinity. This technique has been combined with other treatments and used by Fyson *et al*. (1998), Lychie- Solheim *et al*. (2001) and McCullough & Lund (2007a), to increase pH and primary productivity.

2.5.2.3 Rapid filling

After mining ceases, accelerated filling diverts a large volume of water of good quality into the pit from an adjacent source such as rivers or natural runoff. Lund (2001) highlighted that the accelerated filling helps reduce ground water infiltration. Therefore water that has come in contact with oxidised geologies, containing high concentrations of metals and salts and of low pH will not enter ground water.

Trettin *et al*. (2007) assessed rapid filling of an OCM for successful prevention of AMD at the Goitsche pit lake in Germany. This pit was flooded with water from the Mulde River which neutralised the pit surface water due to a thermocline effect present within the lake. The lower layers were neutralised further as a result of

erosion of carbonate soils in the catchment area. Phosphorus, required for phytoplankton growth and therefore the incision of a food chain was reduced by 92% due to precipitation with Fe and Al. However, re-dissolution of phosphorus may occur in the future as lake sediments become anoxic.

The rapid filled pit lake remediation technique was assessed in the current research at Lake Kepwari in Collie, Western Australia. The rapid filling occurring at Lake Kepwari used water diverted from the Collie River which flows into the Wellington dam reservoir down stream from the pit lake. This reservoir is becoming increasingly saline due to clearing and farming practices within the catchment area. Additional to this problem rainfall in the entire southwest of WA is reducing and freshwater is becoming scarce leaving the use of this water for filling a mine possibly detrimental to down stream life, and for farmers relying on this water for agriculture. Johnson and Wright (2003) noted that the use of this river water has lead to the ephemeral nature of the river pools, resulting in serious biological consequences, both on animal populations and the ecology of the river system. The current use of this river water is then possibly the greater of two evils.

2.5.2.4 Rapid Catalytic Oxydation

Information for the Rapid Catalytic Oxydation (RCO) filtration treatment is via personal communications with Gheorghe Duta from Advanced Water Technologies Pty Ltd (2007). The RCO is new technology utilised as an amelioration process developed by Advanced Water Technologies Ltd Pty to treat ground or surface water to drinking water quality. The aim of the RCO is to remove metal contaminants including Fe, Mn, Al, and As using low cost oxidants and high energy efficiency so that the process can be utilised in the rural areas of developing countries. An advantage of the RCO process is that treatment takes only minutes instead of hours or days that may be restrictive for other water treatment processes. The RCO requires a pH just above 7 to operate efficiently which involves the pre use of an alkaline agent such as limestone or NaOH. Practical treatment of acidic mine pit lake water, would have used the fluidized limestone reactor (FLR detailed in 2.5.2.1) or other limestone treatment for pH correction followed by RCO. Furthermore, the requirement of an oxidising agent such as sodium hypochlorite (active constituent Cl^-

) or stable chlorine dioxide (active constituent ClO_2^-) is needed to completely oxidise metals before filtration. However, chlorine dioxide is a more powerful oxidant than sodium hypochlorite (Barbeau *et al*. 2005). Nevertheless both oxidants can leave residues toxic to the aquatic environment (Junli *et al.* 2001). Consequently other oxidants such as air at an operating pressure on average less than 1 bar (100 kPa) can be used when water will be released to the environment. The cost of oxidants can be low since air can be used as input (not always possible) or in general oxidants of much lower cost than traditional processes require. However, after RCO treatment further reduction of contaminants and in particular residual chlorine and chlorine compounds would need to be achieved. The removal of chlorine could be achieved by passing the filtered water through a shallow pond containing algae and macrophytes before being released to the environment. The RCO may be combined into sequential permeable reactive barriers for environmental remediation of AMD in the future.

Choice of remediation modality depends on the source and quantity of AMD, limnology, climate, budget, stakeholders, location, end use and environmental guidelines. Different pit lakes will therefore require different remediation approaches (Lund *et al.* 2006). The three remediation treatments chosen for assessment in this research; limestone addition, Phosphorus addition and the RCO were ones that were currently under investigation in the test area (Collie Basin) and may not be the best or quickest treatment for those pit lakes. Nevertheless, they have been successful elsewhere (Yokom *et al.* 1997; Koschorreck *et al.* 2007), except for the RCO which is new technology for mine pit lake water amelioration.

2.6 Significance of research

Environmentally sensitive areas can be affected by mixing of local water resources with contaminated mine pit waters leading to loss of biodiversity or ecosystem function (McCullough & Lund 2006). Furthermore even if mixing does not take place, native terrestrial animals may be adversely impacted by pit lake water quality. Additionally within arid regions the pit lake may also act as a source of water to feral animal populations (McCullough *et al.* 2007a). However, to achieve the goal of

relinquishment, significant rehabilitation of both adjacent terrestrial and in lake aquatic environments may be a necessity. Assessing the risk to the environment and if necessary remediation of pit lake waters is an essential process for relinquishment to stakeholders. Consequently mine pit lakes will require permanent ongoing assessment and management to continually evaluate the risk to the environment.

Western Australian fresh water resources are few and far between. The opportunity given to Western Australian communities through the relinquishment of freshwater mine pit lakes allows for the possible development of many end uses. Aquaculture world wide is expanding rapidly and is becoming a necessity to fill the gap of the declining natural fisheries (Viadero Jr *et al.* 2004). Furthermore aquaculture can provide benefits through community involvement, tourism, diversification as well as a food source. Therefore the use of well managed aquaculture projects as an end use for mine pit lakes should be considered as a valuable commodity to communities. Consequently ecotoxicity tests were used to assess remediated pit lake water for residual toxicity, so that it may be of use in future for aquaculture.

Biological assays are commonly used around the world for evaluation of toxicity of effluents and receiving waters. Currently there are no such tests specifically designed for assessment of acidic mine pit lakes or treatment ameliorated mine pit lake waters (Deanovic *et al.* 1999). Therefore the development of bioassays for mine pit lake water will play a significant role in toxicity abatement effectiveness of remediation techniques to these waters. Furthermore the use of a battery of Australian representative species to assess water quality within mine pit lakes will give a clearer picture of the overall potential effects of a mine pit lake water on the aquatic environment (SETAC 2004).

BIOASSAY ASSESSMENT OF CHICKEN CREEK ACID MINE PIT LAKE WATER

3 Bioassay assessment of Chicken Creek acid mine pit lake water

3.1 Abstract

Abandoned mine pit lakes are a legacy of the mining industry. Mine pit lakes present a unique freshwater resource to Australia which has few fresh surface waters. However, many mine pit lakes maintain a low pH and are contaminated with heavy metals, therefore requiring amelioration before realisation of any end use. *Ceriodaphnia* cf *dubia* and *Tetrahymena thermophila* bioassays combined with chemical analysis were used to assess the remediation effectiveness of three limestone treatments to acid mine pit lake water. Untreated pit lake water was acutely toxic to *C.* cf *dubia* and *T. thermophila*. Limestone addition of ≥1 g/L to the pit lake water removed toxicity to the two species indicating that limestone remediated water could be used for biodiversity conservation within treated mine pit lake water.

3.2 Introduction

Acidification is one of the most severe causes of contamination in aquatic ecosystems (Lopes *et al*. 1999). Acidification commonly comes from anthropogenic disturbance of sulphatic rocks and soils, allowing contact with air and water. Mining is the most common cause of acidification, by means of both underground and Open Cast Mines (OCM). Acidic mine pit lakes form from the abandonment of OCM and have become a legacy in Australia. OCM pit-related impacts are a long-term concern for Western Australia, as there are currently more than 1800 existing mine pits and more than 150 mines operating below the water table (Johnson & Wright 2003).

Natural freshwater bodies within Western Australia are a limited and diminishing resource (Johnson & Wright 2003). Pit lakes have the potential to become a valuable

freshwater resource with many end uses incorporating social, environmental and economic benefits. By assessing pit lake water quality and using water remediation techniques, future uses of this freshwater resource can be realised.

There have been seven beneficial end uses described for abandoned pit lakes in Australia (Doupé & Lymbery 2005). The end uses were biodiversity conservation, chemical extraction, an industrial water source, a potable water source, a livestock water source, aquaculture, recreation and tourism and irrigation. Of the seven end uses described, biodiversity conservation was considered the most beneficial through having the lowest risk. Nonetheless, the possibility of combining biodiversity conservation with one of the other end uses may be feasible and therefore of greater benefit (Evans *et al.* 2005). However, many mine pit lakes have low pH and high metal concentrations and without amelioration, restrict most end uses.

Chicken creek is an abandoned mine pit lake located in the southwest of Western Australia (Latitude 33°21’ S Longitude 116°09’ E) near the regional town of Collie. This lake has naturally filled with ground and surface waters. The Collie Basin is a coal-mining region and has many active and abandoned mine pits. Chicken Creek mine void is an abandoned acid pit lake with a ca. pH 3 and heavy metal contamination typical to other pit lakes in the Collie Basin. Concentrations of Al, Cd, Cu, Ni and Zn are commonly elevated above guidelines for water end uses.

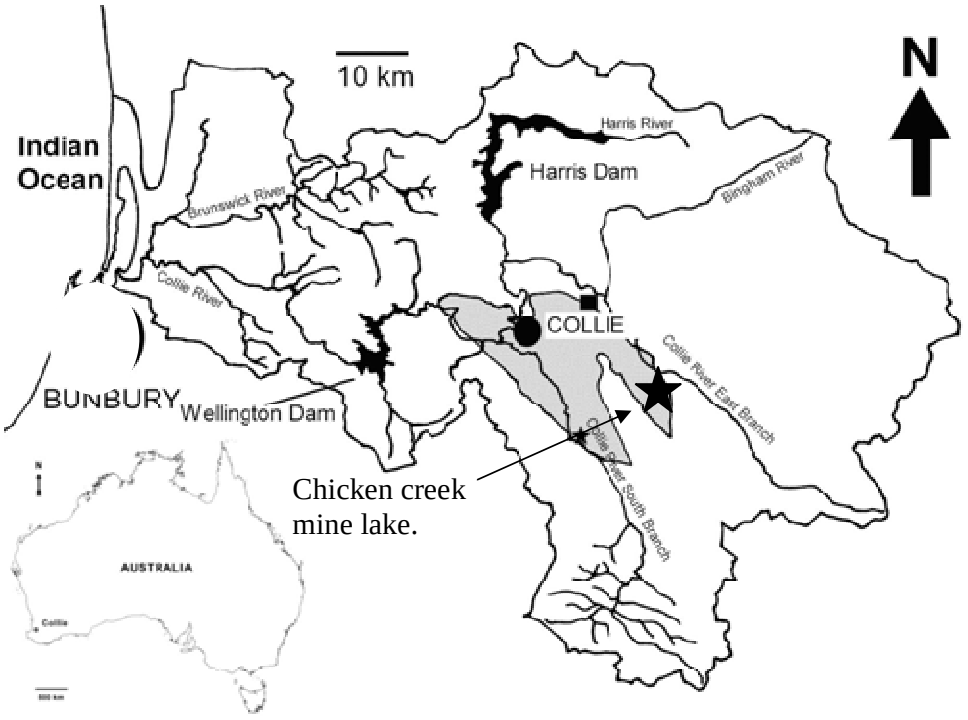


Figure 1. Location of Chicken Creek mine pit lake in Western Australia. Collie Basin indicated by grey shading. Figure adapted from Lund *et al.* (2006).

Current guidelines for assessing aquatic toxicity follow a decision tree approach for assessing risk (ANZECC/ARMCANZ. 2000b). Aquaculture guideline trigger values are based on toxicological data and the interpretation and experience of professional scientists and industry experts. Measuring total concentrations may not reflect the actual bioavailable component of the toxicant (Frömmichen *et al.* 2004). In aqueous systems, bioavailability is often correlated with the free-metal ion concentration, because the free ion is often the most bio-available form of a dissolved metal (Peakall & Burger 2003). Trigger values that are developed from toxicity tests on simple inorganic complexes, without the natural interactions of hardness and dissolved organic matter (synergistic effects) may therefore be overprotective. Nonetheless, they may also be under protective when additive (antagonistic) effects of toxicity to the aquatic environment occur.

Modification of the guidelines to account for the toxicity reducing effect of hardness on metals has been incorporated into the guidelines to increase their relevance (ANZECC/ARMCANZ. 2000b). However, there are many other factors that control

toxicity of complex mixtures found in effluents such as Acid Mine Drainage (AMD). Assessing the bioavailable portion of the effluent can be achieved by analytical measurement and thermodynamic modelling. Although these procedures can identify with some accuracy the bioavailable constituents of effluents they are still problematic (Mitchell 2002). Biological assessment using representative species can confirm the presence of toxicity in effluents, whereby chemical speciation measurements may then be taken to understand what the toxicant may be. The guidelines allow for the use of either, finding the bioavailable toxicants through calculating speciation of the mixture or through the use of biological measurement. Determining the assessment path that is followed will depend on the aims of the assessor. Ultimately biological measurement will provide absolute confirmation of toxicity from chemical measurements (Mitchell 2002).

Crustaceans are frequently used as bio-indicators and bio-monitors for various aquatic systems. Their reproduction strategies can be highly important for the interpretation of data from bio-indicator studies and for the development of ecotoxicity endpoints and guidelines. Crustaceans are a very successful group of animals distributed in marine, terrestrial and freshwater environments and provide informative candidates for comparative toxicological investigations (Rinderhagen *et al*. 2000). The crustacean *Ceriodaphnia dubia* is widely used in toxicity testing of surface waters and effluents and is found world wide as well as in Australia (Sherry *et al*. 1997; Deanovic *et al.* 1999; Kosmala *et al*. 1999; Gensemer *et al*. 2002). Tetrahymena, free-living ciliates in the class Oligohymenophorea, subclass Hymenostomatia, have been used in toxicology for over 30 years (Ruppert & Barnes 1991). *Tetrahymena thermophila* has been used as a model cellular system for studying the toxicology of pharmaceuticals for human medicine, as a bioassay tool to evaluate the safety of products for human consumption, and for an environmental or ecotoxicological purpose in order to consider the impact of ecotoxicants on different groups of organisms (Dayeh *et al.* 2005b). *Tetrahymena thermophila* is also ubiquitous in freshwater ecosystems. Therefore bioassay results obtained from these geographically and scientifically representative species will be comparable to other toxicity studies.

Currently there is research being conducted by the Centre of Excellence for Sustainable Mine Lakes (CSML) on the use of limestone ($CaCO_3$) remediation for Chicken creek pit lake. Limestone treatment is expected to remediate the toxicity of acidic pit lake water cheaply and efficiently, allowing for future uses such as biodiversity conservation, recreation, tourism, aquaculture and irrigation (Evans *et al*. 2005).

The aim of this experiment was to assess the effectiveness of a bioassay battery in differentiating remaining toxicity in acidic pit lake water remediated with three limestone treatments. Effective remediation was concluded by normal survival, growth and reproduction of the two aquatic species used when compared to the control. As well as bioassay assessment a chemical analysis of remaining metals after limestone treatment was made to compare with guidelines for the protection of aquaculture species and aquatic ecosystems.

3.3 Materials and Method

Samples were collected from approximately 15cm below the lake surface of Chicken Creek mine pit lake. Chicken Creek Lake is an abandoned open cast coal mine that has naturally filled with ground and surface water and is located in the Collie Coal basin, Western Australia. Acid Mine Drainage (AMD) has caused the water to become highly acidic (pH~3). The acidic conditions have dissolved metals from the rock strata making up the pit walls and floor, resulting in high concentrations of the metals Al, Cu, Ni and Zn within the pit lake.

3.3.1 Treatments

3.3.1.1 Trial 1. Serial dilution of Chicken Creek Lake water

A trial was conducted to determine the Lethal Concentration to 50% of the test organism's in 48 h (LC_{50}) and the Concentration Inhibiting the organisms 24 h final density by 50% (IC_{50}) for Chicken Creek lake water for *Ceriodaphnia cf dubia* and

Tetrahymena thermophila respectively. The serial dilutions of Chicken Creek mine pit lake Water (CCW) for *C.* cf *dubia* were 0% (control), 6.25, 12.5, 25, 50 and 100% replicated 4 times and for *T. thermophila* 0% (control), 5.625, 11.25, 22.5, 45 and 90% replicated 3 times. The reason for not including 100% pit lake water was due to the volume of *T. thermophila* and PPYS medium added to the *T. thermophila* test. Diluent water for the *C.* cf *dubia* test was High Hardness Combo (HHC) medium. The control for *T. thermophila* was 0.5xPPYS medium and the diluent was Milli-Q water. Mill-Q water was also used to make the HHC for *C.* cf *dubia*, because high mortality was found when using distilled water. Milli-Q or Millipore water (conductivity 0.5µS/cm) was sourced from the School of Applied Chemistry at Curtin University. All treatments were filtered to 0.2 µm before being used in toxicity testing.

3.3.1.2 Trial 2. Limestone remediation

Collie River Water (CRW) was utilised as a representative control for the pit lake region. High-Hardness Combo (HHC) medium was utilised as a control for intra and inter-laboratory comparisons in future similar studies (Baer *et al*. 1999). EDTA was removed from HHC when used for dilution due to it being a strong chelating agent (Bossuyt & Janssen 2003). Remediation treatments on the Chicken Creek acid mine pit lake water used limestone ground to a fine powder. The ground limestone was added to conical flasks and made up to 5 L with unfiltered CCW and mixed with a magnetic stirrer for 24 h. Stirred water was left to stand for 24 h to allow the resultant precipitates to settle to the bottom of the flask and for pH to stabilise. All controls and treatments were filtered to 0.45 µm using glass filter papers. Controls and treatments are stored in acid washed high density polyethylene bottles and refrigerated at <4°C until needed. Test waters are incubated to reach required test temperature and aerated for 15 minutes in 400ml conical flasks before use. The *C.* cf *dubia* bioassay controls were Collie River Water (CRW) (geographically representative control) and HHC medium (chemically defined synthetic water control) and the *T. thermophila* control was 0.5xPPYS.

Both species bioassays testing the effectiveness of the limestone amelioration used the following treatments;

LT1 = Limestone Treatment 1, addition of 1.4 g/L limestone

LT2 = Limestone Treatment 2, addition of 1.0 g/L limestone

LT3 = Limestone Treatment 3, addition of 0.6 g/L limestone

CCW = Untreated Chicken Creek acid mine pit lake water (mine pit lake control)

3.3.2 *Ceriodaphnia* cf *dubia* test protocol

3.3.2.1 Ceriodaphnia cf dubia culture procedure

Culturing of *C.* cf *dubia* is a well-known practice amongst toxicologists. Nevertheless, different protocols are still employed by different organisations, with variations mainly in diluent and culture waters. Utilising a specific diluent/control water that can be used for interlaboratory comparison tests or analysis is paramount (Grippo & Dunson 1996; ASTM 2002). The culture water used for *C.* cf *dubia* was High-Hardness COMBO (HHC) after (Baer *et al.* 1999). The HHC medium is a variation of the COMBO medium originally developed by (Kilham *et al*. 1998) for toxicity testing of algae, invertebrates and fish. By using HHC medium the need for only one culturing and diluent medium would be necessary, therefore saving time and reducing the cost of testing. The HHC medium contains EDTA which is a well known chelating agent, and can modify the toxicity of metals (Sauvant *et al*. 2000). Therefore, when HHC is used as a diluent EDTA was not added to the HHC medium.

Currently a need to culture *C.* cf *dubia* for toxicity testing has arisen from not being able to import ephipia (dormant eggs) into Australia from Belgium. The use of ephipia would remove the need for continual culture of *C.* cf *dubia*. However, assessing the animals health may require culturing for a short period before testing (EPA 2004). Protocol for the culturing of *C.* cf *dubia* for this testing is based on Orr and Foster (1997) and NSW EPA (2004).

The *C*. cf *dubia* cultures were fed two species of green algae, *Ankistrodesmus sp*. and *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata* respectively) (EPA 2004). The algae were sourced from the New South Wales Environmental Protection Agency (NSW EPA) laboratory. Both algae are grown axenically in 100 mL conical flasks for up to 7 days. Algae were transferred to 19 L carboys containing 8 L of HHC medium. Aeration was supplied via an Eterna IV aquarium air pump and filtered to 0.2µm. Algae at the exponential growth phase (5–7 days old) were centrifuged in 50ml centrifuge tubes at 4000rpm for 5mins at 20°C for *P. subcapitata* and 8min for *Ankistrodesmus* sp. The supernatant was poured off and distilled water added, with the procedure being repeated three times. The pellet was resuspended in Milli-Q water to a concentration of 6000 x 10^4 cells/ml and refrigerated at <4°C in the dark. Each algae were fed to *C.* cf *dubia* cultures at a density of 50 x 10^4 cells/ml for a combined total concentration of 100 x 10^4 cells/ml.

3.3.2.2 Ceriodaphnia cf dubia toxicity testing

Protocol for the acute testing followed the methods of Orr and Foster (1997) with the following minor modifications. *Ceriodaphnia* cf *dubia* were not fed in the 48 h test. Each treatment was replicated 4 times and contained 10 neonates born within 12 h of each other and <24 h old at the commencement of the test. Neonates were pooled from cultures into a rinse trough of their respective treatments. The neonates were checked for health (broken appendages and mobility), then randomly placed into their respective treatment wells in a plastic micro plates and covered with parafilm and a plastic lid. Plates were then placed into an incubator at 25°C with a photoperiod cycle of 16:8 h light:dark and a light intensity of 5000 lux. Mortality was assessed at 24 and 48 h with deceased animals removed at the 24 h inspection.

Mortality resulting directly from the WO5H pit lake water after amelioration with limestone was assessed by the *C*. cf *dubia* chronic three brood test. Chronic testing followed ASTM (2002) standard protocol. The three brood test used 10 replicates, with each replicate containing one neonate born within 12 h of each other and <24 h old at the commencement of the test. Test containers were 50 mL plastic sample jars containing 40ml of treatment or control water with no aeration. The *C.* cf *dubia* were

kept in an incubator at 25°C ± 1°C with a photoperiod cycle of 16:8 h light and dark. *Ceriodaphnia* cf *dubia* were individually added to test containers containing their treatment water after previous rinsing in their respective treatment waters. *Ceriodaphnia* cf *dubia* were fed *Ankistrodesmus sp*. algae at a density of $50x10^4$ cells/ml daily. Water changes were made every second day. On water change days water quality measurements were made for renewal water and for old water from test containers. The number of mortalities and the number of neonates (live and dead) produced were counted daily and deceased animals removed. The test was terminated after 8 days or when three brood had been produced in 60% of the controls (Muyssen & Janssen 2002).

3.3.3 *Tetrahymena thermophila* protocol

3.3.3.1 Tetrahymena thermophila culture procedure

A stock culture of the test subject *T. thermophila* was established from dormant animals in the test kit. *Tetrahymena thermophila* were cultured using the PPYS medium described by (Plesner *et al.* 1964; Sauvant *et al.* 2000). The stock culture media consisted of;

Component	**Amount**	**Source**
Proteose Peptone (PP)	200.0g	Sigma #123k0119
Yeast Extract (Y)	20.0g	Fluka Biochemika 70161
Sequestrene(S) (Na/Fe form)	0.30g	Sigma #063k0120

Method for making stock culture of *T. thermophila* in PPYS:

1. Combine PP with, Y and S in a one litre flask adjust to 1000ml using distilled water heat to 80°C and stir until all components are dissolved. This solution can be made to any volume by reducing all components.

2. After cooling centrifuge at 3000rpm for 20mins. Pour off the supernatant into plastic test tubes and store at –20°C until needed.

3. Before use thaw one test tube and transfer 5ml to a 250ml flask. Then add 20 ml of distilled water. This solution is now at the right concentration for culturing the ciliates (2xPPYS).

4. Add a steristopper to the flask and autoclave at 121°C for 15 mins. Allow to cool for at least 24 hours before use.

5. Inoculate the flask/test tube aseptically with ciliates from an axenic culture. Turn over cultures every 3 days into upright test tubes. If logarithmic growth is required then transfer ciliates every day and use slanted test tubes at 30°C in the dark (Gerson 1995).

The stock media was 10 times the PPYS medium recommended for culturing and 20 times that used for toxicity testing. This allowed for a dilution factor when adding the PPYS to the test containers. The PPYS medium acts as a food source as well as a medium suitable for growth and survival of *T. thermophila* (Rasmussen & Kludt 1970). The ingredients were added to 1L of MillQ water sourced from the Chemistry Department, Curtin University of Technology, Bentley, WA. Distilled water was originally trialled as a diluent but was acutely toxic to *T. thermophila*. PPYS was modified for use in testing by removing Sequestrene and dilution by 50%.

3.3.3.2 Tetrahymena thermophila toxicty testing

The protocol for this experiment was adapted from the Prototoxkit F TM Freshwater Toxicity Test with a Protozoan Standard Operational Procedure (Prototoxkit F^{TM} 1998). The same 24 h chronic test was used to assess toxicity from CCW dilutions and CCW ameliorated with limestone. Modifications to the Prototoxkit test were culturing *T. thermophila* in PPYS medium, addition of an extra replicate per treatment and the use of 0.5xPPY for toxicity testing. Addition of the extra replicate was used for both statistical robustness and to allow for of one replicate being greatly different to the other, which sometimes occurs with toxicity tests (Sprague 1969). The use of the 0.5xPPY medium provided an optimal food source for the *T. thermophila* in the 0.2 μm filtered test water which require particulate matter for food (Plesner *et al.* 1964; Rasmussen & Kludt 1970; Pauli & Berger 1997). Leaving out

the Sequestrene when used for toxicity testing removed the possibility of sequestrene from modifying metal toxicity (Sauvant *et al.* 2000).

The toxicity test was a 24 h IC_{50} with an endpoint of *T. thermophila* population density or Final *T. thermophila* Density (FTD). The test procedure was as follows;

1. Add 1.8 mL of sample effluent to test cell from dilution containers.
2. Add 100 µL of PPY to all treatments including control.
3. Add ciliate inoculum 100 µL from stock culture to equal $1x10^4$ ciliates/mL.
4. Incubate for 24 h at 30°C in the dark (gently agitate cuvettes at 20 h).

The stock culture of ciliates was counted under a compound microscope at 100 times magnification using a haemocytometer. At least 200 *T. thermophila* were counted to estimate the density. The mean from these counts was calculated and the result was recorded as $x10^4$ ciliates/mL.

3.3.4 Algae feed trial

By reducing the number of species or the amount of algae that needs to be grown for a test organism both time and money can be saved, thereby increasing the cost-benefit of the toxicity testing. The aim of this trial was to examine the effectiveness of two types of algae on growth, reproduction and survival of *C.* cf *dubia*. Both *C.* cf *dubia* and the algae were cultured in High-Hardness COMBO in separate containers. The COMBO medium was originally developed by (Kilham *et al*. 1998) as a medium for growing algae and zooplankton. However, the hardness of this medium is less than what is found in most natural freshwater environments so the HHC was developed (Baer *et al*. 1999).

The Total Organic Carbon (TOC) content was analysed by Murdoch University Marine and Freshwater Research Laboratory (MAFRL), WA. The process used the assessment of non-purgeable organic carbon (NPOC) content of the total carbon in the algae. The aim of this experiment was to determine if the recommended use of the two algae species *P. subcapitata* and *Ankistrodesmus sp.* (NSW EPA 2004) is significantly better for growth than the use of only one alga. The chronic three brood test described above for *C.* cf *dubia* was used to assess the effectiveness of the feed regime.

3.3.5 Water quality analysis

Water quality for the dilution testing was measured in each treatment before transferring to each replicate container at time 0. A single measurement was performed due to the small size of the test wells and cuvettes used for testing making water quality measurement very difficult. Water quality recorded for *C.* cf *dubia* three brood testing was measured at each water change and a mean figure was calculated for each parameter, except for hardness and alkalinity which were only measured at time 0. Total hardness and alkalinity were measured by a Haggen Aquatic Research Station (HARS), Nutrafin test kit. The pH was measured by a hand held Eco–Scan pH6 portable pH meter. The pH was calibrated daily using pH 4, 7 and 10 standards. Temperature was also measured by the pH meter to an accuracy of 0.1°C. The dissolved oxygen was measured via a Jenway Benchtop Electrochemical Analyser Model 3410. Treatment water metal and metaloid concentrations were analysed by the Marine and Freshwater Research Laboratory (MAFRL), located at Murdoch University Western Australia by inductively coupled plasma–atomic emission spectrometry (ICP–AES). The metals analysed were As, Al, Ca, Cd, Cr, Co, Cu, Fe, Hg, Mg, Mn, Ni, Pb, Se and Zn. Samples were acidified before analysis.

Guideline trigger values used in data analysis are for slightly–moderately disturbed aquatic ecosystems for the protection of 95% of species with 50% confidence as set by ANZECC/ARMCANZ (2000b). Hardness adjustment of the trigger values was calculated by equation 2,

Equation 2. Hardness modifying algorithm

$$HMGV = GV*(H/30)^{a}$$

where HMGV is the hardness modified guideline value (μg/L), GV is the guideline value (μg/L) at a hardness of 30 mg/L as $CaCO_3$, H is the measured hardness (mg/L as $CaCO_3$) of a fresh surface water and a is a constant (ie. slope factor) (Markich *et al*. 2001).

3.3.6 Data analysis

Results for mortality of *C.* c.f. *dubia* bioassays were arcsine transformed before statistical analysis (USEPA 1998). When *C.* c.f. *dubia* data were still not normally distributed ($P<0.05$), data were transformed to rankits before analysis by ANOVA (Pereira *et al*. 2000). Calculation of the Lethal Concentration and Inhibition Concentration values for the two species was done by probit analysis by computer program (Tsvetnenko unpublished) using equations developed by Christensen & Nyholm (1984). Calculation of the intrinsic rate of natural increase for *C.* cf *dubia* was also by computer program after Taberner *et al* (1993).

Statistical analysis of bioassay results was performed using the statistical program SPSS 14.0 for windows (2005). Normality was checked using the Shapiro-Wilks test (Shapiro & Wilk 1965). Non-normally distributed data was checked for homogeneity of variances using Levene's test (Levene 1960). Significant differences found from statistical tests were all at the $P <0.05$ level. Means, standard errors and confidence intervals were calculated by SPSS or Microsoft Excel. When the assumption of normality was met for bioassay data, a one-way analysis of variance (ANOVA) was

performed where appropriate to explore for significant differences between treatments and controls. When significant differences were found, a pair-wise post-hoc test was performed to identify where the differences occurred. The parametric Tamhane post-hoc test was used when variances were not equal and the Dunnett (Dunnett 1980) and Student-Newman-Keul (S-N-K) Range Test (Newman 1939; Keuls 1952) when variances were equal. When data were not normally distributed the non-parametric Kruskal-Wallis H Test was used to find median differences between treatments and controls.

The no observable effect concentration (NOEC) is the highest concentration not giving a statistically significant result compared to the control and the lowest observable effect concentration (LOEC) is the lowest concentration giving a significantly lower effect than the control (OECD/OCDE 1998). The NOEC and LOEC values were calculated from Dunnett's test. Significance levels were tested at the $P<0.05$ level unless otherwise indicated in the results.

3.4 Results

3.4.1 Algae feed trial

The TOC content of *P. subcapitata* was higher than that of *Ankistrodesmus* sp. Both nomographs produced from serial dilutions of each alga had R^2 values of 1.00 (Figure 2). Results from the *C.* cf *dubia* three brood assessment of algae feed efficacy are presented in Figure 2. The intrinsic rate of natural increase was calculated for each treatment with both the mean number of brood and the IRNI showing *P. subcapitata* alone to be less effective for reproduction of *C.* cf *dubia* (Table 2). Using both species of algae produced the equal highest intrinsic rate of natural increase (IRNI) followed by *Ankistrodesmus* sp. alone. The combination of both algae is used by others as a food source in *C.* cf *dubia* and *Daphnia magna* culturing (NSW EPA 2004). A previous test result using Collie River water and only *P. subcapitata* algae gave the result of 0.203 ± 0.033. This is comparable to the result

obtained for *P. subcapitata* from this current study. The combination of the two algae species used in the test almost doubled the IRNI when compared to using *P. subcapitata* alone. Survival with only *P. subcapitata* was also lower that that of the other two treatments.

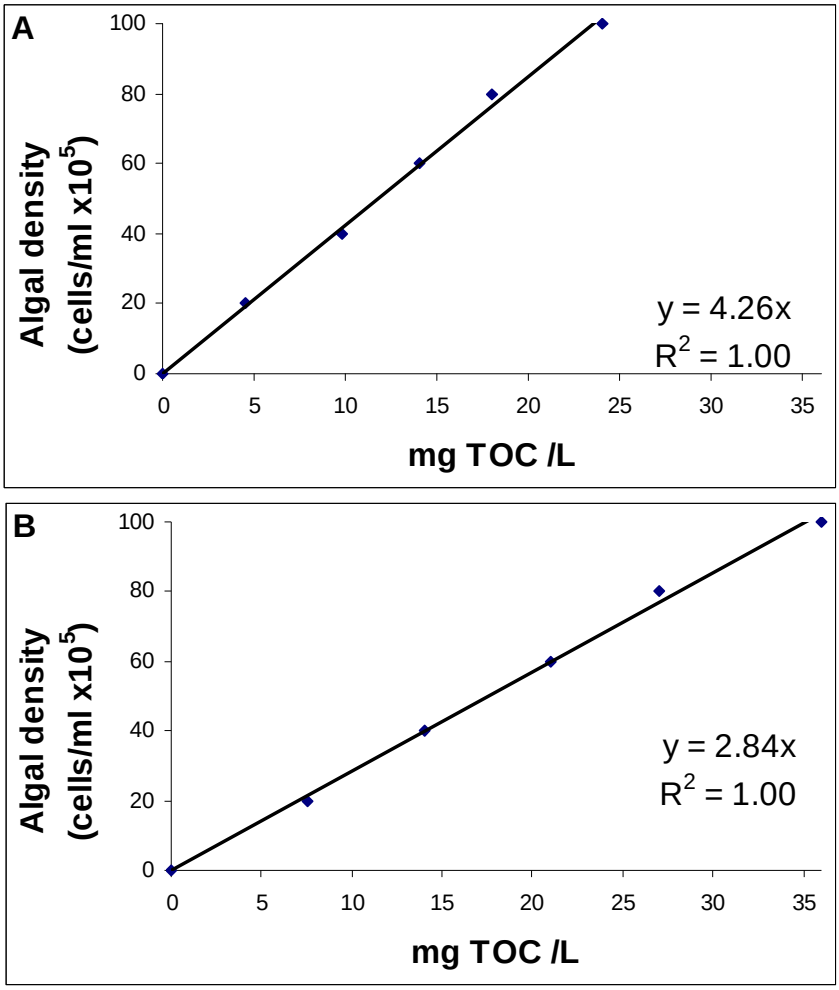


Figure 2. Nomograph of A) *Ankistrodesmus* sp. and B) *Pseudokirchneriella subcapitata*.

Table 2. Results from the *C.* cf *dubia* three brood algae food test. Mean brood produced at 8 days ± standard error. Different lowercase letters indicate significant differences. IRNI is Intrinsic Rate of Natural Increase.

Treatment (food type)	Mean number per brood	Survival (number)	IRNI
P. subcapitata & *Ankistrodesmus sp.*	21 ± 2.1 [a]	10	0.436 ± 0.22[a]
P. subcapitata	11 ± 3.4 [b]	8	0.225 ± 0.29[b]
Ankistrodesmus sp.	20 ± 2.9 [a]	10	0.416 ± 0.19[a]

3.5 Conclusion of algae test

Ankistrodesmus was nearly as effective a feed for C. cf dubia for reproduction and survival. Therefore the use of only Ankistrodesmus algae only was deemed more efficient than the use of both algae for feeding during experiments.

3.5.1 Water quality

3.5.1.1 Bioassay water quality

Water quality from the filtered CCW dilution 48 h *C.* cf *dubia* and 24 h *T. thermophila* bioassay testing at time 0 is presented in Table 3 and water quality from the *C.* cf *dubia* three brood and 24 *T. thermophila* limestone amelioration testing is presented in Table 4. DO was close to 100% in all treatments. Temperature for both species tests was controlled by an incubator which maintained temperature to within 0.1°C of their respective required temperatures. Alkalinity in the serial dilutions of CCW was very low at <10 mg/L in all dilutions except for in the *C.* cf *dubia* 6.25% CCW. The standard error for pH in the *C.* cf *dubia* three brood test did not vary by more than 0.2 units. Hardness, pH and alkalinity were not matched to that of the original lake water as overall toxicity due to all these factors combined without altering speciation and water chemistry, not just chemical toxicity was the aim of assesssing the treated water.

Table 3. Water quality from the 48 h *C.* cf *dubia* and 24 h *T. thermophila* serial dilution tests measured at time 0.

Treatment	pH	Temperature (℃)	Dissolved Oxygen (%)	Alkalinity (mg/L)	Total hardness (mg/L)
C.* cf *dubia					
CRW	7.7	25 ± 0.1	98	70	170
HHC	7.2	25 ± 0.1	101	20	130
6.25% CCW	6.1	25 ± 0.1	99	20	160
12.5% CCW	5.7	25 ± 0.1	101	<10	200
25% CCW	5.4	25 ± 0.1	97	<10	240
50% CCW	4.9	25 ± 0.1	98	<10	280
100% CCW	3.4	25 ± 0.1	93	<10	420
T. thermophila					
TTC	7.5	30 ± 0.1	97	<10	60
5.625% CCW	6.9	30 ± 0.1	98	<10	140
11.25% CCW	6.4	30 ± 0.1	95	<10	140
22.5% CCW	5.9	30 ± 0.1	99	<10	160
45% CCW	5.3	30 ± 0.1	96	<10	240
90% CCW	3.5	30 ± 0.1	96	<10	400

Table 4. Water quality from the *C.* cf *dubia* three brood test (mean ± SE) and from the 24 h *T. thermophila* test. Alkalinity and hardness are measured at time 0.

Treatment	pH	Temperature (℃)	Dissolved Oxygen (%)	Alkalinity (mg/L)	Total hardness (mg/L)
C.* cf *dubia					
CRW	7.7 ± 0.2	25 ± 0.1	98 ± 7	70	170
HHC	7.2 ± 0.1	25 ± 0.1	101 ± 5	20	130
LT1	8.0 ± 0.1	25 ± 0.1	97 ± 5	80	640
LT2	7.8 ± 0.1	25 ± 0.1	95 ± 8	80	640
LT3	7.7 ± 0.2	25 ± 0.1	99 ± 6	50	540
CCW	3.4	25 ± 0.1	93 ± 6	<10	420
T. thermophila					
TTC	7.5	30 ± 0.1	94	<10	60
LT1	7.9	30 ± 0.1	93	60	620
LT2	7.8	30 ± 0.1	96	60	620
LT3	7.7	30 ± 0.1	94	50	500
CCW	3.5	30 ± 0.1	95	<10	400

3.5.1.2 Chemical analysis

At the commencement of the trial Al, Cd, Cu, Ni and Zn concentrations in untreated Chicken Creek lake water were above the aquatic ecosystem guideline values and all but Cd were also above the aquaculture guidelines (Table 5) (ANZECC/ARMCANZ. 2000b).The Al, Cd, Cu, Ni and Zn concentrations were higher in CCW than that in CRW, which only had an elevated Cu concentration above the aquaculture guideline. Some dissolved concentrations of metals were still above the trigger values for the protection of aquaculture species and for the protection of aquatic ecosystems after limestone treatment. None of the limestone treatments reduced Zn and Ni concentrations to below the aquaculture or aquatic ecosystem guideline values and Cd was not reduced to below the aquatic ecosystem guidelines. Limestone treatments ≥1 g/L did not reduce the Al concentration to below the aquaculture guideline values. The LT2 Cu concentration and LT1 Al and Cu concentrations also remained above the aquatic ecosystem guideline values.

After HMGV were applied, Cu, Ni and Zn concentrations still remained elevated in CCW (Table 5). The Cu concentration remained higher than the HMGV in CRW but all other metals were below the guidelines in CRW. The limestone treatments did not reduce the Ni and Zn concentrations to below the HMGV, except for LT1. The concentration of Ca was lower in treatment LT3 than that of treatments LT1 and LT2. The Ca concentration in CCW was lower than that in CRW and the limestone treatments.

Table 5 Dissolved metals and metaloid concentrations in µg/L, except for Ca and Mg which are mg/L. Values exceeding guideline values for the protection of aquaculture species are denoted by a *, protection of 95% of a slightly–moderately disturbed freshwater aquatic environment are denoted by bolding, HMGV for aquatic ecosystems are denoted by a subscript † preceding the figure. Guidelines that are missing are not yet available.

Treatment	Al	Ca	Cd	Cu	Mg	Ni	Zn
CRW	<10	24	<0.6	†**10***	50	<4	<2
LT1	**80***	140	**0.8**	**2**	75	**140***	**100***
LT2	40*	140	**0.8**	**2**	78	†**160***	†**170***
LT3	<10	110	**1.2**	1	76	†**170***	†**440***
CCW	**18000***	18	**1.6**	†**19***	63	†**200***	†**990***
Aquaculture	30 (pH>6.5) 10 (pH<6.5)	-	0.2 - 1.8	5	-	100	5
Aquatic ecosystems	55	-	0.2	1.4	-	11	8
HMGV for Aquatic ecosystems							
CRW	-	-	1	6	-	46	33
LT1	-	-	3	19	-	148	108
LT2	-	-	3	18	-	144	96
LT3	-	-	2	15	-	120	87
CCW	-	-	2	13	-	104	75

3.5.2 *Ceriodaphnia* cf *dubia* bioassay results

3.5.2.1 Ceriodaphnia cf dubia serial dilution test (48 h LC50)

The *C.* cf *dubia* 48 h LC_{50} and 95% Confidence Limits in parenthesis, calculated from the serial dilution of CCW were 23% (19–27%) and the LC_{10} was 7% (6–9%) (Figure 3). The 48 h NOEC was 6.25% CCW and the LOEC was 12.5% CCW.

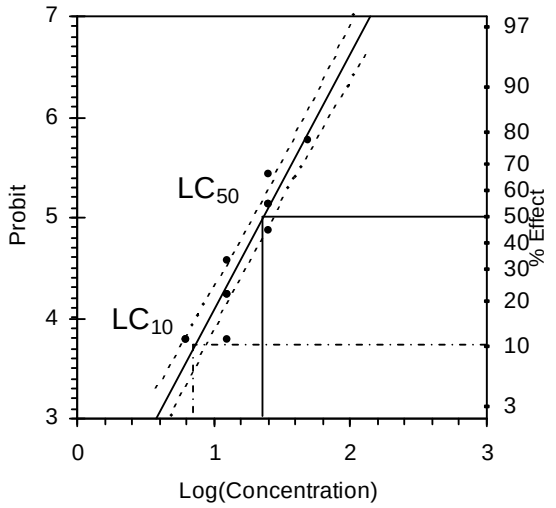


Figure 3. *Ceriodapnia dubia* 48-h serial dilution test mean mortality (log concentration) and 95% confidence interval located either side of the predicted mortality line. The LC_{50} is indicated by a solid line and the LC_{10} is indicated by a dotted and dashed line.

3.5.2.2 Ceriodaphnia cf dubia 48 h acute limestone treatment assessment

Ceriodaphnia cf *dubia* survival results in limestone treated CCW are presented in Figure 4. Complete *C.* cf *dubia* mortality was exhibited in the CCW sample no treatment within 24 h. Survival of *C.* cf *dubia* in the two controls was similar to each other with 95±3% in CRW and 90±6% in HHC. *Ceriodaphnia* cf *dubia* survival in LT3 (72.5±6%) was significantly lower ($F_{(4, 15)}$=4.2, p=0.018) than that of the other limestone treatments and the controls.

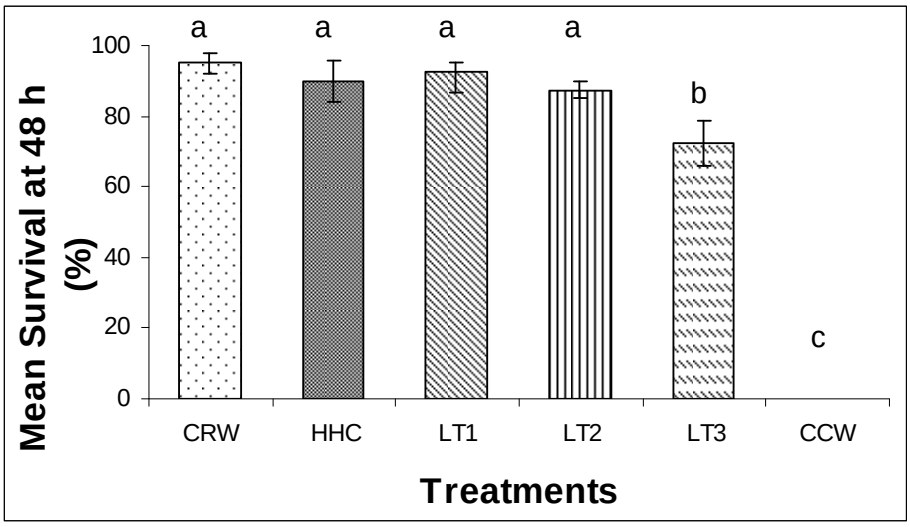


Figure 4. *Ceriodaphnia* cf *dubia* 48-h % mean mortality ± standard error %. Different lowercase letters indicate significant differences.

3.5.2.3 Ceriodaphnia cf dubia three brood test

The mean number of brood, survival and the IRNI at day 8 were similar in the controls and the treatments LT1 and LT2 (Table 6). The first offspring were noted on day 3 in all controls and treatments except LT3 where offspring were not produced until day 5. The controls and treatments met the criteria for a successful test with the exception of LT3. Even though there was a low survival of *C.* cf *dubia* in LT3 some offspring were still produced in the 8 day period.

Table 6. *C.* cf *dubia* three brood test mean brood produced at 8 days± standard error. Different lowercase letters indicate significant differences. IRNI is Intrinsic Rate of Natural Increase.

Treatment	Number of brood	Survival	IRNI
CRW	17±2.2[a]	9	0.490±0.026
HHC	20±2.4[a]	9	0.497±0.028
LT1	18±2.6[a]	9	0.497±0.026
LT2	15±2.8[a]	8	0.454±0.035
LT3	2±1.7[b]	3	0.125±0.208

3.5.3 *Tetrahymena thermophila* chronic testing

3.5.3.1 Tetrahymena thermophila serial dilution of CCW

The calculated *T. thermophila* 24 h IC_{50} was 36% CCW with a 95% CL of 29–45% and the IC_{10} was 5% CCW with a 95% CL of 4–7% (Figure 5). The NOEC was 5.6% CCW and the LOEC was 11.2% CCW. All FTD in CCW dilutions were significantly lower ($F_{(5,12)}$=108, p=0.001) that of the control with the exception of the 5.6% CCW dilution.

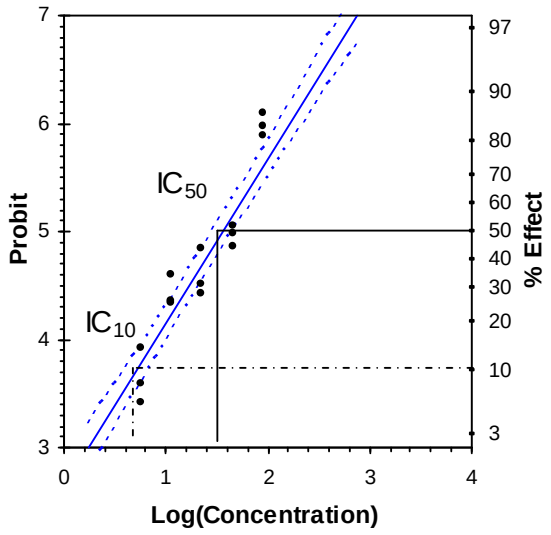


Figure 5. *Tetrahymena thermophila* 24 h IC_{50} (bolded solid line) and IC_{10} (dot and dashed line). Predicted growth inhibition is indicated by solid blue line and is bounded by the 95% confidence levels (dashed lines).

3.5.3.2 Tetrahymena thermophila limestone treatment test.

The results for the limestone treatment test on *T. thermophila* are displayed in Figure 6. The FTD in the treatments LT1 and LT2 was similar to that of the control. The FTD in LT3 was significantly lower ($F_{(4,10)}$=519, p=0.001) than that of the control and the other treatments. The FTD in CCW did not increase from the time 0 density of $1x10^4$ *T. thermophila* /mL.

