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Highlighted Article

Toxicity of acid mine pit lake water remediated with limestone and phosphorus

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ABSTRACT

Pit lakes are increasingly common worldwide and have potential to provide many benefits. However, lake water toxicity may require remediation before beneficial end uses can be realised. Three treatments to remediate AMD (pH ~4.8) pit lake water containing elevated concentrations of Al and Zn from Collie, Western Australia were tested in mesocosms. Treatments were: (a) limestone neutralisation (L), (b) phosphorus amendment (P), and (c) combined limestone neutralisation and phosphorus amendment (L+P). Laboratory bioassays with *Ceriodaphnia* cf. *dubia*, *Chlorella protothecoides* and *Tetrahymena thermophila* assessed remediation. Limestone neutralisation increased pH and reduced heavy metal concentrations by 98% (Al) to 14% (Mg), removing toxicity to the three test species within 2 months. Phosphorus amendment removed toxicity after 6 months of treatment. However, phosphorus amendment to prior limestone neutralisation failed to reduce toxicity more than limestone neutralisation alone. Low concentrations of both phosphorus and nitrogen appear to limit phytoplankton population growth in all treatments.

1. Introduction

Mining pit voids are a common legacy of open cut mining worldwide (Castro and Moore, 1997). These voids may fill with ground and surface water to create a pit lake having a post-mining potential for a range of beneficial end uses (McCullough et al., 2009; McCullough and Lund, 2006). However, the end use is generally restricted by poor water quality in the pit lake (McCullough and Lund, 2006), for example, acid mine drainage (AMD) (Geller et al., 1998). AMD 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) and remediation may be required to allow the water to be used for a beneficial end use.

Although sulphate reduction is one such commonly used approach (Frömmichen et al., 2003) the low sulphur content in many moderately acidified water bodies (e.g., the mine pit lakes of South-Western Australia (Lund and McCullough, 2008), restrict this remediation technique (Lund et al., 2006). Limestone neutralisation is another 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 (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 (Lund et al., 2006; Totsche et al., 2006; McCullough et al., 2008). For example, phosphorus amendment has been used to remediate mine lake P limitation (Bittl et al., 2001; Kopacek et al., 2000; McCullough, 2007) and has been shown to remediate low pH by stimulating phytoplankton alkalinity production due to algal uptake of H^+ when assimilating NO_3^- (Davison et al., 1995b). Addition of P to moderately affected acid lakes may only require a small amount of P addition for a significant increase in pH to be made (Lychie-Solheim et al., 2001).

Aluminium is the most abundant metallic element in the lithosphere, but has little or no known biological function (Gensemer and 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 and 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

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phosphorus limitation of phytoplankton (Bittl et al., 2001). Consequently, aquatic biotic community colonisation in aluminium-buffered lakes is often limited by toxicity of low pH, elevated inorganic reactive aluminium and heavy metal concentrations and phosphorus limitation (Nixdorf et al., 2003).

For instance, even at moderate pH, heavy metal concentrations may still exceed environmental protection water quality guidelines (Markich et al., 2001). The potential impacts of these toxicants on aquatic environments are often studied using mesocosms to 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 (McCullough, 2009).

This study aimed evaluated 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. 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 were assessed using laboratory-based acute and chronic toxicity test protocols for three aquatic organisms from different taxonomic and trophic levels, the water flea *Ceriodaphnia* cf. *dubia*, the microalga *Chlorella protothecoides* and the ciliated protozoan *Tetrahymena thermophila*. Bioassays were 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; McCullough, 2009).

2. Materials and methods

2.1. Study site

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. However, even after rapid filling by diversion of the Collie River over several winters, the 24 GL Lake Kepwari has a low pH of ca. 4.8, and elevated metal concentrations of Al and Zn (Table 1). Acid pit lake water used in the mesocosm trial was collected from Lake Kepwari, a rapid-filled mine pit lake in the Collie Coal Basin in the South West of Western Australia (33.36°S, 116.15°E) (Lund and McCullough, 2009).

2.2. Mesocosm treatments

Using water and sediment collected from Lake Kepwari, twelve 1200 L pit lake mesocosms were established at Edith Cowan University, Perth, in August 2005 with lake sediment and water. Three treatments and an untreated control were replicated three times and arranged in a randomised block design (Lund and McCullough, 2009).