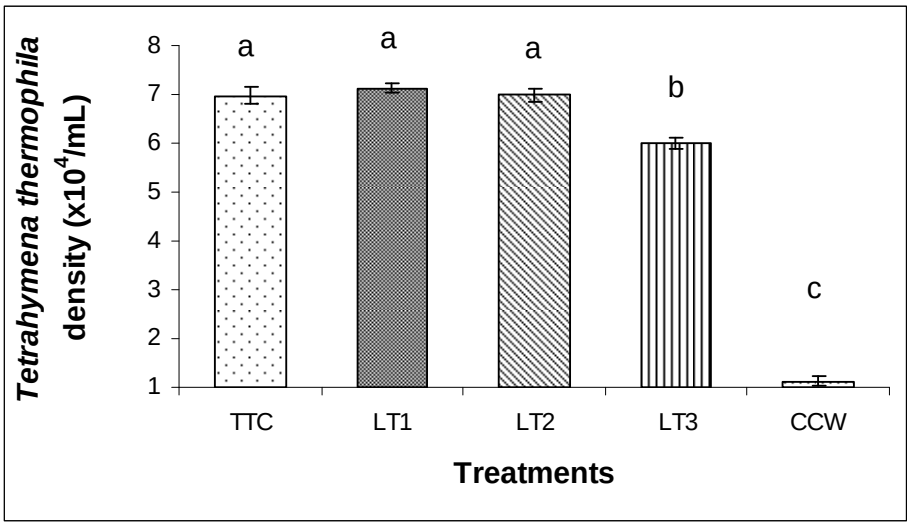


Figure 6. *Tetrahymena thermophila* mean final density (number x10^4/mL) ± standard error. Different lowercase letters indicate significant differences. Graph starts at 1x10^4/mL due to it being the starting density.

3.6 Discussion

3.6.1.1 Water quality and chemical analysis

The aquaculture guidelines for Australia and New Zealand are a relatively new development (ANZECC/ARMCANZ. 2000b). They have drawn extensively on overseas' guidelines for aquaculture as well as on the personal experiences of a number of local scientific and industry specialists. There are many toxicants, metals and chemicals that do not have guidelines and those that do are adapted from guidelines elsewhere. Both the aquaculture and aquatic ecosystem guidelines are missing trigger values for commonly occurring metals and still require investigation into the construction of these trigger values. The new addition of incorporating site specific hardness into the guidelines increases the relevance of the guidelines to the natural environment (Markich *et al.* 2001). Nevertheless, inclusion of more site specific parameters such as dissolved organic matter and pH into the guidelines will have to eventuate, in order to significantly reduce over or under protection of the aquatic ecosystem as well as for other aquatic end uses.

Bioassay parameters of temperature and DO were within those recommended by ASTM (2002) for *C.* cf *dubia*. The pH fluctuated more than that recommended (0.15

units) over the three brood test by up to 0.3 units. However, this was still considered acceptable without adding acids or bases to maintain the pH. The 48 h acute *C.* cf *dubia* test met the criteria for an acceptable test (Orr & Foster 1997). Further the three brood test met the criteria for an acceptable test including <20% of the first generation organisms dying in the control and >60% of the control organisms producing on average 15 brood within 8 days (ASTM 2002). Other than temperature which was maintained to within 0.1°C, ideal parameters for the *T. thermophila* testing were not suggested by (Prototoxkit F^{TM} 1998). Nevertheless, due to the short duration of testing, water quality should not have changed significantly over the 24 h test.

The three limestone treatments to CCW maintained the pH at above 7, which is within the range recommended for aquatic ecosystems. Raising the pH to the circum-neutral value precipitates most heavy metals and lowers the bioavailable proportion of the remaining metal concentrations. Nevertheless, some metals including Zn may still have an adverse affect to aquatic organisms at pH between 7 and 8 (Hyne *et al.* 2005).

The concentrations of the metals Al, Cd, Cu, Ni and Zn in untreated Chicken Creek mine pit lake water were elevated above the aquatic ecosystem guideline values. A combination of the elevated concentration of some metals with a low pH was most likely responsible for toxicity to the test species. The free cadmium ion (Cd^{+2}) is the form of cadmium primarily responsible for eliciting a toxic response in aquatic organisms with toxicity reducing with increasing hardness (Campbell 1995). Cd^{+2} is the predominant species of dissolved cadmium in fresh surface waters at pH ≤8.5 (Spry & Wiener 1991). The acute toxicity of copper for ten Australian species including *C. cf dubia* was found to range from 200 µg/L to 7800 µg/L (Skidmore & Firth 1983) which are far higher concentrations than that found in the CCW or the CRW. Although the Cd and Cu concentrations in the limestone treated CCW were above the Aquatic ecosystem guideline values they would not likely cause toxicity individually. However, recent research on C. cf dubia has shown that hardness may not be effective in reducing the toxicity from these metals {Markich, 2001 #360}. Ni

is moderately toxic to freshwater organisms, with acute LC_{50} values ranging from 510 μg/L for a cladoceran to 43 000 μg/L for fish at low hardness (ANZECC/ARMCANZ. 2000a). In natural waters at pH ≤8.5, the predominant Zinc species is Zn^{+2} which the form of zinc primarily responsible for eliciting a toxic response in aquatic organisms. Both Ni and Zn concentrations in LT2 and LT3 were still above the HMGV indicating the possibility of remaining toxicity at the pH between 7 and 8.

The dissolved concentration of Al (18 000 μg/L) was 1 800 times the aquatic ecosystem guideline value for Al at a pH <6.5. This large Al concentration would be acutely toxic to most freshwater life (Gensemer & Playle 1999) which was demonstrated in both bioassays. The Al concentration remained elevated in ≥1 g/L limestone treated CCW for aquaculture guidelines, but only LT1 was above the aquatic ecosystem guideline and therefore, may still be capable of causing toxicity to aquatic ecosystems.

The water used in the testing was filtered to 0.45 μm to remove algae and bacteria that may interfere with test results, but also removed most suspended solids. In the natural environment suspended organic matter has the ability to adsorb dissolved metals consequently removing them and reducing their bioavailability and possible toxicity (Spry & Wiener 1991). However, if the suspended particulates are consumed by the organism, such as in the case of both the species used in this testing, this fraction may then contribute further to toxicity (ANZECC/ARMCANZ. 2000a).

3.6.1.2 Ceriodaphnia cf dubia food test

An assessment of the feeding regime for *C.* cf *dubia* was made to find which of two algae species both separate and together produced the highest survival and reproduction from a three brood test. A nomograph of TOC results showed that *P. subcapitata* contained the largest proportion of TOC per cell density. Nevertheless, this higher carbon content in *P. subcapitata* did not result in a higher IRNI than that produced by *Ankistrodesmus sp.* alone. The use of both algae as food for *C.* cf *dubia* did not produce a significantly better number of brood, survival or IRNI than

Ankistrodesmus sp. alone. Therefore, to save time and money only *Ankistrodesmus sp.* was fed to *C.* cf *dubia* during three brood testing.

3.6.1.3 Bioassay responses to untreated and limestone treated CCW

The 48 h LC_{50} for *C.* cf *dubia* was lower than the 24 IC_{50} for *T. thermophila*, indicating that the 48 h *C.* cf *dubia* bioassay is slightly more sensitive to the CCW, even though the 24 h *T. thermophila* test is a chronic test. Nevertheless, the LOEC and the NOEC for both species was similar. Results from the dilution test indicate that a high dilution of the CCW would be required to remove toxicity to these organisms. Rapid filling of pit lakes from captured surface water and from river diversion has occurred in several countries to dilute and reduce oxidation of pyritic rock strata (Lund *et al.* 2006; Trettin *et al.* 2007). Previous rapid filling of a mine pit in the Collie Basin produced a lake with pH 4.8 and with lower metal concentrations than adjacent similar pit lakes that were not rapid filled (Lund 2001). Nonetheless, in areas that already have small and diminishing freshwater resources the use of rivers for rapid filling may cause further detrimental affects to the natural aquatic environment (Johnson & Wright 2003). Therefore, the use of other remediation techniques that don't affect adjacent natural aquatic or terrestrial ecosystems would be preferable.

Although all limestone treatments of the Chicken Creek pit lake water brought the pH to above neutral, there was still an underlying toxicity in the lowest limestone treatment. The bioassay results showed LT3 to be significantly lower for *C.* cf *dubia* survival and reproduction and significantly lower for *T. thermophila* final density. Although Al was still above the guidelines in ≥1 g/L limestone treated CCW it did not exhibit toxicity to either species when compared to the controls. In acidified Al rich freshwater, positively charged Al-species are the key toxic components due to accumulation in gills (Teien *et al.* 2006). The uptake and toxicity of aluminium in freshwater organisms generally decreases with increasing water hardness under acidic, neutral and alkaline conditions indicating that the high hardness >600 mg/L would have reduced the bioavailability of the Al. The Al concentration in LT3 was below the detection limit (<10 μg/L) and would therefore not have contributed to the

toxicity exhibited from this treatment to both organisms. Al solubility is the lowest between pH 6 and 7 with 80% existing as a colloidal solid $Al(OH)_3$ (Peakall & Burger 2003) further indicating that the Al present in ≥1 g/L limestone treated CCW would not be toxic.

The Ni concentration remaining in LT2 and LT3 was above the HMGV and therefore is likely to have caused toxicity. However, the LT2 Ni concentration did not cause toxicity in any of the bioassays. Acute LC_{50} values for Ni range from 510 μg/L for a cladoceran to 43 000 μg/L for fish at low hardness (ANZECC/ARMCANZ. 2000a), which are concentrations from 5 to 300 times greater than the concentration remaining in the limestone treated CCW. Even though the Ni concentration was elevated above the HMGV it most likely did not cause the toxic response in LT3 alone.

The Zn concentration remaining in LT3 may have been sufficient to cause toxicity to *C*. cf *dubia* and *T. thermophila* alone. Acute toxicities for Australian freshwater species ranged from 140 μg/L to 6900 μg/L (ANZECC/ARMCANZ. 2000a) which was comparable with the 440 μg/L remaining in LT3. Zinc toxicity for *C*. cf *dubia* has been reported to be reduced by a factor of two, with an increase in water hardness from 44 to 374 mg $CaCO_3$/L (Hyne *et al.* 2005). The NOEC for Zn to *C.* cf *dubia* has been established as 5.5 μg/L (ANZECC/ARMCANZ. 2000a). Therefore indicating that with a factor two increase the trigger value would be approximately 10 μg/L, which is five times less than the expected HMTV calculation. This suggests that high hardness may not be contributing significantly to protecting C. cf dubia from the measured concentration of Zn. Zn has been found to increase in toxicity from pH 7 to 8.2 due to the decrease in competition with H^+ (Hyne *et al.* 2005). Therefore, it is considered that the combination of elevated Zn and Ni were the most likely contributors of toxicity to the bioassay species in LT3 which was not exhibited in ≥1 g/L limestone treated CCW. Bioaccumulation of Zn is also a problem in aquatic environments, and even though toxicity was not displayed in the ≥1 g/L limestone treated CCW long term problems may occur especially in bivalves and vertebrates (Peakall & Burger 2003).

Liming of acidic water bodies is known to cause periodic toxicity to aquatic organisms (Teien *et al.* 2006). As an amelioration technique, liming is used to increase the pH and reduce the concentration of Al-species; in particular low molecular mass Al-species by hydrolysis. However, very toxic high molecular mass Al polymers can form in the unstable mixing zone immediately after liming (Teien *et al.* 2004). Therefore, if liming in pit lakes was to occur organisms already within the lake may be adversely affected. Further if liming was continued in the future to maintain lake water quality, any benefits to the aquatic environment from the original liming may be lost through adverse affects of the continued liming. If commercial aquaculture was taking place within the lake major legal issues may arise. A preferable approach is to lime off-take water and culture fish in purpose built ponds.

The HHC medium produced similar results to the CRW for the *C.* cf *dubia* bioassays, therefore, indicating that HHC would be suitable for future tests as inter and intra laboratory control/diluents. A major problem in any heavy metal toxicity investigation is to ensure that there is nothing present in the experimental medium which is capable of affecting that toxicity to the test organisms, such as EDTA (Tevlin 1978).

Pauli *et al.*(1993) noted that *T. thermophila* proliferation rate and maximal population density give almost identical responses to toxicant exposure. Therefore either test could be utilised. Population growth impairment is one of the most sensitive and often-used sublethal toxic endpoints. *T. thermophila* doubling time is approximately 4 h, thereby allowing shorter tests than 24 h but they may not differentiate toxicity effectively to low concentrations or chronic toxicants (Plesner *et al.* 1964). Therefore the 24 h growth test is far more sensitive than shorter tests. Both reproduction and population density of an organism are important indicators of suboptimal environmental conditions and reflect the sum of a variety of sublethal cytotoxic effects (Pauli *et al.* 1993).

The use of PPYS medium is necessary to provide a food source for the *T. thermophila (*Sauvant *et al. 2000).* The 24 h FTD from PPY in the testing (7×10^4 ciliates/mL was comparable to that obtained by (Pauli *et al.* 1993) at 48 h (9.8×10^4 ciliates/mL) in a proteose peptone media. By not feeding the *T. thermophila* toxicity expressed in 24 h may be more due to starvation than to toxicity present in a sample and the end point would be mortality not final density. The use of a synthetic media for *T. thermophila* has been shown to reduce proliferation and therefore final density indicating that it is not ideal for toxicity testing with *T. thermophila* (Pauli *et al.* 1993). The modification of PPY by dilution to 0.5xPPY decreased possible toxicity modifying effect while still producing sufficient growth to differentiate the toxicity between dilutions. Proteose Peptone media have been recommended for toxicity testing as they have very little effect on toxicity of metals (Sauvant *et al.* 2000).

Both species in the bioassays were able to differentiate toxicity from the limestone treated CCW. *Ceriodaphnia* cf dubia has been shown to be a more representative cladoceran than other larger species such as *Daphnia magna* (Koivisto 1995). Further, *C.* cf *dubia* was one of the species used in the construction of the ANZECC/ARMCANZ (2000b) guidelines. Protozoa are an ecologically relevant supplement to the common basic aquatic acute toxicity tests on fish (Larsen *et al.* 1997).

The response from each chronic and acute bioassay was the same, which was a toxic response from LT3 and no toxic response from ≥1 g/L limestone treated Chicken Creek water. The use of both acute and chronic end points with the two different species representing two different trophic levels is a sound approach to assessment of toxicity from the pit lake water. Similar studies involving invertebrates on acidic waters have shown the usefulness of bioassays with both acute and chronic endpoints for assessing toxicity (Hyne *et al.* 1996; Manusadzianas *et al.* 2003).

3.7 Conclusion

Bioassays are typically used to determine metal-organism interactions and ultimately show the toxicity from complex mixture such as acid pit lake water. The two species used in the bioassays are widely used in toxicology and are representative of both temperate Australian freshwater, as well as two significant trophic levels in aquatic ecosystems.

Limestone treatment of the Chicken Creek pit lake water did not reduce all metals to below the aquaculture, aquatic ecosystem or even HMGV. Nevertheless, toxicity was not exhibited in ≥1 g/L limestone treated Chicken Creek water. However, possible future problems higher up the food chain may occur in this water due to the high Zn concentration allowing for bioaccumulation, especially in bivalves and vertebrates. Therefore, continued monitoring of in lake organisms would have to occur if the lake was treated with limestone. A pilot trial at mesocosm scale is suggested in the Chicken Creek mine pit lake for ≥1 g/L limestone addition. Toxicity monitoring of treated Chicken Creek pit lake water is also suggested with the same bioassays used in this study.

TOXICITY ASSESSMENT OF LIMESTONE REMEDIATED ACIDIC MINE PIT LAKE WATER WITH SILVER PERCH (*BIDYANUS BIDYANUS* MITCHELL 1838) EARLY LIFE STAGES

4 Toxicity assessment of limestone remediated acidic mine pit lake water with Silver perch (Bidyanus bidyanus Mitchell 1838) early life stages

Ethics Statement

This study was conducted with the authorisation of the Australian Ethics Committee (AEC) under approval number R16-05. All fish were handled in accordance with the AEC approval.

4.1 Abstract

Three different limestone treatments were used to ameliorate acid and metal toxicity from Chicken Creek mine pit lake water. Early life stage fish bioassays were used to assess remaining toxicity in the limestone treated Chicken Creek mine pit lake water. The limestone treatments used were 1.4, 1.0 and 0.6 g $CaCO_3$/L. All limestone treatments raised pH to circum-neutral and reduced metal concentrations. Nevertheless, some metal concentrations remained above the guideline values for slightly-moderately disturbed ecosystems the protection of 95% of aquatic ecosystems and aquaculture species with 50% confidence (ANZECC/ARMCANZ. 2000b). Remaining toxicity in the limestone treated acid mine pit lake water was assessed by hatching, growth and survival success of *Bidyanus bidyanus* eggs, sac-fry and post sac-fry life stages. Limestone treatments of 1.4 g/L and 1.0 g/L removed toxicity to all life stages of *B. bidyanus* tested when compared to the controls. Limestone treatment of 0.6 g/L did not successfully remove toxicity with significantly lower survival being observed compared to that of the ≥1.0 g/L limestone treatments. The hardness concentration of 620 mg $CaCO_3$/L was probably the major influence in buffering toxicity to *B. bidyanus* in the two highest limestone treatments, compared to the lowest limestone treatment of 0.6 g/L with a hardness of 520 mg $CaCO_3$/L. *B. bidyanus* as an ecotoxicity test species was successful in assessing toxicity from ameliorated acid pit lake water and is recommended for use in the ecotoxicological assessment of water quality in mine lakes.

4.2 Introduction

For decades, researchers have been working to better understand the effects of acid and metal toxicity to aquatic organisms and to develop ways to effectively mitigate these detrimental effects (Cole *et al*. 2001). Both surface and underground mining have the potential to create acid mine drainage (AMD) therefore adversely affecting the local and adjacent environments (Cocos *et al*. 2002). The formation of acidic leachates from acid sulphate soils is also well documented around the world (Cranston *et al*. 1997; Hyne & Wilson 1997; Poleo *et al*. 1997; Lopes *et al*. 1999; Mays & Edwards 2001).

Use of constructed wetlands as biogeochemical AMD treatment systems has developed rapidly over the last few decades. Although hundreds of wetlands have been constructed to treat AMD, treatment effectiveness continues to be highly variable and often unpredictable (Kilborn 1996; Mays & Edwards 2001; Kalin 2004; Johnson & Hallberg 2005a). A number of constructed AMD wetlands incorporate limestone ($CaCO_3$) to increase pH and precipitate metals before the water is then 'polished' through the wetland. Variability in remediation success for limestone treatments on AMD has also been displayed (LeFevre & Sharpe 2002). There are many technologies incorporating limestone remediation including the use of direct liming (Howells *et al*. 1992), anoxic limestone drains (Cravotta III & Trahan 1999), pulsed fluidised limestone beds (Maree & du Plessis 1994; Cole *et al.* 2001) (Cole *et al.* 2001)and limestone sand reactors (Watten *et al*. 2005). Limestone remediated acid pit lake water has been used for aquaculture (Storer *et al.* 2004). Limestone has the lowest material cost, is the safest, easiest to handle of the AMD remediation chemicals and produces the most compact and easy to handle sludge material (Skousen *et al.* 1998). Therefore, limestone is the most commonly used material for increasing pH and removing metals and metalloids by adsorption, precipitation and co-precipitation processes (Maree *et al.* 1999; Lee *et al.* 2002).

Although treatment with limestone has been found to remediate toxic aspects of AMD such as pH and heavy metal concentrations, toxicity may still remain (Kilborn 1996; Kalin 2004). Historically, analysis of acid and metal polluted waters have been based mainly on chemical characterisation (Peakall & Burger 2003). However, chemical analysis alone cannot adequately demonstrate the many chemical interactions to and responses of exposed aquatic organisms (SETAC 2004) as many pollutants exert their effects as components of complex mixtures by means of additive (synergistic) or less than additive (antagonistic) interactions (Mitchell 2002). Further, the toxicity of metal species, especially that of bioavailable forms, cannot always be characterised accurately or through technological limitations (Sauvant *et al*. 2000).

The use of aquatic organisms in bioassays as indicators of toxicity has been well researched (Chapman 2000; Mitchell 2002). The general consensus among authors using bioassays is that they are essential to determine direct toxic effects to the organisms themselves (Rojickova-Padrtova *et al*. 1998; Deanovic *et al*. 1999). Aquatic organisms have the ability to manipulate chemical uptake on an intra-specific cell level as well as at the cell surface (Worms *et al*. 2006), therefore either reducing or magnifying toxic effects. Bioassays give advantages over chemical analysis alone by expressing potential antagonistic and synergistic effects from interactions with all chemical species present (Kilham *et al*. 1998; Chapman & Simpson 2005).

Fish bioassays are representative for aquaculture and fisheries toxicity assessments (Pyle *et al.* 2002; Viadero Jr & Tierney 2003; Sibrell *et al.* 2006). Aquaculture is increasingly common in mine pit lake water, requiring the use of bioassays representative of the mine pit lake end use (Whisson & Evans 2003; Viadero Jr *et al.* 2004; Storer 2005). When selecting a fish bioassay species it should be recreationally, commercially or ecologically important, be widely available and amenable to routine maintenance in the laboratory (Mitchell 2002). Fish also have a duel value as bioindicators as they are consumed by both humans as well as other predators such as birds (Peakall & Burger 2003).

Bidyanus bidyanus are currently being trialled as a potential aquaculture species in limestone treated mine pit lake water. Therefore, the use of *B. bidyanus* as an ecotoxicity test species would introduce an Australian aquaculture representative fish species (Thurstan & Rowland 1994; Storer 2005) to assess these mine pit lakes for aquaculture suitability. Furthermore, *B. bidyanus* was one of the species used to develop the Australian guidelines for the protection of aquaculture species (ANZECC/ARMCANZ. 2000b).

Fish are used as indicators of toxicity from AMD and for Direct Toxicity Assessment (DTA), also known as Whole Effluent Toxicity (WET) tests (Poleo *et al.* 1997; Sherry *et al.* 1997; Barron & Albeke 2000; Clearwater *et al.* 2002; Pyle *et al.* 2002; Viadero Jr & Tierney 2003). Fish bioassay acute and chronic endpoints are considered the most relevant to instream water quality conditions when compared to invertebrate bioassays (Diamond & Daley 2000). Many different acute and chronic endpoints are used, but the use of growth rate parameters assessing effects on the organism's size and weight (Effect Concentrations) have proven to be very sensitive and reproducible endpoints (Stauber *et al.* 1994; Mitchell 2002). The use of more than one endpoint in the bioassay may also increase the test's sensitivity to a broad range of toxicants or effluents (Fochtman *et al.* 2000).

Many fish bioassays with rigorously developed guidelines are currently in use (Vittozzi & De Angelis 1991; Hyne *et al.* 1996). One of the most prominently used fish species in bioassays is the fathead minnow (*Pimephales promelas*), which has been used to assess toxicity of mercury (Grippo & Heath 2003), captan toxicity (Hermanum *et al.* 1973), refinery effluents (Sherry *et al.* 1997), AMD (Pyle *et al.* 2002) and induced acidification of streams (Zischke *et al.* 1983). However, Vittozzi & De Angelis (1991) reviewed *P. promelas* and other common bioassay fish species and found that there were major differences amongst the responses of these fish to different metals and chemical compounds. This indicates that the use of fish relevant to the geographical location and toxicant should be included when assessing toxicity

and not just species that are convenient or conventional (Hyne *et al.* 1996; Chapman 2002; Mitchell 2002).

Although relevant species are necessary for good toxicity testing, the relevance and suitability of control and diluent waters are also crucial. A variety of factors can influence the results in ecotoxicity testing including physical conditions such as the source and quality of control/dilution water (Baer *et al*. 1999). Many natural and synthetic media and combinations of both exist for toxicity testing, most of which are not chemically defined and may vary considerably between experiments (USEPA 1991). Therefore, the use of a chemically defined test medium is necessary for comparison of bioassay results to a control, as well as for intra- and inter-laboratory results. The synthetic Combo medium has been developed for culture and testing of alga and zoo plankton by Kilham *et al*. (1998). The Combo medium was further modified for its use with fish by primarily increasing the hardness of the medium and renaming it High-Hardness Combo (HHC) (Baer *et al*. 1999). The HHC medium was used as a control in this study for comparison with treatment results and for validation as a control for future testing with *B. bidyanus.*

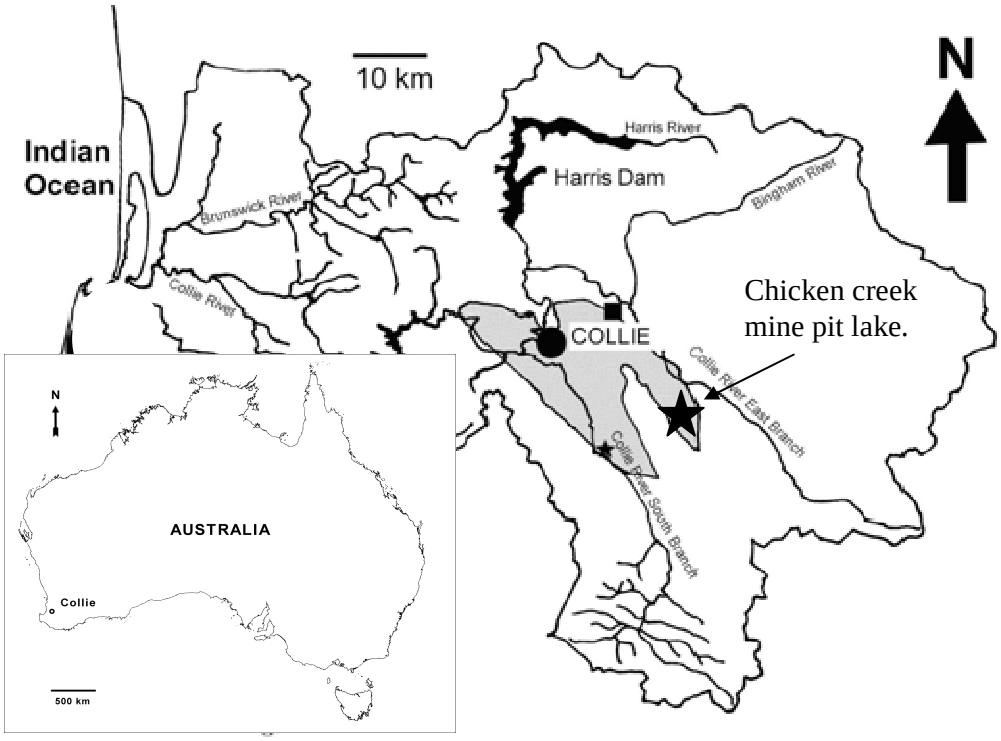


Figure 7. Location of Chicken creek mine pit lake in Collie Western Australia (Latitude 33 21 S, Longitude 116 09 E). Insert of Australia showing location of Collie. Source (Lund *et al.* 2006)

Aquaculture of *B. bidyanus* is currently practiced in the south west of WA, mostly in agricultural farm dams. Also there are aquaculture trials in limestone treated mine pit lake water in the Collie Coal Basin in South-Western Australia (Whisson & Evans 2003). *Bidyanus budyanus* is one of only a few native Australian freshwater species that have the potential to be aquacultured (Rowland 1994a). Further, they are the only Australian native freshwater fish to be commercially aquacultured for food in the south west of WA (Storer *et al.* 2002). *Bidyanus bidyanus* possess many characteristics that make them suitable for aquaculture as well as toxicity studies such as established hatchery techniques and rapid and uniform growth (Rowland 1994a). The use of *B. bidyanus* in toxicity assessments of mine pit lakes for aquaculture will introduce a commercial aquaculture species for toxicity assessment.

This research aimed to assess the toxicity remaining in pit lake water after limestone amelioration through lethal and sub-lethal end points of early life stages of *B. bidyanus*. Toxicity testing was used to assess each of three limestone treatments to water collected from the Chicken Creek acid mine pit lake located within the Collie

Coal Basin. The goal of this study was assessing whether *B. bidyanus* would be a suitable representative of Australian temperate freshwaters. A wider goal was to determine if culture of the early life stages of *B. bidyanus* is possible within limestone treated coal mine pit lake water.

The toxicity assessment aimed to expose various critical life stages of *B. bidyanus* to limestone treated Chicken Creek mine lake water. Hatching, survival and growth endpoints were assessed as representative responses related to aquaculture of *B. bidyanus*. Furthermore the use of *B. bidyanus* critical life stages for direct assessment of the treated Chicken Creek mine lake water was used to show if *B. bidyanus* could be used in future toxicity assessments.

4.3 Materials & Methods

A series of trials using the early life stages of *B. bidyanus* were conducted as follows.

4.3.1 Trial 1: Preliminary exposure of *Bidyanus bidyanus* to limestone treated Chicken Creek water

Trial 1 was a preliminary experiment used to assess methods for *B. bidyanus* testing of acid mine pit lake water and to decide on a suitable protocol for future testing.

Bidyanus bidyanus has been used in toxicity testing previously examining effects to eggs and larvae from salinity (Guo *et al*. 1993), nitrite (Frances *et al*. 1998) and ammonia (Frances *et al*. 2000). However, these tests were used to assess potential benefits or problems associated with water quality parameters in aquaculture and not for ecotoxicological reasons. The *B. bidyanus* bioassay protocol was constructed from fish toxicity assessment procedures employed by Guo *et al*. (1993) and Hyne & Wilson (1997).

4.3.1.1 Treatments

Two different controls were tested. Control one consisted of filtered (0.2 µm) Natural Pond Water (NPW) from a Western Australian commercial silver perch hatchery. A second control, synthetic High-Hardness Combo medium (HHC), designed for multiple species toxicity testing and for intra and inter–laboratory comparisons (Baer *et al.* 1999) was also tested. Treatment one was addition of 1.4 g/L powdered limestone (LT1) and Treatment two was addition of 0.6 g/L powdered limestone (LT3). Treatment three was untreated Chicken Creek mine pit lake Water (CCW) collected from Collie WA, 15 cm below the Chicken Creek mine pit lake surface. All treatment solutions were made up in 5 L conical flasks and magnetically stirred for 24 h at room temperature to be sure the limestone had fully dissolved. Treatment solutions were then left to stand for 24 h to allow the resulting precipitate to settle to the bottom of the flask and filtered to 0.02 µm using membrane filters. Treatment solutions were stored in acid-washed high density polyethylene bottles and refrigerated at <5°C for up to 72 h. Test solutions were vigorously aerated for 15 minutes in 400 mL conical flasks before use.

4.3.1.2 Bidyanus bidyanus broodfish spawning

Bidyanus bidyanus broodfish were sourced from ponds located at a Western Australian commercial silver perch hatchery where they feed on natural food within the ponds such as rotifers, cladocerans, protozoa, frogs and aquatic and terrestrial insects (Rowland 1994b). Broodfish matured naturally but were then artificially induced to spawn. *Bidyanus bidyanus* is not known to spawn naturally in captivity without water level and temperature manipulation which is also not documented.

Females and males were assessed for maturity after Thurstan & Rowland (1994). Mature fish were injected with the hormone, Human Chorionic Gonadotrophin (HCG) at a dose rate of 200 International Units per kilogram of body weight (200 IU/kg) (Thurstan & Rowland 1994). After the hormone injection, the fish were placed in 1 000 L fibreglass tanks to spawn. Spawning occurred approximately 32 to 39 h later. Fish eggs were fertilised naturally by one or more males depending on their size and weight.

4.3.1.3 Egg sourcing and collection

Eggs used in the toxicity study came from two females which were fertilised by four males. Temperatures lower than 24^{o}C result in an extended hatch period and temperatures above 24^{o}C accelerate hatching and can lead to death of larvae. Eggs were removed from the 1 000 L spawning tank by siphon and transported to the lab in 2 L glass beakers and then viewed under a dissecting microscope at 40x magnification to assess if they were viable (fertilised). Viable eggs were characterised by being negatively buoyant, spherical, completely transparent, with no discoloration and of uniform size (Thurstan & Rowland 1994).

4.3.1.4 Source of sac-fry and rearing

Sac-fry larvae were collected from the same spawning batch as the eggs in the hatchability trial detailed above. The eggs were transferred at the age of 1.5 h to one of four 50 L hatching cones. Each cone contained one of the previously defined control or treatment waters. Eggs start to hatch approximately 28–31 h at 24^{o}C after fertilization (Thurstan & Rowland 1994). Hatching can take more than 15 h to complete. Hatched fry were collected by submersing a 1 L beaker into the hatching cone water, where aeration attached to the standpipe keeps the eggs and hatched sac-fry larvae in suspension. Sac-fry collected were no older than 24 h from hatching time at the commencement of the sac-fry trial. Sac-fry were individually assessed for health under a dissecting microscope. Criteria for health were regular movement, presence of a yolk-sac and absence of deformities (e.g. bent spine). Larvae were held in plastic cups containing 100 mL of control water, or treatment water. Optimum growth rates for sac-fry occur between 22–28°C and growth is negligible below 12°C (Thurstan & Rowland 1994). Temperature in the cones was regulated to 24±1°C by 100 W aquarium heaters protected from the larvae by 100µm plastic mesh.

4.3.1.5 Source of post sac-fry and rearing

Sac-fry development continued for approximately five days until the yolk-sac was absorbed. Once the yolk-sac was absorbed a critical stage occurs when the fry start to feed on live food (Thurstan & Rowland 1994). In order to have enough larvae for

the post sac-fry test, sac-fry not used in the sac-fry test were transferred from hatching cones to 100 L tubs containing 30 L of control and treatment waters respectively and mild aeration. Rotifers (5 rotifers/ mL) were added to 100 L tubs starting on day 3 (to accommodate for fast developers) and every day after this. The use of 5 rotifers/mL was chosen as concentrations of zooplankton >1/mL are needed to obtain high survival rates in aquaculture (Thurstan & Rowland 1994). Rotifers were added to bowls containing post sac-fry every 24 h at each water change.

4.3.1.6 Trial 1: egg survival, hatch and hatchability at different liming treatments

Trial 1 was a static non-renewal test in 100 mL plastic cups with lids. Eggs were collected to allow for egg hardening from the spawning tank 1.5 h after spawning (Thurstan & Rowland 1994; Hamilton *et al.* 2005). Aliquots of the controls and treatments (100 mL) were added to plastic cups which are randomly allocated positions on a tray. Three replicates of each treatment and control were used. Test treatment water was not renewed during the test due to the small effective change in water quality parameters experienced in 48 h. Eggs meeting the viability criteria (negatively buoyant, spherical, completely transparent, with no discoloration and of uniform size) were randomly placed into plastic 150 mL cups with wide bore plastic 1 mL pipettes. Each cup received 10 eggs. Cups were placed into an incubator at 24°C±1°C and complete darkness. Mild aeration to keep eggs in suspension was supplied to the treatment containers via an aquarium air pump and micro-pipette ends. Eggs were checked for hatching success after 24, 30, 36 and 48 h. The test ended when either 100% hatching success occurred in all treatments or 48 h had elapsed from trial commencement. Plastic cups were placed under a dissection microscope (40x magnification) to examine for dead eggs, hatched fry, and dead or live fry. Any mortalities, hatched fry and dead eggs were removed at each inspection time. Measurements made in this trial were 48 h survival, hatch and hatchability (hatching success in 48 h). Hatchability was calculated after Koenig (1982). Guo *et al*. (1993) used fish tail twitching 1–2 h before hatching to assess hatchability. However, fry successfully hatching from the egg were used as the end point in this trial. Some fry that were tail twitching in Trial 1 never hatched and consequently died within the allowed hatching time of 48 h.

Randomisation was used for testing in both trials as randomisation is critical when using bioassays to assess toxicity (Mitchell 2002).

4.3.1.7 Trial 1: sac-fry survival

Trial 2 sac-fry for the sac-fry survival test came from the same spawning as the trial 1 egg test. Eggs were transferred to hatching cones which contained their respective treatment and control waters at 1.5 h post fertilisation. Sac-fry were hatched in their respective treatments and controls to better evaluate if these treatments would be capable of raising fish to the juvenile stage. Sac-fry were collected from hatching cones by lowering 2 L glass beakers into the hatching cones. Sac-fry viability was assessed using a dissection microscope. All viable sac-fry were randomly assigned to another 2 L beaker with mild aeration. Test containers used were 100 mL plastic cups with a lid containing 100 mL of treatment or control water. Ten sac-fry were added to each cup and renewal of 100% of the water was made every second day. Mild aeration was used to maintain high dissolved oxygen (DO) and to keep sac-fry in suspension thereby reducing possible bacteria infections. Sac-fry were placed into an incubator at 24±1°C and a 12:12 h light:dark cycle. Mortality and growth was assessed at 12, 24, 48, 72, 96, and 120 h from test commencement. There was no feeding during the sac-fry test.

4.3.1.8 Trial 1: post sac-fry survival

Post sac-fry were hatched in hatching cones containing their respective treatment and control waters. A random selection of approximately 1 000 sac-fry were transferred from hatching cones to 100 L tubs either containing treatment or control waters. Mild aeration was made to each tub to maintain a high DO. Post sac-fry were collected from the same batch, as the eggs and sac-fry used in the previous tests. The criteria for viability of post sac-fry was regular swimming movement, no deformities and a developed mouth. The daily feeding regime for the post sac-fry was 5 rotifers/mL. Fish were assessed for viability using a dissecting microscope at 40x magnification. Test containers consisted of 2 L glass bowls with lids and mild aeration. There were three replicates of each treatment and control, each containing thirty fish. Mortalities

were recorded every 24 h for 120 h (5 days). Mortalities were removed at each inspection time.

4.3.2 Trial 2: Bidyanus bidyanus response to different liming treatments

4.3.2.1 Trial 2: Bioassay treatments

The controls and treatments used were as follows.

CRW = Collie River Water control.

HHC = High-Hardness Combo medium control.

LT1 = Treatment of 1.4 g/L limestone powder to acidic mine pit lake water.

LT2 = Treatment of 1.0 g/L limestone powder to acidic mine pit lake water.

LT3 = Treatment of 0.6 g/L limestone powder to acidic mine pit lake water.

CCW = Untreated acid pit lake water (Chicken Creek).

Collie River water was chosen as a geographically representative control for the lotic waters of the Collie region. The continued use of the defined HHC medium was considered necessary to facilitate intra or inter laboratory comparisons on sensitivity of *B. bidyanus* to other fish species and bioassay results. A 1 g/L limestone powder treatment (LT2) was also added to the previously described two limestone treatments of 1.4 g/L (LT1) and 0.6 g/L (LT3). All concentrations of limestone powder increased the pH to neutral. Untreated Chicken Creek mine pit lake Water (CCW) was used in trial 1 resulting in 100% mortality within 24 h of commencing the trial. Untreated Chicken Creek acid pit lake water was used in trial 2 as a mine pit lake water control. Four replicates of each treatment were used and all controls and treatments were filtered to 0.02 µm before use.

4.3.2.2 Trial 2: egg survival, hatch and hatchability test

Trial 2 used eggs from a second spawning batch following procedures listed above (broodfish section). However, trial 2 used four replicates instead of three and 30 eggs

per cup in place of 10. These modifications were made to increase the statistical power of the test.

4.3.2.3 Trial 2: sac-fry survival and growth

Trial 1 methods were also modified for trial 2 sac-fry survival and growth assessment. Trial 2 modifications were increasing replicates from three to four, test containers from 100 mL plastic cups to 2 L glass bowls with lids containing 1.5 L of treatment or control water and mild aeration, the number of sac-fry from 30 to 100 hundred larvae per bowl instead of thirty and the addition of biometrics. Biometrics for growth were assessed by taking five fish from each bowl at each inspection time and preserving them in 4% buffered formalin. The number of fish left at each inspection time used to derive mortality was calculated by Equation 3.

Equation 3. Mortality calculation

$$N = 100 - [5 \times (I-1)]$$

where N is the number of fish left at each inspection time used to derive mortality and I is the inspection number. Therefore mortality at each inspection time is calculated by N / mortality. Measurements taken were (a) total length, (b) width, (c) height, and (d) yolk-sac diameter. The measurement positions on the fish are displayed in Figure 8. When sac-fry were collected for measuring they were fixed in 4% buffered formalin.

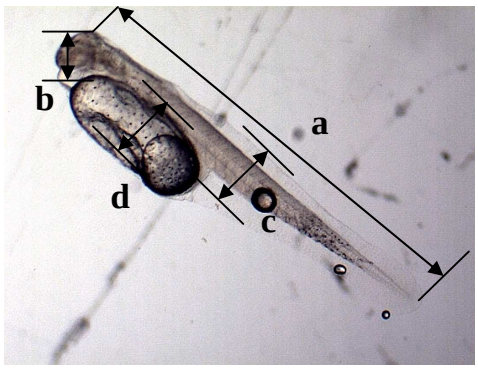


Figure 8. Sac-fry measurements taken at each inspection time.

4.3.2.4 Trial 2: post sac-fry survival and growth

Modifications to trial 1 methods for the post sac-fry test were; increase number of fish used from 30 to 100 per replicate, inclusion of the growth measurements as per trial 2 sac-fry test and the addition of measuring gape (mouth height) (e) (Figure 9). All other methods were as in the trial 1 post sac-fry test.

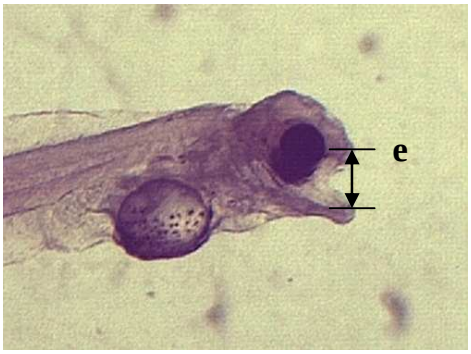


Figure 9. Gape measurement of post sac-fry

4.3.3 Water quality measurements

Ammonia measurements were made with a Hagen Aquatic Research Station (HARS), Nutrafin NH_3-NH_4^+ test kit by constructing a regression line for total ammonia with a Shimadzu UV-1201 Spectrophotometer at a wavelength of 750 nm. Unionised ammonia concentrations (UAN) were calculated from Equation 4 (Johansson & Wedborg 1980).

Equation 4. Free ammonia calculation

$$\%NH_3 = 100 / [1+10^{(\log K_1 - pH)}]$$

$$\text{Log}K_1 = -0.0467 + 0.00113 \times S + 2887.9 / T$$

where K1 is the dissociation constant, S (in g/L) the salinity, and T the temperature in °K.

The pH of test water was measured by a hand held EcoScan pH6 portable pH meter, calibrated daily using pH 4, 7 and 10 standards. Temperature was also measured by the pH meter to an accuracy of 0.1°C. An analysis for metals and metalloids was made by Inductively Coupled Plasma – Atomic Emission Spectrophotometry (ICP-AES) for Ag, Al, As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb and Zn. The analysis was conducted by the Marine and Freshwater Research Laboratory (MAFRL), located at Murdoch University Western Australia. Samples were acidified before analysis.

4.3.4 Data analysis

Hatch, hatchability and survival are presented as mean (%) ± standard error (%) unless otherwise stated. An acceptable level of mortality for a control in toxicity testing is 20% (USEPA 1994). Water quality and metal results are mean mg/L ± standard error mg/L. Statistical analysis of all bioassay and water quality data was performed using the statistical program SPSS (2005). Normality was assessed by the Shapiro-Wilk test. When absence of non-normality was proven, homogeneity of variances was tested using Levene's test. Significant differences between treatments and controls existed when $P < 0.05$.

When the assumption of normality was met, a one-way analysis of variance (ANOVA) was performed to test for significant differences between means of

experimental controls and treatments. When data were not normally distributed, the non-parametric Kruskal-Wallis H Test was instead used to find median differences between controls and treatments. When significant differences were found, a post-hoc test was performed to identify where the pair-wise differences occurred. The parametric post-hoc Tamhane Test was used when variances were not equal. When variances were shown to be equal, Dunnett's Test was used to compare the control T0 to the control T1 and the limestone treatments. The post-hoc Student-Newman-Keuls (S-N-K) range test was used to show homogenous sub-sets (Newman 1939; Keuls 1952). A T-Test was performed to find differences between growth and survival results of limestone treatments. Hatchability was calculated by dividing the % hatched by the % survival (Koenig 1982; Hamilton *et al*. 2005). Growth rate was calculated using the slope of the growth parameters' data. Linear regression was used to find correlations between treatments.

Hardness Modified Guideline Values (HMGV) for the protection of aquaculture species are calculated after (ANZECC/ARMCANZ. 2000b; Markich *et al.* 2001). The Hardness dependant algorithm used was:

Equation 2. Hardness modifying algorithm

$$HMGV = GV(H/30)^{a}$$

where HMGV is the Hardness Modified Guideline Value (μg/L), GV is the Guideline Value (μg/L) at a hardness of 30mg/L $CaCO_3$, H is the measured hardness (mg/L $CaCO_3$) of a fresh surface water and a is a constant (ie. slope factor).

Criteria for successful toxicity testing were used to assess the acceptability of the test data (Riethmuller *et al.* 2003). The criteria were; a consistent temperature (± 2°C), mortality in the control does not exceed 20%, the pH does not change by more than 0.2 from the day 0 reading and the dissolved oxygen is greater than 70% saturation.

4.4 Results

4.4.1 Trial 1: water quality for egg, sac-fry and post sac-fry tests

The water quality results for the egg test were measured at time 0 h and are presented in Table 7. All control and treatment waters were maintained at approximately 25°C. Test water pH of controls and treatments were similar and close to neutral, except in the CCW pH was 3.0.

Table 7. Trial 1 egg test physico-chemical parameters.

Treatment	pH	Temperature (°C)	Alkalinity (mg/L)	Total hardness (mg/L)	Dissolved oxygen (%)
NPW	7.8	25	60	180	96
HHC	7.0	25	<10	100	92
LT1	7.9	25	90	620	94
LT3	7.6	25	40	520	99
CCW	3.0	25	<10	380	95

Spawning occurred approximately 12 h later than expected in trial 1 but, there was still approximately 90% fertilisation of the eggs. There were no hatched larvae or mortalities observed at 24 h post spawning except in treatment CCW where there was total mortality of the embryos. Results for the trial 1 egg test are presented in Table 8. Survival for the control NPW was acceptable at 90%. Nevertheless, the use of this water was not continued in trial 2 due to the low hatchability of 35%. Instead, the geographically representative Collie River water was used in trial 2 as a control. The synthetic control HHC performed well with <20% mortality and hatchability of 99%. Hatchability of 100% was not reached for the control NPW in the 48 h trial. This was probably due to the original low temperature (22°C) that the eggs were spawned and developed in for the first 1.5 h after fertilisation. Number hatched and hatchability was lower in treatment LT3 than that of treatment LT1. Hatch and hatchability was 0% in CCW.

Only survival was measured in the 120 h sac-fry test with >80% survival recorded in NPW, HHC and LT1 (Table 8). Survival of sac-fry was lower in LT3 than the other

controls and treatments except for CCW which had total mortality. Post sac-fry survival in control NPW was also >80%. The control HHC and the treatments LT1 and LT3 showed similar survival in the post sac-fry test. Survival for post sac-fry was lower in treatment LT3 (63%) than the controls and treatment LT1.

Table 8. Trial 1 48 h egg test and 120 h sac-fry and post sac-fry mean results (%) ± standard error (%).

Treatment	Hatched	Hatchability	Embryo survival	Sac-fry survival	Post sac-fry survival
NPW	31±17	35±3	90±20	90±6	87±7
HHC	83±3	99±3	84±1	83±9	87±9
LT1	86±3	99±4	87±1	90±0	87±9
LT3	71±12	86±8	82±13	77±9	63±9
CCW	0±0	0±0	0±0	0±0	0±0

4.4.2 Trial 2: Water quality

Water quality results for the trial 2 egg test are presented in (Table 9). Parameters were measured in test water at times 0 h and 48 h, to identify if there was a change over the test period. Only one replicate from each treatment was chosen at each measurement time for water quality measurements. Calculated UAN concentrations were as high as 0.01 mg/L at 48 h. The CCW maintained the lowest pH, which increased from 3.4–3.9. The other water quality parameters remained relatively constant over the 48 h egg test for the remaining treatments and controls, with a constant temperature of 24°C, DO close to 100% and pH changing less than 0.3 for any treatment. The control and treatment water quality parameters were all acceptable. Total hardness and alkalinity were only measured at time 0.

Table 9. Water quality parameters measured in trial 2 egg test at test commencement and 48 h. Alkalinity and hardness were only measured at time 0.

Treatment	Time (hours)	pH	Temperature (°C)	UAN (mg/L)	Dissolved oxygen (%)	Alkalinity (mg/L)	Hardness (mg/L)
CRW	0	7.6	24	0.00	97	70	160
HHC	0	7.1	24	0.00	101	<10	100
LT1	0	7.9	24	0.01	95	90	640
LT2	0	7.7	24	0.00	99	90	620
LT3	0	7.1	24	0.01	97	40	500
CCW	0	3.4	24	0.01	98	<10	420
CRW	48	7.7	24	0.01	100	-	-
HHC	48	7.3	24	0.00	99	-	-
LT1	48	8.0	24	0.01	97	-	-
LT2	48	7.8	24	0.01	95	-	-
LT3	48	7.3	24	0.01	98	-	-
CCW	48	3.6	24	0.01	97	-	-

Sac-fry water quality results are presented in (Table 10). Sac-fry test water quality fluctuated slightly between water changes. The greatest change occurred between time 0 h and the final water quality measurement at 120 h. The UAN ranged from 0.00–0.02 mg/L, pH ranged from 7.2–8.3 and DO% was close to saturation. Water quality was not measured after 24 h in CCW due to the total mortality of the sac-fry by this time. Only slight changes in the alkalinity and hardness were evident over the 120 h test. All water quality parameters were acceptable in the sac-fry test. Post sac-fry water quality showed similar changes to the sac-fry test (Table 11). The pH and concentration of UAN increased slightly in all treatments. Temperature varied from day to night by approximately 2°C. DO was near 100% saturation at all times.

Table 10. Water quality parameters for the 120 h sac-fry test. CCW is not displayed at 120 h due to the total mortality of sac-fry before 24 h. UAN measurements that are 0 had a detection limit of 0.01 mg/L.

Treatments	Time	pH	Temperature (°C)	UAN (mg/L)	Dissolved oxygen (%)	Alkalinity (mg/L)	Total hardness (mg/L)
CRW	0	7.8	23	0	98	70	170
HHC	0	7.2	23	0	100	<10	100
LT1	0	8.1	24	0.01	98	90	660
LT2	0	8.0	23	0.01	97	90	660
LT3	0	7.8	23	0.01	98	40	540
CCW	0	3.2	22	0.01	98	<10	420
CRW	120	7.8	24	0.01	100	70	170
HHC	120	7.4	25	0.02	102	20	120
LT1	120	8.3	25	0.02	98	80	640
LT2	120	8.0	25	0.01	99	80	640
LT3	120	7.8	24	0.01	99	50	540

Table 11. Water quality parameters for the post sac-fry test. CCW is not displayed at 120 h due to the total mortality of sac-fry before 24 h.

Treatment	Time (hours)	pH	Temperature (°C)	UAN (mg/L)	Dissolved Oxygen (%)	Alkalinity (mg/L)	Total hardness (mg/L)
CRW	0	7.7	23	0	98	70	170
HHC	0	7.2	23	0	101	<10	120
LT1	0	8.0	23	0	97	80	640
LT2	0	7.8	24	0	95	80	640
LT3	0	7.7	23	0	99	50	540
CCW	0	3.4	23	0	93	<10	420
CRW	120	7.8	24	0.01	96	70	170
HHC	120	7.4	24	0.01	98	20	120
LT1	120	8.0	23	0.02	98	80	640
LT2	120	8.0	24	0.02	98	80	640
LT3	120	7.8	23	0.01	96	50	520

4.4.2.1 Trial 2: Chemical analysis

All three CCW limestone treatments contained dissolved concentrations of metals above the trigger values for protection of 95% of the freshwater aquatic environment (Table 12) (ANZECC/ARMCANZ. 2000b). The concentration of Zn in treatment CCW was higher than that in the control CRW and the treatments LT1 and LT2 and the concentration of Ca was lower in treatment LT3 than that of treatments LT1 and LT2. The concentrations of the metals Al, Cd, Cu, Ni and Zn in treatment CCW were higher than that in the CRW and that in the limestone treatments. The concentration of Ca in CCW was lower than that in the control CRW and the limestone treatments.

At the commencement of the trial Al, Cd, Cu, Ni and Zn concentrations in untreated Chicken Creek lake water were above the aquatic ecosystem guideline values and all but Cd were also above the aquaculture guidelines (Table 12). The only concentration of a metal in CRW above the aquaculture guideline was Cu. The limestone treatments did not reduce concentrations of Zn and Ni to below the aquaculture or aquatic ecosystem guideline values and Cd was not reduced to below the aquatic ecosystem guidelines. Treatments LT1 and LT2 did not reduce the Al concentration to below the aquaculture guideline values. The concentration of Cu in treatment LT2 and the concentrations of Al and Cu in LT1 also remained above the aquatic ecosystem guideline values. After modifying the aquaculture guideline values to the HMGV the concentration of Zn still remained elevated in CCW and all limestone treatments.

Table 12. Dissolved metals and metaloids of significance to toxicity responses from *B. bidyanus.* Values exceeding guideline values for the protection of aquaculture species are denoted by a *, protection of 95% of a slightly–moderately disturbed freshwater aquatic environment are denoted by bolding HMGV for aquaculture species is denoted by a subscript † preceding the figure. Guidelines that are missing are not yet available.

Treatment	Al	Ca	Cd	Cu	Mg	Ni	Zn
CRW	<10	24	<0.6	**10**	50	<4	<2
LT1	**80***	140	**0.8**	**2**	75	**140***	†**100***
LT2	40*	140	**0.8**	**2**	78	**160***	†**170***
LT3	<10	110	**1.2**	1	76	**170***	†**440***
CCW	**18000***	18	**1.6**	**19***	63	**200***	†**990***
Aquaculture *	30 (pH>6.5) 10 (pH<6.5)	-	0.2 - 1.8	5	-	100	5
Aquatic ecosystems	55	-	0.2	1.4	-	11	8
HMGV for Aquaculture							
CRW	-	-	8	21	-	415	21
LT1	-	-	27	67	-	1348	67
LT2	-	-	27	66	-	1312	60
LT3	-	-	22	55	-	1093	55
CCW	-	-	19	47	-	942	47

4.4.3 Trial 2: egg survival, hatch and hatchability test

4.4.3.1 Trial 2: embryo survival

Mean survival recorded at 48 h for the CRW was 88 ± 4% (Figure 10). Mean survival in HHC was 77 ± 7%. The survival in CCW was 0% and significantly lower ($F_{(5,18)}$=16.4, p=0.001) than all other treatments and controls. At the first inspection time (24 h) the survival was 0% for CCW. The standard error for LT3 was high at 18.7% compared to the controls and other treatments.

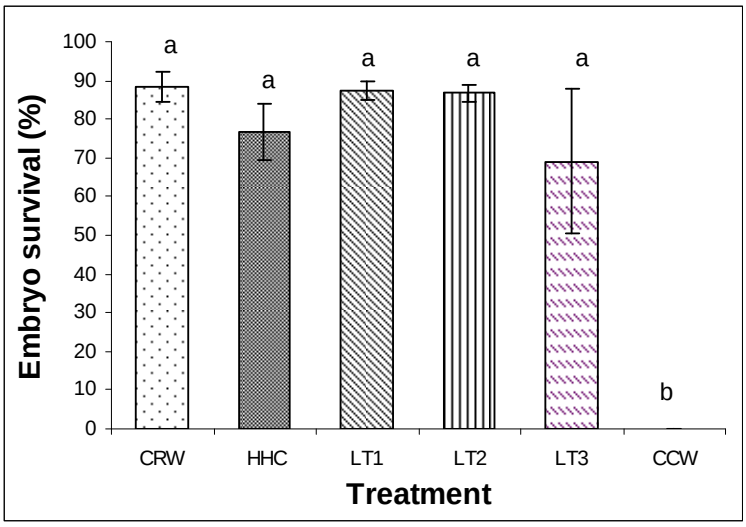


Figure 10. Mean egg survival % ± standard error % after 48 h. Different lowercase letters indicate significant differences.

4.4.3.2 Trial 2: egg hatch and hatchability

Percent hatch in the controls and treatments is displayed in Figure 11. A linear relationship ($R^2 = 0.78$) of hatch for treatments LT1, LT2, LT3 and CCW exists. However, treatments LT1, LT2 and LT3 % hatch did not significantly differ ($F_{(2,9)}$=0.89, p=0.44) from each other. Hatch time varied with hatching in CRW completed by 26.5 h, LT1 by 27 h, LT2 by 30 h, HHC by 33.5 h, and in LT3 by 36 h.

Hatchability at 48 h in the Controls CCW and HHC was 100% (Figure 11) Hatchability in treatments LT1, LT2 and LT3 was not significantly different ($F_{(4,15)}$=4.7, p=0.001) from each other or the controls. However, results from a T-test showed % hatchability for LT3 to be significantly lower ($t_{(6,3)}$=3.96, p=0.03) than the controls CRW and HHC. Due to total mortality within the first 24 h hatchability in the CCW was 0%. Mean hatch and hatchability (%) showed a similar trend. There were no deformities found in trial 2 embryos or hatched sac-fry.

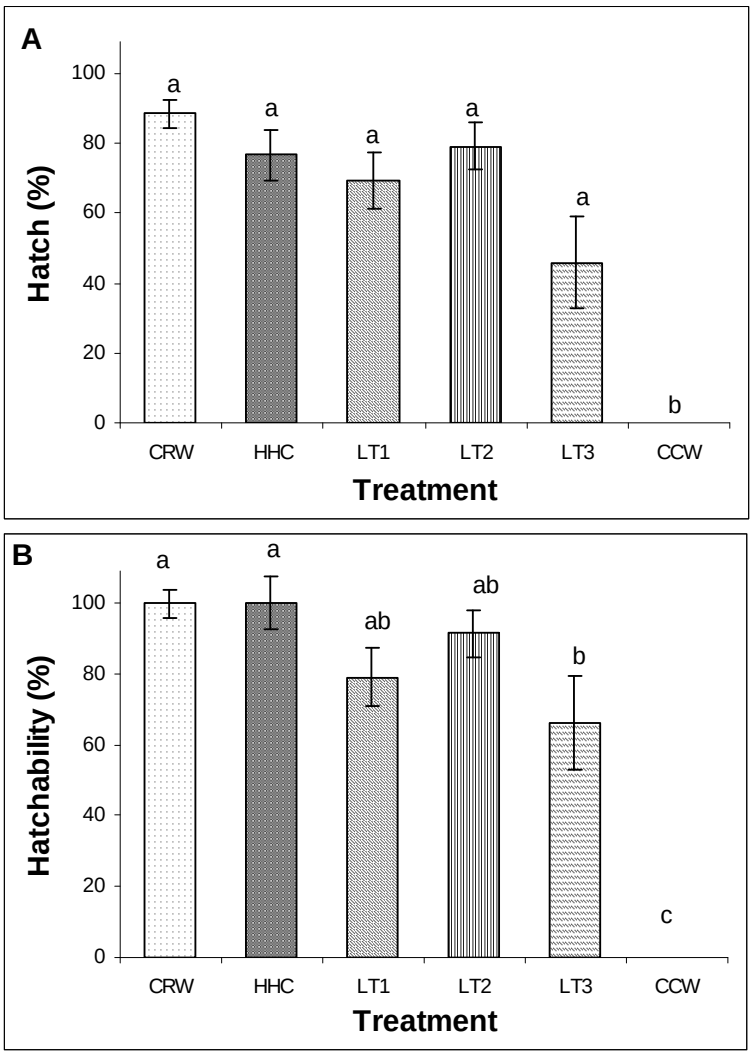


Figure 11. Mean % hatch (A) and hatchability (B) ± % standard error after 48 h test duration. Different lowercase letters indicate significant differences between treatments and controls.

4.4.4 Trial 2: sac-fry survival and growth test

Mean survival at 72 h in CRW, HHC, LT1 and LT2 was greater than 80% (Figure 12). Mean survival in treatment LT3 at both 72 and 120 h was lower than that of the controls and other treatments LT1 and LT2. The mean survival at 120 h in HHC and the treatments LT1 and LT2 was still greater than 80%. Mean survival at 120 h in treatment LT3 was 43±3% which was significantly lower ($F_{(4,15)}$=44.5, P=0.001) than that of the controls and other treatments.

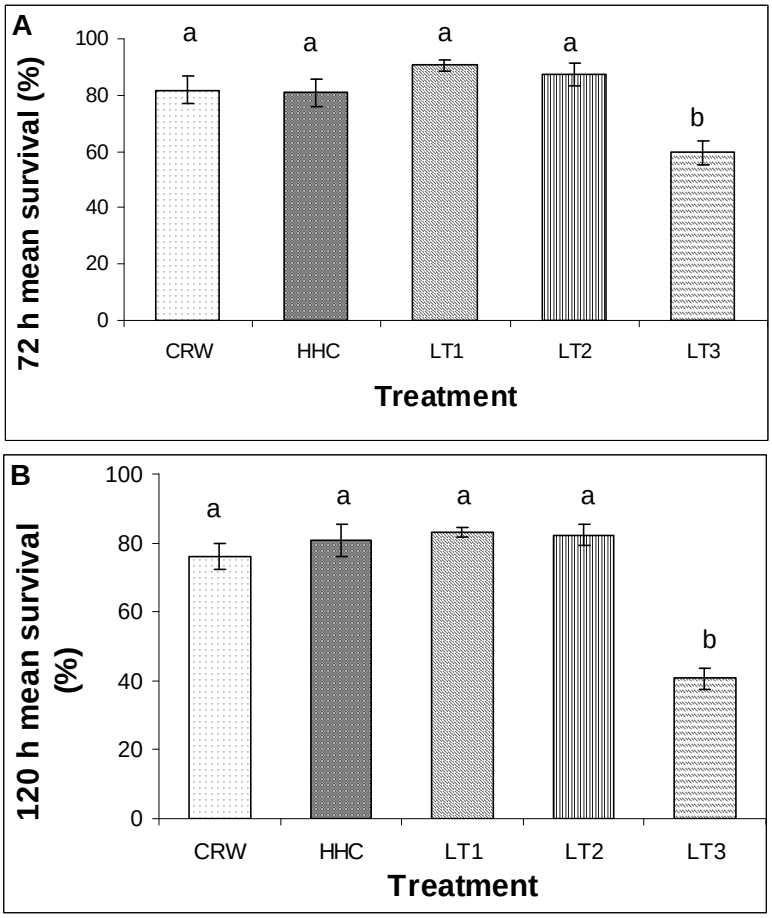


Figure 12. Sac-fry mean survival % ± standard error % for sac-fry at 72 h (A) and at 120 h (B). Different lowercase letters indicate significant differences.

4.4.4.1 Mean growth of sac-fry

Sac-fry length, width and height did not increase after the 72 h inspection time for controls or treatments (Figure 13). Therefore the 72 h inspection time was used as the comparison point for analysis of these parameters. Yolk-sac diameter and gape are compared at 120 h. A lower mean length and yolk-sac diameter were produced in LT3 than the controls and treatments LT1 and LT2. Treatment LT3 also produced sac-fry with a small height and gape and a narrow width similar to control HHC. Treatments LT1 and LT2 produced sac-fry with a significantly higher ($F_{(4,95)}$=7.04, p=0.001) width at 72 h than the controls and treatment LT3. The sac-fry height produced in these treatments was similar to the mean height in the CRW. Gape development started at 72 h post hatch.

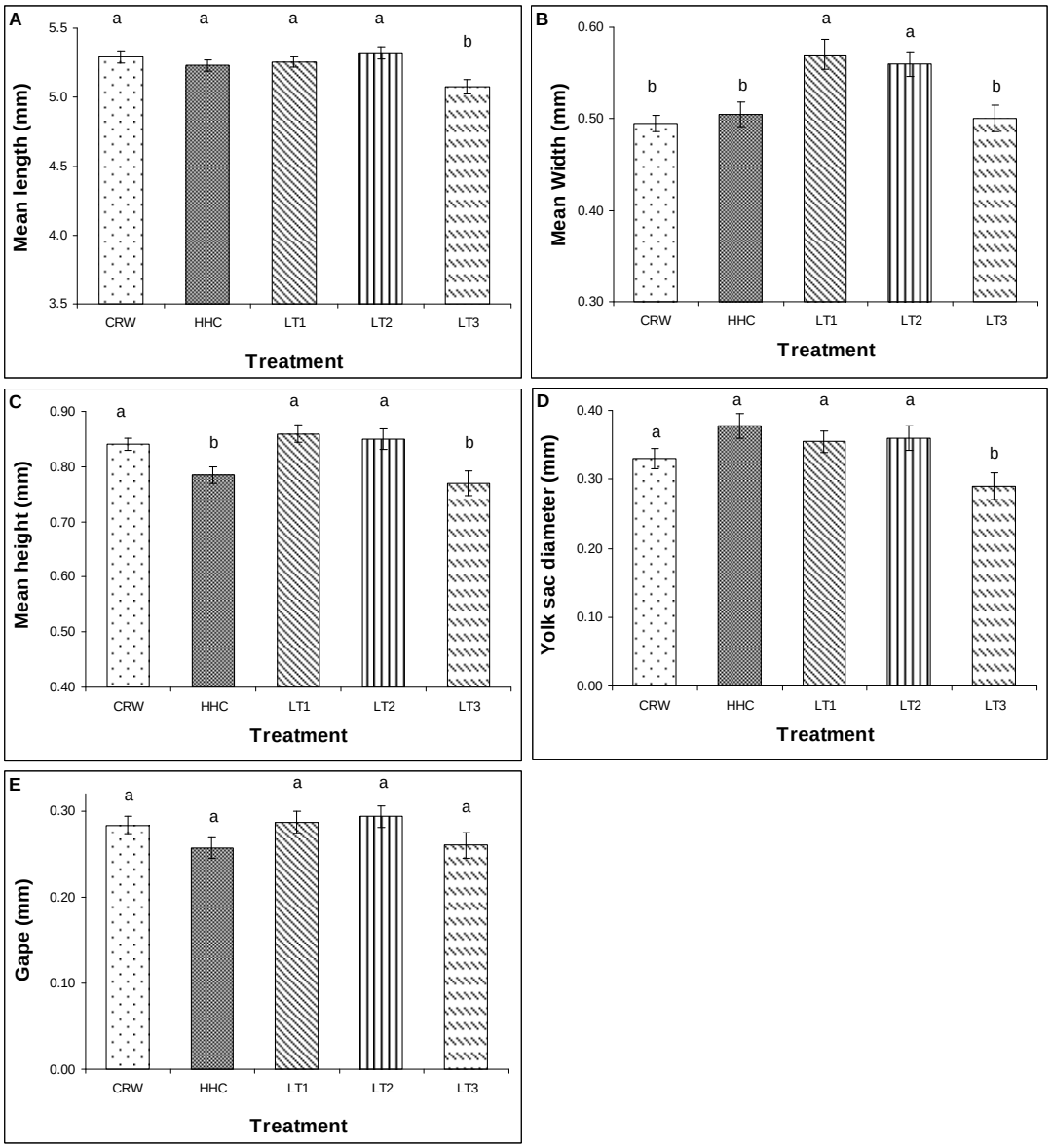


Figure 13. Sac-fry mean growth ± standard error for A) length, B) width, and C) height at 72 h, D) yolk sac at 120 h, and e) gape at 120 h. Different lower case letters indicate significant differences.

Growth rate

No limestone treatment consistently produced a high sac-fry growth rate or low growth rate across the growth parameters (Figure 14). However, the CRW and treatment LT2 produced the highest mean length growth rate for sac-fry of 0.8 mm/day. Control CRW also produced the highest yolk-sac absorption rate of 0.1

mm/day. The lowest sac-fry length growth rate of 0.6 mm/day occurred in the HHC. Sac-fry yolk sac absorption in treatment LT2 was lower than that of the controls and other treatments. Sac-fry mean width, height and gape growth rate was similar in all controls and treatments. Treatments LT1 and LT2 showed the trend of having the highest or equal highest growth rate for all parameters except yolk-sac absorption.

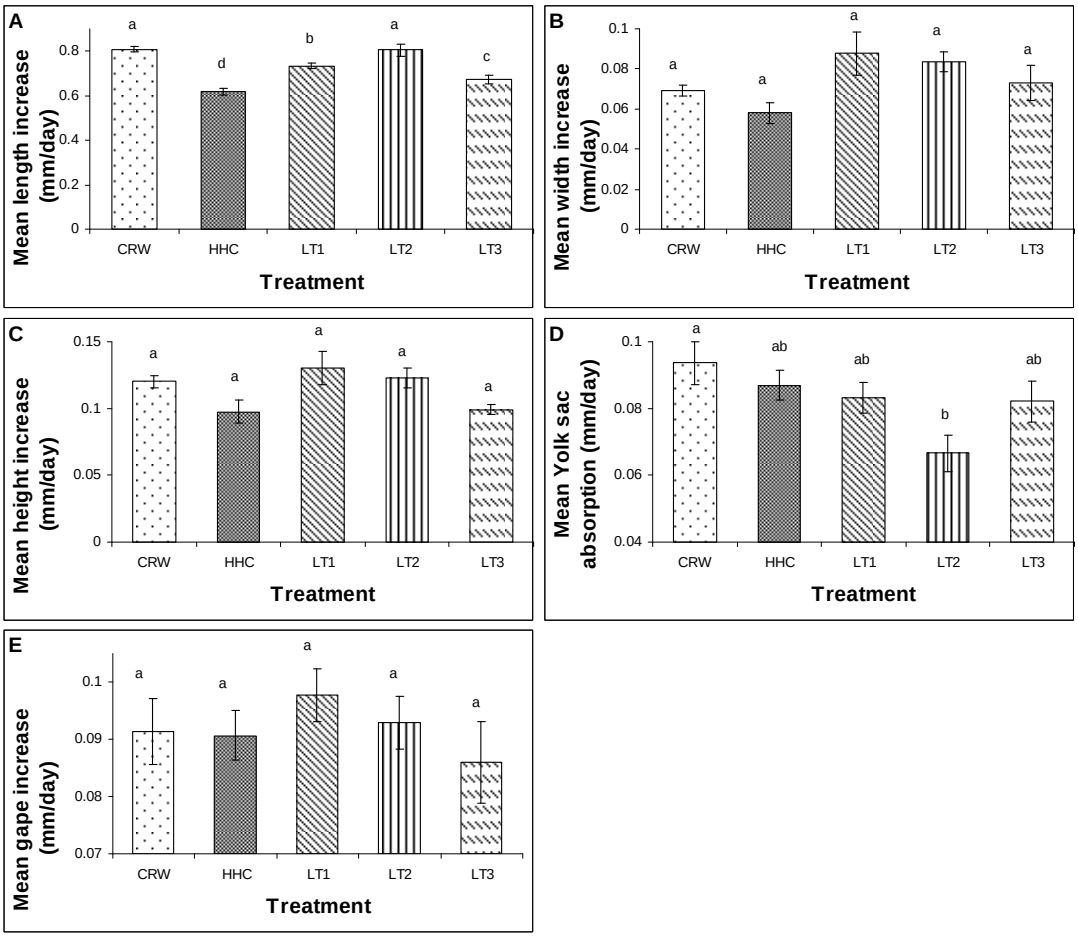


Figure 14. Sac-fry mean growth rate ± standard error of A) length, B) width, and C) height at 72 h, and D) yolk sac at 120 h, and E) gape from 48 h to 120 h. Different lower case letters indicate significant differences

4.4.5 Trial 2: post sac-fry survival and growth test

4.4.5.1 Survival of post sac-fry

Survival at 72 h in the controls and treatments LT1 and LT2 was greater than 80% (Figure 15). Survival in LT3 at 72 h was lower than that of the controls and other

treatments. Survival in the controls at 120 h was greater than 70%. A significantly lower ($F_{(4,15)}$=18, p=0.001) survival (41%) time than that of the controls and treatments LT1 and LT2 was displayed in treatment LT3 at 120 h.

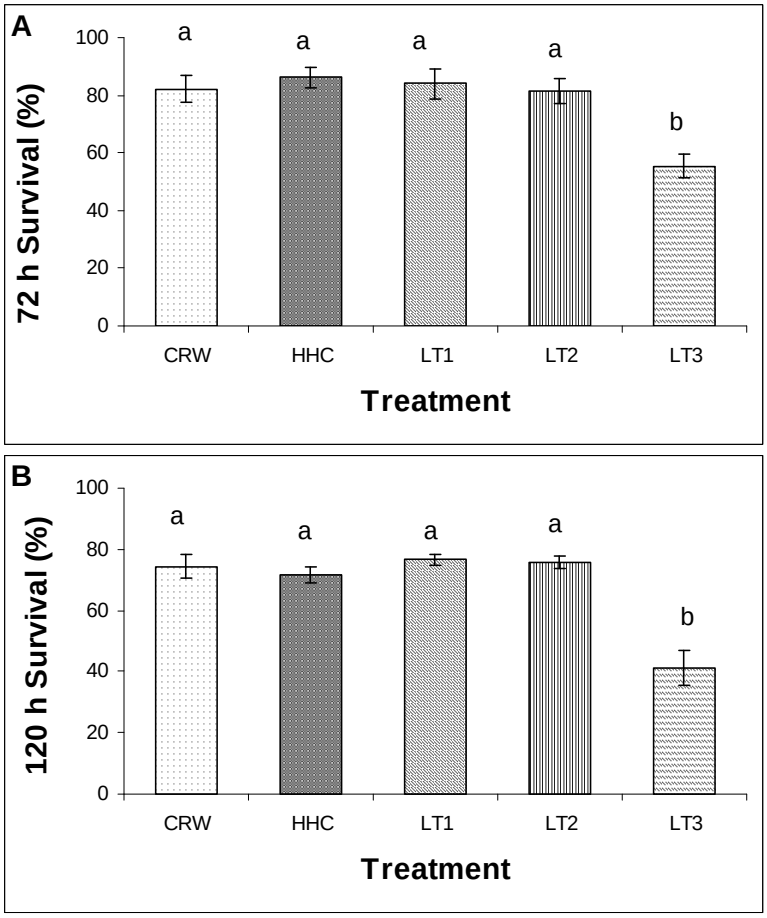


Figure 15. Post sac-fry mean survival ± %standard error for A) at 72 h and B) at 120 h. Different lowercase letters indicate significant differences.

4.4.5.2 Mean growth of post sac-fry

Mean 120 h growth results for the post sac-fry test are presented in Figure 16. The CRW maintained the equal highest mean growth for all parameters. Mean length ($F_{(4,95)}$=62.7, p=0.001) and width ($F_{(4,95)}$=3.0, p=0.023) of post sac-fry in treatment LT3 was significantly lower than that of the other treatments and controls. Post sac-fry height was significantly less ($F_{(4,95)}$=6.1, p=0.001) in treatment LT3 than that of treatments LT1 and LT2 and CRW. Treatment LT3 post sac-fry had a smaller gape

than that of LT1. Mean yolk sac diameter was not included in the post sac-fry results due to the complete yolk sac absorption by 72 h.

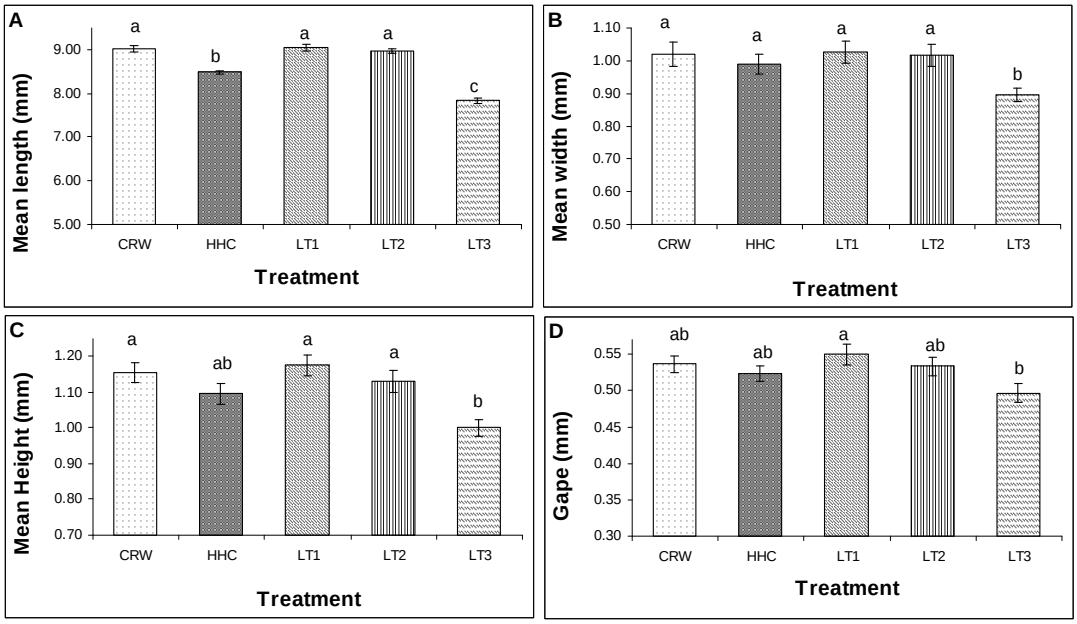


Figure 16. 120 h post sac-fry mean growth (mm) ± standard error (mm) for A) length, B) width, C) height and D) gape. Different lower case letters indicate significant differences.

4.4.5.3 Growth rate of post sac-fry

CRW produced the equal highest growth rate of post sac-fry for all parameters except length where post sac-fry were third longest (Figure 17). Post sac-fry length growth rate was highest in treatment LT1 (0.88 mm/day) followed in descending order by LT2 (0.84 mm/day), CRW (0.78 mm/day), HHC (0.72 mm/day and LT3 (0.64 mm/day). All controls and treatments showed significantly different length growth rates ($F_{(4,15)}$=53.3, p=0.001). Post sac-fry length ($F_{(4,95)}$=28.6, p=0.001) and height ($F_{(4.15)}$=4.9, p=0.01) growth rate were both significantly reduced by LT3. Treatment LT3 also displayed a slower gape development rate than treatment LT1. Controls and treatments LT1 and LT2 displayed a trend for width increase rate, in increasing order from CRW<HHC<LT1<LT2. Treatment LT3 did not follow this width growth rate increasing trend and was lower than the other treatments. The controls and treatments all had a similar effect to post sac-fry height growth rate. Post sac-fry in the controls and treatment LT3 had completely absorbed their yolk

sacs by 48 h. Whereas, fry in treatments LT1 and LT2 did not absorb their yolk sacs until 72 h. Absorption rate and gape increase rate were similar between controls and treatments with the exception of the gape increase rate in treatment LT1 which was significantly higher ($t_{(6,4)}$=3.4, p=0.014) than that of LT3.

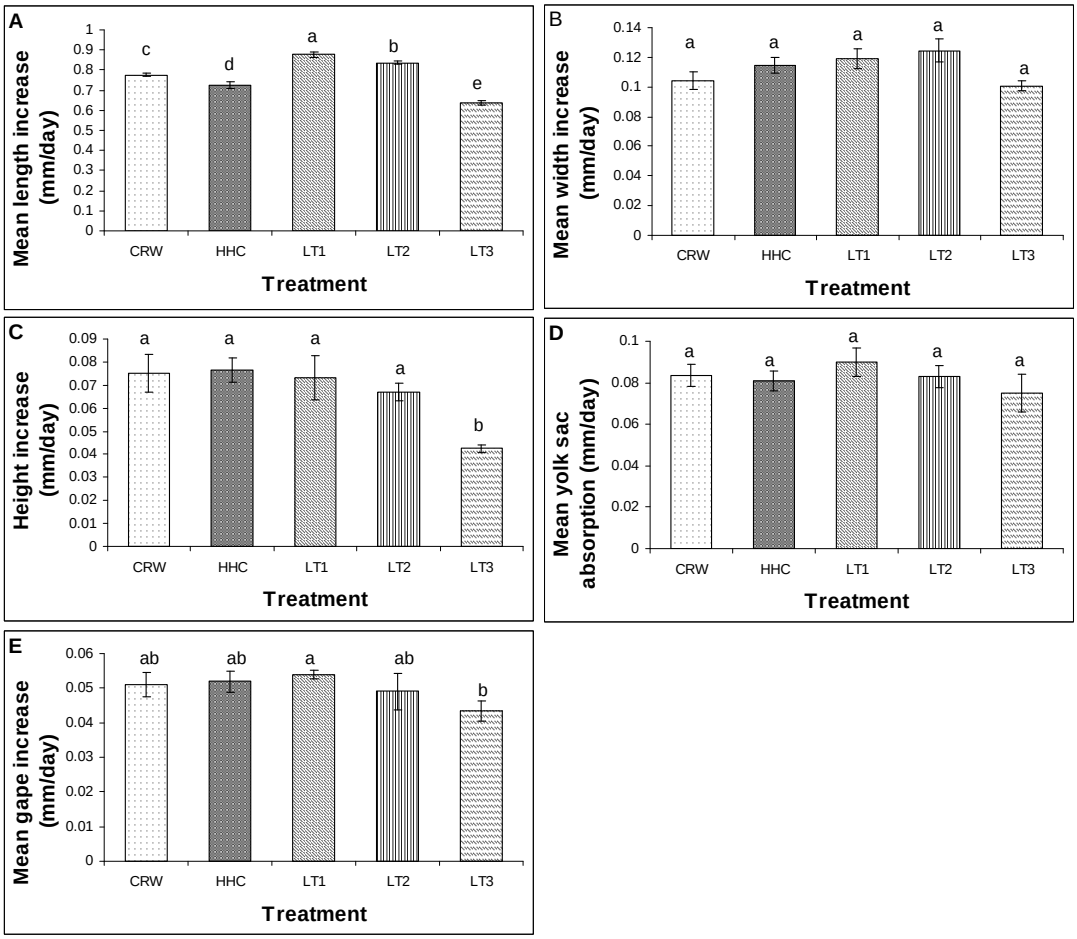


Figure 17. Post sac-fry mean growth rate (mm) ± standard error (mm). All parameters are 120 h except yolk sac absorption which is 72 h. Different lower case letters indicate significant differences.

4.5 Discussion

Three limestone acid mine pit lake water neutralisation treatments 1.4 g/L, 1.0 g/L and 0.6 g/L were used to remediate the toxicity exhibited from Chicken Creek pit lake. Bioassays using *B. bidyanus* were then employed to determine the efficacy of these remediation treatments at reducing toxicity to an aquaculture species. A

synthetic control High Hardness COMBO medium was also trialled for its suitability as a possible control in future toxicity testing with *B. bidyanus*.

4.5.1 Trial 1

Trial 1 was a preliminary experiment to assess whether the methods used would be adequate for the testing of *B. bidyanus* to treated acid pit lake water. The reason for the lateness of the spawn can be attributed to the low temperature of 21±2°C in the spawning tank. As expected mortality was 100% within the untreated Chicken Creek water. Survival results from the egg test in trial 1 natural pond water were acceptable however the hatchability was not and therefore this water was not used in Trial 2. Differentiation between survival results in treatments was not possible from trial 1. Therefore, to increase the statistical power of the testing, more replicates, a higher number of fish and eggs per replicate and the measuring of growth parameters were introduced in trial 2.

High mortality before hatching has been noted by a silver perch hatchery manager in WA on many occasions, especially late in the spawning season (C. Schmidt, silver perch hatchery manager Pers comm. 2004). As a result, it was decided to assess hatchability in trial 2 after embryos emerged from the egg and not before (Buhl & Hamilton 1990).

The yolk sac supplies fry nutrients for the first 3–7 days of their life, depending on temperature(Thurstan & Rowland 1994). During this time, the sac fry develop a mouth and gut and will begin to feed from the environment. Sac-fry food in the natural environment consists of algae, protozoans, cladocerans, rotifers and copepods (DoPIF 2004). This complex food web is very difficult to replicate within an enclosed aquaculture or laboratory situation, therefore only one live food source, rotifers, was chosen. Rotifers were chosen due to their nutritional quality, body size and relatively slow motility all contributing to their usefulness as good prey for actively feeding larvae (Guo *et al*. 1993; Fengqi 1996). Rotifers are fed *Chlorella protothecoides* algae which is grown in HHC medium. The algae assimilate nutrients from the HHC, thereby being enriched with the specific nutrients the fish need to

survive and grow. Rotifers (*Brachionus* spp.) then assimilate the nutrients from eating the algae. Fish receive the required nutrients by eating the rotifers that have in turn eaten the algae. Fry would be weaned onto microencapsulated pellet feeds as soon as possible in an aquaculture situation, but the addition of this feed has the potential to greatly alter the toxicity of the acid pit lake water, confounding the experiment. Because of this, only 5 rotifers/mL were used as the feed source. The use of 5 rotifers/mL proved to be adequate for normal growth and survival of the *B. bidyanus* post sac-fry in trial 1 and therefore 5 rotifers/mL feeding was used in trial 2.

The post sac-fry time period is the critical stage where larvae mouth and gut development enables the fish to start feeding from the environment, and as such is a very sensitive stage in the development of finfish where high mortality can be expected (Buhl & Hamilton 1990; Guo *et al*. 1993). By feeding from the environment, the likelihood of ingesting toxicants may also be increased adding to the other toxic pathways the fish is already exposed to (Peakall & Burger 2003). The high mortality shown at the post sac-fry stage in LT3 showed the sensitivity of post sac-fry to relatively low concentrations of toxicants.

4.5.2 Trial 2 water quality

The (ANZECC/ARMCANZ. 2000b) aquaculture guidelines for Australia and New Zealand are a relatively new development. The guidelines have drawn extensively on overseas' guidelines for aquaculture as well as on the personal experiences of a number of local scientific and industry specialists (ANZECC/ARMCANZ. 2000b). Several Australian representative species were used to modify the guidelines for use in Australia. *Bidyanus bidyanus* was one of the fish species used for the guideline development and therefore the guidelines are quite relevant to the use of this species. However, toxicity testing of specific chemicals and toxicants to back up the chosen guideline values has not yet been included. Consequently there are many toxicants, metals and chemicals that do not have guidelines and those that do are adapted from guidelines elsewhere. Furthermore, where specific water quality guidelines cannot be

given for the protection of aquaculture species, the use of the guidelines for the protection of aquatic ecosystems are suggested in its place (ANZECC/ARMCANZ. 2000b). Therefore, both aquaculture and aquatic ecosystem guidelines are used in this assessment of CCW toxicity and the limestone remediated CCW to *B. bidyanus*.

Analysis of metals and metaloids showed that the control CRW was within the guideline limits set for aquaculture by ANZECC/ARMCANZ (2000b) with the exception of Cu. The absence of a toxic response displayed by any of the hatching, survival or growth parameters measured indicates CRW to be a good representative control for natural waters of the Collie Coal Basin. The growth of fry was comparable and the survival was higher in CRW than that normally found in aquaculture of *B. bidyanus* (Thurstan & Rowland 1994). High *B. bidyanus* survival relative to normal aquaculture survival rates, can be attributed to the careful management of the physical parameters temperature, pH, light, dissolved oxygen and the filtering of the water that may not occur so rigorously in aquaculture within ponds or large tanks.

The concentrations of the metals Al, Cd, Cu, Ni and Zn in untreated Chicken Creek mine pit lake water were elevated above the aquatic ecosystem guideline values. A combination of the elevated concentration of some metals with a low pH was probably responsible for the acute mortality of the *B. bidyanus* eggs and fry in the untreated water. The concentrations in parenthesis of Cu (20 μg/L) and Zn (990 μg/L) found in CCW were above the 96 h LC_{50} values for Cu (10 μg/L) and Zn (315 μg/L) found by Buhl & Hamilton (1990) for artic grayling fry, indicating the potential for toxicity to the *B. bidyanus* embryo and fry. Furthermore, the LC_{50} values reduced with an increase in the age of the artic grayling juveniles indicating that it is possible that latter stages of the early life stages may still be very sensitive to toxicants. Although the arctic grayling were exposed to Cu and Zn in 'clear water' the CCW was filtered and would have removed nearly all colloidal organic matter that may have acted as a potential buffer to the toxicity. Suspended organic matter has the ability to adsorb dissolved metals consequently removing them from bioavailability and reducing possible toxicity (Spry & Wiener 1991). That is, unless

the suspended particulates are not consumed by the organism. Therefore, in the filtered water the concentrations of Cu and Zn were probably adequate to cause toxicity, even without a low pH.

Ni is not known to be highly toxic to fish and decreases in toxicity with increasing hardness (ANZECC/ARMCANZ. 2000a). No effect on larval fathead minnow was shown from growth after a 168 h exposure to a Ni concentration of 2000 μg/L in hard water (hardness 140 mg/l as CaCO3) (Pyle *et al.* 2002). Furthermore, Alam and Maughan (1992) exposed 3.2 cm and 6.0 cm common carp (Cyprinus carpio) to Ni (pH 7.1) and reported 96-h LC50s of 1300–1540 μg/L and 1640–2300 μg/L respectively. These studies show that Ni is acutely toxic to fish at concentrations significantly higher than the highest concentrations observed in this study of 200 μg/L, therefore indicating that at the high hardness (>500 mg/L) in the treated water Ni would have contributed little toxicity to *B. bidyanus*.

The elevated concentration of Al (18000 μg/L) in CCW was more than thirty times higher than the 380–490 μg/L giving high mortality in 97 h to seven freshwater fish species tested in acidic water (Poleo *et al*. 1997). Furthermore, the Al concentration in LT3 would not have been the cause of toxicity in hatching, hatchability, survival, growth and growth rate because it was below both guideline levels and detection levels. The toxicity of Al to fish is primarily due to effects on osmoregulation by deformation of the gill surface. Little absorbed Al is found in blood serum or internal organs, therefore the embryo stage should be the least affected (Poleo *et al*. 1997). The most sensitive stage should be the post sac-fry stage when gill development is occurring and with sensitivity decreasing then on with ontogenetic development (Peakall & Burger 2003).

However, Gellert & Heinrichsdorff (2001) noted that the most sensitive stage is when the egg was not hardened at 0–1.5 h of age for zebrafish, the 0–1.5 h egg age was not tested due to the high mortality that can occur when moving eggs that have not yet hardened, therefore, removing possible errors of survival and or growth occurring in the limestone treatments. Furthermore, high mortality has been recorded

(60%) for *B. bidyanus* embryos before 1.5 h post fertilisation in water with a 6 ppt salinity (Guo *et al*. 1993). High mortality was attributed to the eggs water hardening process not being completed indicating either a high sensitivity to the salinity, or possibly previous damage and stress sustained from movement of eggs before hardening, thereby exaggerating the response.

Metal concentrations were all reduced in limestone treated CCW from the original concentration in the CCW. Toxicity present in the pit lake water was removed completely from limestone treatments LT1 and LT2, most likely through increasing pH and reducing metal concentrations. Nevertheless, concentrations of some metals present in the treated water were still above the guideline values for these metals and were more likely to be causing toxicity than pH. Toxicity from low pH (H^+ ions) is not considered as deleterious as toxicity resulting from dissolved metals (Stephens & Ingram 2006). For example, hydrogen ions can compete with metals for binding sites, consequently reducing metal uptake due to competition at cell surfaces (Hyne *et al*. 2005). The pH was increased to above 7 in all three limestone treatments showing that the remaining toxicity seen in LT3 to be due to remaining elevated metal concentrations. The concentration of Zn remaining in mine pit lake water after treatment with LT3 was higher than that of treatments LT1 and LT2 and could have been the metal causing toxicity to the fry in this treatment. The remaining concentration of Zn in LT3 was 440 μg/L which was higher than the 96 h LC_{50} of 315 μg/L for arctic grayling shown by (Buhl & Hamilton 1990). There would be a high proportion of Zn^{+2} at the circum-neutral pH in the treated CCW which is the main toxic form of Zn (ANZECC/ARMCANZ. 2000a). The Zn toxicity has been shown to increases when pH increases from 6.5 to 8.2 (Hyne *et al*. 2005). Furthermore, Hyne *et al*. (2005) noted that the Zn toxicity did not reduce significantly with increasing Ca concentrations. Therefore, the remaining elevated Zn concentration is likely to be toxic to *B. bidyanus.* Even if Zn doesn't exhibit toxicity from these bioassays, Zn is known to bio-accumulate and cause long term problems especially in bivalves and long lived vertebrates (Barron & Albeke 2000).

Hardness is known to reduce the toxicity of metals to fish and other aquatic animals through competition for binding sites (Brown & Markich 2000; Morgan *et al*. 2005). The permeability of fish-gill membranes to divalent metal ions is commonly inversely related to aqueous calcium concentrations (Spry & Wiener 1991). The high total hardness created by the LT1 and LT2 (660 mg $CaCO_3$/L) could have been sufficient to buffer potential toxicity from the metal concentrations remaining above the guideline values. The total hardness resultant in the pit lake water from LT3 (540 mg$CaCO_3$/L) was not as high as the other limestone treatments and therefore may not have completely buffered toxicity to *B. bidyanus*. Very little research on the effects of hardness concentrations to metal toxicity above 400mg/L has been completed. Therefore only assumptions on the hardness effects can be made above 400mg/L (Markich *et al.* 2001; Morgan *et al.* 2005).

Several metal concentrations remained elevated above the aquaculture and aquatic ecosystem guideline values in all the limestone treatments of CCW. Even when applying the HMGV, the total concentration of Zn was still elevated and was probably the major toxicity causing metal in LT3. However, when applying the HMGV caution has to be used due to the way the HMGV were constructed. Firstly the hardness concentrations used in the construction of the HMGV were between 20–400mg/L (Markich *et al*. 2001). Secondly, the HMGV were only developed for the protection of aquatic ecosystems and not for aquaculture or other aquatic end uses (ANZECC/ARMCANZ. 2000b). Thirdly, although the use of hardness algorithms probably increases the usefulness of the guidelines they are likely to be inaccurate without the consideration of other crucial factors such as DOM, pH and most importantly the complex antagonistic and synergistic effects of metals on aquatic organisms in complex mixtures (Peakall & Burger 2003). Therefore, caution should be applied with regard to HMGV use and especially when hardness is outside the 20–400mg/L range.

Assessment of Al toxicity to Vendace (*Coregonus albula* L.) by Duis & Oberemm (2001) showed increasing Ca concentration from 111–117 mg/L to 233–256 mg/L had no influence on hatching and survival percentages. However increasing the total

hardness from 300 mg$CaCO_3$/L in CCW to >540 mg$CaCO_3$/L through treatment with limestone is an increase by nearly a factor of 2 and would probably succeed in buffering toxicity from the remaining metals to *B. bidyanus* eggs and fry. Barron & Albeke (2000) noted that calcium reduces zinc uptake through both biological acclimation and chemical processes, and that the protective effects of calcium are approximately additive. Therefore, indicating that the increase of hardness from 540 mg/L in LT3 to 640 mg/L in LT1 and LT2 may be sufficient to reduce toxicity from the remaining elevated concentration of Zn.

Some static toxicity tests over estimate toxicity indicating that the concentrations of metals still present within the limestone remediated water may be even less toxic *in situ* when fish are to be aquacultured (Lowell *et al*. 1995; Dilks & Pendergast 2000). Another reason for natural aquatic environments commonly showing lower toxicity of metals to aquatic organisms than laboratory experiments can be attributed to natural Dissolved Organic Carbon (DOC) (Hunn 1985; De Schamphelaere & Janssen 2004). Therefore, concentrations of metals derived as toxic in the laboratory using artificial diluents can be overprotective for aquatic environments. Risk assessment guideline values are often over protective to aquatic environments due to there derivation from *in vitro* bioassays, further indicating the effectiveness in the limestone treatments of LT1 and LT2 to removing toxicity to eggs and fry of *B. bidyanus* (Chapman 2000; Chapman 2002). However, guideline values have also been noted to be under protective in some instances where additive toxicity effects are encountered with more than one toxicant (ANZECC/ARMCANZ. 2000b; Mitchell 2002)

The water quality parameters of temperature, and DO measured during trial 2 were within the range suggested necessary for good egg hatching and survival by Thurstan & Rowland (1994). Ammonia and urea are the two main nitrogenous products excreted by teleost fish in aquaculture and are highly toxic to aquatic animals (Frances *et al*. 2000). Ammonia usually represents 75–90% of nitrogenous excretion and is mainly excreted as the unionised form NH_3 (UAN) (Lemarie *et al*. 2004). The EC_5 of wet weight gain for *B. bidyanus* juveniles was found to be 0.06 mg UAN/L

and survival was not affected below 0.36 mg UAN/L (Frances *et al*. 2000). Furthermore, growth of *B. bidyanus* was not reduced in ponds until concentrations of UAN reached levels of more than 0.65 mg/L (Rowland 1994b). In the present study, the measuredtotal ammonia was at insignificant levels for toxicity from either of the two forms of ammonia. Therefore, it was concluded that although fry are possibly more susceptible to ammonia toxicity, the small concentration of UAN present (<0.03 mg/L) would not have had a significant affect to larval growth or survival during the experiment.