Representative control water samples of Collie River water (CRW) were collected upstream from the Collie River diversion site. The Mesocosm Control (MC) contained 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 maintained 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 was a concentration that could be achieved in an large mine pit lake (Lychie-Solheim et al., 2001) and 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 depth was then maintained by regular additions of deionised water.

Table 1
Select Lake Kepwari metal concentrations (μg/L) after Lund and McCullough (2009).

Al	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
1213	61	< 10	< 10	150	79	243	60	6	440

2.3. Water quality analyses

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 of 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 chemistry samples from each mesocosm were collected, two were immediately filtered through 0.5 μm filters (PALL 'Metrigard') the remainder unfiltered. All samples were stored frozen in acid washed high-density polyethylene bottles. Filtered samples were analysed for soluble reactive phosphorus (SRP) 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.

2.4. Water toxicity testing

Water samples were collected on each bi-monthly sampling occasion and were tested for toxicity using; the water flea *C.* cf. *dubia*, the microalga *C. protothecoides* and the ciliated protozoan *T. thermophila*. All mesocosm bioassay water samples were filtered to 0.5 μm filters (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 culture water.

2.5. C. cf. dubia bioassay

2.5.1. 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) (Kilham et al., 1998) with EDTA not included to remove the possibility of modifying toxicity of metals present. A static acute toxicity test for *C.* cf. *dubia* was conducted with five sub-replicates from each mesocosm as well as CRW and HHC waters over 48 h to incorporate slower metal toxicity processes.

2.5.2. Culture of test organism

Stock cultures of *C.* cf. *dubia* were cultured in HHC medium, made up using distilled water as a diluent. *C.* cf. *dubia* cultures were fed *Ankistrodesmus* sp. algae daily at a density of 100×10^4 cells/mL. The feeding regime was then altered (Hyne et al., 2005) as there was no significant difference (Student *T*-test, $n=5$, $P > 0.05$) in the intrinsic rate of natural increase (IRNI) of *C.* cf. *dubia* when two algae, *Pseudokirchneriella subcapitata* and *Ankistrodesmus* sp., were used as compared to use of a single alga.

2.5.3. Test protocol

C. cf. *dubia* 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 and 48 h with any deceased animals being removed at the 24 h mortality count with no feeding of the neonates during the 48 h test period (Orr and Foster, 1997).

2.6. C. protothecoides bioassay

2.6.1. 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 (C) with nutrients added after Stauber et al. (1994), and pH neutralised (N) Lake Kepwari mesocosm control water. The N water was neutralised by drop wise addition of 0.1 M NaOH 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 the test to remove suspended particles that could alter *C. protothecoides* growth results (Parent and 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 to differentiate between reduced growth rate resulting from the presence of toxicants, and reduced growth rate

resulting from a lack of nutrients. An interaction of P with Al could reduce the effective Al concentration altering toxicity of the sample therefore leaving K_2HPO_4 out removed the possibility of Al concentration changes.

2.6.2. Test protocol

The 72 h *C. protothecoides* 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. protothecoides* at the beginning of the test was approximately 7×10^4 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. Similarly to the *C.* cf. *dubia* test, the *C. protothecoides* test was extended to 72 h to incorporate slower metal toxicity processes.

2.7. T. thermophila bioassay

2.7.1. 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* as a chronic toxicity test completed in a short period of time (24 h) due to a fast reproductive rate. 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) with analytical-grade PPY added to all samples.

2.7.2. Test protocol

T. thermophila were cultured in slanted 10 mL plastic test tubes using analytical-grade water with the addition of 2 × PPYS. The concentration used in bioassays was 0.5 × PPYto decrease the likelihood of reducing, masking or increasing toxicity of the mesocosm samples to *T. thermophila*.

Stock culture *T. thermophila* were counted to 200 ciliates prior to inoculation of test samples then *T. thermophila* were diluted with reagent grade water to a density of 20×10^4*T. thermophila*/mL. Test containers consisted of 4 mL clear plastic cuvettes containing 1.8 mL of mesocosm or control water with 0.1 mL inoculation of ciliates added and 0.1 mL 10 × PPY added to make a concentration of 0.5 × PPY totalling 2 mL. A final density of 1×10^4 *T. thermophila/mL* was achieved at the commencement of the test. Three sub-replicates of each mesocosm and T were used in toxicity assessment (n=36). All cuvettes were covered with a lid and placed into an incubator for 24 h at 30 °C in the dark. Ciliate population growth was estimated by counting of ciliates as described above.