The optimal pH range for most aquatic organisms falls between the range of 6.5–9.0 (Thurstan & Rowland 1994). However, *B. bidyanus* fry in ponds frequently deal with pH values from 6–10.2 with normal survival rates showing a high resilience to pH fluctuation (Rowland 1994b). This pH fluctuation usually results from high biomasses of algae producing and using CO_2 and therefore decreasing and increasing pH respectively. Fluctuation in pH was not experienced during the testing and pH was well within the range 6.5–9.0. Therefore, the pH (7–8.2) that resulted from treatment of CCW with limestone should not have had an adverse effect on growth rates. However, as stated above, the remaining Zn concentrations may still have been toxic to *B. bidyanus* at the pH range 7–8.2.

A high sensitivity to toxicants has been described for post sac-fry at time of first feeding due to subtle decreases in feeding resulting in future mortality and growth problems (Cleveland *et al.* 1991). This may be attributed to the high oxygen demand the fish exhibits at the first feeding stage (Ronnestad *et al*. 1998). The oxygen was artificially maintained at above 90% at all times during the testing and therefore would not be seen as a factor in reduced survival or growth for post sac-fry. Therefore, any toxicity would likely have occurred from remaining concentrations of metals in the limestone treatments.

4.5.3 Trial 2 egg survival, hatch and hatchability test

This study's testing was conducted late in the *B. bidyanus* spawning season. Lateness in the season (February) can affect results due to poorer quality and less eggs being produced. The change in egg production is due to the female starting to reabsorb the roe (Rowland 1994b). Furthermore, the availability (seasonal) of eggs or fry for testing may be a problem unless fish can be artificially matured and spawned all year round in temperature controlled tanks. Maintaining a large fish species such as *B. bidyanus* year round for spawning would be expensive, that is, if it is even possible. These factors may all restrict the quality, reproducibility and timing of the toxicity test.

Results from the egg test showed that CCW caused 100% mortality in unhatched embryos. This result supports the original hypothesis that CCW would be unsuitable for hatching *B. bidyanus* without some form of remediation. Embryos showed high survival in the CRW which was similar to that of HHC. This indicates that HHC could be used as a control in future testing of acid pit lake water treatments. Modification of HHC medium to increase hardness and alkalinity could increase its comparability to the Collie River Water and other natural waters (Baer *et al*. 1999). LT1 and LT2 showed similar results to the controls CRW and HHC and therefore would be suitable for hatching of *B. bidyanus*. Survival of embryos in LT3 was low and variable indicating that it was not suitable as a successful remediation treatment of CCW. However, there was no statistical difference between treatments showing that the post-egg hardening stage is not as sensitive to toxicants as the sac-fry and post sac-fry stages.

Trial 2 hatch and hatchability gave similar results to each other with the parameter hatchability slightly more sensitive in finding differences between the treatments and controls. This indicates that the endpoint of hatchability could be used as a good parameter for differentiating toxicity by itself in future testing. The effectiveness of hatchability as an end point has been shown by others (Guo *et al*. 1993; Morgan *et al*. 2005). The controls CRW and HHC both displayed hatchability of 100%, supporting the utilisation of HHC in further testing as a control. LT3 showed a lower hatchability than the controls indicating further that this treatment is not sufficient in

removing toxicity from CCW. Hatchability of *B. bidyanus* was a more sensitive parameter than survival in the egg test.

The survival of embryos in the controls (>70%) was higher than that experienced by Hamilton *et al.* (2005) using razor back sucker fish (survival 66%). Percent hatch was also higher than that recorded by Hamilton *et al*. (2005), however hatchability in controls was similar. The sac-fry stage has been noted as the most sensitive for Australian bass (*Macquaria novemaculeata*) (Hyne & Wilson 1997). Nevertheless *B. bidyanus* sac-fry and post sac-fry survival showed similar sensitivity. The high survival recorded in trial 2 controls is a good indicator that *B. bidyanus* would be a useful species in future toxicity testing.

The recorded high embryo survival and hatchability indicates that commercial scale hatching success with limestone treated CCW of *B. bidyanus* is probable with CCW limed to at least 1.0g/L. Hatching at the pit lake, in place of buying in fry or spawning at a separate location and transporting them to the pit lake for culture, could reduce possible incidences of high mortality from transport and acclimation stress as well as reducing logistical problems and costs. Furthermore, fish have been noted to take approximately two weeks to completely acclimate to temperature changes and this may be longer for chemical acclimation (Sprague 1971). Instead *B. bidyanus* can be hatched in limestone treated pit lake water and transferred straight to tanks or ponds containing the same treated pit lake water at a similar temperature. Juvenile and adult *B. bidyanus* have been successfully grown in limestone treated CCW previously to this study (Storer *et al.* 2004) showing the good prospect for rearing of eggs and fry within this water. The methods utilised here on *B. bidyanus* egg survival, hatch and hatchability for assessing the limestone treated CCW would also be useful in assessing effectiveness of other acid mine pit lake amelioration treatments.

4.5.4 Trial 2 Sac-fry survival and growth test

Survival of *B. bidyanus* sac-fry in the controls and limestone treatments LT1 and LT2 was above 80% at 72 h, demonstrating HHC to be a satisfactory control for future testing. Survival resulting from exposure of sac-fry to LT1 and LT2 demonstrated that these treatments remediate toxicity sufficiently. Survival of sac-fry resulting from exposure to treatment LT3 was approximately half that of the other limestone treatments. In an aquaculture situation, survival of sac-fry would not be expected to be as high as recorded in this test which is commonly only 30–50% (Rowland 1994b). Therefore, if the survival ratio stayed approximately the same (2:1 for CRW:LT3), normal aquaculture sac-fry survival in LT3 would be approximately 15–20%. Aquaculture aims at achieving the highest survival and growth rates therefore excluding the use of LT3 for sac-fry rearing. Similar results were seen for sac-fry survival at 72 and 120 h. Therefore, the test could be terminated at 72 h with the same result not only reducing the duration of the test but also the cost.

Growth of sac-fry in controls was similar for all growth parameters except height indicating HHC to be sufficient in sustaining growth in sac-fry. The mean growth of sac-fry in LT3 was lower than that of the other limestone treatments for most parameters. This lower growth demonstrates that LT3 is not effective in completely removing toxicity to the sac-fry. All the growth parameters measured were able to show that LT3 treated CCW was toxic to the sac-fry except for gape. Therefore the use of length, width, height and yolk-sac absorption could be used in future testing as sensitive parameters to differentiate treatment success. Yolk sac absorption and length increase are common biometrics for assessing toxicity to fry development (Hyne & Wilson 1997; Ronnestad *et al.* 1998; Carnevali *et al.* 1999).

The parameter yolk sac diameter can be compared between treatments as an indication of energy expenditure for fry growth (Vuorinen *et al*. 1993). Vuorinen *et al*. (1993) showed that Al and low pH reduced pike and roach sac-fry growth, which was coincident with a slower absorption of the yolk due to a lower metabolism. However, results for *B. bidyanus* in this study displayed quicker yolk-sac absorption

coincident with lower growth of the parameters. High yolk-sac absorption rates in the presence of a weak toxicant could be attributed to the need for higher energy expenditure for normal metabolic processes. The toxicant may only be sufficient to increase metabolic processes slightly and not enough to affect any parameters measured in this testing, except energy consumption. Therefore, the smaller yolk-sac result from fry in limestone treatment LT3 could indicate that more energy has been expended in sac-fry from LT3 for normal metabolic processes. However, more investigation into measuring the metabolic processes is required to understand why yolk-sac absorption was greater in slower growing sac-fry.

The reduction in growth of sac-fry in LT3 was not displayed in the results from the growth parameter gape. Therefore gape size at these early stages of growth may not be a good indicator of toxicity.

Growth rate of sac-fry was similar in controls for all parameters except length where HHC was lower than CRW. Even though the mean growth at the end of the test was similar for CRW and HHC, if growth rate trends continued further, a significant difference in mean growth may be seen in later stages. If this is the case, HHC may not be suitable for longer test durations. Although, in the case of testing sac-fry, the test cannot run any longer without feeding due to the complete absorption of the yolk-sac within 3 to 7 days. Growth rates were similar in all treatments for width, height and gape but still resulted in different mean growth at 72, 72 and 120 h respectively. The significant differences found between the controls and limestone treatments in growth rate of length can be attributed to the large number of fry per replicate and four replicates for finding statistical differences between treatments. However, the growth rate differences only lead to a difference in mean length of sac-fry in LT3. Utilising a longer test duration, significant differences in the mean growth result may be detected. Longer tests are not possible with sac-fry, but continuing sac-fry testing into the post sac-fry stage may further produce significant differences. Therefore, only fry length was considered suitable in differentiating growth rates of different limestone treatments for sac-fry.

4.5.5 Trial 2 Post sac-fry survival and growth test

Feeding >1 rotifer/mL is needed to obtain *B. bidyanus* survival of 30% or more (Thurstan & Rowland 1994). The survival of post sac-fry in LT3 at both 72 and 120 h was less than half the survival for the controls and other limestone treatments. This high survival for post sac-fry was similar to that of the sac-fry test. High survival above 50% would not be expected in an aquaculture situation and is attributed to the stable laboratory conditions and lack of predation or disease. However the high post sac-fry survival is useful in the testing of toxicants and whole effluent testing. High survival (>80%) shows that HHC would be adequate for use as a control in future tests on post sac-fry. Future mortality occurring after the post sac-fry test would be expected to be low as fry develop into juveniles (Guo *et al*. 1993).

The mean length at 120 h in HHC was lower than that in the CRW. This trend did not follow the other growth parameters indicating that this control would still possibly be suitable for growth of post sac-fry. Longer testing of this stage up to the juvenile stage (day 18) should be possible with HHC as a control, but would need to be investigated further. Post sac-fry in LT3 exhibited toxic responses, with lower growth rates for all growth parameters except gape. Further indicating that LT3 is not suitable for rearing of post sac-fry. Post sac-fry in LT1 and LT2 exhibited good mean growth similar to the controls and therefore liming of CCW >1g/L should be considered for rearing of *B. bidyanus* post sac-fry.

Bidyanus bidyanus post sac-fry growth rate for length when feeding on natural food in aquaculture is normally between 0.6 mm and 1 mm per day (Thurstan & Rowland 1994). The growth rate results for length for post sac-fry fed rotifers were within this growth rate range for all controls and limestone treatments. Growth rate of length varied between all treatments and controls with a range of 0.61–0.82 mm/day. The variation in growth rate could lead to a difference in mean growth rate if the test was extended or if the limestone treatments were used for aquaculture. HHC and LT1 and LT2 exhibited similar growth rates with the exception of length. The post sac-fry test could be extended to find more sensitive differences or trends between the treatments

and controls if growth rate was used as the test endpoint. HHC displayed equally good growth rates for all parameters when compared to CRW indicating that it could be used for a control or diluent in further testing. Post sac-fry growth rates in LT1 and LT2 followed the trend of being higher than that of post sac-fry LT3 for all parameters excluding LT3 from rearing of post sac-fry.

Yolk sacs were absorbed by fry in all treatments and controls by 144 h post hatch, which is slightly longer than average for *B. bidyanus* (120 h) Thurstan & Rowland (1994). Different fish species commonly exhibit different yolk-sac absorption times with temperature being a major contributing factor (Guo *et al*. 1993). Yolk-sac absorption in *B. bidyanus* is usually completed by 120 h at between 20–25°C. The Yolk-sac was absorbed last in limestone treatments T2 and T3 in direct contrast to what would be expected in relation to absorption and growth increase as indicated by Ronnestad *et al*. (1998). The yolk-sac absorption/growth phenomenon found in this testing is explained in the trial 2 sac-fry section.

Juveniles and adult *B. bidyanus* have been successfully grown in limestone treated pit lake water previously to this study (Storer *et al*. 2004). The results from this study show that rearing *B. bidyanus* from the egg stage through to the post sac-fry stage is possible in LT1 and LT2. Therefore, rearing of *B. bidyanus* from egg to adult is possible at a commercial scale in limestone treated CCW.

Fry used in both the sac-fry and post sac-fry stages were collected from treatment or control water that had already exposed the previous life stages to the respective treatment waters. Previous natural selective forces may have resulted in weaker fish dying before being introduced into the actual bioassay tests. Therefore, fry tested are not a representation of the normal population but are actually the strongest or most resilient of the population. Consequently, survival and growth rates calculated from exposure to treated water are possibly higher than would be found if the fish came from a normal population not affected by the treatments. However, by allowing the hatching and development to take place in the respective treatments the fry are exposed for a longer period and therefore show effects from a chronic exposure over

the tested life stage as well as the preceding ones. This also is more representative if aquaculture of *B. bidyanus* was to be cultured in the limestone treated mine pit lake water.

The most sensitive growth stage of *B. bidyanus* may possibly be before egg hardening (<1.5 h after spawning) (Guo *et al*. 1993), however, eggs were not spawned in treated pit lake water. Further testing to assess the possibility of this pre-egg hardening stage could be beneficial due to the small time frame and low cost of the experiment. This experiment utilised an intermediary choice of these qualities but, with more treatments, endpoints and a longer duration may have given more sensitive results.

Animal ethics approval is becoming more stringent and tests using vertebrates may eventually be prohibited. Consequently work to establish substitutes for vertebrate tests are becoming more common. However, currently no substitute has been developed that can completely remove the need to use fish in an ecosystem representative battery or for biological indicators of toxicity for aquaculture purposes (Mitchell 2002). There is no doubt that animal ethics policy is a significant restriction to aquatic vertebrate studies and will eventually exclude these studies from ecotoxicology.

4.6 Conclusions

Chicken Creek acid pit lake water was acutely toxic to all three *B. bidyanus* early life stages tested in these trials. The sac-fry and post sac-fry life stage toxicity tests for *B. bidyanus* displayed similarly high survival in limestone treatments LT1 and LT2, higher than that found commercially, indicating the possible use of ≥1g/L limestone to treat CCW for commercial scale aquaculture. The fry survival results indicated that limestone treatment for <1g/L was not suitable for rearing *B. bidyanus* at these early life stages. The limestone treatments of ≥1g/L removed toxicity to *B. bidyanus*

from the CCW allowing for growth rates equivalent to those found in commercial aquaculture.

The limestone treatments ≥1g/L ameliorated toxicity from the Chicken Creek acid mine pit lake water through increasing pH and dissolved metal reduction. However, some concentrations of metals remained elevated above guideline values for the protection of aquaculture species and for protection of 95% of aquatic ecosystems as (ANZECC/ARMCANZ (2000b). The increase in concentration of total hardness and alkalinity from the limestone treatments was probably capable of buffering the antagonistic toxicity from the remaining metal concentrations. Therefore, as all other concentrations of remaining metals were similarly low in all limestone treatments the primary remaining toxic agent in LT3 was probably Zn. Also toxicity exhibited in LT3 could occur from additive toxicity from all the concentrations of remaining metals in the presence of the lower total hardness concentrations compared to the ≥1g/L limestone treatments (Warne & Hawker 1995; Mitchell 2002). However, this is less likely to be the case as small increases in concentration of hardness do not have an equivalent effect in buffering toxicity (Morgan *et al*. 2005).

Survival was a good indicator of remaining toxicity for the post-sac and sac-fry life stages by showing significant differences between limestone treatments. Nonetheless, test duration for assessing growth rate of post sac-fry could also be extended to find significant differences or trends. However, mean height at 72 h for sac-fry and 120 h for post sac-fry gave a good indication of remaining toxicity and differentiation between the limestone treatments. The sac-fry yolk-sac diameter at 120 h and length at 72 h also gave a good indication of remaining toxicity in the limestone treatments. Hatchability was able to differentiate toxicity remaining in LT3 compared to CRW but hatch percentage could not.

The HHC medium gave promising results for survival of the early life stages tested but gave variable results to some growth parameters of sac-fry and post sac-fry when compared to that in Collie River water. This indicates that HHC may need further testing before being used as a permanent control. Increasing the Ca hardness

concentrations of this medium may have a positive influence for consistency of the growth and survival parameters. However, this could affect its usefulness as a diluent by reducing or buffering toxicity from some ions and complexes through competition with binding sites (Morgan *et al.* 2005).

Bidyanus bidyanus met the criteria for being a representative aquaculture toxicology test species through having the following attributes; established culturing and hatchery techniques, known optimum physical parameters, rapid and uniform growth, known diseases under hatchery conditions, can be held in captivity in high densities, is amenable to artificial feeds, is an Australian aquaculture species, is an Australian native fish and is non-cannibalistic (Rowland 1994a; Riethmuller *et al.* 2003). *Bidyanus bidyanus* proved to be a good candidate for future toxicity testing of remediated pit lake waters for aquaculture through meeting criteria for a good toxicity test species, with >80% survival in control water, comparable growth rates to commercial aquaculture obtained in laboratory conditions and by using sufficiently sensitive biometrics was able to differentiate toxicity from the ameliorated mine pit lake water.

Through the damming of rivers, malignant mining and farming practices together coupled with climate change, fresh surface water resources are rapidly being contaminated and reduced within Australia (Johnson & Wright 2003). Mine pit lakes offer a large freshwater resource that if managed correctly may sustain significant environmental, social and economic benefits from selected end uses for the future. A commercial scale experiment, culturing *B. bidyanus* within >1g/L limestone remediated CCW will confirm the effectiveness of this ameliorated water for the end use of aquaculture.

BIOASSAY ASSESSMENT OF RAPID CATALYST OXIDISED ACIDIC MINE PIT LAKE WATER

5 Bioassay assessment of rapid catalyst oxidised acidic mine pit lake water

5.1 Abstract

This study used bioassays and chemical analysis to assess the effectiveness of an active filtration/remediation technique on acid mine pit lake water (abandoned flooded mine pit) contaminated with heavy metals. Quantitative indices of acute and chronic toxicity in a serial dilution approach were generated from three Australian representative species, *Chlorella* protothecoides, *Ceriodaphnia* cf *dubia* and *Tetrahymena thermophila*. LC_{50} and IC_{50} dilution values and 95% confidence intervals (CI) in order of decreasing sensitivity were *C.* cf *dubia* 48 h LC_{50} 11% (9–14%), *T. thermophila* 24 h IC_{50} 16% (14–17%) and *C. protothecoides* 72 h IC_{50} was 29% (27–30%). A toxicity assessment of NaOH and rapid catalytic oxidation (RCO) treatments to the acid mine pit lake water was also made using the same three species. All three treatments increased pH and significantly reduced metal concentrations. Cd and Cu concentrations were the only metals still remaining elevated after treatment with the RCO, but the source of Cd was found to be from the RCO itself. Residual toxicity still remained after treatment with the RCO as shown by all but the *T. thermophila* bioassay and was most likely due to the Cd concentration which did not come from the mine pit lake water .Therefore, the RCO treatments were considered capable of removing all metals to below guideline levels and also to remove toxicity to the three test species.

5.2 Introduction

Acidification is one of the most severe causes of contamination to aquatic systems, not only as a toxicant itself, but also through its effects on the speciation, mobility, and bioavailability of metals (Sauvant *et al.* 2000). Studies on water quality in areas affected by acid precipitation, have shown that concentrations of Al, Mn, Cd and Zn normally increase along an environmental gradient of decreasing pH (Parent &

Campbell 1994). By increasing the pH to neutral, most metal bioavailability to aquatic organisms can be removed or reduced to below water quality guidelines. Some metals however still exhibit toxicity even at pH levels higher than neutral (e.g. Al, Zn, Mn) (Hallberg & Johnson 2005). Removal techniques of these metals at circum-neutral pH levels have been slow, expensive or unsuccessful in the past (Gillespie *et al.* 1999; Fyson *et al.* 2003; Viadero Jr & Tierney 2003; Bulusu 2005; Hallberg & Johnson 2005).

Open Cast Mines (OCM) are a major cause of environmental acidification through the exposure of pyritic strata to oxygen. The pyritic rock oxidises and when in contact with water results in Acid Mine Drainage (AMD). AMD further dissolves metals from the OCM walls and overburden. The result is a mine pit lake with high acidity and elevated concentrations of metals, such as those found in the Collie Basin coal mining area in Western Australia (Figure 18).

Bioavailability of metals to aquatic organisms is most often correlated to the free-metal concentrations, because the free metal ion is often the most bioavailable form of a dissolved metal (Peakall & Burger 2003). Historically, water quality studies have been directed towards obtaining physical and chemical data on effluents and toxicants (Calevro *et al*. 1999). From these physical and chemical parameters collected from air, soil and water, inferences are made on potential effects to aquatic organisms (Peakall & Burger 2003). This is not always acceptable as a full means of defining toxic effects and risk to aquatic organisms (Heijerick *et al*. 2002a).

Development of models to explain the interactions and to predict toxicity to aquatic organisms from complex mixtures is an area of intensive study. The bioavailability of metals has been explained by the biotic ligand model (BLM) and the free ion activity model (FIAM). A review of the BLM by Slaveykova & Wilkinson (2005) has shown the model to be very useful in assessing bio-uptake of metals but still does not completely explain the interactions of toxicants to aquatic organisms. More recently bio-uptake has been explained by incorporating biological receptor theory (BRT) into the FIAM resulting in the extended FIAM (Brown & Markich 2000).

Even with modification to the FIAM many limitations still exist due to the complexity of mixtures and bio- and physico-chemical interactions with organisms. Worms *et al* (2006) highlighted that "biological (transport across membrane), chemical (dissociation kinetics of metal complexes) and physical (diffusion) limitation can be demonstrated occurring in the immediate proximity of the biological surface. Furthermore, the organism can employ a number of biological internalization strategies to get around limitations that are imposed on it by the physio-chemistry of the medium. Finally, once inside the micro-organism, cells are able to employ a large number of strategies including complexation, compartmentalization, efflux or the production of extracellular ligands to minimize or optimize the reactivity of the metal". Therefore, chemical analysis alone cannot predict toxicity and the use of representative species in a bioassay format should still be used in risk assessment of contaminated waters.

Bioassays are increasingly used as sensitive indicators of pollutant toxicity and aquatic environmental risk assessment, since they are rapid, inexpensive, applicable to a variety of toxicants and allow several acute and chronic endpoints to be assessed simultaneously (Calevro *et al*. 1999). Direct assessment of toxicity is still the only assessment method to accurately demonstrate affects to aquatic organisms from effluents and toxicants in the environment (Mitchell 2002). When combined with data on physico-chemical parameters, bioassays are a powerful tool for displaying and understanding actual toxicity to the aquatic environment (Peakall & Burger 2003). Therefore the combination of both chemical and biological analysis has been implemented in this study to assess amelioration of acid mine pit lake water.

The aim of this study was to assess the effectiveness of an active amelioration/filtration technique to WO5H acidic pit lake water contaminated with heavy metals. The acidic pit lake water is sourced from the Collie Coal Basin in Western Australia (Figure 18). This is a coal-mining region with active and non-active OCM voids below the water table. Three aquatic species were utilised in bioassays in an assessment of the acidic pit lake water toxicity before and after the filtration/remediation processes.

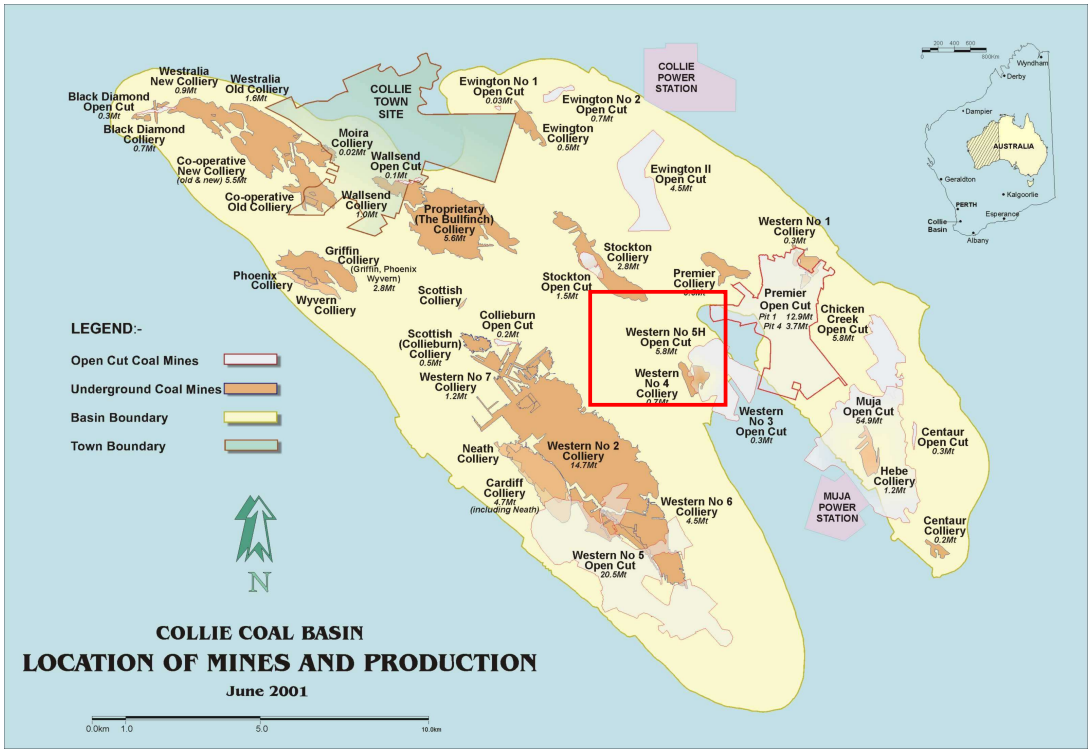


Figure 18. Location of WO5H mine pit lake in Western Australia, indicated by a red square.

5.3 Materials and Methods

5.3.1 Description of the study area and sample location

The mine lake WO5H is located near the town of Collie in Western Australia. Collie is in the higher rainfall region of southern Western Australia, with an average rainfall of 850 mm/yr and an evaporation rate of 1600 mm/yr. The Collie Basin is a small, shallow intra-cratonic Permian sedimentary outlier within Archaean granitic rocks (Johnson & Wright 2003). WO5H mine pit lake is one of several open cast mines (OCM) for coal no longer operational in this area. WO5H pit lake water has a pH of approximately 3. This is typical of other mine pit lakes (eg. Chicken Creek mine pit lake ~pH 3) in the Collie Basin.

5.3.2 Amelioration/filtration process

The amelioration/filtration process is a patented process developed to remove elevated concentrations of metals from contaminated waters. The process was designed to especially remove Mn which can be difficult to remove at environmentally low pH and even at higher pH close to neutral (Lee *et al.* 2002). The filtration/amelioration process has been developed by Gheorghe Duta (Advanced Water Technologies Pty Ltd) to remove most metal contaminants to allow water to be used for different end uses including potable water in developing countries. The water treatment process has been named Rapid Catalytic Oxidation ("RCO"). The RCO filtration process uses three steps; 1) increasing pH to above 6 (if necessary), 2) oxidation with an oxidising agent and 3) filtration through the catalyst sand filter.

Water from the WO5H acid mine pit lake was delivered to the filtration site (Canning Vale, Perth, WA) and filtered within 24 h of delivery. The pH of the water to be treated needs to be increased to >6 for the filtration process to work efficiently. Therefore adjustment of pH to around neutral with NaOH or another alkaline substance is necessary before filtration of the sample can be achieved. In the field, neutralisation by limestone is cheaper but can be less effective, depending on retention time and or armouring effects (Kalin 2004). NaOH was utilised to treat WO5H pit lake water before oxidation and filtration took place as it is relatively cheap, doesn't leave toxic residues and is very effective in increasing pH. After neutralisation the water is treated with an oxidising agent. The oxidising compounds trialled were pool chlorine (active constituent Cl^-) and chlorine dioxide (ClO_2^-). These chemicals came in the form of pool chlorine NaOCl and stabilised chlorine dioxide solution of $NaClO_2$ and $NaHCO_3$. Both of the oxidising agents utilised in the study are currently in use for domestic potable water disinfection treatment worldwide. The addition of Cl^- or ClO_2^- concentration is regulated with an electronic dose applicator on the filtration unit designed to only inject the necessary quantity of oxidising agent. The oxidising agent addition to water at a circum-neutral pH precipitates any remaining Fe, Al and some Mn and Zn. The oxidised water then reacts with the catalyst in the sand filter to remove remaining metals in the water.

The catalyst is patented and manufactured in Western Australia by Quantum Filtration Pty Ltd (now Advanced Water Technologies Pty Ltd).

5.3.3 Controls and treatments for bioassays

The control used in the *C. protothecoides* tests was synthetic soft water (SSW) (Stauber *et al*. 1994). The *C. dubia* testing used Collie River water (CRW) sourced from up stream of the Collie mining area and a synthetic medium High-Hardness Combo (HHC) (Baer *et al*. 1999). The *T. thermophila* control (TTC) was proteose peptone and yeast extract medium at a 50% concentration, diluted with Milli Q water (Pauli & Berger 1997).

Water used for toxicity tests was acidic pit lake water, sourced from the WO5H mine pit lake in Collie WA. Dilutions of W05H pit lake water for LC and IC values were 50, 25, 15, 10, 5 2.5 and 0% for *C. protothecoides*; 100, 50, 25, 12.5, 6.25 and 0% for *C*. cf *dubia*; and 90, 45, 22.5, 11.25, 6.125 and 0% for *T.thermophila.* Three different amelioration treatments to remediate pH and metal toxicity were applied to the WO5H pit lake water. Treatment one was WO5H acid pit lake water with the amelioration treatment of using NaOH only and no filtration; treatment two was WO5H acid pit lake water amelioration treatment using addition of NaOH, Cl^- and RCO filtration (RCOCl); treatment $RCOClO_2$ was WO5H acid pit lake water amelioration treatment using addition of NaOH, ClO_2^- and filtration ($RCOClO_2$); untreated WO5H acid pit lake water was used as a pit lake control. All treatment samples used for testing were filtered to 0.2 μm before testing due to possible remaining suspended colloidal particles affecting spectrophotometer readings.

5.3.4 *Chlorella protothecoides* test protocol

5.3.4.1 Culturing

The *C. protothecoides* stock culture was sourced from the CSIRO Marine and Atmospheric Research Microalgae Supply Service in Tasmania, Australia. The algae

were cultured in Woods Hole MBL medium plus Nutrient Broth Oxoid number two (NB_2) (Nichols 1973). Culture conditions were after Stauber *et al*. (1994). Using the NB_2 for algae growth in stock cultures reduced the lag phase that occurs at the beginning of toxicity tests (Tsvetnenko 2003). Algae were grown in glass 250ml conical flasks at 23°C and re-inoculated to new medium every 5-7 days to keep algae in the exponential growth phase ready for testing. Lighting was from standard white flouresent lights providing approximately 8000 lux. *Chlorella protothecoides* test inoculum was prepared 2-3 h before incubation in the toxicity test. The algal cells were centrifuged in an Eppendorf (swing out rotor) Centrifuge 5804-R, in 50 ml plastic tubes at 2500 rpm at 20°C for 7 mins. The supernatant was discarded and the cell pellet resuspended in deionised water by gentle vortex mixing. The centrifugation and washing was repeated 3 times to remove culture medium. The algal pellet was then resuspended in synthetic soft water (SSW).

5.3.4.2 Toxicity testing

The growth inhibition test protocol followed Stauber *et al*. (1994) with some minor modifications as listed below (Tsvetnenko 2003). All treatments and controls were filtered to 0.2μm before the algae test as colloidal matter may still be present at 0.45μm. Test containers used were 150ml glass flasks containing 50ml of test solution and alga inoculum. The density of *C. protothecoides* at the beginning of the test was approximately $7x10^4$ cells/ml in all flasks. The alga density was measured via absorbency readings from a spectrophotometer (Shimadzu UV-1201 spectrophotometer) set at wavelength of 750 nm. Individual flask densities were measured at times 0, 24, 48 and 72 h. Photometric measurement in a 5cm cuvette instead of a 1cm cuvette was used because it increases the sensitivity and accuracy of the measurement (Tsvetnenko 2003). Flasks were shaken once daily by hand to keep algae suspended and to promote gas exchange. Flasks were kept within a light cabinet with a photoperiod cycle of 12 h light and dark at a temperature of 23°C ± 2°C. The flasks were randomly allotted positions within the light cabinet, and then randomly allocated new places every day after spectrophotometer readings were taken. The *C. protothecoides* control was Synthetic Soft Water (SSW) buffered with HEPES buffer to approximately pH 7.

The alga inhibition test is a 72-h chronic test using serial dilutions of WO5H pit lake water with each dilution replicated three times. The diluent used was the control synthetic soft water without addition of the HEPES buffer which would have changed the pH of the dilutions. Nutrients for exponential growth of alga were added to all flasks as per Stauber *et al* (1994). The five dilutions of the WO5H pit lake water were used to find IC_{50} and IC_{10} values for the untreated WO5H pit lake water, defined as the effective concentration giving 50% or 10% reduction respectively in algal growth rate over 72 h compared to the control. Testing the direct toxicity effect of treated pit lake water was conducted to find if the amelioration/filtration process had removed toxicity to *C. protothecoides*.

5.3.5 Ceriodaphnia cf dubia tests

5.3.5.1 Culturing

Ceriodaphnia cf *dubia* were cultured in High-Hardness Combo synthetic medium made up with distilled water (Kilham *et al*. 1998; Baer *et al*. 1999). Milli Q water was the preferred choice of diluent for the culture water but was not available in large enough quantities. High-Hardness Combo medium has been designed for culturing and toxicity testing with both algae and zooplankton.

The need to culture *C.* cf *dubia* for toxicity testing arose from an inability to import ephippia (dormant eggs) into Australia from Belgium. The use of *C.* cf *dubia* ephippia removes the need for live cultures which take time and money to maintain. However, culturing of *C.* cf *dubia* is a well-known practice amongst toxicologists, but many varied methods and culture mediums are currently in use. Protocol for the culturing of *C.* cf *dubia* was based on NSW EPA (2004) and Orr & Foster (1997) with some modification as indicated below.

Ceriodaphnia cf *dubia* cultures were maintained in 3 L glass beakers, holding 2 L of HHC medium. Culture water was renewed every 2 days mid week (Monday,

Wednesday and Friday). The cultures were fed green algae, *Ankistrodesmus sp*. at a density of $50x10^4$ cells/ml. *Ceriodaphnia* cf *dubia* 48 h acute reference tests with K_2CrO_4 were performed to assess health and sensitivity of *C.* cf *dubia* cultures.

5.3.5.2 Ceriodaphnia cf dubia 48 h acute mortality test

The test protocol was based on Orr & Foster (1997) and ASTM (2002) with some modifications as indicated below. *Ceriodaphnia* cf *dubia* were not fed in the 48 h test. Serial dilutions of the WO5H pit lake water were replicated 4 times. Controls used were Collie River water (CRW) and HHC medium. Natural Collie River water is a geographically representative control water from the Collie region giving good survival and reproduction of *C.* cf *dubia*. Control HHC was used as the diluent for testing and was modified by removing EDTA (Baer *et al.* 1999). EDTA was removed from HHC due to it being a strong chelating agent with the capability to modify toxicity exhibited from metal concentrations. An acute 48-h LC_{50} and LC_{10} was derived from WO5H dilutions and 48-h mortality resulting directly from the WO5H pit lake water amelioration treatments.

5.3.5.3 Ceriodaphnia cf dubia three brood chronic testing (8 day)

The *C.* cf *dubia* were kept in an incubator at 25°C ± 1°C with a photoperiod cycle of 16:8 h light and dark. *Ceriodaphnia* cf *dubia* were individually added to test containers containing their treatment water after previous rinsing respective treatment waters. *Ceriodaphnia* cf *dubia* were fed *Ankistrodesmus sp*. algae at a density of $50x10^4$ cells/ml/day. Water changes were made every second day. On water change days water quality measurements were made for renewal water and for old water from test containers. The number of mortalities and the number of neonates (live and dead) produced were counted daily. The test was terminated after 8 days or when three brood have been produced in 60% of the controls (Muyssen & Janssen 2002).

5.3.6 *Tetrahymena thermophila* test

5.3.6.1 Tetrahymena thermophila culturing

Culturing protocol was based on techniques used by Plesner *et al*. (1964) and Pauli & Berger (1997). Ciliates were cultured in a medium containing proteose peptone, yeast extract and sequestrene (NaFe form) (PPYS) (Gerson 1995). The culture medium is similar to that used by Pauli & Berger (1997) but with the addition of sequestrene. Sequestrene increases the growth rate of the *T. thermophila* through making essential metals bioavailable to the animal.

Component	**Quantity**	**Source**
Proteose Peptone (PP)	200.0g	Sigma #123k0119
Yeast Extract (Y)	20.0g	Fluka Biochemika 70161
Sequestrene(S) (Na,Fe form)	0.30g	Sigma #063k0120

The method for preparation of the stock medium was as follows

1. Combine PP with, Y and S in a one litre flask adjust to 1000ml using distilled water heat to 80°C and stir until all components are dissolved. This solution can be made to any volume by reducing all components i.e. By 1/20th therefore PP 10g, Y 1g and S 0.015g per 50ml of distilled water.
2. After cooling centrifuge at 3000rpm for 20mins. Pour off the supernatant into plastic test tubes and store at –20°C until needed.
3. Before use thaw one test tube and transfer 5ml to a 250ml flask. Then add 20 ml of distilled water. This solution is now at the right concentration for culturing the ciliates (2xPPYS).
4. Then add a steristopper to the flask and autoclave at 121°C for 15 mins. Allow to cool for at least 24 hours before use.
5. Inoculate the flask/test tube aseptically with ciliates from the axenic culture. Turn over cultures every 3 days into upright test tubes. If logarithmic growth is required then transfer ciliates every day and use slanted test tubes (Gerson 1995).

A concentration of 10 times PPYS and PPY medium was stored in 10 mL plastic test tubes in the freezer until needed. *Tetrahymena thermophila* were axenically cultured in slanted 10 mL test tubes containing PPYS with a small number transferred to fresh PPYS medium every 3 days. Transferring every 3 days keeps the *T. thermophila* in the exponential growth phase ready for toxicity testing. PPYS was modified by removing Sequestrene and a 50% dilution with milli-Q water to increase responsiveness to toxicants when used in the bioassay.

5.3.6.2 Tetrahymena thermophila 24-h Chronic testing

Due to *T. thermophila* generation times being less than three hours, multi-generation chronic tests were performed within a time span shorter than most acute tests (Pauli & Berger 2000). The *T. thermophila* bioassay control (TTC) was the culture growth medium PPY diluted with mill-Q water to 0.5xPPY. Test containers were 3 mL cuvettes containing 2 mL of treatment or control water. The bioassay experiment used 5 serial dilutions of the WO5H pit lake water as per the *C.* cf *dubia* bioassay, replicated 3 times. The same amount of PPY was added to all treatments from a stock solution with a concentration of 10xPPY to make 0.5xPPY final concnetrtion. The test was conducted for 24 h in complete darkness at 30℃ within an incubator. Shaking of the cuvettes was done 1 hour before test end. Calculation of density was done by fixing animals in a 4% buffered formalin solution and using a microscope at 100 times magnification to manually count the *T. thermophila*. A minimum of 200 *T. thermophila* were counted in each sample before a mean density was calculated.

5.3.7 Water Quality

Total hardness and alkalinity were measured by a Haggen Aquatic Research Station (HARS), Nutrafin test kit. The pH was measured by a hand held Eco–Scan pH6 portable pH meter. The pH was calibrated daily using pH 4, 7 and 10 standards. Temperature was also measured by the pH meter to an accuracy of 0.1°C. The dissolved oxygen was measured via a Jenway Benchtop Electrochemical Analyser Model 3410. Treatment water metal and metalloid concentrations were analysed by

the Marine and Freshwater Research Laboratory (MAFRL), located at Murdoch University Western Australia by inductively coupled plasma–atomic emission spectrometry (ICP–AES).

Guideline trigger values used, are for the protection of 95% species in slightly–moderately disturbed aquatic ecosystems as set by ANZECC/ARMCANZ (2000b). Hardness adjustment of the trigger values is calculated by the equation

$$HMGV = GV*(H/30)^{a}$$

where HMGV is the hardness modified guideline value (μg/L), GV is the guideline value (μg/L) at a hardness of 30 mg/L as $CaCO_3$, H is the measured hardness (mg/L as $CaCO_3$) of a fresh surface water and a is a constant (ie. slope factor) (Markich *et al*. 2001).

5.3.8 Data analysis

Statistical analysis of bioassay results was performed using the statistical program SPSS (SPSS Inc. 14.0 for windows. 2000). Normality was checked using the Shapiro-Wilks test (Shapiro & Wilk 1965). Non-normally distributed data was checked for homogeneity of variances using Levene's test (Levene 1960). Significant differences found from statistical tests were all at the $P < 0.05$ level. Means, standard errors and confidence intervals were calculated by SPSS or Microsoft Excel.

Results for mortality of *C.* c.f. *dubia* bioassays were arcsine transformed before statistical analysis (EPA 1998). When *C.* c.f. *dubia* data were still not normally distributed ($P<0.05$), data were transformed to rankits before analysis by ANOVA (Pereira *et al*. 2000). Calculation of the EC_{50} values for all species was done by probit analysis on a computer program using equations developed by Christensen &

Nyholm (1984). Calculation of the intrinsic rate of natural increase for *C.* c.f. *dubia* was after Taberner *et al* (1993).

Chlorella protothecoides absorbence data were transformed by natural log to achieve a linear relationship (LN of absorbance at 750nm). Therefore algae density units are represented by LN absorbance (750nm). Growth rates over the 72 h were estimated from the slope of this transformed data. Growth inhibition compared to that of controls following LN transformation was calculated using the formula:

$$100 \times (1-Gt/Gc)$$

where Gt is the growth rate for the treatment and Gc is the growth rate for the control.

When the assumption of normality was met for all species bioassay data, a one-way analysis of variance (ANOVA) was performed where appropriate to explore for significant differences between treatments and controls. When significant differences were found, a pair-wise post-hoc test was performed to identify where the differences occurred. The parametric Tamhane post-hoc test was used when variances were not equal and the Dunnett (Dunnett 1980) and Student-Newman-Keul (S-N-K) Range Test (Newman 1939; Keuls 1952) when variances were equal. When data were not normally distributed the non-parametric Kruskal-Wallis H Test was used to find median differences between treatments and controls.

The no observable effect concentration (NOEC) is the highest concentration not giving a statistically significant result compared to the control and the lowest observable effect concentration (LOEC) is the lowest concentration giving a significantly lower effect than the control (OECD/OCDE 1998). The NOEC and LOEC values were calculated from the Dunnett test. Significance levels were tested at the $P<0.05$ level unless otherwise indicated in the results.

5.4 Results

5.4.1 Water quality

5.4.1.1 Bioassay water quality

The parameters of temperature and dissolved oxygen (DO) were within those recommended for *C. protothecoides* (Stauber *et al*. 1994) and *C.* cf *dubia* (Orr & Foster 1997). Suitable water quality parameters were not suggested for *T. thermophila* by the Prototoxkit F^{TM} (1998). Due to the short duration of the *T. thermophila* test, water quality was not expected to change significantly.

Physico-chemical results are presented in Table 13, 14 and 15. Dilution of WO5H pit lake water with HHC increased the pH from 3.4 to 7.6 and decreased the hardness concentration from 160 to 120 mg/L in 100% WO5H water and 6.25% WO5H water respectively. The amelioration treatments of NaOH, RCOCl and $RCOClO_2$ all raised the pH to around neutral. The RCOCl and $RCOClO_2$ treatments maintained a high total hardness of 180 mg/L similar to that of the untreated WO5H pit lake water. The water quality parameters measured during testing of *C. protothecoides* and *T. thermophila* are presented in Table 13. Alkalinity in all dilutions lower than 12.5% and the treated WO5H pit lake water was <10 mg/L. Temperature in both the *C. protothecoides* and *T. thermophila* tests did not fluctuate due to temperature regulation by the incubation unit.

Table 13. Water quality parameters for *Ceriodaphnia* cf *dubia* testing.

Treatment	pH	Temperature (℃)	DO%	Hardness (mg/L)	Alkalinity (mg/L)
CRW	7.6	25±1	97	260	60
HHC	8.2	25±1	98	120	50
WO5H 6.25%	7.6	25±1	97	120	20
WO5H 12.5%	7.2	25±1	98	120	20
WO5H 25%	6.3	25±1	97	130	<10
WO5H 50%	4.7	25±1	97	150	<10
WO5H 100%	3.4	25±1	94	160	<10
NaOH	7.2±0.1	25±1	97	100	<10
RCOCl	6.8±0.1	25±1	96	180	<10
$RCOClO_2$	6.6±0.1	25±1	100	180	<10

Table 14. Water Quality parameters for *Chlorella protothecoides* bioassay.

Treatment	pH	Temperature (℃)	DO%	Hardness (mg/L)	Alkalinity (mg/L)
SSW	7.6	23±2	99	40	20
WO5H 2.5%	7.2	23±2	98	50	<10
WO5H 5%	7.1	23±2	98	70	<10
WO5H 10%	7.1	23±2	99	80	<10
WO5H 15%	6.5	23±2	94	90	<10
WO5H 25%	6.0	23±2	97	90	<10
WO5H 50%	4.8	23±2	97	100	<10
NaOH	7.2	23±1	99	100	<10
RCOCl	6.8	23±1	101	180	<10
$RCOClO_2$	6.6	23±1	99	180	<10

Table 15. Water quality for 24 h *T. thermophila* test.

Treatment	pH	Temperature (℃)	DO%	Hardness (mg/L)	Alkalinity (mg/L)
TTC	7.8	30±1	97	20	20
WO5H 5.125%	7.1	30±1	97	80	<10
WO5H 11.25%	7.0	30±1	98	90	<10
WO5H 22.5%	6.2	30±1	97	90	<10
WO5H 45%	4.8	30±1	97	100	<10
WO5H 90%	3.4	30±1	94	160	<10
NaOH	7.2	30±1	99	100	<10
RCOCl	6.8	30±1	96	180	<10
$RCOClO_2$	6.6	30±1	98	180	<10

5.4.1.2 Chemical analysis

The total and dissolved metal concentrations for untreated and treated WO5H mine pit lake water are presented in Table 16. The dissolved Cu concentration (10 μg/L) in the control Collie River water was above its trigger value of 1.4 μg/L. Dissolved and total concentrations of Al, Cd, Cr, Cu, Ni and Zn in untreated WO5H pit lake water were above the aquatic ecosystem trigger values. Treatment with NaOH displayed concentrations of total Cd, Cr, Cu, Ni and Zn and dissolved concentrations of Ni and Zn above the trigger values. The $RCOClO_2$ treatment retained a total and a dissolved concentration of Cd above the guideline value. RCOCl and $RCOClO_2$ both contained total concentrations of Cu and Zn and the dissolved concentration of Cu above guideline values for aquatic ecosystems.

Some total and dissolved metal concentrations also remained elevated above aquaculture guidelines (Table 16). The total Al, Cu, Fe, Mn, Ni and Zn concentrations in the NaOH treatment remained above the aquaculture guidelines. The only dissolved concentration above the aquaculture guidelines in the NaOH treatment was Mn. The dissolved and total Cd concentration in $RCOClO_2$ was higher than the guideline and higher than the original concentration in the WO5H water.

Converting the guideline values to the hardness modified guideline value (HMGV) did not affect the interpretation of Cd and Cr concentrations being above the standard

guideline value. However the dissolved concentration of Zn remaining in WO5H and Ni remaining in the NaOH treatment were below the HMGV value. Further, the remaining dissolved and total concentration of Cu for RCOCl and $RCOClO_2$ and the total concentration of Zn were below the HMGV (Table 16).

Table 16. Metal concentrations of significance to toxicity responses in bioassays. Where no data is presented data was not available. Bolded figures are above trigger values for the protection of 95% of slightly–moderately modified freshwater aquatic environments, figures with an * are above aquaculture guidelines. Hardness adjusted trigger values are only available for Cd, Cr, Cu, Ni and Zn and concentrations exceeding these are preceded by a subscript †.