2.8. Data analysis

Statistical analysis of all bioassay and water quality data was performed with SPSS (2000) at the $P < 0.05$ level. Results for mortality of *C.* cf. *dubia* data were transformed to rankits (Pereira et al., 2000). *C. protothecoides* absorbency data were transformed by natural log with algae density units represented by LN absorbance (550 nm) and growth rates at 72 h (day 3) estimated from transformed data slope. Growth inhibition compared with that of controls was calculated using the formula:

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

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.

A repeated measures one-way analysis of variance (ANOVA) tested for significant differences between treatments and control mean test responses. When ANOVA assumptions data were not meet, the non-parametric Kruskal–Wallis *H* Test tested for median differences between mean treatment and control test responses. The parametric Tamhane Test *post-hoc* test identified where differences occurred 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 in Primer software (Clarke and Ainsworth, 1993; PRIMER-E Ltd, 2006). Test species responses were standardised against their highest replicate response to enable a comparison with positive bioassay growth rates (Fig. 1).

Hardness modified guideline values (HMGV) for aquaculture and aquatic ecosystem protection were calculated after ANZECC/ARMCANZ (2000). Speciation was determined for Al at each sample month by the computer program PHREEQC (Version 2.13.07) (Parkhurst and Appelo, 1999).

3. Results

3.1. Water quality changes

Mesocosm control pH rose from around 5 at months 2 and 4, to around 6 at months 6 and 8 (Table 2). 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. ORP 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

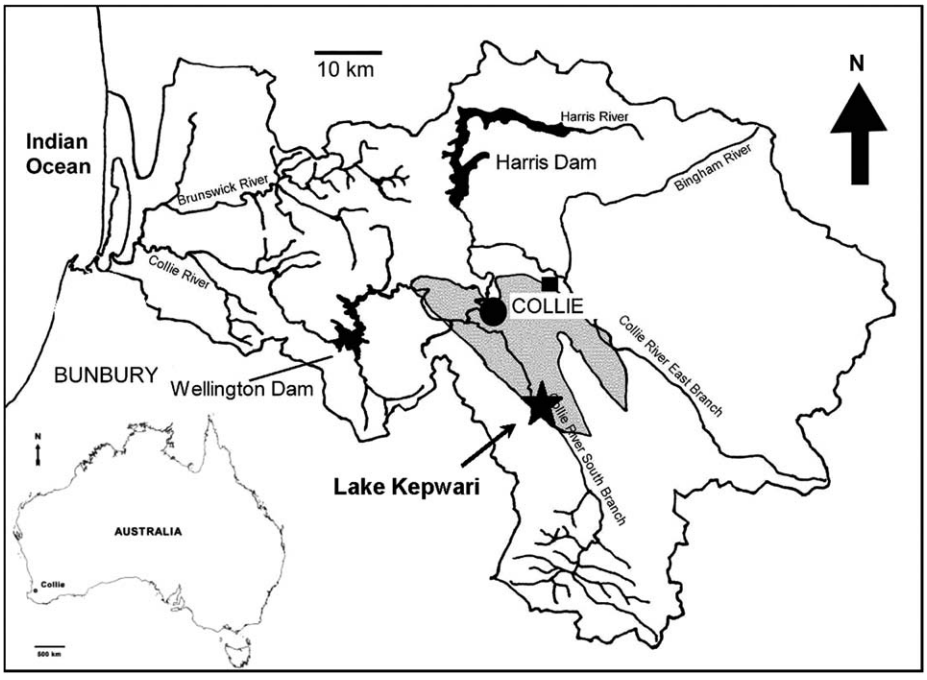

Fig. 1. Location of Lake Kepwari (indicated with an '*') in the south-western lobe of the Collie Coal Basin, Western Australia.

Table 2
Physico-chemistry of mesocosm at toxicity testing months 2, 4, 6 and 8.

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 (0.10)	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)

Values are means (standard error).