	Al		**Ca**		**Cd**		**Cr**		**Cu**		**Fe**		**Mn**		**Ni**		**Zn**	
Treatment	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total	Disolved	Total
CRW	<10	-	24	-	<0.6	-	<1	-	†**10***	-	6	-	1	-	<4	-	<2	-
Untreated WC5H	**11000***	**13000***	17	19	†**13***	†**13***	†**7**	†**7**	†**25***	†**26***	2100*	2600*	960*	1100*	†**170***	†**180***	**20***	†**980***
NaOH	10	**12000***	14	15	<0.6	**1**	<1	†**7**	<1	**22***	<2	2700*	670*	850*	**19**	†**140***	<2	†**900***
RCOCl	<10	<10	21	24	<0.6	<0.6	<1	<1	**2**	**2**	<2	<10	0.7	0.7	<4	<4	<2	**12***
RCOClO2	<10	<10	20	22	†**22***	†**25***	<1	<1	**2**	**2**	<2	<10	0.4	0.4	<4	<4	<2	**10***
Aquaculture guidelines	30 (pH>6.5) 10 (pH<6.5)		-		0.2 - 1.8		20		5		10		10		100		5	
Aquatic ecosystem guidelines	55 (pH>6.5)		-		0.2		1		1.4		-		1900		11		8	
Hardness modified guidelines																		
CRW					1.4		5.9		8.8						69.0		50.1	
Untreated WC5H					0.6		2.7		3.9						30.6		22.3	
NaOH					0.6		2.7		3.9						30.6		22.3	
RCOCl					1.0		4.3		6.4						50.4		36.7	
RCOClO2					1.0		4.3		6.4						50.4		36.7	

5.4.2 *Chlorella protothecoides* results

5.4.2.1 Chlorella protothcoides 72 h chronic serial dilution test

The *C. protothecoides* 72 h growth rate IC_{10} and IC_{50} values for the serial dilution of WO5H pit lake water with 95% confidence intervals were 17% (15–18%) and 28% (27–30%) respectively (Figure 19). The *C. protothecoides* growth rate in the control was significantly higher ($F_{(6, 14)}$=593.9, p=0.001) than that of the 50% dilution of WO5H pit lake water. The NOEC was 25% WO5H pit lake water and the LOEC was 50% WO5H pit lake water. Therefore the LOEC is not of any use due to it being higher than the $IC_{50.}$ The 72 h growth rate exhibited in the 25% WO5H treatment was lower than that of 2.5, 5, and 15% WO5H pit lake water treatments.

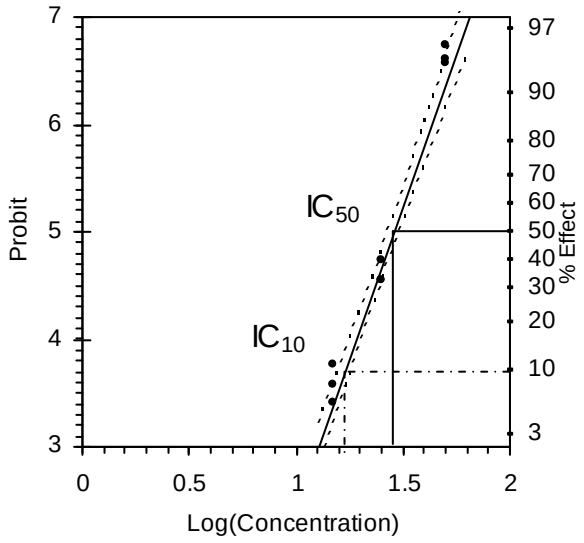


Figure 19. *Chlorella protothecoides* 72 h growth rate with confidence intervals (dashed line), inhibition effect from WO5H pit lake water diluted with Milli Q water. IC_{50} indicated by a solid line, IC_{10} indicated by a dotted and dashed line.

5.4.2.2 Chlorella protothecoides 72 h growth rate response to WO5H amelioration treatments

Growth rate means and confidence intervals for the SSW control and the treatments NaOH, RCOCl and $RCOClO_2$ are presented in Table 17. Treatments NaOH and $RCOClO_2$ had large confidence intervals compared to that of the control SSW and

the treatment $RCOClO_2$. The mean growth rate exhibited in the control was higher than that of the WO5H pit lake water treatments.

Table 17. *Chlorella protothecoides* 72 h growth rate means and confidence intervals for amelioration treatments to WO5H pit lake water

Treatment	Mean	Confidence interval
SSW	1.15	1.11-1.19
NaOH	1.08	0.87-1.29
RCOCl	1.03	0.81-1.26
$RCOClO_2$	1.07	1.05-1.09

The *C. protothecoides* 72 h growth rate exhibited in the control SSW was significantly higher ($t_{(4,\ 3.1)}$=8.6, p=0.001) than that in the $RCOClO_2$ treated WO5H pit lake water (Figure 20). There was no difference in growth rate between the three WO5H treatments.

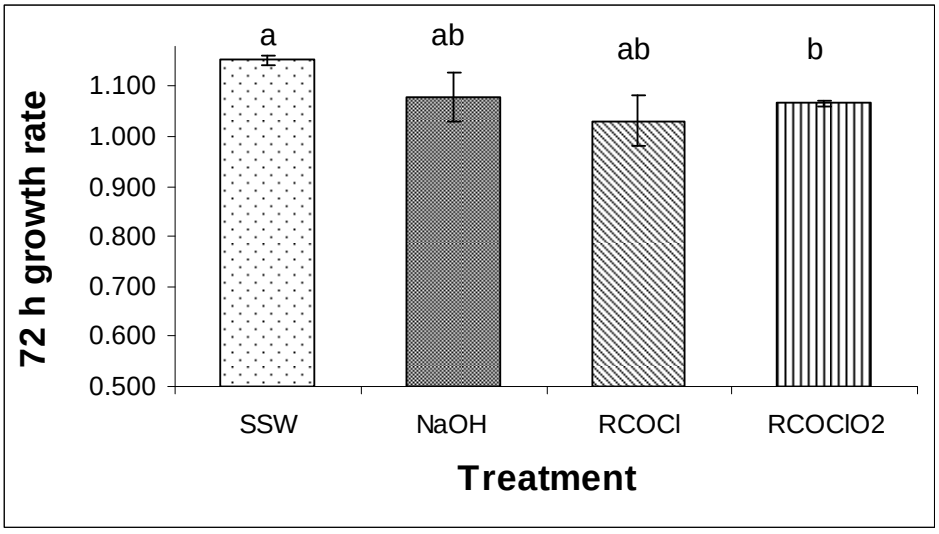


Figure 20. *Chlorella protothecoides* 72 h growth rate ± standard error (LN absorbance at 750nm) for amelioration treatments to WO5H pit lake water. Different lowercase letters indicate significant differences.

5.4.3 Ceriodaphnia cf dubia results

5.4.3.1 Ceriodaphnia cf dubia serial dilution test

Ceriodaphnia cf *dubia* 48 h mortality in the controls CRW and HHC was less than 10% (Figure 21). The *C.* cf *dubia* 48 h LC_{10} and LC_{50} values with confidence intervals in WO5H mine pit lake water were 3.7% (2.8–4.7%) and 11.4% (9.3–14.0%) respectively. Mortality exhibited in all the WO5H pit lake water dilutions was significantly higher ($F_{(6, 28)}$=93.9, p=0.001) than that of the controls with the exception of the 6.25% WO5H pit lake water concentration. The 48 h mortality LOEC was 12.5% and the NOEC was 6.25% WO5H mine pit lake water.

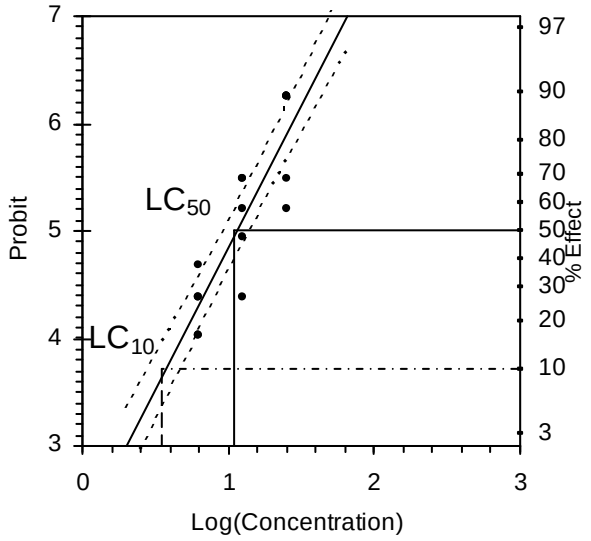


Figure 21. *Ceriodapnia dubia* 48-h mean mortality data with confidence levels displayed either side of the predicted mortality line. The LC_{50} is indicated by a solid line and the EC_{10} is indicated by a dotted and dashed line.

5.4.3.2 Ceriodaphnia dubia acute 48 h response to the treated WO5H pit lake water

Figure 22 displays the survival in the treated WO5H pit lake water. The controls CRW and HHC both exhibited less than 10% mean mortality. Treatments NaOH, RCOCl and $RCOClO_2$ displayed significantly lower ($F_{(4,20)}$=6.0, p=0.002) survival

than the controls. There was no difference between the 48 h mortality that occurred in the three treatments used on the WO5H mine pit lake water.

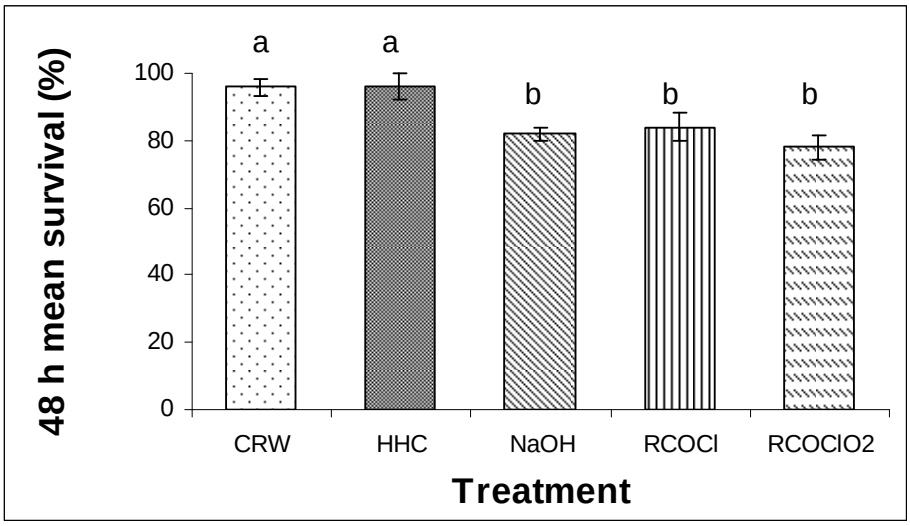


Figure 22. *Ceriodaphnia* cf *dubia* 48 h mean mortality ± standard error for controls and three treatments of WO5H water. Lowercase letters indicate significant differences (P<0.05) between treatments.

5.4.3.3 Ceriodaphnia cf dubia three brood test

The controls both met the requirements for a successful test as recommended by the ASTM (2002). The RCOCl treatment also passed the requirements for a successful test, foremost the production of three brood totalling over 15 in 60% of the remaining population, with no more than 20% mortality. The HHC gave a significantly higher ($F_{(4, 45)}$=24.9, p=0.001) number of brood in the eight days than the control CRW and the pit lake water treatments. Treatment $RCOClO_2$ caused total mortality in all *C.* cf *dubia* within five days of the test commencement (Table 18).

The eight day *C.* cf *dubia* 3 brood test IRNI result was similar for CRW, HHC and RCOCl (Table 18). Treatment NaOH produced a lower *C.* cf *dubia* IRNI than the controls and treatment RCOCl. The IRNI for *C.* cf *dubia* in the $RCOClO_2$ treatment could not be calculated due to the total mortality of all *C.* cf *dubia* by day 5.

Table 18. Results for the *C.* cf *dubia* 3 brood test. Different superscript lowercase, indicate significant differences. N/C = non-calculable due to total mortality

Treatment	Mean ± standard error	Survival (No.)	IRNI
CRW	15.4±1.8^{b}	9	0.547±0.038
HHC	24.4±1.7^{a}	10	0.586±0.038
NaOH	8.9±2.1^{c}	10	0.413±0.056
$RCOClO_2$	12.6±2.4^{bc}	8	0.519±0.054
$RCOClO_2$	0±0.0^{d}	0	N/C

5.4.4 *Tetrahymena thermophila* population growth results

The *T. thermophila* 24 h final population (FTD) IC_{10} and IC_{50} (inhibition concentration) with 95% confidence intervals for the serial dilution of WO5H pit lake water were 2.5% (2.2–2.8%) and 15.6% (14.1–17.3%) respectively. The confidence intervals were small indicating accurate results for the IC values (Figure 23). The resultant 24 h mean final *T. thermophila* densitty (FTD) for all WO5H pit lake water dilutions were significantly lower than that of the control FTD. The LOEC was 6.25% WO5H pit lake water.

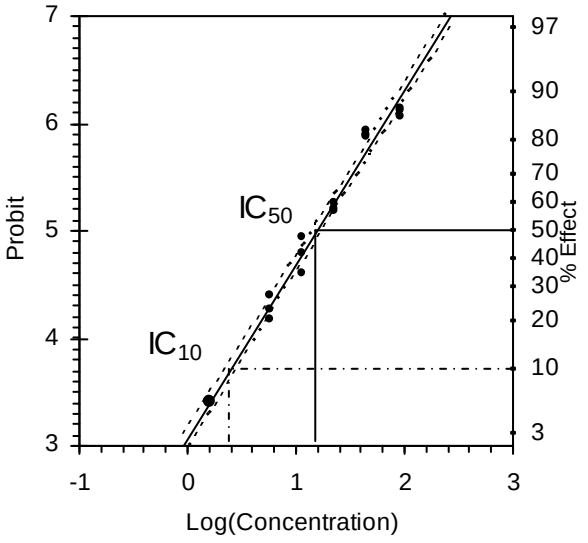


Figure 23. *Tetrahymena thermophila* 24 h final population with confidence intervals for inhibition effect from WO5H pit lake water diluted with milli Q water. The EC_{50} is indicated by a solid line and the EC_{10} is indicated by a dotted and dashed line.

The *T. thermophila* 24 h FTD means exhibited from the treatments RCOCl and $RCOClO_2$ were similar to that of TTC (Table 19). The FTD in the NaOH treatment was lower than the rest of the treatments and control FTD.

Table 19. *Tetrahymena thermophila* 24 h FTD means and confidence intervals.

Treatment	**Mean (FTD x10^4)**	**Confidence interval (FTD x10^4)**
TTC	7.1[a]	6.7 - 7.4
NaOH	6.2[b]	5.7 - 6.7
$RCOClO_2$	7.1[a]	6.8 - 7.3
$RCOClO_2$	7.0[a]	6.3 - 7.6

The FTD for the NaOH amelioration treatment was significantly lower ($F_{(3,8)}$=14.8, p=0.001) than that of the control and the other treatments. All other treatments displayed a FTD similar to that of the control Figure 24.

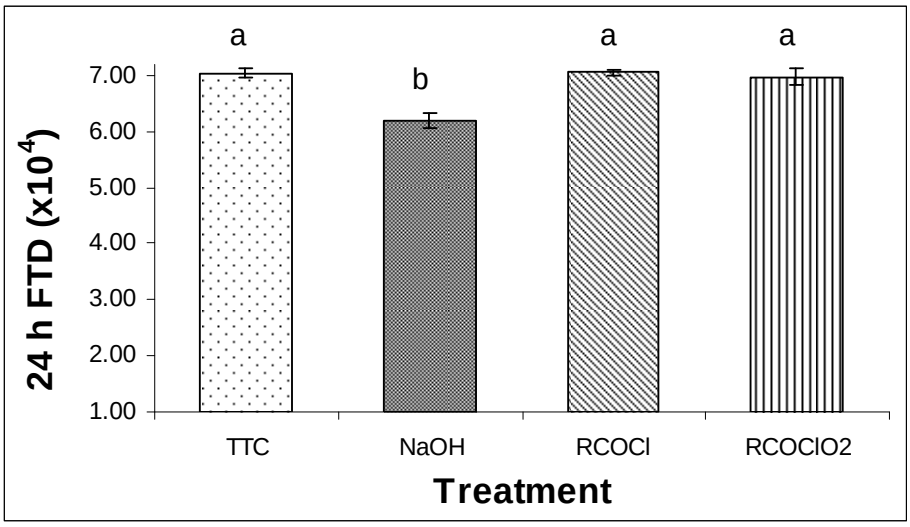


Figure 24. *Tetrahymena thermophila* 24 h FTD mean ± standard error. Different lowercase letters on top of columns show significant differences.

5.5 Discussion

A chemical and biological assessment of toxicity from untreated and treated WO5H pit lake water was made in this study. Untreated WO5H pit lake water was acutely toxic to the three bioassay species. All three amelioration treatments increased pH and reduced metal concentrations thereby, reducing toxicity to the three bioassay species.

The Al, Cd, Cr, Cu, Ni and Zn concentrations in the untreated WO5H pit lake water were above guideline values for the protection of 95% of slightly to moderately modified aquatic environments (ANZECC/ARMCANZ. 2000b). The amelioration treatments were successful in reducing metal concentrations for all the above metals and some to below the reporting level limits. The most effective treatment was RCOCl, but none of the three amelioration treatments reduced all the dissolved and total concentrations of metals to below the guideline values.

After treatment with RCOCl and $RCOClO_2$ the dissolved concentration of Cd, Ca and Cu increased slightly, possibly due to metals being oxidized and consequently reducing the pH of the water to below pH 7. Therefore, the slight change towards

acidity may have caused some calcium dissolution (minimum solubility at pH = 9.3) from deposits in the RCO from previous experimental work. The concentration of Cd remaining in treatment $RCOClO_2$ was above that found in the untreated WO5H mine pit lake water, which may have occurred through the treatment and filtration process. Chlorine dioxide is a more powerful oxidant than sodium hypochlorite and subsequently could cause corrosion of metal components. The release of cadmium in $RCOClO_2$ is most probably from the metallic ball valves used in the experimental RCO. Metals are known to have an additive effect to toxicity and could therefore still exhibit some toxicity to the aquatic environment (Mitchell 2002). With knowledge of how sensitive the entire system becomes when dealing with trace element removal at the μg/L level, modification to the RCO to avoid contamination is currently being conducted. This will allow for a more consistent and effective removal of metal toxicants.

The hardness measured in the treated waters was between 100 and 180 mg/L. High hardness has the potential to reduce toxicity of aquatic toxicants (Gensemer *et al*. 2002). The hardness concentration was the lowest in the water treated with only NaOH. This indicates that treating this pit lake water with NaOH actually reduced the dissolved concentration of Ca in the mine pit lake water.

The only site specific water quality adjustment currently used with the guidelines is a hardness algorithm (Markich *et al*. 2001). By adjusting the guideline values to get the HMGV some of the concentrations of metals present in the treated WO5H pit lake water were then below these values. This indicates that the toxicity from the RCO treated water should be quite low. Treatment with NaOH did reduce the dissolved concentration of Ni but not the total Mn and Ni concentrations to below the HMGV. This indicates that toxicity (that is generally connected to free ion concentrations) would not be likely at these metal concentrations (Peakall & Burger 2003). However, some metals that are not dissolved can still cause toxicity to aquatic organisms, especially fish through smothering of gills (Poleo *et al*. 1997). The only other metal concentration remaining above the HMGV in the treated WO5H pit lake water was the total and dissolved Cd concentrations in the $RCOClO_2$ treatment. Nonetheless, the bioavailability of Cd has been shown to decrease with pH from 7.5–6.0 to water

flea, amphipod and fish (Markich *et al*. 2001). That is because the increase in H^+ concentration can competitively inhibit the binding of the free metal ion at the cell membrane surface reducing metal bioavailability (Brown & Markich 2000).

5.5.1 Bioassay responses to treated and untreated WO5H acidic pit lake water

5.5.1.1 Chlorella protothecoides bioassay tests

Unicellular algae are the foundation of most aquatic food chains, are sensitive to a wide range of toxicants and are highly representative of freshwater environments, therefore making them useful for direct toxicity testing (Franklin *et al*. 1998). The growth rate inhibition 72 h EC_{50} for *C. protothecoides* was 28% WO5H pit lake water indicating a high toxicity towards *C. protothecoides* even with the naturally high hardness present in the mine pit lake water. The total concentration of Al at the 28% (72 h EC_{50}) dilution of WO5H mine pit lake water would have been ~3200 µg/L at a pH of 6, which is more than three orders of magnitude higher than the 72 h EC_{30} for *C. pyrenoidosa* at the same pH (Parent & Campbell 1994). The concentration of 3200 µg Al/L should have shown a higher toxicity effect to *C. protothecoides* but the high proportion of solid Al to free Al would have reduced toxicity. Furthermore, the addition of KH_2PO_4 as a nutrient in the test is known to bind with Al making it non-bioavailable (Kopacek *et al.* 2000; Ulrich & Pöthig 2000; Lychie-Solheim *et al.* 2001). Although not measured, dissolved organic matter (DOM) is also known to reduce the toxicity from aluminium to aquatic organisms and could have been a contributing factor to the reduced toxicity response from *C. protothecoides* (Markich *et al*. 2001; Gundersen & Steinnes 2003).

The concentration of Cu at the 72 h EC_{50} (28%) dilution of WO5H pit lake water would have been approximately 7 µg/L at a pH of 6, which is similar to the EC_{50} of Cu found by Franklin *et al.* (2000) of 1.4 µg/L at pH 6.5 and 35 µg/L at pH 5.7. However, the study by Franklin *et al*. (2000) was conducted in soft water with a total hardness of ~40 mg $CaCO_3$/L whereas in this study the hardness was ~90 mg $CaCO_3$/L, indicating that the higher hardness was not having a significant effect

in reducing the toxicity from the Cu at pH of 6. The high toxicity shown by *C. protothecoides* in the presence of the 90 mg $CaCO_3$/L total hardness could be attributed to the low alkalinity (<10 mg/L) present in the WO5H pit lake water not assisting in buffering of the toxicity (Markich *et al*. 2001).

A lower growth rate for *C. protothecoides* was exhibited in treatment $RCOClO_2$ on the WO5H pit lake water than that of the synthetic control (SSW) indicating remaining toxicity in the treated WO5H pit lake water. Treatment $RCOClO_2$ contained Cd and Cu concentrations that may have contributed to the lower growth rate. Although the concentration of Cu was above the guideline value its not likely that it would be toxic to the algae on its own according to the HMGV. Adverse effects in fish and other wildlife are probable when concentrations of Cd exceed 3 µg/L (Peakall & Burger 2003). Therefore, the dissolved Cd concentration (22 µg/L), that was still above the HMGV probably contributed to the reduced growth rate in WO5H pit lake water treated by $RCOClO_2$. Treatment RCOCl had similar concentrations of metals with the exception of Cd and a similar pH to $RCOClO_2$, but displayed a similar algal growth rate to the control SSW. Therefore, the concentration of Cd was probably causing the decreased growth rate in treatment $RCOClO_2$. Franklin *et al*. (1998) has noted that the end point of growth rate is more sensitive than that of photosynthesis and respiration in many freshwater algae species. Therefore, if another endpoint other than growth rate was used a toxic effect may not have been displayed in treatment $RCOClO_2$. Although the Cd concentration may be contributing to toxicity, with modifications to the RCO to remove components containing Cd, toxicity may no longer be present.

Algal growth rate in the NaOH treatment was similar to that in SSW even with elevated concentrations of total Al, Cu, Ni and Zn still being present. The similar *C. protothecoides* growth rate displayed in the control (SSW) as that in the NaOH treatment could be attributed to the small proportion of free metal ions present at the neutral pH. The neutral pH exhibited in the NaOH treatment would mean most of the total metal concentrations would be non-bioavailable to the algae and would therefore not exhibit a toxic effect (Peakall & Burger 2003).

5.5.1.2 Ceriodaphnia cf dubia tests

The WO5H pit lake water caused complete mortality to *C.* cf *dubia* even at 50% dilution with HHC. The HHC as a diluent was able to show the high toxicity of this water with *C.* cf *dubia.* The toxicity to *C.* cf *dubia* exhibited at the calculated 48 h EC_{50} dilution value of 11.4% WO5H pit lake water was probably from the dissolved concentrations of metals remaining at this dilution. The calculated total concentration of Al (1400 μg/L) in 11.4% WO5H pit lake water dilution is in excess of 20 times the guideline value for Al. However, the pH at the EC_{50} dilution of 11.4% would have been approximately 7 and Al exists as 90% colloidal solid at this pH (Peakall & Burger 2003). When HHC was added to the WO5H water a white precipitate was observed which would have been mainly solid $Al(OH)_3$ (Parent & Campbell 1994; Lychie-Solheim *et al.* 2001). Therefore the Al concentration may not have been bioavailable but may have been able cause toxicity through smothering of the *C.* cf *dubia* gills (Alstad *et al*. 2005).

Calculated total Cd, Cu and Ni concentrations for the 48 h EC_{50} were 2, 3, and 19 μg/L respectively and were above the guideline values. The Cd, Cu and Ni concentrations would probably have contributed to the *C.* cf *dubia* 48 h EC_{50} toxicity. The *C.* cf *dubia* 48 h EC_{50} for Cu was found to be 1.6 μg/L by Hyne *et al*. (2005) which is lower than that estimated from the *C.* cf *dubia* EC_{50} Cu concentration in WO5H pit lake water. The toxic effect of Cu has been found to decrease as pH increases indicating that Cu may not have had a toxic effect at the neutral pH. Nevertheless, Zn has been shown to increase its toxicity to *C.* cf *dubia* concurrent with an increase in pH from 6.5 to 7.5 with 48 h EC_{50} values of 413 and 200 μg/L respectively (Hyne *et al*. 2005). Calculated total Zn concentration for the *C.* cf *dubia* 48 h EC_{50} would have been approximately 112 μg/L at pH 7, and may have contributed most of the toxicity to *C.* cf *dubia*. The toxicity increasing trend exhibited by Zn with increasing pH values can be attributed to a decrease in competition between H^+ ions with Zn^{2+} ions for the receptor binding sites described in the biotic ligand model (Heijerick *et al*. 2002b). Furthermore even if Zn wasn't causing toxicity it is known to bio-accumulate and could be a serious problem to higher organisms with long term exposure (ANZECC/ARMCANZ. 2000a).

Calculated total Zn and Al concentrations for the *C.* cf *dubia* 48 h NOEC were above the aquatic ecosystem guideline values. However, the pH of 7 and the total hardness of 120 mg/L would have made the effective toxicity from these metals very low (Markich *et al.* 2001). Raising the pH to neutral removes most toxicity from the aquatic environment by precipitation (Peakall & Burger 2003) further indicating that Al would not have been contributing greatly to toxicity at pH 7 (Sauvant *et al.* 2000).

The three treatments on the WO5H pit lake water showed lower *C.* cf *dubia* survival than the two controls. This finding indicated that toxicity still remained in the treated waters. The total concentrations of Al, Cd, Cu, Ni and Zn in WO5H pit lake water treated only with NaOH were all above the HMGV. The pH of the NaOH treated WO5H pit lake water was approximately 7.2 indicating that Zn would have been the only toxicant remaining in the sample (ANZECC/ARMCANZ. 2000a). The *C.* cf *dubia* three brood test gave similar results to the 48 h acute test except for the total mean brood produced in NaOH and RCOCl treated WO5H pit lake water not being different to that in the Collie River control. However, brood production in treatment RCOCl did not meet the required mean 15 brood per replicate (ASTM 2002). Therefore, it can be concluded from the *C.* cf *dubia* three brood test that all three treated waters still exhibited toxicity to *C.* cf *dubia.* The *C.* cf *dubia* 3 brood IRNI was lower in the NaOH treatment than that in the controls, and $RCOClO_2$. IRNI was not calculable due to total mortality before day 8 probably due to the concentration of Cd and Zn remaining in that treatment. However, *C.* cf *dubia* IRNI calculated in treatment RCOCl was similar to the controls, therefore showing that the use of IRNI as an endpoint may not be as sensitive as that of total brood produced. Although toxicity is reduced and concentrations of metals are almost all decreased to below HMGV by the three treatments they are not effective in completely removing toxicity to *C.* cf *dubia.*

The background concentration of Cu present in the Collie River water was above the guideline trigger value and may have had an adverse effect on the reproduction of *C.* cf *dubia* in the chronic three brood test. Previous collections of Collie River water in

winter did not contain detectable levels (<1 μg/L) of Cu. However, the sample utilised as a control for *C.* cf *dubia* in the current experiments was collected in summer. Johnson & Wright (2003) have noted adverse changes in the water quality of the Collie River from seasonal fluctuations. This indicates that the Collie River water may be naturally toxic to sensitive species at some times of the year. However, the biota currently living within the Collie River may have adapted to chemical changes over time and would therefore not show toxic responses to the river waters seasonal effects (Kilham *et al*. 1998). The Collie River water was successful as a representative control for the *C.* cf *dubia* in the present testing even with the elevated concentration of Cu. However, the number of brood produced in the three brood test was lower than that in the HHC indicating the reduced fecundity of the *C*. cf *dubia.*

Ceriodaphnia cf *dubia* reproductive success in Collie River water for the 3 brood test was different to that in the HHC medium. Reproductive success is essential for species success and is therefore an important endpoint to monitor with lower reproduction indicating toxicity (Mahar & Watzin 2005). The results from the *C.* cf *dubia* three brood test were within the parameters for a successful test (ASTM 2002), but the number of brood produced was significantly lower than that produced in the HHC medium. However, there was no significant adverse effect (mortality) seen in the *C.* cf *dubia* 48 h acute serial dilution test in the CRW control. These observations indicate that both controls are suitable for *C.* cf *dubia* toxicity testing.

The Collie River water hardness measured in summer was almost twice that of the winter sample. The total hardness and alkalinity concentrations may have had an effect in reducing the toxicity from the elevated concentration of Cu. However, even when the HMGV was used, the total concentration of Cu in CRW was still high. The HMGV does not take into account the alkalinity which could also buffer some of the Cu toxicity (Markich *et al*. 2001). The IRNI calculated in the control CRW from the three Brood Test was similar to that in the HHC medium suggesting that there was no toxicity exhibited from the CRW. The DOM concentration may also have played a part in reducing toxicity from the elevated Cu concentration. The toxicity of Cu is reduced linearly by the addition of natural DOM such as fluvic acid (Hyne *et al.*

2005). However, the DOM was not measured in this study so no conclusion on its effect to Cu toxicity can be concluded. De Schamphelaere & Janssen (2004) have noted that, in general, too little is known about how water chemistry affects chronic copper toxicity to cladocerans.

Many different mediums are currently employed in toxicity testing, both synthetic and natural (with many chemically undefined) adding to variability and uncertainty of results (Baer *et al*. 1999). Use of de-chlorinated tap water, mineral water, ground water and USEPA synthetic water (medium hardness) did not provide sufficient reproduction and survival for culturing. After experimentation showing no difference in survival for *C.* cf *dubia* in HHC to that in CRW and demonstration of three brood test acceptability for HHC ASTM (2002) it was decided to use HHC as a control/diluent for culturing and toxicity testing of *C. dubia*. The use of HHC for a culture medium and a diluent in toxicity testing may have an effect on the resulting toxicity displayed compared to other synthetic toxicity control mediums. However, the culturing of test animals in media lacking certain trace elements/metals could give rise to animals that are unnaturally sensitive to those same metals during toxicity tests (Muyssen & Janssen 2002). Using HHC as a culture medium, which contains trace metals necessary for most freshwater algae and zooplankton growth and reproduction could reduce unwanted sensitivity (Kilham *et al*. 1998). Several internationally recommended culture and test media for daphnids contain only a few salts and it may be questioned if their composition is adequate to meet the animal's nutrient requirements (Muyssen & Janssen 2002).

Test solutions used in a toxicity test should have a minimal effect to the chemical characteristics of the effluent (Tevlin 1978) so that water toxicity due to low pH as against that due to other contaminants can be discriminated (Lopes *et al*. 1999). EDTA (ethylene diamine tetra-acetic acid) is part of the HHC medium known to form complexes with almost every metal ion, including cadmium (Tevlin 1978). By removing the chelating agent EDTA from HHC it can then be employed in diluent testing of toxicants or effluents containing heavy metals (Baer *et al*. 1999).

5.5.1.3 Tetrahymena thermophila tests

Tetrahymena thermophila are a eukaryote but are cultured both easily and economically, just like the prokaryotic bacteria, making them highly suitable for toxicity testing (Pauli & Berger 2000). Shorter tests than the 24 h growth test used in this research are also possible but may not provide the sensitivity needed for toxicity testing on samples with very low concentrations of toxicants as found in the treated pit lake water (Sauvant *et al.* 2000). The calculated *T. thermophila* 24 h EC_{50} (15.6%) WO5H pit lake water dilution meant that the total concentrations of Al, Cu, Ni and Zn would still be above the guideline values. The concentrations at the 24 h EC_{50} dilution of Al and Zn especially, were elevated above the guidelines. Aluminium is not expected to cause toxicity at a circum-neutral pH (ANZECC/ARMCANZ. 2000a; Sauvant *et al.* 2000), indicating that Zn may be the major contributor to toxicity from the WO5H pit lake water. Nevertheless, Gallego *et al.* (2007) found the 24 h LC_{50} for Zn at a pH of 6.8 was 3580 µg/L showing that *T. thermophila* has a naturally high resilience to Zn. At the LOEC (6.25%) WO5H pit lake water dilution, calculated total concentrations of Zn and Al would have still been above the guideline values and may still have contributed toxicity towards *T. thermophila*.

The only treatment on the WO5H pit lake water to exhibit remaining toxicity to *T. thermophila* was treatment with NaOH only. This corresponds with the numerous metals still remaining above the guideline values as well as the HMGV. However, the high remaining Cd in treatment $RCOClO_2$ would have been expected to cause toxicity, as Cd is known to adversely affect *T. thermophila* at low concentrations (24 h LC_{50} of 195 µg/L) (Gallego *et al.* 2007). The chronic 24 h *T. thermophila* bioassay showed that there was no toxicity in the $RCOClO_2$ and therefore risk to *T. thermophila* was removed in both the treatments RCOCl and $RCOClO_2$.

5.5.1.4 Bioassay toxicity response comparison

Short term sublethal algal tests have been found to be 6–10 times more sensitive than acute lethality tests with animals (Stauber *et al.* 1994). Nevertheless, in this research the relative sensitivity (highest to lowest sensitivity) of the three test species bioassays to the WO5H pit lake water was *C.* cf *dubia* (48 h EC_{50} 11%), *T.*

thermophila (24 h EC_{50} 15%) and *C. protothecoides* (72 h EC_{50} 28%). The toxicity exhibited from the WO5H pit lake water was less than additive for the total concentrations of metals remaining for all three species (Mahar & Watzin 2005). Although this is the least common effect to be displayed from complex mixtures of chemicals ii has been reported on several other occasions (Mitchell 2002). The less than additive mixture toxicity result indicates that the total hardness, DOM, and pH were playing a significant buffering role within the diluted WO5H pit lake water that allowed for only a small portion to be bioavailable to the three species. The circum-neutral pH exhibited could also indicate competition of H+ with the other metals reduced bioavailability and hence toxicity (Markich *et al*. 2001).

After treating the WO5H pit lake water with each of the three treatments, toxicity remaining was highest in treatment $RCOClO_2$ for *C. protothecoides*, similar in all treatments for *C.* cf *dubia* and highest in the NaOH treatment for *T. thermophila*. The different results demonstrate the different responses and sensitivity expressed to chemical mixtures from different species and trophic levels as noted by (Manusadzianas *et al*. 2003). Furthermore, the different results between the species indicates the difference in sensitivity and therefore usefulness in using species from different trophic levels (Mitchell 2002). The qualitative assessment of the responses to the treated WO5H pit lake water indicates that the 48 h *C.* cf *dubia* test may be the most sensitive of the three tests used. If only *T. thermophila* or *C. protothecoides* were used for toxicity assessment, water from two of the treatments would have been shown to be non toxic. By using a battery of species it was possible to demonstrate residual toxicity following treatment. Due to the different toxic responses seen from the different treatments all three tests would be recommended to be used in conjunction with each other for future testing.

Models (FIAM and BLM) designed to predict bioavailability of metals don't take into account a deficiency of trace elements that may exist in an aquatic ecosystem. This can be a natural phenomenon or the result of reactions with anthropogenic contaminants. Essential trace metals are often highly regulated in aquatic organisms in order to avoid situations of trace metal deficiency or overload (Worms *et al*. 2006)

but in the case of a prolonged shortage of trace elements adverse effects may still occur. The chronic deficiency of common ions can be as detrimental to aquatic organisms as excessive ions (SETAC 2004). The treatments of RCOCl and $RCOClO_2$ may have removed trace metals necessary for growth reproduction and survival in the 3 test species. Toxicity was exhibited in these two treatments that cannot be completely explained by the measurable concentrations of metals remaining and may be due to a deficiency of trace elements from the filtration/amelioration treatment.

5.5.2 RCO: Neutralising, oxidising and filtration

The RCO process was originally developed to treat groundwater to drinking water quality. The aim was to remove metal contaminants including Fe, Mn, Al, As using low cost oxidants and high energy efficiency so that we could apply this process in the rural areas of developing countries. Practical and cost effective pH treatment of mine pit lake water would have been achieved using a fluidized limestone reactor (FLR) or another limestone derived treatment.

The RCO filtration removed most but not all toxicity to the three bioassay species. Therefore, indicating that modification to the treatment would need to be done if water were to be released to the environment. Nonetheless, treated water may be able to be utilised for other end uses such as industry and horticulture. Mn in the pit lake water is a problematic metal due to the brown discolouration it can leave and may adversely affect its use in industry. In freshwater systems the speciation and solubility of manganese is highly pH dependent and does not readily form sulfidic minerals and requires elevated pH (~8) for abiotic oxidation of Mn (II) to insoluble Mn (IV) (Peakall & Burger 2003; Hallberg & Johnson 2005). Wetland and other passive treatments can remove amounts of Mn from the affected water but the speed and variation at which the process occurs detracts from the usefulness of the process. Variation due to seasons can greatly affect and even prevent the removal of Mn from solution (Hallberg & Johnson 2005). Seasons where biological activity is reduced (winter, southern Australia) due to temperature and photosynthetic rate usually

coincide with high rainfall times where acid leaching can be increased. Therefore, when the highest rate of Mn removal is needed the rate of removal will be at its lowest (Hallberg & Johnson 2005). The use of a catalyst sand filter increases the speed and effectiveness of Mn removal compared to conventional wetland or anoxic limestone drain methods without having to raise the pH to above neutral and with a relatively small retention time. The remaining concentration of Mn in the WO5H pit lake water treated with RCOCl and $RCOClO_2$ was less than 1 μg/L at a pH of 6.6 and 6.8 respectively. The filtration results indicate that the RCO filtration/amelioration treatment may be effective at removing Mn from water contaminated with higher concentrations.

Utilising free chlorine (Cl-) can leave residual toxicity to aquatic organisms that may still be acceptable in drinking water for human consumption (Kim *et al*. 2002). Utilising ClO_2^- does not leave residual toxicity from halogenated by-products and would allow for the use of the treated water to be returned to the natural environment or for use in aquaculture (Junli *et al*. 2001). Therefore it would be preferable to use ClO_2^- instead of Cl- for oxidation prior to filtration. Although the cost of ClO_2^- is higher than that of Cl-, but the oxidation effectiveness is greater from ClO_2^-. Before release to the environment or use in aquaculture RCO filtered water would have to be passed through a shallow pond with vegetation which would have allowed further reduction of contaminants and in particular residual chlorine and chlorine compounds. Further research in collaboration with the government department Conservation and Land Management (now Department of conservation (DEC)) on the RCO has shown that with the use of air as the oxidising agent toxicity to aquatic organisms is completely removed from treated water personal communication Gheorghe Duta (2007).

Further research into what applications the filtration may be used for and the quantity and cost of different oxidising agents and neutralising agents all need to be assessed to quantify the cost effectiveness of the filtration technique. This research is currently being undertaken by Advanced Water Technologies.

5.6 Conclusion

The acute 48 h *C.* cf *dubia* mortality bioassay was the most sensitive test for the untreated pit lake water followed by the 24 h *T. thermophila* test of population inhibition and the *C. protothecoides* 72 h growth rate inhibition. The results from the 48 h *C.* cf *dubia* bioassays indicate that there is still an amount of residual toxicity in the treated WO5H pit lake water. Even after applying the HMGV water treated with NaOH alone still contained several metals above the HMGV. The bioassays did differentiate between toxicity in the three treatments indicating that the RCOCl was most effective. Therefore, the bioassay results combined with the metals analysis indicate that there is still an element of risk to the aquatic environment after treatment with any of the three treatments on the WO5H pit lake water. The three Australian representative bioassays species used, combined with chemical analysis was considered effective at assessing the remaining environmental toxicity/risk in the treated WO5H mine pit lake water.

The use of chemical analysis alone has some well-known limitations, especially the inability to account for the bioavailability of the toxicants and to predict the interactive effects in complex mixtures (Manusadzianas *et al*. 2003). Complex models designed to account for bioavailability and consequent responses of aquatic organisms are becoming better at predicting toxicity (Worms *et al*. 2006). Furthermore, not all authors are in agreeance that the knowledge of mode of toxic action is required in order to predict toxicity successfully (Mitchell 2002). Ultimately biological measurement will provide absolute confirmation of toxicity from chemical measurements (Markich *et al*. 2001).

CHEMICAL AND PHYSICAL ANALYSIS OF PIT LAKE WATER TREATED IN MESOCOSMS WITH LIMESTONE AND PHOSPHORUS

6 Chemical and physical analysis of pit lake water treated in mesocosms with limestone and phosphorus

6.1 Abstract

Lake Kepwari is a rapid filled abandoned mine pit lake with a moderately low pH 4.8 and is contaminated with high metal concentrations. Lake Kepwari is proposed for use as a recreational lake but does not meet guidelines for this use. A mesocosm approach was used to apply three amelioration treatments to the lake water in order to permanently raise pH and reduce metal concentrations. The treatments were limestone, phosphorus and limestone and phosphorus. All three treatments raised the pH to neutral and reduced metal concentrations to below guidelines with the exception of Zn over the eight month trial. Limestone addition alone was the quickest in increasing pH and reducing metal concentrations by month 2.

6.2 Introduction

Mine pit lake end uses are influenced by physical and chemical parameters within the lake and from adjacent terrestrial sources. The water quality in mine lakes, in particular acidity, salinity, hardness and metal composition differ depending on the geology, hydrology and geochemistry of the local and surrounding areas. One or more remediation techniques are generally required on Australian coal mine lakes, given that water quality in these lakes rarely meets ANZECC/ARMCANZ (2000b) water quality guidelines for many end uses.

Acidity produced in these pit lakes from oxidation of pyrite (FeS_2) in adjacent overburden and pit lake walls mobilises metals of toxic concern including, Al, As, Co, Cr, Cu, Fe, Ni, Mg, Mn, Pb and Zn. Continual disturbance of pyritic rocks and soils, leaching of overburden from precipitation events and pit lake water level

changes also result in acidic water leaching solutes into adjacent aquatic environments. Acidification itself not only causes severe toxicity through lowered pH but also causes further toxicity through its effects on the increased speciation, and therefore bioavailability, of other toxicants, particularly heavy metals (Lopes *et al.* 1999).

Metals in natural waters are found in equilibrium between different forms such as dissolved, complexed, or associated phases with colloids and particles. The distribution between these physicochemical forms determines potential metal bioavailability (Hyne *et al*. 2005). Acid pit lakes are prevalent with the conditions necessary for increasing metal bioavailability and therefore toxicity and need to be remediated to a level where end uses can be realised.

Acidic mine lakes found in the Collie Basin in Western Australia contain a number of metals especially Al, Mn, and Zn in concentrations that exceed ANZECC/ARMCANZ (2000b) toxicant guidelines for the protection of fresh water aquaculture species and (Sappal *et al*. 2000). These acidic pit lakes occur through exposure of pyrite and other sulphide minerals associated with coal seams to water and oxygen (DeNicola & Stapleton 2002).

Aluminium is mainly responsible for buffering and toxicity (directly or indirectly) for these acidic lakes, due to the low alkalinity often less than 20mg/L. Elevated Al concentrations in acidified lakes affect biota and nutrient cycling, through reducing bioavailability of phosphorus, thus causing, phosphorus nutrient limitation (Bittl *et al*. 2001; Lychie-Solheim *et al.* 2001). Kopacek *et. al.* (2000) gave evidence that Al disturbs in-lake phosphorus cycling therefore affecting natural remediation processes within acidified pit lakes. Consequently aquatic species colonization in aluminium-buffered lakes is complex and mostly controlled by the sensitivity of the organisms towards both protons and to inorganic reactive aluminium species (Nixdorf 2003).

Manganese and Zn also play a role in toxicity to aquatic biota within these pit lakes. Soluble manganese adds to the acidity in coal mine pit lakes and is usually found to be elevated when compared to natural waters (Hallberg & Johnson 2005). Toxicity of Mn to aquatic organisms is not fully understood but when water high in dissolved Mn is used for human consumption, agriculture and industry, problems with Mn (IV) precipitates occur. The removal of manganese from pit lake water can be difficult due to the high pH (>8) required for precipitation but co precipitation with other metals such as Al and Fe does occur at lower pH levels (Lee *et al*. 2002). Aquatic toxicity from Zn is better understood than Mn and well documented for many species (Barron & Albeke 2000; Duan *et al.* 2001; Heijerick *et al.* 2002a). Although like most metals, concentrations of Zn are not the only parameter dictating toxicity but are reliant on other factors such as pH, alkalinity, hardness and dissolved organic matter (Muyssen & Janssen 2002). Within coal pit lakes mainly acidity hardness and pH drive this toxicity through their influence on speciation.

Potential impacts of acidification and metal contamination on aquatic environments are often studied using mesocosms (Fyson *et al*. 1998; Woelfl *et al.* 2000; Tostche *et al*. 2003; Koschorreck *et al.* 2007). These can be used to assess water remediation techniques, conduct toxicity studies of effluents and to study other aspects or parameters of aquatic environmental effects (Bortnikova *et al*. 2001). Mesocosms allow for a large-scale measurement of remediation techniques and are easily replicated, an approach that is sometimes difficult to achieve in the field. Mesocosms have particular application in the assessment of toxicity of water and sediments collected from mine lakes and in studies of potential in-lake remediation approaches (Koschorreck *et al.* 2007).

Rapid filling of mine lakes can be a quick solution to the reduction or prevention of further oxidation of pit lake walls. This technique has been used worldwide with varied success (Trettin *et al*. 2007). The accelerated filling also helps reduce ground water infiltration, from water that may have come into contact with oxidised geologies causing low pH and containing high concentrations of metals and salts (McCullough & Lund 2007a). The pit lake water used in this trial was obtained from

a rapid filled open cut mine pit lake in Collie, WA, Lake Kepwari. After rapid filling by diversion of the Collie River, Lake Kepwari still maintains a low and reducing pH (pH 4.8), and elevated Al, Mn and Zn total concentrations of 1 130, 260 and 450 μg/L respectively.

The Collie Coal Basin is a site of significant environmental acidification. Abandoned voids with pH levels of ≤ 4 are found throughout the region, the result from over 100 years of coal mining operations. The aim of this study was to evaluate, using mesocosms, three different water treatment approaches for mine lake water - limestone chip (L), phosphorus (P) and limestone and phosphorus (L&P) additions. These studies are aimed at remediation of lake water toxicity through reducing metal solubility at increased pH and through bacterial and planktonic growth stimulation. Limestone was utilised to increase pH to levels suitable for biotic growth and survival through reducing dissolved metal and other contaminant concentrations. Mine lake water was phosphorus amended as it is known to be a limiting nutrient within freshwater systems, especially those affected by acidic conditions (Parent & Campbell 1994; Kopacek *et al.* 2000; Bittl *et al.* 2001).

6.3 Materials and Methods

6.3.1 Study site

Sediment and water used in this study were collected from a mine lake located in the Collie Coal Basin in the south west of Western Australia (Latitude 33°21’ S Longitude 116°09’ E) and transported to Edith Cowen University, Perth, WA. Lake Kepwari, originally an open cut coal mine, is being developed as a recreational lake (Lund 2001).

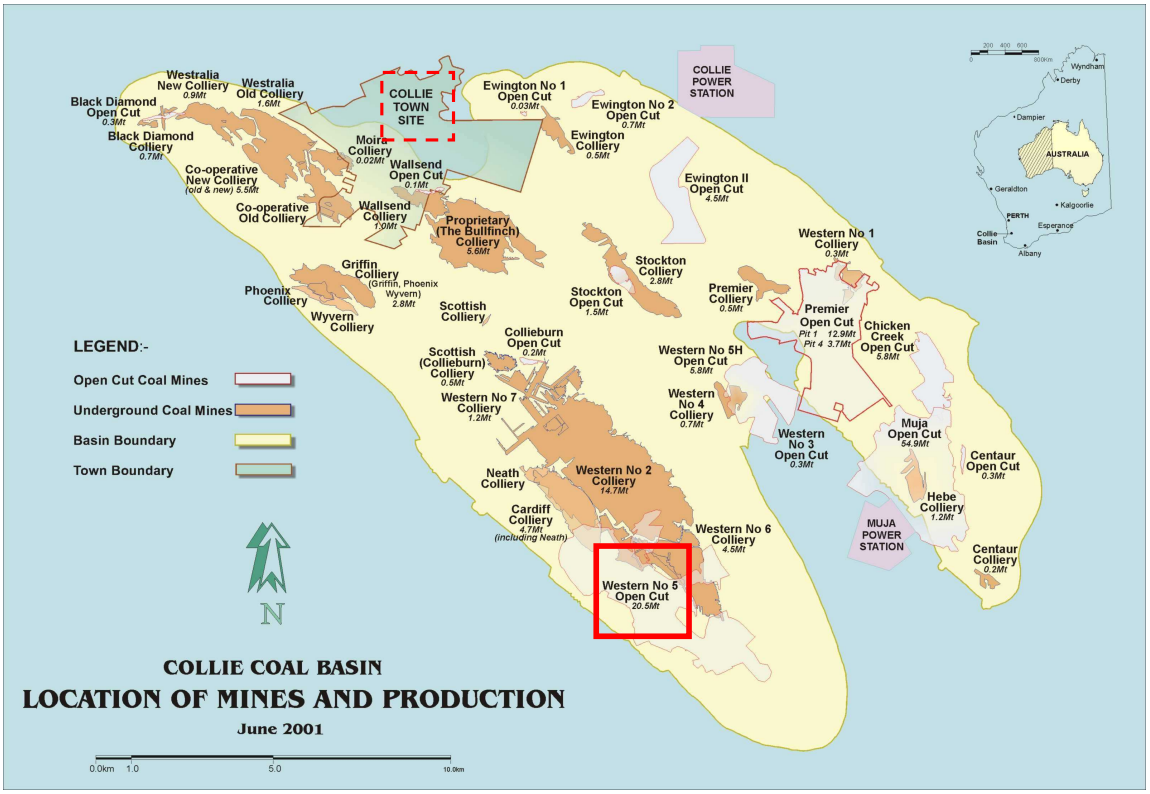


Figure 25. Location of Lake Kepwari in Collie, Western Australia. Collie town site encompassed by box with a broken line and Lake Kepwari encompassed by box with unbroken line.

Lake Kepwari is a mine lake developed from a final mine void at the Premier Coal mine site located in Collie, Western Australia. Mining was ceased at Lake Kepwari in the 1990s (Figure 25). Overburden dumps and exposed coal seams were covered, battered and revegetated with endemic flora by direct seeding. In order to further reduce wall exposure and rates of consequent acid production, the lake was rapid-filled by a diversion from the South Branch of the Collie River over three winters until 2005. The volume of Lake Kepwari is 24 GL, with a maximum depth of 65 m and surface area of 10 ha. A proposal to develop the lake as a recreational resource has received strong community support. This development is predicated on the lake being relinquished by the mining company to the State, a scenario that is presently under consideration. Due to the accelerated filling from the river diversion the Lake pH is approximately 4.8 and contains elevated levels of dissolved metal concentrations, in particular Al, Mn and Zn that are lower than lakes in the same area (Johnson & Wright 2003). The lake straddles the original watercourse of the Collie River, which is being diverted around the lake through a diversion channel most of

the year. Lake Kepwari is an iridescent blue, mostly due to the high aluminium concentration which may be a tourist attraction to the lake (Plate 5).

Plate 5. Lake Kepwari after filling by diversion of the Collie River. (original source unknown)

6.3.2 Mesocosm treatments

Using water and sediment collected from Lake Kepwari, mesocosms were established at Edith Cowan University, in August 2005. Each mesocosm contained 1 200 L of mine pit lake water along with sediment also collected from the lake littoral benthos. The sediment was collected using a sludge pump and transported in a steel tank.

During the course of the trial precipitation events did not exceed evaporation and therefore with the freeboard at the top of the tanks water did not overflow at any time. However, the mesocosm tanks were topped up with deionised water to compensate for evaporation therefore, removing error arising from possible evapoconcentration of chemicals within the mesocosms.

The 12 mesocosms contained three treatments and an untreated pit lake control, each replicated three times. The twelve mesocosms were arranged in a 4 x 3 randomised block design (Plate 6).

Plate 6. Mesocosm setup at Edith Cowan University (source: McCullough 2006)

Samples from the mesocosms were collected for chemical analysis prior to the commencement of the trial (Month 0) and at bimonthly intervals thereafter (Months 2, 4, 6 and 8 respectively). Control mesocosms (MC) contained intact Lake Kepwari water while treated mesocosms contained: 1) Lake Kepwari water, amended with limestone chips, to pH neutral (L); 2) the addition of di-potassium orthophosphate (K_2HPO_4) at a maintained soluble reactive phosphorus (SRP) concentration of 10-20 µg/L (P); and 3) a combination of both limestone and phosphorus treatments (L&P). Further phosphorus additions utilised in maintaining a stable phosphorus level were made 3 days after Month 2 sampling, 8, 19 and 33 days after Month 4 sampling and 12 and 27 days after month 6 sampling.

6.3.3 Water quality analysis

Water quality parameters of each mesocosm were measured on November 2005 (month 2) and at bi-monthly intervals thereafter; January 2006 (month 4), March 2006 (month 6) and May 2006 (month 8).

Water samples collected from mesocosms for analysis were immediately filtered to 0.5 μm and stored in acid washed high-density polyethylene bottles at <5°C before analysis. Measurements for temperature, pH, dissolved oxygen (% saturation and mg/L), specific conductance, and ORP (platinum reference electrode) were collected *in situ* with a Hydrolab Datasonde 4a. A filtered mesocosm water sample was analysed for SO_4 and Cl on an ion chromatograph (Metrohm model 7961), ammonia, NOx and filterable reactive phosphate (FRP) were analysed on a Skalar Autoanalyser after APHA (1998). The remaining filtered sample was then acidified with reagent grade HCl and selected metals analysed by Inductively Coupled Plasma Atomic Emission Spectrophotometry (ICP-AES) for Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cu, Fe, K, Mg, Mo, M, Na, Ni, Sb, Se, Sn and V. Unfiltered samples were digested using a persulfate digestion and then analysed as per FRP and NOx on a Skalar Autoanalyser to determine total P and total N respectively according to APHA (1998).

6.3.4 Data Analysis

Statistical analysis of water quality data was performed using the statistical program SPSS©. Normality was assessed by the Shapiro-Wilk test at $P>0.05$ and if met, homogeneity (variance) was tested using the Levene statistic $P>0.05$. A parametric one-way analysis of variance (ANOVA) or repeated measures ANOVA was performed to demonstrate if significant differences between treatments or samples existed. When significant differences were shown by the ANOVA ($P<0.05$), a post-hoc test was performed to show where the differences occurred. The *post-hoc* test used when variances were not equal was the parametric Tamhane Test. When variances were shown to be equal the *post-hoc* Student-Newman-Keuls (S-N-K) Range Test was performed to show homogeneity sub-sets. When data was not normally distributed the non-parametric ANOVA Kruskal-Wallis H Test was used to

find median differences. T-Tests were used for pair-wise comparisons, between test treatments, between individual mesocosms and between samples.

Data presented in the results section for water quality parameters is mean concentration (mg/L) ± standard error, unless otherwise stated. Significant differences found from statistical tests are all at the $P<0.05$ level.

6.4 Results

6.4.1 Mesocosm Physical Parameters

Initial results (month 0) for the physical parameters were taken from mesocosms before the initialisation of treatment regimes, with the first sample collected after treatments implementation occurring at month 2. Temperature in the mesocosms was relatively constant between 24 to 27°C over the late spring and summer period of sample Months 2, 4 and 6 with no variation between individual mesocosm replicates. Temperatures for the mesocosms during early spring (Month 0) and late autumn (Month 8) were similar to each other (15-17°C) but were significantly lower than those for Months 2, 4 and 6 (Table 20).

At the commencement of the trial, pH measurements were all below the recommended pH guideline range of 5-9 for freshwater aquaculture species (ANZECC/ARMCANZ. 2000b). The Mesocosm Control (MC) and treatment P exhibited increases in pH over the 8 month trial period, with both rising from 4.6 to 6.4 and 6.7 respectively. Treatments L and L&P increased the pH through neutralisation (as per treatment regime) of the pit lake water, with pH remaining significantly higher than that of MC and treatment P from month 2 onwards. A pH close to neutral was maintained in treatment L at all months while that of treatment L&P showed a significant fall from Months 2 to 4 but was constant thereafter. By Month 8 pH in all treatments were all circum neutral, but were also all significantly different from each other.

The oxidation-reduction potential (ORP) values (mean mV ± standard error) at months 2, 4 and 6 were lower in treatments L and L&P than those of MC and treatment P. ORP values for MC and P decreased over the sampling period from 190 and 192 to 143 and 125 respectively. Treatments L and L&P showed no significant changes from month 2 onwards with one exception, a significantly lower ORP value observed in treatment L&P in the month 6 sample. There was no significant difference between MC or any of the treatment ORP values at month 8 (Table 20).

Table 20 Physical parameters for treatments from all sample months. Mean values ± standard error. Different superscript letters indicate significant differences within a treatment between months. Different superscript numbers indicate significant differences between treatments within a sample month.

Treatment	Month 0	Month 2	Month 4	Month 6	Month 8
Temp (℃)					
MC	$^{1}15.6 \pm 0.1^{a}$	$^{1}24.4 \pm 0.2^{b}$	$^{1}24.8 \pm 0.2^{b}$	$^{1}26.8 \pm 0^{b}$	$^{1}16.2 \pm 0.4^{a}$
L	$^{1}15.6 \pm 0.1^{a}$	$^{1}24.6 \pm 0.1^{b}$	$^{1}24.9 \pm 0.1^{b}$	$^{1}26.9 \pm 0.1^{b}$	$^{1}16.4 \pm 0.6^{a}$
P	$^{1}15.5 \pm 0.2^{a}$	$^{1}24.6 \pm 0.2^{b}$	$^{1}25.1 \pm 0.2^{b}$	$^{1}26.9 \pm 0.1^{b}$	$^{1}16.6 \pm 0.7^{a}$
L&P	$^{1}15.7 \pm 0.1^{a}$	$^{1}24.9 \pm 0.2^{b}$	$^{1}25.1 \pm 0.1^{b}$	$^{1}26.8 \pm 0.1^{b}$	$^{1}16.7 \pm 0.4^{a}$
pH					
MC	$^{1}4.6 \pm 0.0^{a}$	$^{1}5.2 \pm 0.1^{a}$	$^{1}5.3 \pm 0.1^{a}$	$^{1}6.0 \pm 0.1^{ab}$	$^{1}6.4 \pm 0.1^{b}$
L	$^{1}4.7 \pm 0.0^{a}$	$^{2}7.4 \pm 0.4^{a}$	$^{2}6.9 \pm 0.1^{a}$	$^{2}7.0 \pm 0.1^{a}$	$^{2}7.3 \pm 0.1^{a}$
P	$^{1}4.6 \pm 0.0^{a}$	$^{1}5.1 \pm 0.1^{a}$	$^{1}5.6 \pm 0.2^{a}$	$^{1}6.4 \pm 0.2^{b}$	$^{3}6.7 \pm 0^{b}$
L&P	$^{1}4.9 \pm 0.4^{a}$	$^{2}7.4 \pm 0.1^{a}$	$^{2}6.6 \pm 0.1^{b}$	$^{2}6.9 \pm 0.1^{bc}$	$^{4}7.0 \pm 0.1^{c}$
ORP (mV)					
MC	$^{1}190.7 \pm 3.9^{a}$	$^{1}192.0 \pm 4.9^{a}$	$^{1}187.7 \pm 4.9^{a}$	$^{1}126.3 \pm 3.8^{b}$	$^{1}142.7 \pm 9.8^{b}$
L	$^{1}190.0 \pm 3.2^{a}$	$^{2}114.7 \pm 14.7^{a}$	$^{2}133.3 \pm 7.4^{a}$	$^{2}91.3 \pm 6.3^{a}$	$^{1}120.3 \pm 7.2^{a}$
P	$^{1}192.3 \pm 4.4^{a}$	$^{1}203.0 \pm 9.5^{a}$	$^{1}178.0 \pm 5.5^{a}$	$^{3}110.0 \pm 2.0^{b}$	$^{1}125.3 \pm 2.7^{b}$
L&P	$^{1}185.3 \pm 10.2^{a}$	$^{2}122.3 \pm 10.3^{a}$	$^{2}141.3 \pm 4.3^{a}$	$^{2}91.0 \pm 2.6^{b}$	$^{1}126.3 \pm 4.4^{a}$

6.4.2 Mesocosm Water Chemistry

At the commencement of the mesocosm trial, concentrations of several metals (Al, Mn and Zn) in the Kepwari pit lake water exceeded toxicant guidelines for the protection of freshwater aquaculture species (ANZECC/ARMCANZ. 2000b). The measured Al concentration of Lake Kepwari water exceeded the guidelines for pH less than 6.5 (sample pH 4.8) by two orders of magnitude. Similarly the Zn

concentration was elevated by almost two orders of magnitude. Manganese was more than 25 times the recommended guideline concentration (Figure 26). Al and Zn Concentrations were also elevated when using the guidelines for the protection of aquatic ecosystems but Mn was not.

Figure 26. Lake Kepwari dissolved metal concentrations (μg/L), that exceed ANZECC/ARMCANZ (2000) guidelines for the protection of freshwater aquaculture species or aquatic ecosystems.

Metal	Aquaculture	Aquatic ecosystems	Measured concentration
aluminium	10 (pH <6.5)	55	1125
manganese	10	1900	265
zinc	5	8	450

The Al, Mn and Zn concentrations showed decreases over the 8 month period in all mesocosm treatments (L, P and L&P) as well as in MC (Figure 27). The concentrations of these three metals in L and L&P showed sharp decreases by month 2. The Al, Mn and Zn concentrations in MC and P were higher than those of treatments L and L&P at all sampling months, with the exception of Al after month 4 and Mn levels in P at month 8. The Mn concentration in MC and Zn concentration in all treatments exceeded aquaculture guideline levels at the completion of the trial while the Al concentration fell to below the guideline in MC and all treatments after month 6.

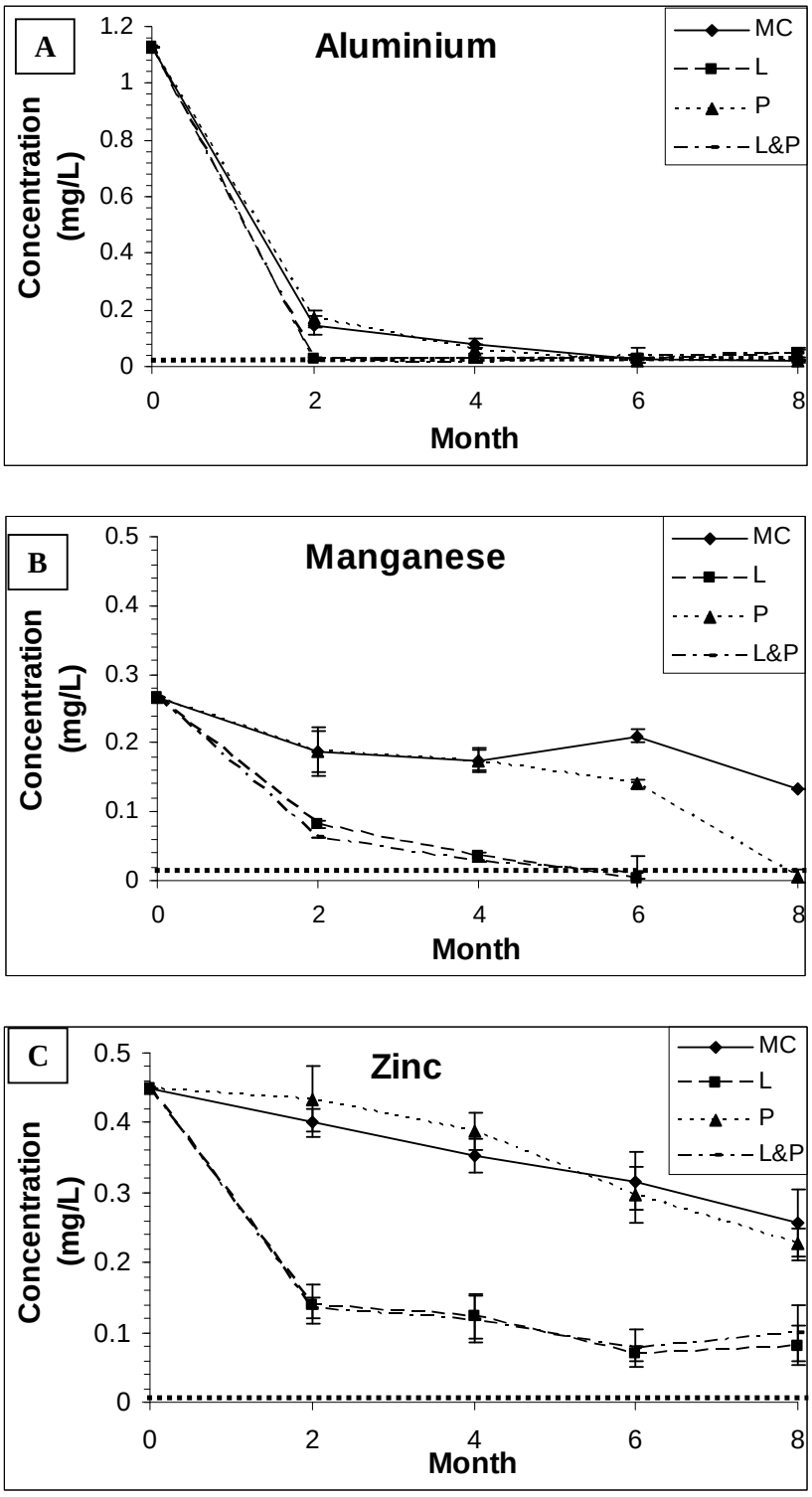


Figure 27. Mean concentrations ± standard error for metals exceeding ANZECC/ARMCANZ (2000) toxicant guidelines for the protection of freshwater aquaculture species. Horizontal broken line indicates guideline concentration level.

Metal concentrations at month 0 of Al, Mn and Zn all exceeded the ANZECC/ARMCANZ (2000) freshwater aquaculture guidelines in all mesocosms. By month 8 all treatments and including the MC had reduced the concentration of Al to below guideline values for a pH >6.5. Manganese was reduced to below guideline levels in all treatments but remained elevated in MC. The concentration of Zn was not reduced to below the guideline value by any treatment or by MC by the end of the 8 month trial (Table 21).

Table 21 Month 0 and 8 mean metal concentrations ± SE exceeding ANZECC/ARMCANZ (2000) toxicant guidelines for the protection of freshwater aquaculture species. Bolded concentrations are significantly (P<0.05) elevated above guideline values.

Metal	Treatment	Aquaculture	Aquatic ecosystems	Month 8 mean concentration ± SE
Al	MC	10 (pH <6.5)		17 ± 0
	L	30 (pH >6.5)	55 (pH >6.5)	43 ± 20
	P			20 ± 10
	L&P			43 ± 20
Mn	MC	10	1900	**133 ± 30**
	L			<10
	P			<10
	L&P			<10
Zn	MC	5	8	**257 ± 50**
	L			**81 ± 30**
	P			**227 ± 20**
	L&P			**100 ± 40**

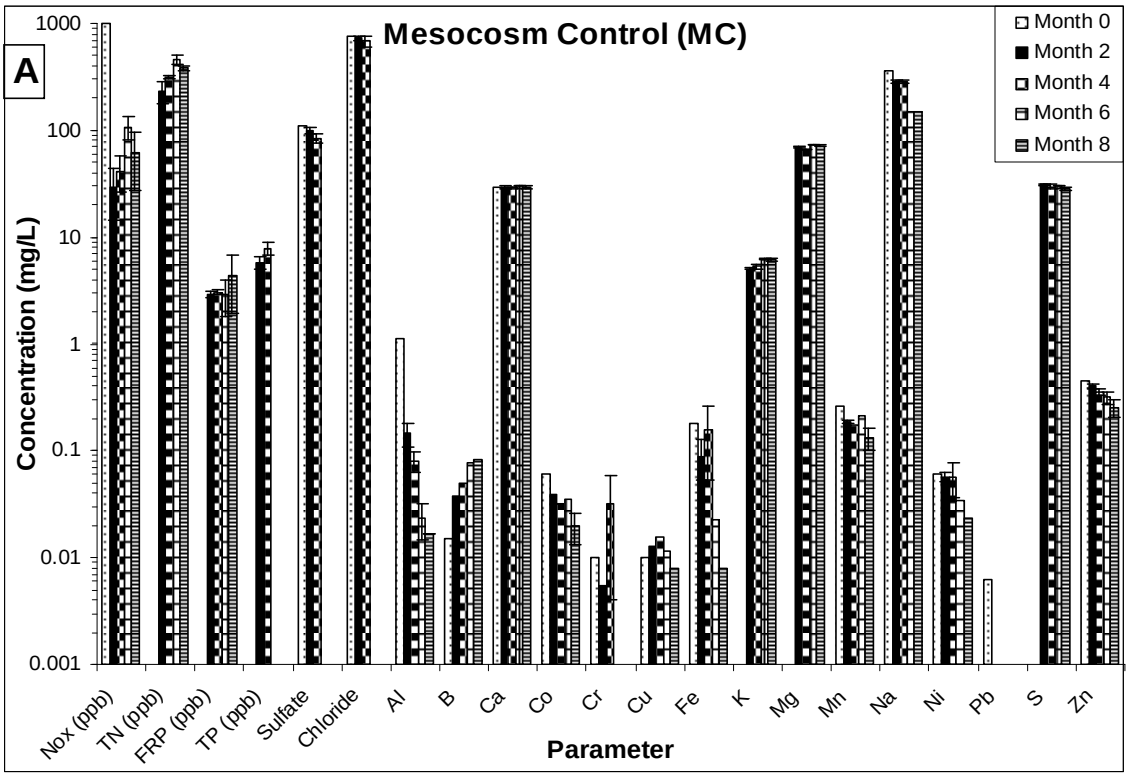
Physico-chemical parameters and metal concentrations measured in the mesocosms over the 8 month trial are shown in Figure 28 A, B, C and D. Variations in the three metals exceeding guidelines Al, Mn and Zn have already been discussed. With respect to the other water chemistry components, the concentrations of the elements B, Ca, Cr, Cu, K, Mg, Na, Pb and S, and of the water quality parameters total nitrogen (TN), free reactive phosphorus (FRP), total phosphorus (TP), sulphate and chloride showed no significant differences either between treatments or within treatments over the 8 month trial period. Decreasing trends were observed in Co, Fe and Ni concentrations, as well as significant decreases in the levels of NOx.

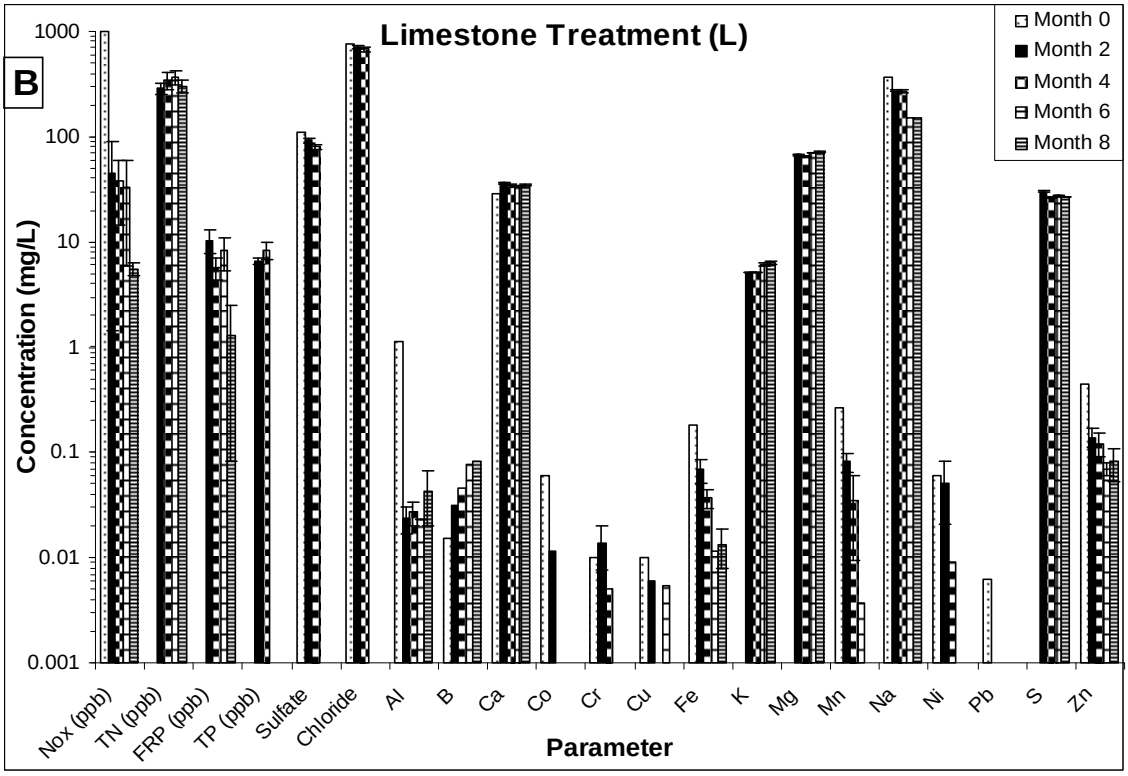
The reduction in concentration of the elements Co, Fe and Ni in the MC tended to be similar to that seen in the treatment mesocosms. Co and Fe concentrations decreased over the 8 months in all treatments with a differing pattern of decrease for the two elements. The Mesocosm Control Co concentration showed a decreasing trend with a significant concentration decrease by month 8 from 60 to 20 μg/L, while Fe showed a significant decrease after 6 months from 180 to 20 μg/L. In contrast, both elements displayed a significant decrease of concentration in treatments L and L&P by month 2 to 70 and 50 μg/L respectively for Fe and 10 μg/L for Co. The Co concentration in treatments L and L&P showed no further significant decrease from month 2 to month 8, while the Fe concentration in treatment L only, significantly declined again by month 4 to 10 μg/L. In treatment P, the Co concentration decreased significantly by month 8 to <10 μg/L, while Fe levels showed a significant decrease by month 6 to 10 μg/L. Ni concentrations fell significantly over the 8 months in treatments L and L&P to <10 μg/L but were unchanged in treatment P. A decreasing concentration trend for Co, Ni and Fe was displayed in MC but Fe and Ni did not decrease until months 6 and 8, falling to levels of 20 and 30 μg/L respectively. The Co concentration was significantly reduced in MC by month 2 to 30 μg/L.

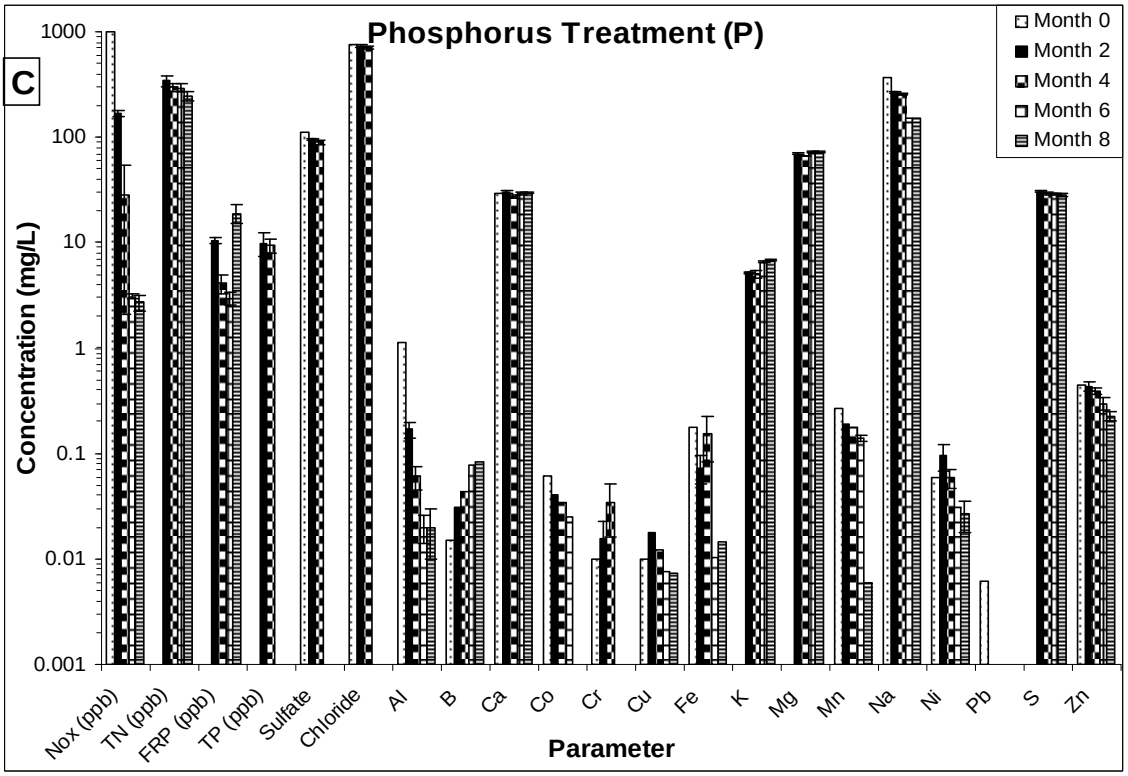
The only other significant reduction in a physico-chemical concentration was that of NOx. The concentration of this parameter showed a significant decrease in MC with a similar decrease in all three treatments. NOx concentration in MC decreased from 1000 to 29 μg/L by month two and treatments L and L&P reacted similarly decreasing to 46 and 2 μg/L respectively. There were no further changes in NOx for treatments L and L&P or MC after Month 2. Treatment P showed a decreasing trend with a decrease in concentration by Month 2 to 170 μg/L and again by Month 4 to 37 μg/L.

When comparing the effect of different treatments on metal concentrations in the mesocosm samples several differences were observed. The limestone and limestone plus phosphorus treatments (treatments L and L&P respectively) produced a greater reduction in metal concentrations compared to that achieved with the phosphorus treatment (P) alone. Overall, there was little difference after 8 months between the

reduction in concentrations observed with the limestone and phosphorus compared to the limestone alone. However, when additional phosphorus was added after month 6 a further reduction in some metals was observed.




B
Limestone Treatment (L)
Month 0
Month 2
Month 4
Month 6
Month 8
Concentration (mg/L)
1000
100
10
1
0.1
0.01
0.001
Nox (ppb)
TN (ppb)
FRP (ppb)
TP (ppb)
Sulfate
Chloride
Al
B
Ca
Co
Cr
Cu
Fe
K
Mg
Mn
Na
Ni
Pb
S
Zn
Parameter


C
Phosphorus Treatment (P)
Month 0
Month 2
Month 4
Month 6
Month 8
Concentration (mg/L)
1000
100
10
1
0.1
0.01
0.001
Nox (ppb)
TN (ppb)
FRP (ppb)
TP (ppb)
Sulfate
Chloride
Al
B
Ca
Co
Cr
Cu
Fe
K
Mg
Mn
Na
Ni
Pb
S
Zn
Parameter

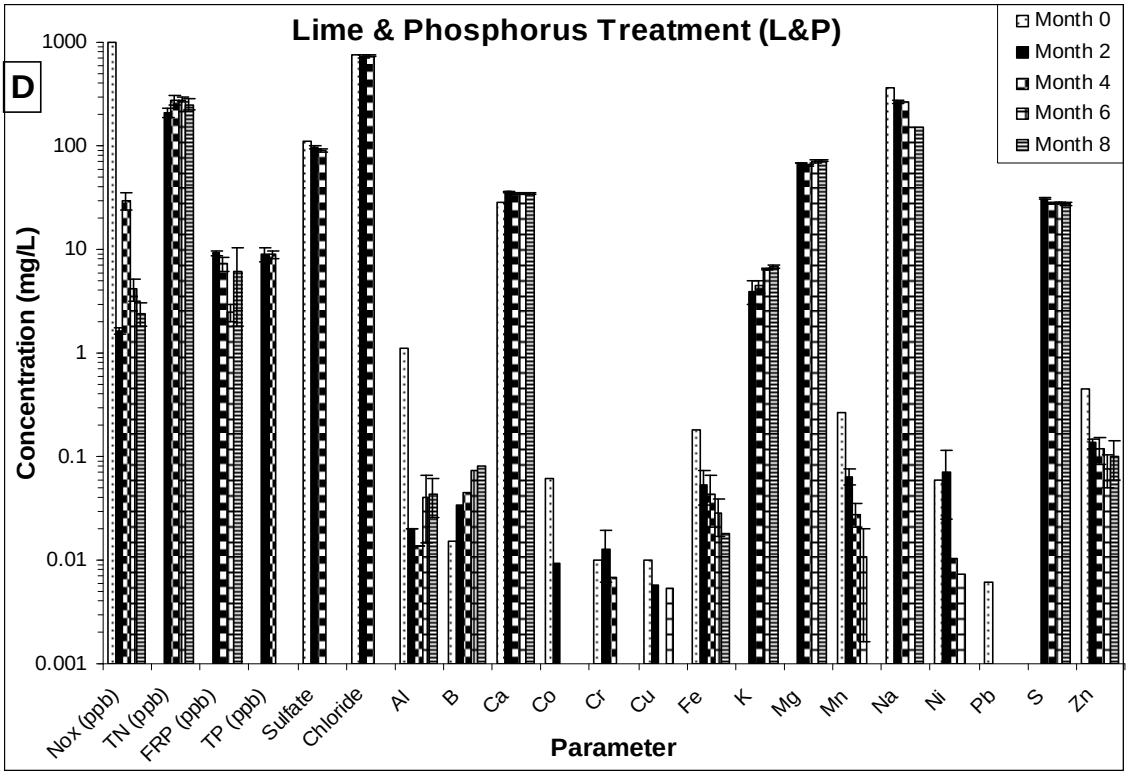


Figure 28. Mean chemical concentrations ± standard error for Mesocosm Control (MC) (A), treatment L (B), treatment P (C), and for treatment L&P (D). Y-axis is a log scale. Data missing from Month 0 for any parameter was not collected. Data shown as 0 for any other month was below the detection limit except for TP, sulphate and chloride which were not measured at months 6 and 8.

6.5 Discussion

The aim of this study was to evaluate Lake Kepwari water toxicity relating to chemical changes from implementation of treatments on the Lake water in mesocosms over an 8 month period. The three different water treatment approaches used on the mine lake water were; limestone chip, phosphorus and limestone chip plus phosphorus additions which were monitored over an 8 month period. The limestone addition was utilised with the intent of increasing pH and therefore removing metal bioavailability. Phosphorus addition was utilised to stimulate phytoplankton growth, owing to the low presence of phosphorus generally being a limiting factor in freshwater lakes (Kopacek *et al.* 2000; Lychie-Solheim *et al.* 2001) and its effect in increasing alkalinity through stimulated biological nitrate uptake.

The mesocosm trial lasted 8 months over which time included spring, summer and autumn seasonal temperatures and daylight durations. These seasonal changes no doubt have had an effect upon the treatments used on the Lake Kepwari water, firstly by having a low temperature at the beginning of the trial, slowing biological processes especially affecting treatment P which relies on biological uptake of nitrate to produce alkalinity. This in turn would have affected the rate of amelioration from these natural biological processes and could explain partly the initial sluggish response of treatment P in reducing toxicity. Secondly the shorter light duration due to cloud and daylight hours occurring in early spring could have also slowed the biological processes, especially phytoplankton growth, required for amelioration. This effect may not have been as pronounced in the limestone treatments due to the limestone not relying on biological activity for pH remediation.

Several physical and chemical parameters in the Lake Kepwari water exceeded (ANZECC/ARMCANZ. 2000b) freshwater aquaculture guideline values at the commencement of the mesocosm trial month 0. Lake Kepwari pH measured at month 0 before treatment was outside the guideline range of pH 5-9. Aluminium, Mn and Zn concentrations all exceeded the freshwater aquaculture guideline concentrations (ANZECC/ARMCANZ. 2000b) indicating the need for further assessment of the pit lake water. Lake Kepwari does not have a low pH when compared to other mine lakes in the Collie Coal Basin (WO5H pH ~3, Chicken Creek pH ~3) but the toxicity to aquatic life is still prevalent (Lund *et al.* 2006). The amelioration regimes utilised on the pit lake water of L and L&P increased to and maintained a circum-neutral pH for the duration of the 8 month trial, indicating that limestone addition alone is suitable as a pH remediation strategy for this water. Treatment P did not exhibit a quick pH response by month 2 but pH did progressively increase during the 8 month trial to an acceptable level for use in aquaculture. The pH measured in the Mesocosm Control and treatment P were not significantly different from each other over the 8 months, indicating that the pH may increase over time due to other natural processes or rain dilution effects without P addition. The increase in pH from limestone addition was also probably responsible for the decrease in Al, Mn and Zn concentrations in treatments L and L&P by month 2. Al, Mn and Zn will precipitate or co-precipitate at pH levels between 6 and 8 by

adsorption, cluster formation, homogenous solid solution, heterogeneous solid solution or a combination of these processes as shown by Lee *et al.* (2002) and Jackson & Bistricki (1995).

Concentrations of the three metals exceeding the aquaculture guidelines reduced in the three treatments and MC, with limestone addition showing the quickest reduction in these metals. The concentrations of metals in most waters are controlled by adsorption and coprecipitation processes with which limestone can play a significant role (Lee *et al.* 2002). The mean Al concentration in treatments L and L&P was reduced to below the guideline value by month 2 and only fluctuated slightly over the following 6 months showing that limestone addition alone effectively reduced the concentration of Al from this pit lake water. By month 8 mean Al concentrations had reduced from 1125 µg/L to <50 µg/L for all treatments. The Al concentrations in treatments MC and P were reduced to below the guideline concentration but were not significantly lower than that of Treatments L and L&P indicating that the Al present in L and L&P may no longer be influencing toxicity. Aluminium in the aquatic environment is found mostly in the free ion Al^{3+} form from pH 4.5 to 5.5 and forms solid $Al(OH)_3$ precipitates between the pH range of 5.2-6.5 (Sauvant *et al.* 2000). By Month 4 the pH for all treatments and control had increased to above the pH of 5.2 required for $Al(OH)_3$ precipitation reactions to occur resulting in a significant decrease of mean Al concentrations. Al once precipitated, is no longer in a bio-available form and therefore reduces toxicity to aquatic biota. However, if solid $Al(OH)_3$ precipitates onto gills asphyxiation and adverse cell interactions may occur, especially in fish (Cleveland *et al.* 1991; Buckler *et al.* 1995).

Manganese is frequently present at elevated concentrations in waters draining from and within coal mine lakes (Hallberg & Johnson 2005), which is the case in Lake Kepwari. The mean concentrations of Mn within treatments L, P and L&P all reduced from 265 µg/L (>25 times aquaculture guidelines) to below the aquaculture guideline concentration of 10 µg/L by Month 8. The mean concentration of Mn within Treatment I was 132 µg/L at Month 8 which was still more than one order of magnitude higher than the aquaculture guideline concentration. Mn is present as the

Mn^{2+} species below pH 8, but can co-precipitate with Al, Fe and Zn at pH levels below 8 (Lee *et al*. 2002). Limestone addition increased the pH to above 6.5 for L and L&P and Mn was removed effectively possibly co-precipitating with other metals (Lee *et al*. 2002). Phosphorus addition also removed Mn effectively, indicating that phosphorus also has an effect on reducing the Mn present in this pH range of 4.8 to 7.

Mean Zn concentrations decreased in all treatments with treatments L and L&P having significantly lower concentrations than MC and P at Month 8. This showed that the addition of limestone alone could significantly reduce zinc concentrations from the Kepwari lake water. According to (Hyne *et al*. 2005) Zn toxicity can increase as a function of increasing pH (6.5–7.5). The Zn concentration exceeding the aquaculture guidelines in treatments L and L&P, therefore, may be capable of toxicity to aquaculture species. Zn is also known to readily bioaccumulate in aquatic animals and may be a cause for concern if aquaculture is to take place in the limestone treated water (ANZECC/ARMCANZ. 2000a).

Although chemical analysis can give a picture of what toxicants may cause toxicity within an aquatic system, the complex nature of natural systems means that this analysis does not always provide accurate conclusions (Lahr *et al*. 2003). Even with the comparison of chemical data to the guidelines the requirement of biological assessment is necessary for absolute confirmation of toxicity (Mitchell 2002).

6.6 Conclusion

All three treatments reduced metal concentrations and increased pH with L and L&P being the most effective in the initial months of 2 and 4. Limestone treatment alone is effective in increasing the pH to neutral and reducing metals significantly and to below aquaculture guidelines with the exception of Zn and therefore, stands as being the most successful remediation regime. Treatment P was not as effective in removing metals from the Kepwari pit lake water as L but may still be an option as a

remediation technique with further study. Even though metal concentrations have been reduced, bioaccumulation of metals could still be a problem to humans if fish, crustaceans and or molluscs farmed in this water are to be consumed (other option; ornamental species) (ANZECC/ARMCANZ. 2000a; Storer 2005).

As long as any terrestrial sources of pyrite-bearing overburden and the void walls are kept from being further oxidized, limestone treatment of the Kepwari pit lake water may be effective in removing toxicity through increasing pH and consequent precipitation of metals.

Results show that the addition of phosphorus to the Kepwari water does not make a significant difference to metal results when compared with no P addition, with the exception of Mn. Adding of L&P to the Kepwari water compared with just adding limestone also showed no difference in physico-chemical results. This could be explained three possible ways, abiotic Al-PO_4 interactions in the extracellular environment, Al inhibition of PO_4 uptake and interference with intracellular phosphorus metabolism (Parent & Campbell 1994). All three of these pathways for P removal or interference can affect not only remediation of water quality by micro-organisms, but also bioassay results when using PO_4 as a nutrient for algal growth (Kopacek *et al.* 2000).

Using chemical data and water quality trigger values in assessing toxicity to aquatic biota can be restrictive and may require extrapolation and site specific modifications (Markich *et al.* 2001). As stated within the guidelines they are only trigger values and when exceeded require the use of further risk assessment with the decision tree (ANZECC/ARMCANZ 2000). These guidelines are generally overprotective due to the complex nature of natural systems reducing the overall chance of a toxic agent being able to have a direct affect to an organism. Nevertheless, being over protective of our natural aquatic environments is appropriately conservative. When complexes of many different chemicals together combine to increase a toxic effect these trigger values may not be protective enough. By incorporating metal speciation,

bioavailability and ecological host factors into guidelines a more accurate guide can be realised (Peakall & Burger 2003).

BIOASSAY TOXICITY ASSESSMENT OF MINING PIT LAKE WATER REMEDIATED WITH LIMESTONE AND PHOSPHORUS.

7 Bioassay toxicity assessment of mining pit lake water remediated with limestone and phosphorus.

7.1 Abstract

Pit lakes are a common legacy worldwide and are being increasingly recognised for their potential to provide benefits to local communities. Toxicity of many pit lake waters may require remediation before some beneficial end uses can be realised. Three treatments to remediate acidic (pH 4.8) pit lake water containing elevated concentrations of Al and Zn from Collie, Western Australia were tested in replicated 1200L mesocosms. The treatments were: a) limestone neutralisation (L), b) phosphorus amendment (P), and c) combined limestone neutralisation and phosphorus amendment (L&P). Bioassays using *Ceriodaphnia* c.f. *dubia, Chlorella protothecoides* and *Tetrahymena thermophila* were utilised to assess treatment remediation success. Limestone alone was found to increase pH and remove heavy metal concentrations, thereby removing toxicity to the three test species within 2 months of treatment. Phosphorus addition alone removed toxicity to the test organisms after 6 months of treatment. Adding phosphorus to liming failed to reduce toxicity more than liming alone. Low concentrations of nutrients also appeared to limit phytoplankton population growth in all treatments.

7.2 Introduction

Mining pit voids are a common legacy of open cut mining worldwide (Castro & Moore 1997). Unless backfilled, many of these voids will eventually fill with ground and surface water to create a pit lake. The water quality in pit lakes, in particular acidity, salinity, hardness and metal composition differ depending on the hydrology and geochemistry of the local and surrounding areas (Miller *et al.* 1996). Pit lakes have the post-mining potential to be used for a range of beneficial end uses including recreation, biodiversity conservation, stock and plant irrigation, aquaculture, extraction of industrial chemicals and as sources of potable and/or industrial water

(Doupé & Lymbery 2005; McCullough & Lund 2006). The end use is generally restricted by poor water quality in the pit lake (Grünewald & Uhlmann 2004).

For example, geological acidification is a common problem in pit lakes by mining activities enhancing weathering of ore bed geologies, and consequently rates of oxidation and hydrolysis of pyritic (FeS_2) rocks. These processes produce acid, which then dissolves a range of solutes creating Acid Mine Drainage (AMD) (Geller *et al.* 1998). This acidification may cause toxicity to aquatic biota not only through lowered pH, but also through increased bioavailability of other toxicants, particularly heavy metals (Lopes *et al.* 1999). Until recently acid pit lakes have received little attention for remediation for future end uses (Koschorreck *et al.* 2007).

Due to toxicity, remediation of pit lake water quality is required to allow the water to be used for a range of beneficial end uses (Doupé & Lymbery 2005; McCullough & Lund 2006). Sulphate reduction is one such commonly used approach via stimulation of naturally occurring microbial processes in lakes with high sulphate concentrations (Frömmichen *et al.* 2003). Combinations of organic carbon material can be a cheap effective means to increase alkalinity and thereby reduce heavy metal concentrations (Frömmichen *et al.* 2004; Bozau *et al.* 2007).

However, the low sulphur content in many moderately acidified water bodies (e.g., the mine pit lakes of South-Western Australia (Johnson & Wright 2003; Lund *et al.* 2006)), restrict this remediation technique (Lund *et al.* 2006). Limestone neutralisation is a common remediation method for such moderately acidic waters, by increasing pH to levels suitable for biotic growth and survival and by reducing dissolved metal and other contaminant concentrations (Cravotta III & Trahan 1999; Maree *et al.* 1999; Cole *et al.* 2001; Kalin 2004; Watten *et al.* 2005). Passive remediation techniques using *in-situ*, biologically-based treatment approaches have also been suggested as practical techniques for remediation of moderately acidic pit lake water quality (Woelfl *et al.* 2000; 2003; Lund *et al.* 2006; Totsche *et al.* 2006). Phosphorus amendment has been used to overcome phosphate limitation (Parent & Campbell 1994; Kopacek *et al.* 2000; Bittl *et al.* 2001) and has been shown to

remediate low pH by stimulating phytoplankton alkalinity production through nitrate assimilation (Davison *et al.* 1995). Addition of P to moderately affected acid lakes only requires a small amount of P addition for a significant increase in pH to be made. Addition of 4–19 μg/L P has been shown to increase pH from 4.9–5.2, corresponding to a 50% increase in H+ -equivalents from 12–6 μeq/L due to algal uptake of H^+ when assimilating NO_3^- (Lychie-Solheim *et al.* 2001). Furthermore, labile Al concentrations reduced from 150–100 μg/L, possibly due to the increased pH and probably due to the precipitation with phosphate (Lychie-Solheim *et al.* 2001).

Aluminium is the most abundant metallic element in the lithosphere, but has little or no known biological function (Gensemer & Playle 1999). Aluminium is thought to be responsible for pH buffering and toxicity (directly or indirectly) in moderately low pH lakes (pH 3–5) (Stephens & Ingram 2006) and is found in moderate concentrations within many moderately acidic pit lakes. Elevated Al concentrations in acidified lakes may affect biota both through direct toxicity and by disruption of in-lake phosphorus cycling (Kopacek *et al.* 2000), resulting in phosphorus limitation of phytoplankton (Bittl *et al.* 2001). Consequently aquatic community colonization in aluminium-buffered lakes is limited by sensitivity towards protons, phosphorus limitation, and toxicity from inorganic reactive aluminium and heavy metals (Nixdorf 2003).

Even at moderate pH, heavy metal concentrations may still exceed environmental protection guidelines (Rothenhöfer *et al.* 2000; Markich *et al.* 2001). The potential impacts of these toxicants on aquatic environments are often studied using microcosms and mesocosms (Tang 1993; Fyson *et al.* 1998; Frömmichen *et al.* 2003; Collins *et al.* 2005). Replicated mesocosms allow for a larger and more complex-scale measurement of biotic effects than laboratory trials and avoid issues associated with replication at a field scale (Odum 1984).