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 elements were below their detection limits (limit in parentheses) 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 and 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 with the control for months 2 and 4. Nevertheless, month 6 Cr concentrations were generally the same or lower than the control. P had substantially reduced Mn and Co compared with the MC. Liming had higher concentrations of Al in month 8, although this higher concentration was also at a higher pH (Table 2). Liming also had lower levels of Zn compared with control and P. Treatment SRP varied little in MC near the limit-of-detection of 3 μg/L. SRP 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.

3.2. Speciation modelling

Speciation modelling indicated the proportion of free Al^{+3} prior to remediation (month 0) was approximately 53%, and decreased in all the treatments to less than 1% by month 8 (Fig. 2). The Al^{+3} in MC and P was not dominant by month 4 where $Al(OH)_2^+$ was in 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 months 2–8. Zn^{+2} ion was the dominant species months in all mesocosms with greater than 85% present at all.

3.3. C. cf. dubia acute testing

3.3.1. Influence of treatment and exposure duration on toxicity

Bioassay controls CRW and HHC both showed low mortality ($<10\%$) over the 8 months 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 (Fig. 3).

C. cf. *dubia* mortality did not differ between the bioassay controls and treatments L and L+P at any month over the 8 months mesocosm trial with mortality always $<20\%$ for both controls and treatments (Fig. 3). In contrast, *C.* cf. *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.* cf. *dubia* within MC had not decreased over the period of the trial. Mortality of *C.* cf. *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.* cf. *dubia*.

3.4. C. prototheocoides chronic testing

The growth rate for the synthetic soft water (SSW) control was consistent at all months during the 8 months test period. Trials one and two were run simultaneously after month 2 with only one control for both trials at each month (Figs. 4A, 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.

Trial one (*sans* nutrients) displayed a higher mean growth rate in the control than all treatments at all sample months (Fig. 4A). 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 (Fig. 4A). 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 with month 6. All treatments displayed higher growth rates at month 8 than at month 2 (Fig. 4A,B).

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 (Fig. 4A). 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 showed an increasing trend from month 2 to month 8.

3.4.1. Month 2

The 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) (Fig. 5).

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 a high coefficient of variance (Table 3). When 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.