This study aims to evaluate the efficacy of three different *in-situ* remediation treatments for reducing toxicity in moderately acidic (*ca*. pH 4.8) acid pit lake water

at field scales after McCullough & Lund (2007a). These treatments were; a) limestone neutralisation (L), b) phosphorus amendment (P), and c) combined limestone neutralisation and phosphorus amendment (L&P).

Toxicity of these remediated pit lake waters was assessed using laboratory-based acute and chronic toxicity test protocols for three aquatic organisms from different taxonomic and trophic levels, the water flea *Ceriodaphnia* c.f. *dubia*, the microalga *Chlorella protothecoides* and the ciliated protozoan *Tetrahymena thermophila*. Bioassays are used as sensitive indicators of pollutant toxicity, since they are rapid, inexpensive, applicable to a number of toxicants and allow several acute and chronic endpoints to be assessed simultaneously (Calevro *et al.* 1999). The chemical modelling program PHREEQC (Parkhurst & Appelo 1999) was also used to assess the proportion of each species of Al and Zn at each testing month. PHREEQC is used for a wide variety of low-temperature aqueous geochemical calculations and is based on an ion-association aqueous model.

7.3 Materials and Methods

7.3.1 Study site

The pit lake water used in the mesocosm trial was collected from Lake Kepwari a rapid-filled mine pit lake located in the Collie Coal Basin in the South West of Western Australia (33.36° S, 116.15° E) (Figure 29). The Collie Coal Basin of South-Western Australia is a site of significant environmental acidification, resulting from over 100 years of coal mining in pyrite-bearing geologies (Ashton & Evans 2005). However, even after rapid filling by diversion of the Collie River over several winter seasons, the 24 GL Lake Kepwari has a low and declining pH of *ca*. 4.8, and has elevated metal concentrations of Al and Zn (McCullough & Lund 2007a; McCullough & Lund 2007b).

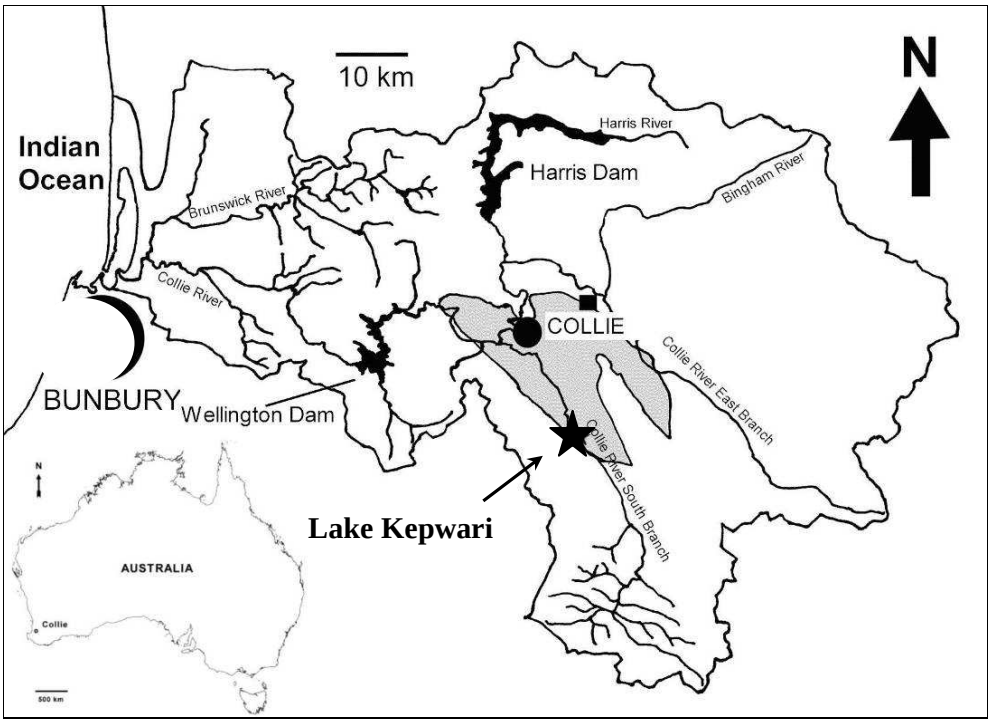


Figure 29. Location of Lake Kepwari (indicated with an '∗') in the south-western lobe of the Collie Coal Basin, Western Australia. Figure modified from Lund et. al. (2006)

7.3.2 Mesocosm treatments

Using water and sediment collected from Lake Kepwari, twelve 1 200 L pit lake mesocosms had been established at Edith Cowan University, Perth, in August 2005. Each mesocosm contained sediment and lake water collected from the lake. Three treatments and an untreated control were replicated three times and arranged in a randomised block design (McCullough & Lund 2007a).

Representative control water samples of Collie River water (CRW) were collected upstream from the Collie River diversion site. The Mesocosm Control (MC) contained intact Lake Kepwari water while treated mesocosms contained: 1) Lake Kepwari water pH amended with limestone chips to pH neutral (L), 2) di-potassium orthophosphate (K_2HPO_4) to an initial soluble reactive phosphorus (SRP) concentration achievable at a field scale of around 20 µg/L (P), and 3) a combination of both the limestone and phosphorus treatments (L&P). Further phosphorus

additions of 10 µg/L P were required to maintain phosphorus levels and were made 3 days after month 2 sampling, 8, 19 and 33 days after month 4 sampling and 12 and 27 days after month 6 sampling. The P concentration chosen is a logistically feasible concentration that could be achieved in a large mine pit lake (Lychie-Solheim *et al.* 2001). This P addition also reflects an adequate concentration for primary productivity stimulation without leading to lake eutrophication. Mesocosm water levels were initially maintained by roof-collected rainwater to accommodate for evaporation. However, rainwater use was discontinued after it was found to be contaminated with P (total contribution of *ca.* 2 µg/L total P to each mesocosm shortly before month 2) Water level maintenance was then made by regular additions of deionised water.

7.3.3 Water quality analysis

Water quality parameters of each mesocosm were measured at bi-monthly intervals of November 2005 (month 2), January 2006 (month 4), March 2006 (month 6) and May 2006 (month 8).

Measurements for temperature, pH, Dissolved Oxygen (DO) (% saturation and mg/L), specific conductance, chlorophyll *a* concentrations and Oxidation Reduction Potential (ORP) (platinum reference electrode) were performed bi-weekly *in situ* with a Hydrolab Datasonde 4a. On each bi-monthly sampling occasion, three surface water samples from each mesocosm were collected, two were immediately filtered through 0.5 µm glassfibre filterpaper (PALL 'Metrigard') and the remainder was left unfiltered. All samples were stored frozen in acid washed high-density polyethylene bottles prior to chemical analysis. Filtered samples were analysed for Filterable Reactive Phosphate (FRP) on a Skalar Autoanalyser after APHA (1998). Remaining filtered mesocosm water sample was acidified with 1% reagent grade HCl and selected metals analysed by Inductively Coupled Plasma Atomic Emission Spectrophotometry (ICP-AES) for As, Al, Ca, Cd, Cr, Co, Cu, Fe, Hg, Mg, Mn, Ni, Pb, Se and Zn.

7.3.4 Water toxicity testing

Water samples were also collected on each sampling occasion for toxicity testing. The samples were tested for toxicity using three test organisms; the water flea *Ceriodaphnia* c.f. *dubia*, the microalga *Chlorella protothecoides* and the ciliated protozoan *Tetrahymena thermophila*. All mesocosm water samples were filtered to 0.5 µm glassfibre filterpaper (PALL 'Metrigard') and stored frozen for less than 7 days before conducting the toxicity bioassays. Water samples were placed into an incubator 24 h before testing to bring their temperature to within 1°C of that of the test organism's culture water. A Cr (VI) reference test was used to assess *C. protothecoides*, *C.* cf *dubia* and *T. thermophila* health and sensitvity changes.

7.3.5 *Ceriodaphnia* c.f. *dubia* bioassay

7.3.5.1 Test samples

Two bioassay controls were prepared from natural Collie River water (CRW); as a representative of Collie Basin surface waters) and a synthetic medium High-Hardness COMBO (HHC). HHC is a synthetic medium developed for use in toxicity testing and culturing of multiple species and is recommended for inter-laboratory comparisons of test results (Kilham *et al.* 1998; Baer *et al.* 1999). The chelating agent EDTA was left out of the HHC medium, to remove the possibility of it modifying toxicity of the metals in the treatments (Tevlin 1978). A 48 h static acute toxicity test for *C.* c.f. *dubia* was conducted on five sub-replicates from each mesocosm as well as CRW and HHC waters.

7.3.5.2 Culture of test organism

Test organism culturing was based on procedures from the New South Wales Environmental Protection Authority (NSW EPA) (NSW EPA 2004). Stock cultures of *C.* c.f. *dubia* were cultured in HHC medium, made up using distilled water as a diluent. *Ceriodaphnia* c.f. *dubia* cultures were fed *Ankistrodesmus* sp. algae daily at a density of $100x10^4$cells/mL. The feeding regime was altered from the original procedure after (Hyne *et al.* 2005) as it was found that there was no significant

difference (Student T-test, n=5, P>0.05) in the intrinsic rate of natural increase (IRNI) of *C.* c.f. *dubia* when two algae, *Pseudokirchneriella subcapitata* and *Ankistrodesmus* sp., were used as compared to use of a single alga. Stock cultures of the alga were sourced from the NSW EPA laboratory.

7.3.5.3 Test protocol

The *Ceriodaphnia* c.f. *dubia* acute toxicity test protocol of Orr and Foster (1997) was used. Neonates were individually transferred to plastic test wells, with each test well containing 10 mL of control or treatment water and 10 neonates. The test plates were placed into an incubator at 25±1°C with a daily photoperiod of 16:8 h light:dark. Mortality was assessed after 24 h and 48 h with any deceased animals being removed at the 24 h mortality count. As recommended by Hyne *et al.* (2005), there was no feeding of the neonates during the 48 h test period.

7.3.6 *Chlorella protothecoides* bioassay

7.3.6.1 Test samples

A 72 h chronic static toxicity test was conducted on mesocosm water samples using *C. protothecoides*. Two bioassay controls were also tested, a synthetic soft water (SW) with nutrients added after Stauber *et al.* (1994), and neutralised (N) Lake Kepwari mesocosm control water. The N treated water was neutralised by drop wise addition of 0.1 M sodium hydroxide solution until the pH reached *ca.* 7 and was then left for 24 h to stabilise before use. Three sub-replicates were tested from each mesocosm and bioassay control. All mesocosm waters were filtered to 0.2 µm before testing to remove all suspended particles including any acidophilic algae or bacteria that could alter the *C. protothecoides* growth results (Parent & Campbell 1994).

Two *C. protothecoides* trials were conducted on each sample; in trial one (*sans* nutrients) nutrients were only added to the synthetic control; in trial two (with nutrients) sodium nitrate ($NaNO_3$) and di-potassium orthophosphate (K_2HPO_4) were added to all test samples and to the control. By using this approach it was possible to differentiate between reduced growth rate resulting from the presence of toxicants,

and reduced growth rate resulting from a lack of nutrients. Furthermore the interaction of P with Al could reduce the effective Al concentration altering toxicity of the sample (Parent & Campbell 1994). Therefore, leaving K_2HPO_4 out would remove the possibility of this occurring.

7.3.6.2 Test protocol

A stock culture of *C. prototheco*ides, sourced from the CSIRO Marine and Atmospheric Research Microalgae Supply Service, Tasmania, was used as the test alga. The 72 h *C. prototheco*ides chronic test protocol followed Stauber *et al*. (1994), with the exception of the absence of nutrient addition to mesocosm samples in trial one. Test vessels were 150 mL conical glass flasks containing 50 mL of test solution and algae inoculant. The density of *C. prototheco*ides at the beginning of the test was approximately $7x10^4$cells/mL in all flasks. Cell densities in all flasks were measured at 0, 24, 48 and 72 h using a Shimadzu UV-1201 spectrophotometer at a wavelength of 750 nm.

7.3.7 *Tetrahymena thermophila* bioassay

7.3.7.1 Test samples

A 24 h chronic static toxicity test was conducted on samples from each mesocosm and a bioassay control using the ciliated protozoan *T. thermophila*. The *T. thermophila* bioassay is a chronic toxicity test completed in a short period of time (24 h) due to the fast reproductive rate of this species. The bioassay *T. thermophila* control (TTC) water used was a combination of analytical grade water and a food substrate consisting of proteose peptone and yeast extract (PPY). Analytical grade PPY after Gerson (1995) was added to all samples to promote growth and reproduction of the *T. thermophila*.

7.3.7.2 Test protocol

A stock culture of *T. thermophila* was sourced from a PROTOXKIT F ™ test kit. *Tetrahymena thermophila* were cultured in slanted 10 mL plastic test tubes, using

analytical grade water with the addition of 2xPPYS as per (Gerson 1995). The concentration used in bioassays was 0.5xPPY. The dilution of PPY decreased the likelihood of reducing, masking or increasing toxicity of the mesocosm samples to *T. thermophila*.

T. thermophila in the stock culture were counted prior to inoculation of test samples using a haemocytometer and a compound microscope at 100 times magnification. A minimum of 200 ciliates were counted to determine the density of the stock culture. *Tetrahymena thermophila* were diluted with Milli Q water to a density of $20x10^4$ *T. thermophila* /mL. Test containers consisted of 4 mL clear plastic cuvettes. Each test cuvette contained 1.8 mL of mesocosm or control water with 0.1 mL inoculation of ciliates added and 0.1 mL 10xPPY added to make a concentration of 0.5xPPY totalling 2 mL. A final density of $1x10^4$ *T. thermophila* /mL was achieved at the commencement of the test. Three sub-replicates of each mesocosm and treatment were used in toxicity assessment (n=36). All cuvettes were covered with a lid and placed into an incubator for 24 h at 30ºC in the dark. There was no mixing or shaking of the test containers during this time except for at 23 h when each cuvette was gently agitated. At the end of the incubation period, ciliate population growth was estimated by counting of ciliates as described above.

7.3.8 Data analysis

Statistical analysis of all bioassay and water quality data was performed using the statistical program SPSS (2000). Normality was checked using the Shapiro-Wilk test (Shapiro & Wilk 1965). Non-normal data was then checked for homogeneity of variances using Levene's test (Levene 1960). Significant differences found from statistical tests were all at the $P < 0.05$ level.

Results for mortality of *C.* c.f. *dubia* bioassays were arcsine transformed before statistical analysis. When *C.* c.f. *dubia* data were still not normally distributed ($P<0.05$), data were transformed to rankits (Pereira *et al.* 2000).

Chlorella protothecoides absorbency data were transformed by natural log to achieve a linear relationship (LN of absorbance at 750nm). Therefore algae density units are represented by LN absorbance (750nm). Growth rate at 72 h (day 3) were estimated from the slope of this transformed data. Growth inhibition compared to that of controls was calculated using the formula:

$$G_i = 100 \times (1-G_t/G_c)$$

Where G_i is the growth inhibition, G_t is the growth rate for the treatment and G_c is the growth rate for the control.

When the assumption of normality was met, a parametric or repeated measures one-way analysis of variance (ANOVA) was performed where appropriate to explore for significant differences between treatments and controls. When data were not normally distributed the non-parametric Kruskal-Wallis H Test was used to find median differences between treatments and controls. When significant differences were found, a pair-wise *post-hoc* test was performed to identify where the differences occurred. The parametric Tamhane Test *post-hoc* test was used when variances were not equal and the Student-Newman-Keuls (S-N-K) Range Test when variances were equal.

An assessment of which water quality variables had most influence on test species response was determined using the "BEST" routine (Clarke & Ainsworth 1993) in Primer software (PRIMER-E Ltd 2006). This programme Spearman Rank correlates test species biotic responses with environmental variables.

To enable an inter-species comparison of sensitivity to treatments test species responses were standardised against their highest replicate response using PRIMER software (standardised response). Percent mortality was converted to '% survivorship' (100%-mortality%) to enable a comparison with positive growth rates reported from the other bioassays of *T. thermophila* and *C. protothecoides*.

Hardness Modified Guideline Values (HMGV) for aquaculture and aquatic ecosystem protection were calculated after ANZECC/ARMCANZ (2000b). HMGV take into account site specific hardness, therefore allowing for the toxicity buffering capacity of Ca (Markich *et al.* 2001). Speciation was determined for Al at each sample month by the computer program PHREEQC (Version 2.13.07) (Parkhurst & Appelo 1999).

7.4 Results

7.4.1 Water quality changes

Mesocosm Control pH rose from around 5 at months 2 and 4, to around 6 at months 6 and 8 (Table 22). Following lime neutralisation, pH of both limed treatments was >7 for month 2 and circum-neutral for later testing months. P treatment pH showed a similar trend to control pH, albeit consistently almost ½ a pH unit higher in test months after month 2. Electrical conductivity of all treatments was similar at around 2.50 mS/cm for all treatments and sample months. Oxidation-reduction-potential was highest in the MC at testing months 2 and 4 at around 190 mV, decreasing to around 125 and 145 mV in months 6 and 8. P treatment showed a similar pattern, with a decrease from an initial high of around 200 mV in month 2, to only 180 mV in month 4, 110 mV in month 6 and increasing again to 125 mV in month 8. Both limed treatments showed similar ORP results with 120 mV in month 2, 130 in month 4, 90 in month 6 and 120 mV in month 8, although L&P ORP was around 6% higher in all but month 6.

Many metals were below their detection limits (in parentheses) in all treatments and testing months, including As (10 µg/L), Cd (0.6 µg/L), Hg (20 µg/L), Pb (10 µg/L) and Se (20 µg/L). Alkalinity was negligible in all mesocosms consistent with the moderately low pH and low hardness. Ca concentrations in un-neutralised treatments were moderately hard 30 mg/L (total hardness 75 mg/L as $CaCO_3$), and for

neutralised treatments were only slightly harder at around 35 mg/L. Al, Cu, Co, Fe, Mn, Ni, and Zn all showed a similar pattern between control and P, with concentrations lower at later months, albeit even lower in P. However, liming treatments showed lower concentrations of these metals, which were then even lower in later testing months. Cr was slightly higher in dosed treatments compared to the control for months 2 and 4. However at month 6 levels were generally the same or lower than the control. P had substantially reduced Mn and Co compared to the MC. Liming had higher concentrations of Al in month 8, although this higher concentration was also at a higher pH (Table 22). Mesocom treatments with liming also had lower levels of Zn compared to control and P. Treatment FRP varied little in MC near the limit-of-detection of 3 μg/L. FRP was generally higher in limed and P treatments at 5–10 μg/L, and was highest in P in month 8 at 20 μg/L.

Table 22 Physico-chemistry of mesocosm at toxicity testing months 2, 4, 6 and 8. Values are means (standard error).

Month	Treatment	pH	EC (mS/cm)	ORP (mV)
2	MC	5.2 (0.1)	2.54 (0.03)	192 (3.5)
	L	7.4 (0.3)	2.51 (0.02)	115 (10)
	L&P	7.4 (0.1)	2.55 (0.02)	122 (8)
	P	5.1 (0.1)	2.57 (0.01)	203 (7)
4	MC	5.3 (0.1)	2.45 (0.02)	188 (4)
	L	6.9 (0.1)	2.44 (0.03)	133 (5)
	L&P	6.6 (0.1)	2.46 (0.01)	141 (3)
	P	5.6 (0.1)	2.47 (0.01)	178 (4)
6	MC	6.0 (0.1)	2.52 (0.01)	126 (3)
	L	7.0 (0.1)	2.47 (0.01)	91 (5)
	L&P	6.9 (0.1)	2.50 (0.04)	91 (2)
	P	6.4 (0.1)	2.47 (0.1)	110 (1)
8	MC	6.4 (0.1)	2.47 (0.02)	143 (7)
	L	7.3 (0.1)	2.49 (0.03)	120 (5)
	L&P	7.0 (0.1)	2.50 (0.03)	126 (3)
	P	6.7 (0.0)	2.45 (0.01)	125 (2)

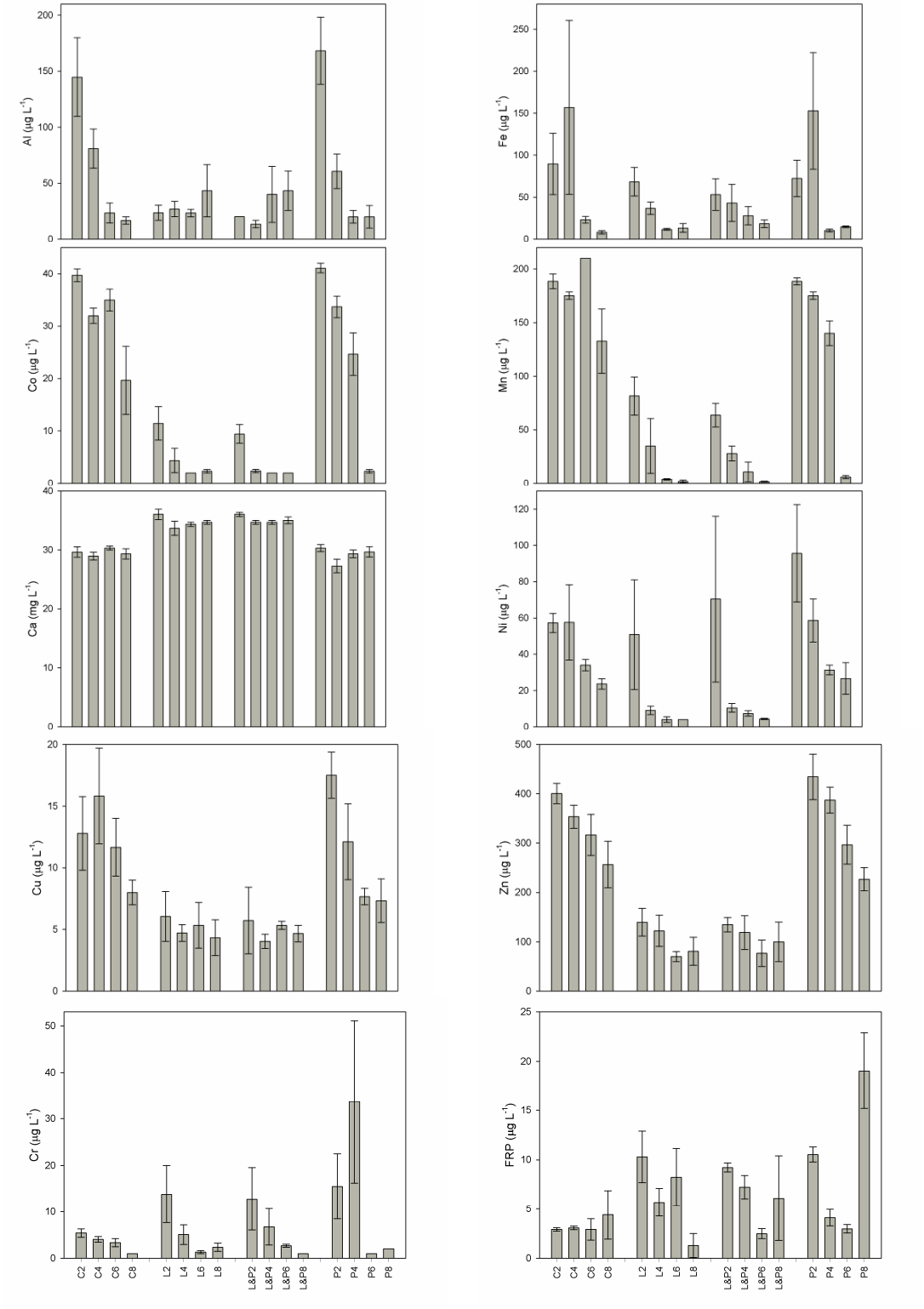


Figure 30. Mean metal concentrations ± standard error from the mesocosms, recorded on months 2, 4, 6 and 8 for the mesocosm control (C), Limestone (L), Limestone and P (L&P) and P only (P) treatments.

Month 0, Al, Mn and Zn concentrations exceeded aquaculture guidelines and Zn exceeded aquatic ecosystem protection guidelines in the untreated MC. Mean Al concentration in the MC and treated mesocosms was reduced to below aquaculture guidelines by month 8. The Mn concentration remained elevated above aquaculture guidelines in the MC but not the treated mesocosms. Mean Zn concentration remained elevated above both guidelines at month 8. HMGV allow the buffering capacity of Ca to be incorporated into guidelines for some metals (ANZECC/ARMCANZ. 2000b). Of the three elevated metals HMGV are only available for Zn with the HMGV for aquaculture and for aquatic ecosystems, 11 μg/L and 14 μg/L respectively for Zn. All Zn concentrations at month 8 were still above the HMGV indicating the possibility of toxicity from Zn.

7.4.2 Speciation modelling

The computer program PHREEQC was used to determine the Al speciation at each sample month in each treatment and control (Figure 31). The proportion of free Al^{+3} at month 0 was approximately 53%, which decreased in all the treatments to less than 1% by month 8. The Al^{+3} in MC and P was no longer dominant by month 4 where $Al(OH)_2^+$ had the highest proportion which continued in MC through to month 8. By month 6 speciation in P was dominated by $Al(OH)_4^-$ with a small remaining proportion of Al^{+3} which continued through to month 8. The speciation trend was similar in both L and L&P with $Al(OH)_4^-$ dominating from month 2–8. The speciation of Zn was also calculated from PHREEQC and it was found that the Zn^{+2} ion was the dominant species with greater than 85% present at all months in all mesocosms.

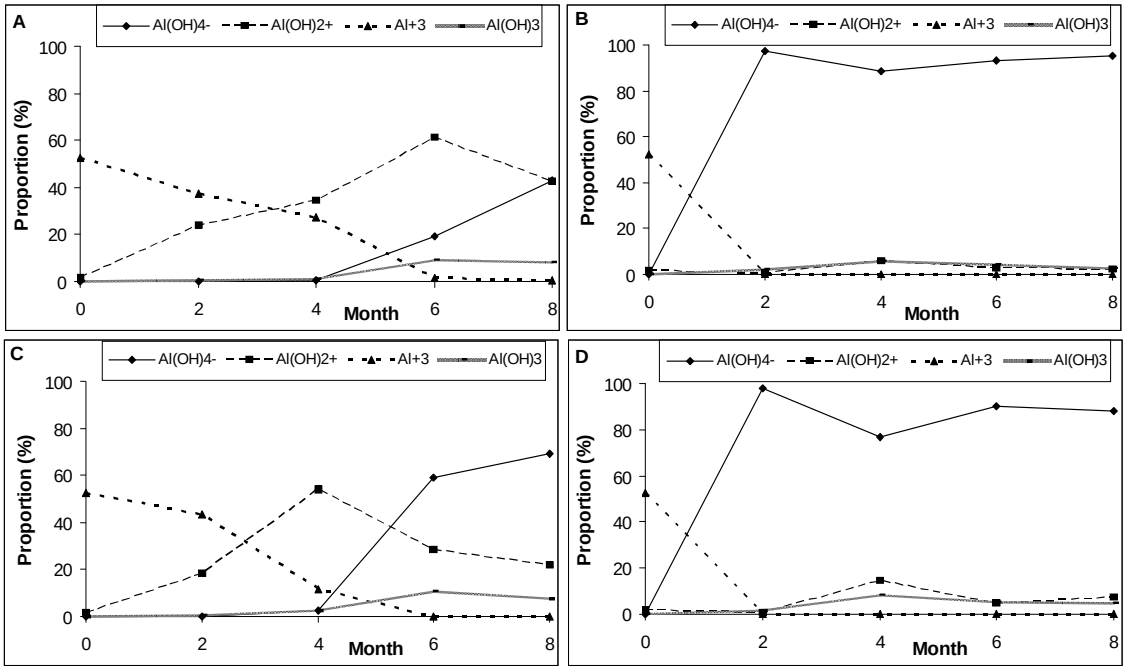


Figure 31. Speciation (%) of Al in A) mesocosm control, B) limestone treatment, C) Phosphorus treatment and C) limestone and Phosphorus treatment from PHREEQC.

7.4.3 Ceriodaphnia c.f. dubia acute testing

7.4.3.1 Influence of treatment and duration on toxicity

Bioassay controls CRW and HHC both showed low mortality (<10%) over the 8 month trial period with the exception of month 2. In this month the mortality in H was 16% but this was not significantly higher ($F_{(3, 6)}$=0.06, P=0.98) than that of CRW (Figure 32).

Ceriodaphnia c.f. *dubia* mortality did not differ between the bioassay controls and treatments L and L&P at any month over the 8 month mesocosm trial with mortality always <20% for both controls and treatments (Figure 32). In contrast, *C.* c.f. *dubia* mortality in MC and P was higher than that of the bioassay controls and treatments L and L&P at every sampling occasion. Mortality in MC fluctuated, increasing markedly from months 2 to 4 and decreasing from months 6 to 8. Mortality in MC at month 8 did not differ from mortality displayed at month 2 indicating that toxicity to *C.* c.f. *dubia* within MC had not decreased over the period of the trial. Mortality of *C.*

c.f. *dubia* in P was similar to MC at months 2 and 4, but mortality in P decreased from then on with month 8 mortality lower than that of month 2 mortality, indicating some reduction of toxicity to *C.* c.f. *dubia*. Some variation between mesocosm replicates was indicated from large standard errors and the increase and decrease in mean mortality from month to month (Figure 32).

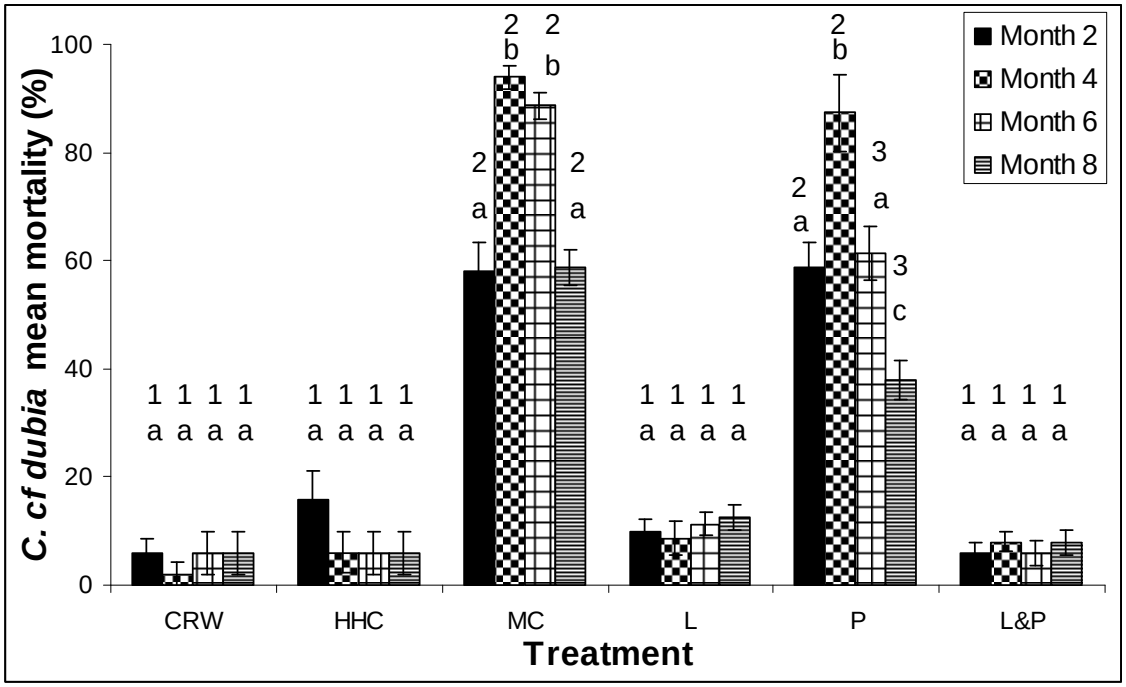


Figure 32. *Ceriodaphnia* c.f. *dubia* 48 h mean % mortality ± standard error for controls and treatments at sample months 2–8. Different lowercase letters indicate significant differences for a control or treatment between sample months. Different numbers indicate significant differences between treatments within a particular sample month.

7.4.4 Chlorella protothecoides chronic testing

The growth rate for the synthetic soft water (SSW) control was consistent at all months during the 8 month test period. Trials one and two were run simultaneously after month 2 with only one control for both trials at each month (Figure 33 A, B). Both trials exhibited growth rates in MC and P lower than that of control C and all other treatments at months 2 and 4. Trial one treatment growth rates were lower than trial two growth rates at every sample month. Growth rates for all treatments in trial

one showed a distinct increase at month 6. Growth rates for L&P reduced in both trials at month 8.

As anticipated, trial one (*sans* nutrients) displayed a higher mean growth rate in the control than all treatments at all sample months (Figure 33). Nil growth was displayed in treatments N, L and L&P at months 2 and 4, but MC and P displayed an algicidal effect at these months. Treatments from trial one all displayed positive growth rates at months 6 and 8 with the growth rate in MC lower than in all other treatments at month 6. All treatments except N displayed reduced growth rates at month 8 compared to month 6. All treatments displayed higher growth rates at month 8 than at month 2.

Trial two growth rates in the control and treatments N, L and L&P were higher than in MC and P at each sample month except for month 6 where P was no longer lower (Figure 33 A). Control and treatments N, L and L&P did not differ from each other at any sample month. Trial two growth rates for MC and P decreased from months 2 to 4 but did show an increasing trend from month 2 to month 8.

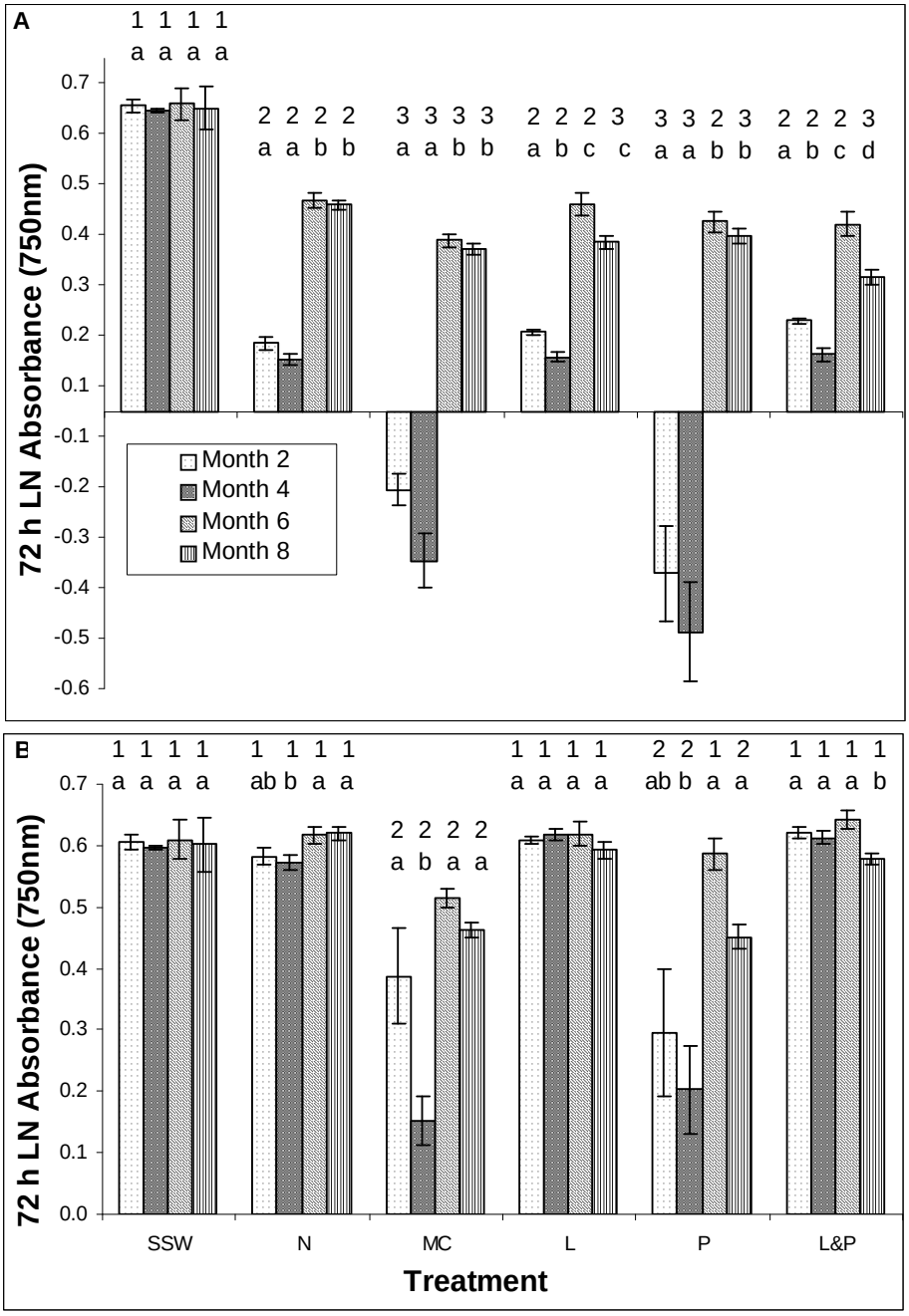


Figure 33. *Chlorella protothecoides* mean growth rate (slope) ± standard error at 72 h for trial 1 (*sans* nutrients) (A), and trial 2 (nutrients added) (B), for months 2-8. Different lowercase letters indicate significant differences for a control or treatment between sample months. Different numbers indicate significant differences between a control or treatment within a sample month.

7.4.4.1 Month 2

The two trials of no-nutrient and nutrient addition to treatments showed similar growth rate trends at month 2 clearly indicating a lower growth response from MC

and P. Treatments N, L and L&P displayed significantly higher growth rates than the MC and P in both trials. The main difference displayed between the two trials was a significantly higher growth rate for all treatments found in trial 2 (nutrient addition) (Figure 34).

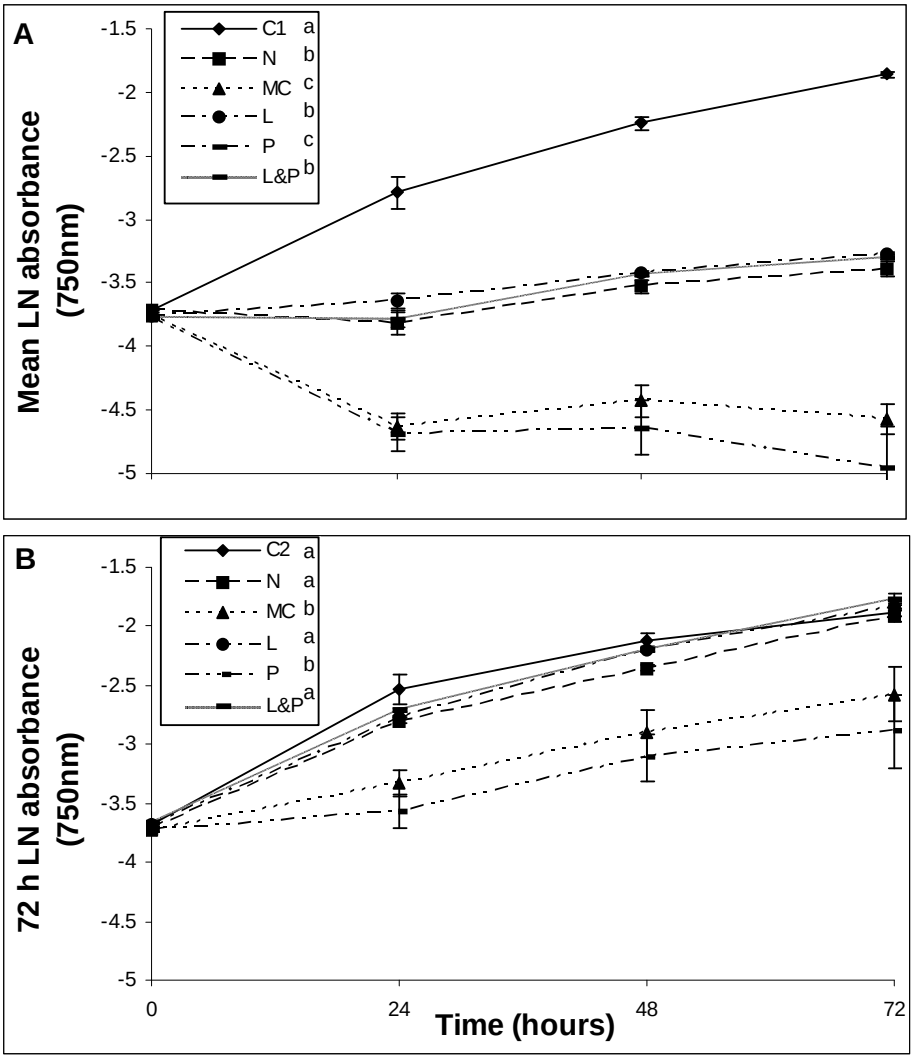


Figure 34. *Chlorella prototheocoides* mean LN absorbance ± standard error for control one trial one (A), and trial two (B), at month 2. Legend abbreviation expansions C1 is synthetic control 1, C2 is synthetic control 2, N is NaOH neutralised lake water, L is limestone treated lake water, P is phosphorus treated lake water and L&P is limestone and phosphorus treated lake water. Different lowercase letters next to treatment abbreviations indicate significant differences between treatments at 72 hours.

Replicates of MC and P showed significant variation in both trials at month 2. The large variation in growth rate for MC and P was shown by the high coefficient of variance (Table 23). When the outliers were removed the mean growth rates in MC and P increased, but were still markedly lower than that of the other treatments and C. Nutrient addition to the treatments N, L and L&P in trial two removed the difference between C and these treatments in growth rates displayed in trial one.

Table 23. Month 2 *C. protothecoides* growth rate and inhibition summary for controls and treatments. S.E. = standard error. CV = % coefficient of variance. Different superscript lowercase letters indicate significant differences between treatment means. N/A = not applicable.

	Trial one (sans nutrients)				Trial two (with nutrients)			
Treatment	**Growth rate mean ± S.E.**	**CV (%)**	**Difference to control, (%)**	**Toxicity effect**	**Growth rate mean ± S.E.**	**CV (%)**	**Difference to control, (%)**	**Toxicity effect**
SSW	0.606±0.01[a]	5	N/A	N/A	0.606±0.01[a]	6	N/A	N/A
N	0.126±0.02[b]	44	-80	Inhibition	0.582±0.01[a]	7	-5	None
MC	-0.222±0.04[c]	54	-136	Algicidal	0.388±0.08[b]	59	-37	Inhibition
L	0.172±0.00[b]	6	-72	Inhibition	0.609±0.01[a]	4	-1	None
P	-0.346±0.1[c]	91	-156	Algicidal	0.295±0.10[b]	105	-52	Inhibition
L&P	0.181±0.01[b]	9	-71	Inhibition	0.620±0.01[a]	5	1	None

7.4.5 *Tetrahymena thermophila* chronic testing

The *T. thermophila* control (TTC) showed a consistent final *T. thermophila* density (FTD) for the 24 h chronic test at each sample month with a mean FTD of $7.1x10^4$ciliates/mL. A similar FTD was also exhibited in treatments L and L&P across each month, both with a mean of $7.0x10^4$ciliates/mL over the 8 month period. An increasing FTD trend was shown in MC over the 8 month trial with large increases occurring at months 6 and 8. However, the FTD for MC was lower than that of TTC, L and L&P at all months. Treatment P followed a similar trend to MC with an increase of FTD at month 6 and again at month 8, with all FTD's lower than those of T and treatments L and L&P. FTD's in treatment P were lower than those of MC at months 2 and 4 but were higher at months 6 and 8 (Figure 35).

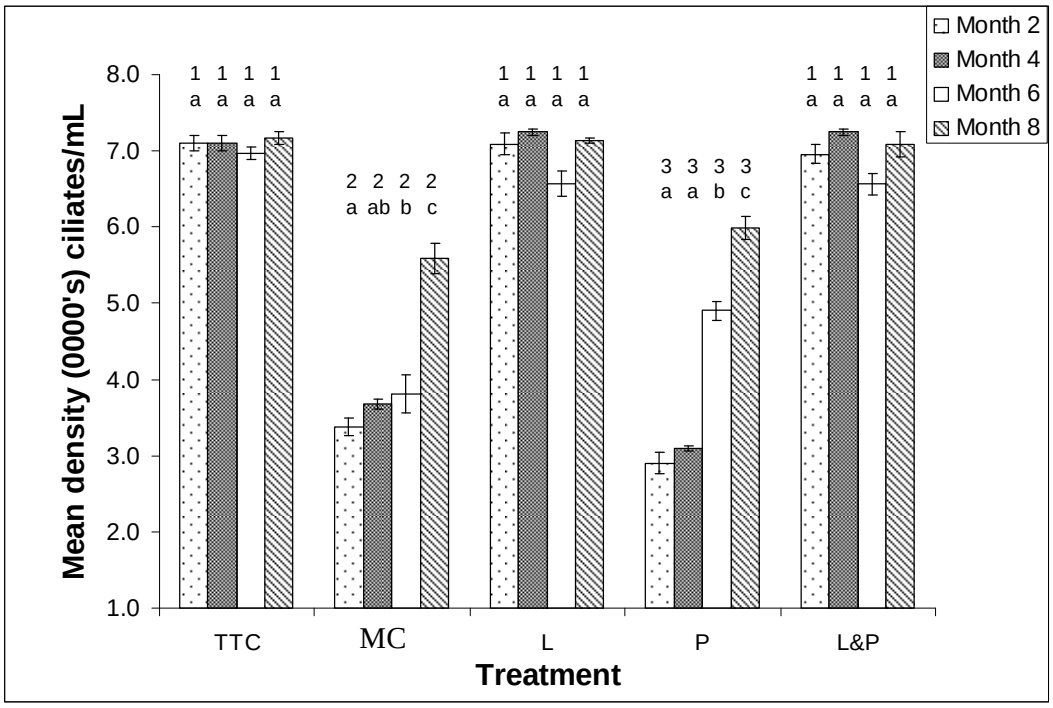


Figure 35. Final mean density of *T. thermophila* after 24 hours of incubation. Different lowercase letters indicate significant differences within treatments (between sample months). Different numbers indicate significant differences between treatments within a particular sample month.

7.4.6 Bioassay parameter relationships

7.4.6.1 Bioassay response to physico-chemical parameters

Toxicity bioassays displayed good correlations with a number of chemical and physical parameters measured during the trial (Table 24). The *C.* c.f. *dubia* bioassay responded at both 24 h and 48 h to NOx with a high negative correlation ($\rho>0.8$) and a positive correlation to Ca. Calcium (true water hardness) has been shown to reduce toxicity and bioavailability through competition with other divalent metals (Gensemer *et al.* 2002). There was also a high positive correlation to pH and a negative correlation to Zn and Mn/Co concentrations ($\rho>0.8$) but their relationship was not as strong as between NOx and Ca.

The *C. protothecoides* bioassay showed a distinctly different correlation between the two bioassay trials of with and without nutrients (Table 24). Trial one (*sans*

nutrients) growth response exhibited a strong correlation of $\rho>0.8$ to pH and a negative correlation to Mn/Co and Ni concentrations. Trial 2 (with nutrients) growth increase response was more closely correlated to Cr, B, Zn and Mg concentrations. Nonetheless, the growth response of trial two did not strongly correlate with these parameters with a $\rho<0.5$.

The *T. thermophila* bioassay population response was similar to the *C. c.f. dubia* 24 and 48 h bioassays with a strong positive correlation of $\rho>0.85$ to pH and the Ca concentration. The *T. thermophila* bioassay population response showed a strong negative correlation to Zn and NOx concentrations (Table 24).

Table 24 Correlation of test species responses at month 4 to physiochemical parameters with X indicating which parameter the bioassay is correlated too.