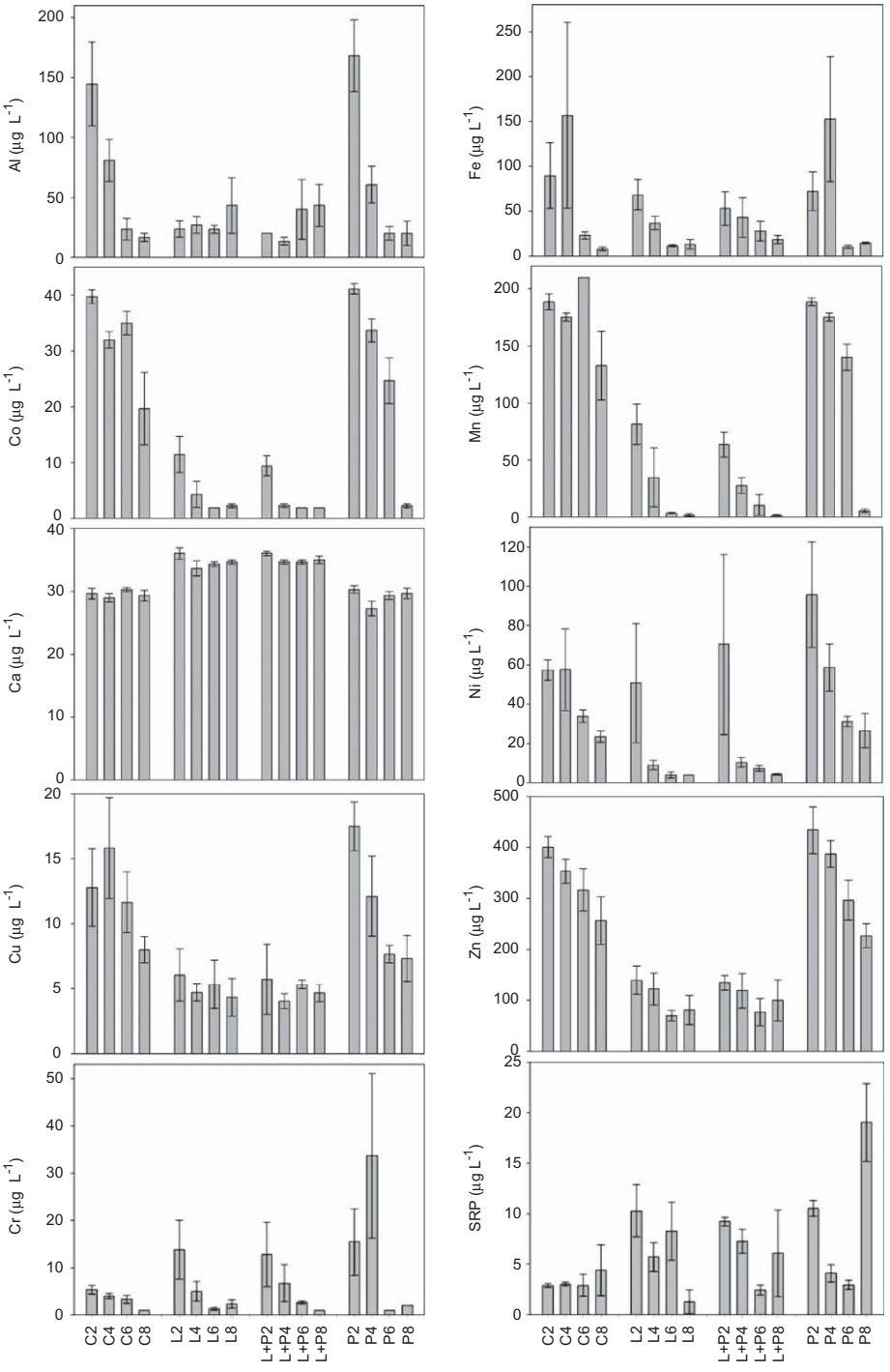

Fig. 2. Mean metal and SRP concentrations ± standard error from the mesocosms, recorded on months 2, 4, 6 and 8 for the mesocosm control (C), limestone (L), limestone and P (L+P) and P only (P) treatments.

3.5. *T. thermophila* chronic testing

The *T. thermophila* control (TTC) showed a consistent final *T. thermophila* mean density (FTD) of 7.1×10^4 ciliates/mL. A similar FTD was also exhibited in treatments L and L+P across each month, both with a mean of 7.0×10^4 ciliates/mL over the 8 months 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 (Fig. 6).

3.6. Bioassay parameter relationships

3.6.1. Bioassay response to physico-chemical parameters

Toxicity bioassays correlated well with a number of chemical and physical parameters measured during the trial (Table 4). The *C.* cf. *dubia* bioassay responded at both 24 and 48 h to NO_x with a