Bioassay	**ρ**	**pH**	**Cr**	**B**	**Zn**	**NOx**	**Ca**	**Cu**	**Mn/Co**	**Ni**	**EC**	**Mg**
Variable												
C. dubia (24 h)												
2	0.86					X	X					
3	0.83				X	X	X					
3	0.83					X	X		X			
3	0.83					X	X	X				
3	0.83	X				X	X					
C. dubia (48 h)												
2	0.90					X	X					
3	0.91					X	X		X			
3	0.90	X				X	X					
3	0.89				X	X	X					
4	0.92				X	X	X		X			
C. protothecoides trial two (with nutrients)												
1	0.81									X		
2	0.82								X	X		
2	0.81	X								X		
3	0.82	X							X	X		
4	0.82	X							X	X	X	
C. protothecoides trial one (*sans* nutrients)												
4	0.40		X	X	X							
4	0.39		X	X								
4	0.38		X	X								X
4	0.37		X	X								X
5	0.42		X	X	X							X
T. thermophila 24 h												
2	0.88	X				X						
3	0.92	X				X	X					
3	0.90	X			X	X						
3	0.89				X	X	X					
4	0.93	X			X	X	X					

7.4.6.2 Comparison of tolerances of test species to mesocosm treatments

The standardised response of the bioassays to the mesocosm control and treatments are illustrated in Figure 36. The bioassay response to the MC was similar to that of treatment P with the exception of a markedly lower response of the *C.* c.f. *dubia* 48 h bioassay to the MC. The MC displayed higher adverse responses across all the bioassays when compared to those of the remediation treatments L and L&P. Similar bioassay responses to treatments L and L&P were observed; excluding the response of *C. protothecoides* in trial one (*sans* nutrients) to treatment L&P which was markedly lower than that of treatment L. The response of the *C. protothecoides* trial one bioassay to treatment P was higher than that of L&P but similar to L and the MC. Bioassay responses to Treatment P were all lower than those to treatments L and L&P with the exception of *C. protothecoides* in trial one.

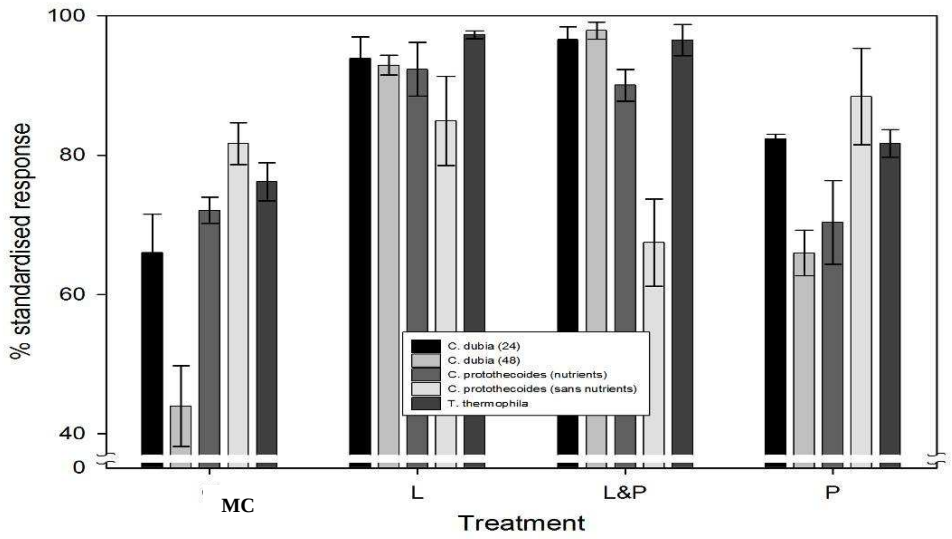


Figure 36. Standardised responses for toxicity bioassays after 8 months of treatment. from left to right within a treatment**.**

7.5 Discussion

7.5.1 Water quality changes an improvements

The pH (*ca*. 4.8) at the commencement of the mesocosm trial, was lower than the aquaculture and aquatic ecosystem guideline range of 6–9, but increased to within this range in all treatments and the mesocosm control by month 8. The pH 4.8 found at Lake Kepwari is common among natural fresh surface waters affected by deposition (Vyhnálec 1994; Poleo *et al.* 1997; Lychie-Solheim *et al.* 2001; Johnson & Hallberg 2005a) suggesting that pH would not cause toxicity on its own. The microalga *C. pyrenoidosa* has previously shown no significant change in growth rate over the pH range of 4.5–6.0 (Parent & Campbell 1994) showing that pH alone may not cause toxicity in that range. However, pH is known to influence metal speciation, with a reduction of pH from neutral commonly coinciding with a dissociation of some inorganic and organic metal complexes (Markich *et al.* 2001). Therefore, increasing the pH may not directly reduce toxicity, but rather increases the non-bioavailable proportion of the metal to aquatic ecosystems.

Saturation indices indicate the thermodynamic tendency of a particular solution to precipitate or dissolve a mineral (Nordstrom and Alpers, 1999; Alpers and Nordstrom, 1999). Negative SI values indicate under saturation with respect to a given mineral and a tendency for the solution to dissolve that mineral, whereas positive SI values indicate that the mineral is likely to precipitate. Geochemical modelling of aqueous solutions is limited by availability of appropriate thermodynamic data, underlying assumptions of geochemical equilibrium that may or may not apply, availability of complete water-chemistry data for the solution to be modelled, and quality of the data (Hammarstrom *et al.* 2005).

The uptake and toxicity of aluminium in freshwater organisms generally decreases with increasing water hardness under acidic, neutral and alkaline conditions (Folsom *et al*. 1986, Playle *et al*. 1989, Reid & McDonald 1991, Gunderson *et al*. 1994). Nevertheless, to date there has not been a hardness dependant algorithm developed

for Al and incorporated into the ANZECC/ARMCANZ guidelines. While generally being over protective, chemical trigger values provide a sound precautionary approach for protection of the ecology of the aquatic environment.

The dissolved Al concentration decreased significantly in the treatments and mesocosm control by month 2, but was still above the guidelines in the mesocosm control and Phosphorus treatment. Aluminium in the aquatic environment is found mostly in the free ion (Al^{+3}) form from pH 4.5–5.5 and solid $Al(OH)_3$ precipitates between the pH range of 5.2–6.5 (Sauvant *et al*. 2000). Therefore, the pH exhibited in the pit lake water (4.8) would mean that a high proportion of the Al present would be Al^{+3} which would be the main cause of toxicity at this pH. The results from PHREEQC supported the existence of a high proportion (53%) of Al^{+3} in the Kepwari pit lake water at the pH 4.8. By Month 6 the pH for all treatments and the control (pH>6) had increased to above the pH of 5.2 required for $Al(OH)_3$ precipitation reactions to occur resulting in a significant decrease of dissolved Al concentration.

The inorganic single unit $Al(OH)_2^+$ complex is a very toxic form of Al (Driscoll *et al*. 1980) and was present as a high proportion of the Al in both the mesocosm control and the Phosphorus treatment in months 4, 6 and 8. Unexpected toxicity of Al at pH 6.4 has been seen in *Salmo trutta,* showing that even with increases in pH close to neutral if concentrations of Al are high, Al can still exhibit toxic effects (Witters *et al.* 1990). Toxicity above pH 6.4 was seen in this testing in the MC at month 8 where the proportion of $Al(OH)_2^+$ was still high.

Zn commonly co-precipitates with other metals at pH values lower than that achieved in the treatments and the mesocosm control by month 8 (Jackson & Bistricki 1995; Lee *et al.* 2002). However, the Zn concentration remained elevated above the guidelines and the HMGV for the entire trial period. Furthermore, and contrary to most metals, the toxicity of Zn to *C.* cf *dubia* has been shown to increase with an increase in pH from 5.5–8.4 (Hyne *et al.* 2005). This is attributed to the

decrease in competition from H^+ ions. Therefore, Zn^{+2} may continue to contribute to toxicity when $Al(OH)_2^+$ decreases.

Cr has been shown to be highly toxic to aquatic animals (Calevro *et al.* 1999). The cause of the increase in Cr at month 4 is not known, but is possibly due to sediment release, and more so in the Phosphorus treatment possibly due to ionic displacement by PO_4^-.

Limestone remediation of the Lake Kepwari water was the only treatment to quickly reduce toxicity for all three test species. Lime remediation is known to decrease acidity and reduce metal bioavailability (Kalin *et al.* 2005; Koschorreck *et al.* 2007). Additions of nutrients (P) can stimulate primary productivity which can produce alkalinity and biogenic metal scavenging (Davison *et al.* 1995). The treatment of P addition was used to stimulate primary productivity resulting in passive alkalinity production. The P treatment had less impact on reducing toxicity to the test species than the limestone treatments. Adding P to the liming failed to reduce toxicity more than liming alone.

The toxicity results from the *C.* cf *dubia* bioassay showed that mean mortality over the 8 month trial in treatments L (10.7%) and L&P (7.0%) was similar. Furthermore, these mean mortalities were not significantly different from the bioassay controls CRW (5.0%) and HHC (8.5%) indicating toxicity to *C.* c.f. *dubia* had been removed from the Kepwari pit lake water in these treatments. Toxicity to *C.* c.f. *dubia* from the pit lake water was not exhibited at month 2 or any month thereafter in the limestone treatments, indicating that limestone addition (L) alone could significantly reduce or completely remove toxicity without the need for phosphorus addition. The absence of *C.* cf *dubia* mortality corresponded with the high proportion of $Al(OH)_4^-$ and low proportion of Al^{+3} and $Al(OH)_2^+$ present in the limestone treatments. Nevertheless, to encourage future primary productivity, phosphorus addition may still need to be considered for continued passive alkalinity production from phytoplankton.

The mesocosm control and the Phosphorus treatment demonstrated equally high but variable mortality (>40%) of *C.* cf *dubia* from month to month showing poor removal of toxicity to *C.* cf *dubia* up until month 6. Toxicity from the mesocosm control from month 2 to 6 corresponded with the high proportion of $Al(OH)_2^+$ indicating that this complex was possibly contributing to toxicity. At month 8 the increase of $Al(OH)_4^-$ and decrease of $Al(OH)_2^+$ corresponded to decreased toxicity to *C.* cf *dubia*. At month 8, treatment P displayed lower mean mortality than previous months and lower mortality than the MC, indicating that the Phosphorus treatment had decreased toxicity to *C.* c.f. *dubia* from the pit lake water. The reduced toxicity from the Phosphorus treatment was most likely due to the decrease in $Al(OH)_2^+$ and the increase in $Al(OH)_4^-$. The remaining toxicity from the mesocosm control to *C.* c.f. *dubia* at month 8 may have been due to remaining $Al(OH)_2^+$ that was removed in the limestone treatments. Further toxicity may have resulted in the mesocosm control from reduced competition of H^+ with the elevated Zn concentration allowing the Zn^{+2} to become more bioavailable to the *C.* cf *dubia* (Hyne *et al.* 2005).

Variation in *C.* c.f. *dubia* mortality was exhibited between mesocosm replicates of the MC and treatment P. This was probably due to small metal concentration differences between replicates of the mesocosm control and the Phosphorus treatment. Blooms of phytoplankton and colonisation of macroinvertebrates were occasionally observed in mesocosm replicates in a highly variable manner. The variation in metal concentration between replicates can be partly attributed to this random colonisation, including founder effects (Futuyma 1998), of bacteria, phytoplankton and zooplankton as well as random colonisation by aquatic biota flying or blown into the mesocosms. Further quantitative analysis of macroinvertebrate, phytoplankton, zooplankton and benthic algae communities will also be performed (McCullough & Lund 2007a). Phytoplankton and benthic algae particularly, have the capability to directly modify water quality through the transformation and assimilation of carbon, various metals and other chemical compounds from the water and sediments (Lychie-Solheim *et al.* 2001). However, whether this biological variation is comparable with the larger scale of pit lakes is not known.

7.5.2 Chlorella protothecoides 72 h chronic bioassay

The microalga *C. protothecoides* is commonly used as a 'sensitive' test species for toxicity studies (Stauber *et al.* 1994; Franklin *et al.* 1998; Franklin *et al.* 2000). *Chlorella protothecoides* was effective in differentiating the toxicity from the three remediation treatments applied to Kepwari pit lake water. Tests without nutrient addition (trial one) and with nutrient addition to treatments (trial 2) in the *C. protothecoides* bioassay were used to differentiate between any effect on toxicity that may eventuate from the addition of nutrients. The use of trial one removed the possible interference to toxicity from Phosphate addition. Another way of removing error from nutrient addition has been suggested through utilising the luxury uptake of Phosphorus exhibited by algae, allowing continued algal growth without extra addition of Phosphorus to experiments (Helliwell *et al.* 1983; Parent & Campbell 1994). However, the use of trial one gives the extra advantage of also not adding nitrate which can modify toxicity.

Chlorella protothecoides test results indicated that both trials were effective in differentiating toxicity from the treatment approaches with limestone addition most effective in removing toxicity. Nevertheless, final mean growth rate was lower in trial one than trial two, most likely due to nutrient deficiency. Trial one could therefore be used to identify the availability or deficiency of nutrients from a pit lake, hence indicating whether or not nutrients would be limiting in the full recovery of that lake.

Furthermore *C. protothecoides* trial one displayed common growth rate trends to trial two. The mesocosm control and the Phosphorus treatment displayed a decrease in growth rate from month 2 to 4 and an increase from 4 to 6. The growth rate decrease from month 2 to 4 from the mesocosm control and the Phosphorus treatment corresponded with an increases in $Al(OH)_2^+$ indicating that this Al form may be causing toxicity. A significant difference occurred between the mesocosm control and the Phosphorus treatment at month 6 which was correlated with the increase of $Al(OH)_4^-$ and the decrease of $Al(OH)_2^+$ in the Phosphorus treatment allowing a

higher growth rate. Trial one growth rate in the mesocosm control at month 8 was similar to that of the treated mesocosms which was probably due to the increase in $Al(OH)_4^-$ and decrease of $Al(OH)_2^+$.

Mean growth results for *C. protothecoides* trial two (with nutrients) were similar for the control C and treatments L and L&P at all months indicating removal of toxicity by these two treatments. The growth rate exhibited for the limestone treatments can be attributed to the reduction in toxicity by the formation of $Al(OH)_4^-$. Therefore, indicating limestone addition alone to be sufficient for toxicity removal. The *C. protothecoides* result was comparable to the result from the *C.* c.f. *dubia* bioassay, indicating again that the limestone treatment of this acidified pit lake water alone was sufficient in remediating water toxicity to *C. protothecoides* and that the phosphorus additions had very little influence in the earlier months of 2 and 4.

The growth rate from the Phosphorus treatment at month 6 was similar to the limestone treatments in both trials indicating that the pit lake water had been remediated. The high growth rate in P correlated with an increase in the formation of $Al(OH)_4^-$ and a decrease in $Al(OH)_2^+$. The significantly lower growth rate in MC again indicated that by month 6 phosphorus additions were having an effect in reducing toxicity to *C. protothecoides*. However this remediation effect was not pronounced at month 8 in trial two when mean growth rates in the bioassay for both MC and P reduced and were again lower than the limestone treatments. This further displays evidence that the dosing regimen of phosphorus used for treatments P and L&P did not make a lasting difference in reducing toxicity. Therefore remaining toxicity is probably due to the elevated concentration of Zn at month 8. Furthermore, the concentration of $Al(OH)_2^+$ at month 8 may have also contributed to the reduced growth rate. Therefore, phosphorus addition may need to be continued to become permanently effective.

The main removal mechanism for FRP is thought to be adsorption to aluminium species (Kopacek *et al.* 2000; Ulrich & Pöthig 2000; Dessouki *et al.* 2005) which can furthermore influence toxicity by removing metal toxicants from bioavailability

(Yang *et al.* 2001). Water column nitrate concentrations can be reduced by ammonification (mineralization) and ammonia volatilization, denitrification and mineral sorption (Yang *et al.* 2001). Algae tests which use treatments with and without nutrient addition can be applied to other pit lake water bioassays where nutrient limitation (especially P) is suspected in pit lake water or where P may influence metal toxicity (Parent & Campbell 1994).

Addition of K_2HPO_4 as a nutrient will only increase phytoplankton growth if added in excess to P precipitation with Al. The toxicity from Al should therefore decrease as Al is removed from the water column to the sediment, although alkalinity increases from phytoplankton will only occur with further addition of P to the water (Fyson *et al.* 1998). Amendment with P will also serve to reduce Al toxicity through precipitation (Lund *et al.* 2006). The proposed role of phytoplankton in bioremediation is to adsorb and absorb metals and to increase pH through nitrate assimilation which produces alkalinity (Davison *et al.* 1995). A secondary role is to maintain a supply of organic carbon and nutrients to fuel the growth and activity of sediment-based alkalinity generating bacteria (sulphate and iron reducers) (Fyson *et al.* 2003; Dessouki *et al.* 2005). The addition of phosphorus in this treatment was used to increase phytoplankton production in the pit lake water leading to a remediation effect. This effect was pronounced in the results of the *C.* c.f. *dubia* bioassay by month 8, suggesting a higher sensitivity for this species to this remediation regime.

7.5.3 *Tetrahymena thermophila* 24 h chronic bioassay

The chronic test using the ciliate *T. thermophila*, was performed in a short period of time (24 h) with a small water sample volume, if necessary allowing for the use of increased replication and quick test repeatability. The *T. thermophila* toxicity bioassay results were consistent with that of the previous two bioassays. That is, the bioassay control presented a consistent FTD over the 8 month trial similar to the limestone treatments. The Mesocosm Control and the Phosphorus treatment gave consistently lower FTD's than the other treatments indicating that treatment L alone

was effective in reducing toxicity to *T. thermophila*. The increasing trend in FTD from the Mesocosm Control still showed that over time the acidic pit lake water toxicity was reducing without treatment, through natural alkalinity producing processes and without further inputs from surrounding acidity producing areas (Totsche *et al.* 2006). How long this would take in the actual pit lake is not known, as contributions from acid producing sources remain unquantified.

The FTD from the Mesocosm Control increased from months 6 to 8 corresponding with a decrease in $Al(OH)_2^+$ and an increase in $Al(OH)_4^-$, indicating that $Al(OH)_2^+$ was possibly contributing to the toxicity to *T. thermophila*. FTD from the Phosphorus treatment was slightly lower but significantly lower than that of the Mesocosm Control at months 2 and 4. Toxicity from the Phosphorus treatment corresponded to a higher Al^{3+} proportion at month 2 and a higher $Al(OH)_2^+$ at month 4 indicating that, not only was $Al(OH)_2^+$ driving toxicity to *T. thermophila* but also Al^{3+} at the lower pH. This significantly higher toxicity from the Phosphorus treatment at months 2 and 4 was not seen in the other species bioassays. Nevertheless, from month 6 to 8 the FTD from the Phosphorus treatment was significantly higher than that of the Mesocosm Control. FTD increase in the Phosphorus treatment corresponded to an increase in $Al(OH)_4^-$ and a decrease in $Al(OH)_2^+$. Therefore, showing that toxicity may have been caused by the $Al(OH)_2^+$ complex at the pH range 5 to 6.5.

The slow effect of the Phosphorus treatment in removing toxicity could be due to the low ambient temperature and light levels present in early spring when the mesocosm experiment began. These physical factors could easily have slowed phytoplankton, bacteria and benthic algae population growth and thus the biochemical processes required to remove toxicity during these cooler months. The delayed amelioration of toxicity may also have been due to the removal of the limiting nutrient phosphorus by Al, inturn, restricting growth of phytoplankton and benthic algae populations (Parent & Campbell 1994).

7.5.4 Comparison of bioassay responses to physiochemical parameters

Correlation of test species responses to physico-chemical parameters can support assessment of potential toxicity factors influencing that bioassay's results. The main physico-chemical parameter combinations influencing the *C.* c.f. *dubia* bioassay at both 24 h and 48 h were negative correlation to NOx and a positive correlation to Ca ($\rho>0.8$) found in all five computed results. A positive correlation to pH, and a negative correlation to Zn and Mn/Co was also found. The *T. thermophila* bioassay population response was similar to the *C.* c.f. *dubia* bioassays with a strong negative correlation of $\rho>0.85$ to NOx and positive correlation to Ca, but with pH and concentrations of Zn found in four and three computed results respectively. The correlation with NOx indicates a possible relationship with alkalinity forming reactions through phytoplankton assimilation of NOx, which may not be pronounced in the *C. protothecoides* bioassay trial two (nutrients added) (Parent & Campbell 1994; Lychie-Solheim *et al.* 2001).

As anticipated by using the two different trials of with and *sans* nutrients to the treatments, the *C. protothecoides* bioassays showed distinctly different correlations to the physico-chemical parameters. Trial one (*sans* nutrients) growth response exhibited a good positive correlation of $\rho>0.8$ to pH and a negative correlation to Mn/Co and Ni concentrations. Trial two (with nutrients) growth increase response showed a negative correlation ($\rho<0.5$) to Cr, B, Zn and Mg concentrations. However, the growth response of trial two did not have a high correlation value with these particular variables. This distinctly different sensitivity to different parameters demonstrates the necessity of a battery of test species to be used as well as nutrient-adjusted and unadjusted algae trials to completely explain toxicity from acid pit lake water.

7.5.5 Comparison of bioassay responses to mesocosm treatments

The standardised response is an indication of how different test species responded to different treatments relative to other test species. All these standardised test species responses therefore demonstrate a battery response, displaying an overall treatment effect as a way of determining a possible overall mode of ecological response to the treatments.

The mesocosm control (MC) displayed a lower response (but still positive) than the three pit lake water treatments when comparing the bioassay responses to the water quality improvement. All treatments on the pit lake water had a positive response to improving water quality of the acidic pit lake water. Although there were generally small differences between bioassay responses within a treatment, there was no corresponding trend of a single bioassay response over all treatments which would have indicated a single more sensitive bioassay. The 48 h *C.* c.f. *dubia* bioassay presented a lower response than all other bioassays in the MC but only continued the trend in treatment P suggesting remaining toxicity of the pit lake water in both. The *C. protothecoides* trial two (with nutrients) showed a lower response in treatment L&P to the water quality improvement in direct contrast to the *C. protothecoides* trial one results, possibly indicating trial two to be a more sensitive test to this treatment regimen. There were no other notable differences for bioassay responses within treatments, but by using the battery response a more insightful evaluation of acid pit lake water toxicity was made.

Responses shown by the bioassays indicated that the amendment of P to acid pit lake water was not as effective in removing toxicity to *C.* c.f. *dubia* as it was in other bioassays, suggesting this species was more sensitive to this treatment of acid pit lake water. Amendment of the acid pit lake water following limestone neutralisation and P addition compared with just adding limestone had no significant effect to bioassay results. This could be explained by three possible modes, abiotic Al-PO_4 interactions in the extracellular environment, Al inhibition of P uptake and interference with intracellular phosphorus metabolism (Parent & Campbell 1994). All three of these pathways for P removal or interference can affect not only

remediation of water quality by micro-organisms, but also bioassay results when using P as a nutrient for phytoplankton growth.

7.6 Conclusion and Recommendations

All remediation treatments had a positive effect in removing toxicity to test organisms in the 8 month period, with L and L&P the most effective in the earlier sample months 2 and 4. Limestone neutralisation alone of the pit lake water was effective in increasing the pH to neutral and reducing metals significantly by month 2. Treatment P was effective in removing some toxicity to *C.* c.f. *dubia, C. protothecoides* and *T. thermophila* from the Kepwari pit lake water, but did not remove toxicity as quickly as limestone neutralisation alone. Although Zn was still elevated in L and L&P it did not exhibit toxicity to either of the test organisms in treatments with limestone neutralisation. Even though toxicity is not always pronounced from bioassays in these treatments, bioaccumulation of metals (ANZECC/ARMCANZ. 2000a; Barron & Albeke 2000) and longer term effects on reproduction and other critical responses may still be an issue for natural ecosystems to develop in pit lakes.

Each of the three species used to assess the toxicity exhibited in this pit lake water displayed a similar response to each of the treatments, with a toxic response to MC and P and a significantly lowered toxicity from treatments L and L&P. The toxicity to all three test species correlated with the proportion of the $Al(OH)_2^+$ complex, with an increase in proportion corresponding with increased toxicity. As $Al(OH)_4^-$ increased toxicity reduced indicating that $Al(OH)_4^-$ was not toxic, and may have competed for binding sites of the organisms when $Al(OH)_2^+$ was present thereby buffering toxicity. Finally Zn^{+2} may have contributed to toxicity in the mesocosm control at month 8 due to the decrease in competition with H^+ increasing bioavailability.

In conclusion by using this battery of different test species, an understanding of toxicity that may remain in an acid pit lake with and without remediation was achieved. It is recommended that a battery of similar multi-species and multi-trophic level bioassays be used in further assessment of acid pit lake water remediation effectiveness. Further assessment on these remediation methods is required to find if they are to be successful at a full-field scale, by being able to mitigate toxicity of this acid pit lake to a standard to allow for a natural ecosystem to develop.

7.7 Contribution of others

The bioassay experiments, analysis of results, discussion and conclusions were all designed, implemented and written by the Thesis author Luke Neil. The author acknowledges the Co-authors for the journal article (McCullough, C., Lund, M., Tsvetnenko, Y., Evans, L.) have contributed to the discussion and conclusion including editing of this chapter. Also the empirical measurements of mesocosm water chemistry at each month were supplied by Clint McCullough and Mark Lund.

A similar but revised version of this chapter has been submitted to the journal of Ecotoxicology and Environmental Safety.

GENERAL DISCUSSION AND CONCLUSIONS

8 General discussion and conclusions

8.1 Research questions

Five questions were developed to guide this research on mine pit lake water toxicity assessment. All five questions were addressed in the research and are discussed below.

8.1.1 Question one

Do any of the pit lake water treatment modalities reduce metal concentrations to below current guideline parameters for aquaculture species and aquatic ecosystems?

While none of the treatments reduced all metal concentrations to below the guidelines for either protection of aquaculture species or for protection of aquatic ecosystems each treatment was effective in reducing some of the metal concentrations. A summary of the mine pit lake metal concentration data and guidelines are presented in Table 25.

The RCO system was the most effective treatment examined, in that all but two of the dissolved metal concentrations were reduced to below both sets of guidelines. Furthermore, if the Cd concentration is not taken into account, due to the source of the Cd most likely coming from the RCO itself, then only the Cu concentration slightly exceeded the guidelines. When the HMGV is applied, the Cu concentration is below this value. Therefore, it can be considered that the RCO was the most effective in reducing metal concentrations to below the guidelines for the protection of aquaculture species and aquatic ecosystems.

The most persistent metal remaining after application of treatment modalities other than the RCO was Zn (Table 25). After application of all treatments to the pit lake water, with the exception of the RCO and NaOH neutralisation, the Zn concentration always remained above both aquaculture and aquatic ecosystem guidelines. Even though toxicity was not expressed in Chicken Creek water treated with ≥1 g/L limestone, Zn is known to be bio-accumulative and is therefore considered a risk to most aquacultured and other higher trophic level animals. It should be noted that the Zn concentration displayed a decreasing trend over the eight months of the Lake Kepwari mesocosm trial within water treated with limestone, suggesting that natural processes in mine lakes may ameliorate elevated Zn levels over time after limestone addition.

8.1.2 Question two

Do any of the treatment modalities completely eliminate toxicity from mine pit lake water as shown by bioassay results when compared to the bioassay control? If not, which treatment was most effective in reducing toxicity?

All of the treatment modalities to the mine pit lake water removed some toxicity as indicated by bioassay results when compared to the bioassay controls (Table 26). However, only treatment with limestone of ≥1 g/L completely removed toxicity from mine pit lake water, as evidenced by the Chicken Creek study with *C.* cf *dubia* and *T. thermophila* bioassays and the early life stage bioassays with *B. bidyanus*. Treatment with the RCOCl system showed complete removal of toxicity to all bioassays with the exception of the 48 h *C.* cf *dubia* test. All bioassays also showed complete removal of toxicity from limestone alone and limestone & phosphorus treated Lake Kepwari water in the mesocosm trial. It can be concluded that limestone addition can completely remove toxicity from mine pit lake water, as shown by bioassay responses in treatment and controls not being significantly different.

The RCOCl system was also effective in reducing toxicity in mine pit lake water although the 48h *C.* cf *dubia* bioassay showed some residual toxicity which was still present following treatment. Although the 48 h *C.* cf *dubia* bioassay showed a significantly lower survival in the treated water to the controls, the survival was still >80% indicating that toxicity removal was relatively effective in the RCO treatment to WO5H mine pit lake water. Taking into account the high survival in the 48 h *C.* cf *dubia* bioassay, and the possibility that the lower responses found in the *C. protothecoides* and *C.* cf *dubia* three brood tests from $RCOClO_2$ treated water were due to Cd being released from the RCO system, it is possible that this system did, in fact, remove all toxic elements from the mine pit lake water. The company marketing this system have conducted modifications aimed at preventing Cd being released during treatment. A further assessment of the effectiveness of the modified RCO system in removing toxicity from mine pit lake water is warranted.

8.1.3 Question three

Do any of the bioassays consistently confirm toxicity in water samples when metal concentrations exceed guideline trigger values for protection of aquaculture species and aquatic environments? Conversely, do any of the bioassays consistently confirm a lack of toxicity when all metal concentrations are below guideline trigger values?

None of the bioassays consistently confirmed toxicity when metal concentrations exceeded guidelines for the protection of aquaculture species and aquatic ecosystems (Table 25). Nevertheless, toxicity was confirmed in a number of bioassays with elevated concentrations of Zn, Mn and Ni, the most persistent metals remaining above guidelines following treatment. For example, *Ceriodaphnia* cf *dubia* 48 h mortality and three brood and 24 h *T. thermophila* bioassays confirmed toxicity in LT3 treated Chicken Creek water which contained elevated Cd, Mn, Ni and Zn concentrations. The same three bioassays confirmed toxicity with NaOH addition to WO5H water which contained elevated Mn and Ni concentrations. The 72 h *Chlorella protothecoides* and *C.* cf *dubia* 48 h and three brood bioassays confirmed toxicity in $RCOClO_2$ treated WO5H water which had an elevated Cd concentration.

Similarly, the *Chlorella protothecoides* 72 h, *C.* cf *dubia* 48 h and *T. thermophila* 24 h bioassays confirmed toxicity in P treated Lake Kepwari water which contained elevated Cu, Ni and Zn concentrations.

No treatment method reduced all metal concentrations to below guideline levels, so a comparison of bioassay results obtained with a treatment in which no metals exceeded guideline levels was not possible.

8.1.4 Question four

Do bioassays increase the robustness of results obtained from mesocosm assessment of mine pit lake water treatment modalities?

The results obtained in the bioassay studies indicate that additional information is obtained when this type of assessment is performed in addition to chemical analysis. Chemical analysis following limestone treatments (L and L&P) showed that Al and Zn concentrations exceeded guidelines for both the protection of aquaculture species and for aquatic ecosystem at the completion of the mesocosm trial. Specifically, without the application of bioassays to assess residual toxicity from the mesocosm treated Lake Kepwari water the treatments using limestone would have been considered unsuccessful in removing toxicity when compared with the guidelines (Table 25). Furthermore, the use of three test organisms with both acute and chronic endpoints broadens the assessment of toxicant bioavailability to species at different trophic levels within the aquatic food web, thereby adding confidence to the accuracy of the prediction of likely adverse effects to the aquatic environment.

8.1.5 Question five

Does *B. bidyanus* meet criteria to be a good bioassay candidate for assessing toxicity from limestone treated mine pit lake water for the end use of aquaculture? The

selected criteria were; survival in control consistently >80%, small variation (standard error) between replicates of controls or treatments (i.e. similar growth, hatching and survival in replicates), no cannibalism and normal growth and survival in the control.

Bidyanus bidyanus did meet the criteria chosen for a good bioassay candidate. Survival was >80% in the Collie River water control and the High-Hardness Combo medium control throughout the testing (Table 27). The variation measured by standard error was small between replicates with <20% for hatchability, <10% for survival and <15% for any growth parameter. Cannibalism was not observed in the testing, and with the high survival (>80%) in the controls and in some treatments it was unlikely to have occurred. Survival and growth in the controls was similar to that recorded from hatchery production of *B. bidyanus* (Thurstan & Rowland 1994).

Table 25. Metal concentrations in mine pit lake water before and after treatment (μg/L) except for Ca and Mg which are mg/L).

Treatment	Al	Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Chicken Creek pit lake water	180	18	2	170	<10	19	11	63	770	200	30	990
Limestone 1.4 g/L	80	140	0.8	130	<10	2	<2	75	640	140	<10	100
Limestone 1.0 g/L	40	140	0.8	150	<10	2	<2	78	680	160	<10	170
Limestone 0.6 g/L	<10	110	1.2	160	<10	1	<2	76	680	170	<10	440
WO5H pit lake water	1100	17	1.3	130	7	25	2100	22	960	170	<10	200
NaOH neutralisation	10	14	<0.6	14	<10	<1	<2	17	670	190	<10	<2
Cl^- and RCO	<10	21	<0.6	<2	<10	2	<2	25	<1	<4	<10	<2
ClO_2^- and RCO	<10	20	22	<2	<10	2	<2	25	<1	<4	<10	<2
Lake Kepwari water	1125	29	-	61	10	10	180	-	265	60	6	450
MC	17	29	<0.6	20	<10	8	8	72	133	24	<10	257
L	43	35	<0.6	<2	<10	<1	13	71	<10	<4	<10	81
P	20	30	<0.6	<2	<10	7	15	72	6	27	<10	227
L&P	43	35	<0.6	<2	<10	<1	18	72	<10	<4	<10	100
***Aquaculture guidelines**	**30**	**-**	**0.2 - 1.8**	-	**20**	**5**	**10**	**15000**	**10**	**100**	**1 - 7**	**5**
***Aquatic ecosystems guidelines**	**55**	**-**	**0.2**	**-**	**1**	**1.4**	**-**	**-**	**1900**	**11**	**3.4**	**8**

* ANZECC/ARMCANZ guidelines for the protection of aquaculture species and aquatic ecosystems.

Table 26. Bioassay results from treated mine pit lake water that were not significantly lower than that of the control. Yes indicates a result equal to or better than the control. No indicates a significantly lower result to the control

Mine pit lake assessed	Treatment modality	*C. protothecoides* (with nutrients)	*C.* cf *dubia* (48 h)	*C. cf dubia* (3 brood test)	*T. thermophila* (24 h)
Chicken Creek	**Limestone 1.4 g/L**	**-**	**YES**	**YES**	**YES**
	Limestone 1.0 g/L	**-**	**YES**	**YES**	**YES**
	Limestone 0.6 g/L	**-**	**NO**	**NO**	**NO**
WO5H	**NaOH neutralisation**	**YES**	**NO**	**NO**	**NO**
	Cl- and RCO	**YES**	**NO**	**YES**	**YES**
	ClO2- and RCO	**NO**	**NO**	**NO**	**YES**
Lake Kepwari	**Limestone**	**YES**	**YES**	**-**	**YES**
	Phosphorus	**NO**	**NO**	**-**	**NO**
	Limestone and phosphorus	**YES**	**YES**	**-**	**YES**

Table 27. Comparison of results from treated Chicken Creek mine pit lake water to the controls. YES indicates that the result was the same or greater than that of the controls. NO indicates the result to be lower than that of the control. Life stages followed by (gr) are growth rate only.

Treatment to Chicken Creek pit lake water	Life stage tested	Survival	Hatch	Hatchability	Length	Width	Height	Gape	Yolk sac diameter
Limestone 1.4 g/L	**Egg**	**YES**	**YES**	**YES**	**-**	**-**	**-**	**-**	**-**
	Sac-fry	**YES**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**YES**
	Sac-fry (gr)	**-**	**-**	**-**	**NO**	**YES**	**YES**	**YES**	**YES**
	Post sac-fry	**YES**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**-**
	Post sac-fry (gr)	**-**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**YES**
Limestone 1.0 g/L	**Egg**	**YES**	**YES**	**YES**	**-**	**-**	**-**	**-**	**-**
	Sac-fry	**YES**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**YES**
	Sac-fry (gr)	**-**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**YES**
	Post sac-fry	**YES**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**-**
	Post sac-fry (gr)	**-**	**-**	**-**	**YES**	**YES**	**YES**		**YES**
Limestone 0.6 g/L	**Egg**	**YES**	**YES**	**NO**	**-**	**-**	**-**	**-**	**-**
	Sac-fry	**NO**	**-**	**-**	**NO**	**YES**	**NO**	**YES**	**NO**
	Sac-fry (gr)	**-**	**-**	**-**	**NO**	**YES**	**YES**	**YES**	**NO**
	Post sac-fry	**NO**	**-**	**-**	**NO**	**NO**	**NO**	**YES**	**-**
	Post sac-fry (gr)	**-**	**-**	**-**	**NO**	**YES**	**NO**	**YES**	**YES**

8.2 General Discussion

Comparisons of the efficacy of different treatment modalities on acidified, metal contaminated mine lake water using an ecotoxicology approach was made through this study. Water from three acid mine pit lakes in the Collie Basin were treated with different amelioration modalities, which were individually assessed for efficacy in reducing toxicity to aquaculture species and aquatic ecosystems with both biological and chemical analysis.

Based on the bioassay and chemical findings on Chicken Creek and Lake Kepwari water, the direct risk to aquaculture species and the aquatic environment could be considered to be minimal following limestone treatment. The results obtained in the RCO treatment studies suggest that the direct risk to aquaculture species and the aquatic environment are small following this treatment, particularly if the modified RCO system is used.

The amelioration treatments used on the pit lakes can be categorised into ‘in-lake treatments’ and ‘off-take treatments’. In-lake treatments are those in which treatment occurs within the lake and off-take treatment is when water is removed from the lake and then treated. The laboratory trials on limestone treatment of Chicken Creek mine pit lake water was used to investigate whether the off-take treatment system operating at the mine site, a Fluidised Limestone Reactor, would remove toxicity to aquaculture species. The RCO is also an off-take treatment system. The mesocosm approach using limestone and phosphorus addition, on the other hand, is representative of an in-lake treatment modality. Removal of toxicity was demonstrated with both the off-take and the simulated in-lake system. Mine pit lakes vary in many aspects including hydrogeology, isolation, climate and ecology. Therefore, choosing a treatment modality is likely to be done on a case by case basis, depending on the end use targeted (Kalin 2004; Johnson & Hallberg 2005a).

Studies performed in this research have enabled a comparison to be made between the application of bioassays based on invertebrate species and those based on observation of adverse effects on early life stages of fish in the assessment of mine lake water treatments. All the responses to ≥1 g/L limestone treated Chicken creek pit lake water, with the exception of length in the sac-fry growth test, gave the same response as *C. dubia* and *T. thermophila*, in that the ≥1 g/L limestone treated Chicken Creek pit lake water did not exhibit a toxic response in either the bioassays or in the early life stages of *B. bidyanus* (Table 26, 28).

Both the *C.* cf *dubia* and *T. thermophila* bioassays and *B. bidyanus* early life stage tests showed that 0.6 g/l limestone treatment did not remove all toxicity from the Chicken Creek mine pit lake water. All three bioassays gave a definite response of toxicity although only the growth parameter of length showed a consistent adverse affect in the *B. bidyanus* assays. The results obtained using *B. bidyanus* fry length as the toxicity endpoint were similar to those obtained with the *C.* cf *dubia* and *T. thermophila* bioassays. This observation suggests that the latter bioassays, which are quicker and easier to perform that the early life stage tests, could be substituted for the *B. bidyanus* early life stage test if desired.

Good survival and growth were achieved in ≥1 g/L limestone treatments to Chicken Creek mine pit lake water even with Al, Mn, Ni and Zn concentrations present above guideline values for the protection of aquaculture species. Co levels were also high in this mine pit lake. Co does not have a guideline trigger value for the protection of aquaculture species, therefore, the low reliability figure for protection of aquatic ecosystems of 1.4 μg/L was used in its place. The Co concentration was elevated above the low reliability figure of 1.4 μg/L by two orders of magnitude. Therefore, in this case using the guidelines alone would be over protective to the early life stages of *B. bidyanus*, and would limit the aquaculture of *B. bidyanus* from the limestone treated water.

Earlier work on another mine pit lake in Eneabba WA showed similar results to that in this research (Tsvetnenko 2003). This study showed that several metal

concentrations were elevated above aquaculture guidelines despite *Chlorella protothecoides*, *Daphnia magna* and *Brachionus calyciflorus* bioassays demonstrating no toxicity from the water. In agreement with a similar report (Markich *et al.* 2001) it was concluded that the elevated concentrations of metals were not bioavailable to the species tested. Synergism and antagonism of toxicants in complex mixtures indicate that comparison of chemical data with guidelines will rarely accurately characterise toxicity and are only triggers for further investigation (Peakall & Burger 2003; Peijnenburg & Jager 2003). Bioassay assessment with sensitive organisms, broadly relevant species and end points, as shown in this research, have the ability to demonstrate direct effects from complex mixtures (Mitchell 2002). Therefore, bioassay assessment is recommended to be included in the future planning of mine lake assessment for the end uses of aquaculture and biodiversity conservation.

Although a complete removal of toxicity to aquatic organisms following limestone treatment was demonstrated in bioassay assessment of both off-take and simulated in-lake treatment systems, the lack of toxicity to a bioassay species cannot be used to assess the threat to human health that could result from consumption of fish cultivated in treated mine lake water. As some metals bioaccumulate, cultured fish may have concentrations of metals that don't directly affect their health but may be problematic to humans when consumed over a period of time and chemical analysis of fish cultured in off-take mine lake water should be performed prior to approval for human consumption. Bioaccumulation could be reduced through in-lake treatment modalities. Further studies on the efficacy of in-lake treatment systems in reducing dissolved metal levels and bioaccumulation of these metals in aquatic organisms growing in the lakes should be performed.

It is recommended that guidelines for mine closure include assessment for possible end uses of the final pit lake. If end uses are planned for, and these include the application of amelioration treatment regimes and biological toxicity assessment, mine pit lakes may then become a legacy attributed to beneficial outcomes and not environmental disasters.

8.3 Conclusions

Mining operations, farming practices, damming rivers and the changing climate are all depleting and/or contaminating freshwater resources (Johnson & Wright 2003). Mine pit lakes offer a large freshwater resource that if managed correctly may sustain significant environmental, social and economic benefits for local and regional communities.

Assessment of mine pit lake physico-chemical parameters and metal concentrations as well as possible implementation of amelioration modalities, may be required before end uses are instigated. Bioassays can play an important role in the assessment of the effectiveness of proposed off-take and in-situ mine lake treatment approaches and in the interpretation of the biological significance of metal concentrations that exceed recommended guideline levels.

The major finding from this study was that chemical assessment should be combined with biological analysis to achieve an accurate toxicity assessment of treated mine pit lake water. The three temperate Australian species used in the study were found to be suitable for use as bioassay species in the assessment of mine pit water toxicity and in the efficacy of treatment systems in removing toxicity. *Bidyanus bidyanus* was shown to be a suitable Australian representative candidate for toxicity assessment for the mine pit lake end use of aquaculture.

The direct risk to aquaculture species and the aquatic environment was shown to be minimal from limestone treated Chicken Creek and Lake Kepwari mine pit lake water, based on the bioassay results and chemical assessment. The direct risk to aquaculture species and the aquatic environment was also shown to be minimal from the RCO treated WO5H mine pit lake water. The phosphorus treated Lake Kepwari water did show a reduction in toxicity in the bioassay tests and also showed a decreasing metal concentration trend, suggesting that with more time, toxicity may be removed and that the metal concentrations may be reduced to below guideline

levels using this treatment approach. Even if phosphorus treatment alone proves to be in-effective, the small addition of phosphorus combined with limestone may decrease the recovery time of the aquatic environment through reduced toxicity and increased nutrients for primary productivity.

The three species used in the bioassays were able to demonstrate different responses to different toxicants within the complex mixture of treated mine pit lake water. The selected species covered primary producers, zooplankton and single celled animals which are essential components of most food webs, making them highly representative of the aquatic environment. Using the different trophic levels allows for a broader, more sensitive toxicity assessment for both aquaculture species and for the aquatic environment than one species can alone. Therefore, the geographically and ecologically representative freshwater species chosen for this research may be applicable for assessment of other mine pit lake waters for aquaculture and biodiversity conservation.

Until now there has not been a freshwater, Australian native and commercially relevant aquaculture fish species used in toxicity assessment of waters for use in aquaculture. *Bidyanus bidyanus* met criteria for a good toxicity test species and may be useful in future assessment of freshwaters for aquaculture. Further assessment of *B. bidyanus* as a representative toxicity test species for aquaculture should be made. Research should include; reference toxicity testing and toxicity responses to common metals and toxicants in Australian temperate freshwaters. Furthermore, research into toxicity assessment with *B. bidyanus* will provide information for further improving freshwater aquaculture guidelines, thereby providing stakeholders a higher confidence when administering guidelines for assessing the potential of freshwater bodies for aquaculture.

8.4 Recommendations and future research

Currently guidelines for OCM closure do not require the pit lake water to be remediated for end uses (Johnson & Wright 2003). Addition of protocol designed to plan for end uses of pit lakes needs to be developed and implemented. Two major components of the protocol need to be choice of relevant end uses that are decided on by relevant current and future stakeholders, and pit lake water assessment for the chosen end uses. If the end use involves interaction of the pit lake water with adjacent aquatic environments, both chemical and biological assessment of risk must be implemented (Deanovic *et al.* 1999; Viadero Jr & Tierney 2003). If biological assessment is to be used on temperate mine pit lakes, the three species *C. protothecoides*, *C.* cf *dubia* and *T. thermophila* are recommended for assessment. Through the development and implementation of a mine pit lake closure protocol, addressing pit lake end uses, realisation of environmental, social and commercial benefits to stakeholders will be made.

Research on hardness effects to Al toxicity using Australian representative species should be made. Al is major element of environmental concern in fresh water environments, especially affected by acidity. Improving the reliability of the Al guideline with a hardness algorithm would allow investigators greater confidence when considering risk from Al concentrations to the aquatic environment.

Future research needs to be made on *B. bidyanus* to develop a protocol for its use in assessment of not only mine pit lake water, but all temperate fresh waters for aquaculture. This will create a standard bioassay directly relevant to Australian waters and aquaculture that can determine accurately is a freshwater source is suitable for aquaculture.

Field scale mesocosm assessment of limestone treatment to Lake Kepwari needs to be trialled to assess the in-situ response to treatment. Biological analysis using the three species from this research could then be used in conjunction with chemical analysis to assess residual toxicity, therefore confirming remediation effectiveness.

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APPENDICES

10 Appendices

10.1 Reference testing

Reference testing was completed for all three species using potassium dichromate (GPR™ 99% pure $K_2Cr_2O_7$) as the toxicant. The use of $K_2Cr_2O_7$ as a reference toxicant is common among toxicity testing and is therefore useful for intra and inter-laboratory comparisons. Concentrations of Cr (VI) are used for the EC calculations. The three bioassays used for toxicity testing covered both chronic and acute tests and were acute 48 h *C.* cf *dubia* mortality bioassay, chronic 72 h *C. protothecoides* growth rate bioassay and 24 h *T. thermophila* final density bioassay. The end point used for *C.* cf *dubia* was mortality, cell division rate (growth rate) for *C. protothecoides* and final density for *T. thermophila*. However, for ease of presentation, LC, and IC values are presented as EC_{50}s. Six tests were completed for each species over the duration of the experimental period.

A summary of the reference toxicity tests for all three species is presented in Table 28. No significant difference was found within any of the six independent tests conducted for each species. The sensitivity of the three species to Cr (VI) varies by more than two orders of magnitude. The mean EC_{50}s, in order of sensitivity were 24 μg/L for 48 h *C.* cf *dubia*, 135 μg/L for 72 h *C. protothecoides* and 7030 μg/L for *T. thermophila*. The large difference in sensitivity gives further support to the use of multi specie bioassay batteries, to cover the naturally large variation of sensitivity to different toxicants in the natural aquatic ecosystem. Nevertheless, the variation between tests within each bioassay was not high with a coefficient of variation of ≤10%. This is a good indication that test organism health was not changing over the test period, therefore showing that the bioassay test precision was acceptable.

Table 28. Summary of reference toxicity tests

Test species	Test	EC_{50} (µg Cr (VI) /L)	95% CI
Chlorella protothecoides	1	140[a]	114 - 173
72 h chronic test	2	144 [a]	118 - 180
	3	130 [a]	103 - 167
	4	119 [a]	99 - 143
	5	128 [a]	108 - 154
	6	150 [a]	119 - 194
	Mean	135	
	SD	12	
	CV%	9	
Ceriodaphnia cf *dubia*	1	25 [a]	21 - 30
48 h acute test	2	25 [a]	19 - 33
	3	21 [a]	16 - 28
	4	22 [a]	18 - 26
	5	23 [a]	18 - 29
	6	28 [a]	23 - 34
	Mean	24	
	SD	3	
	CV%	10	
Tetrahymena thermophila	1	6709 [a]	5470 - 8170
24 h chronic test	2	7220 [a]	5805 - 8918
	3	7266 [a]	5740 - 9120
	4	7241 [a]	6318 - 8276
	5	7015 [a]	5690 - 8596
	6	6728 [a]	5816 - 7758
	Mean	7030	
	SD	257	
	CV%	4	

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