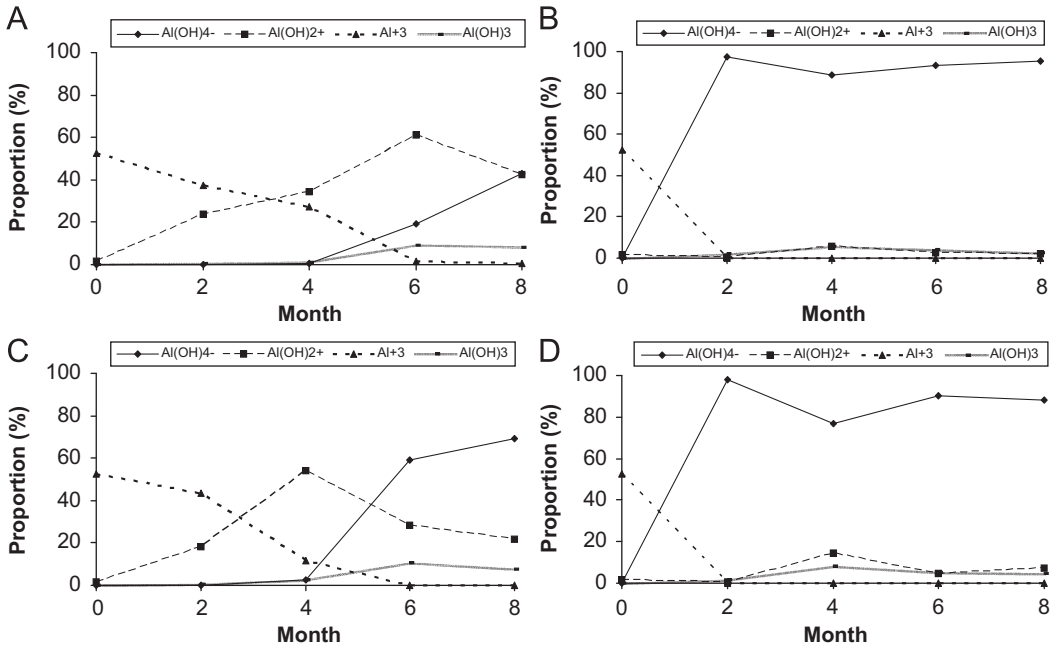

Fig. 3. Speciation (%) of Al in (A) Control, (B) L, C) P and (C) L+P treatments.

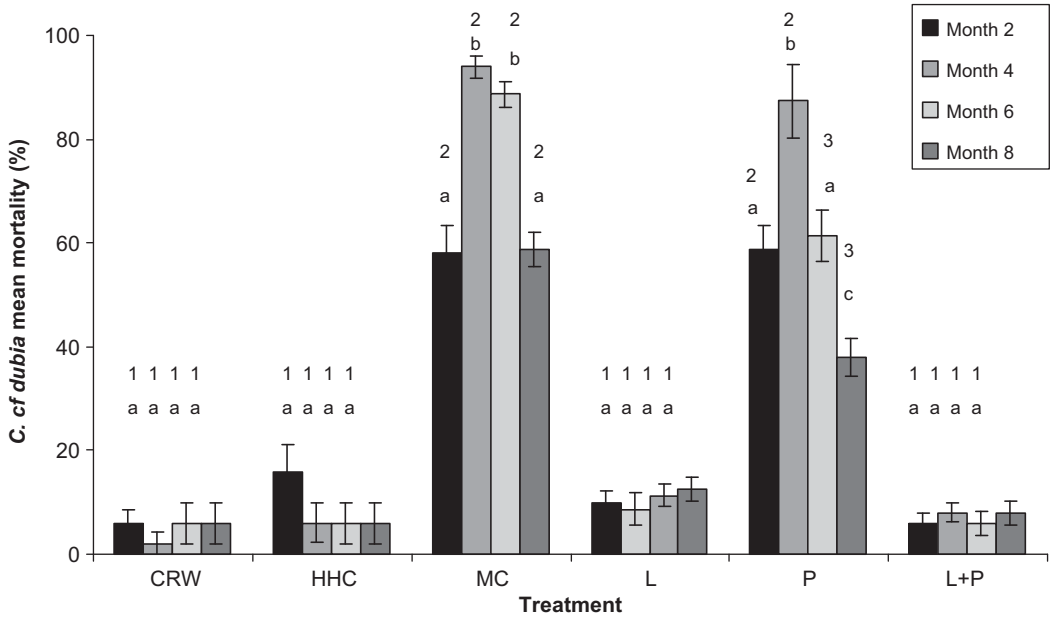

Fig. 4. *Ceriodaphnia* cf. *dubia* 48 h mean % mortality ± standard error for controls and treatments at sample months 2–8. Different lowercase letters indicate significant differences for a control or treatment between sample months. Different numbers indicate statistically significant differences.

high negative correlation ($\rho > 0.8$) and a positive correlation to Ca. Here 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 NO_x and Ca.

The *C. protothecoides* bioassay showed a distinctly different correlation between the two bioassay trials of with and without nutrients (Table 4). 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 but did not strongly correlate with these parameters ($\rho < 0.5$).

The *T. thermophila* bioassay population response was similar to the *C.* cf. *dubia* 24 and 48 h bioassays with a strong positive correlation of $\rho > 0.85$ to pH and the Ca concentration and a strong negative correlation to Zn and NO_x concentrations (Table 4).

3.6.2. Comparison of tolerances of test species to mesocosm treatments

The standardised bioassays response to the MC was similar to that of treatment P with the exception of a markedly lower response of the *C.* cf. *dubia* 48 h bioassay to the MC (Fig. 7). The

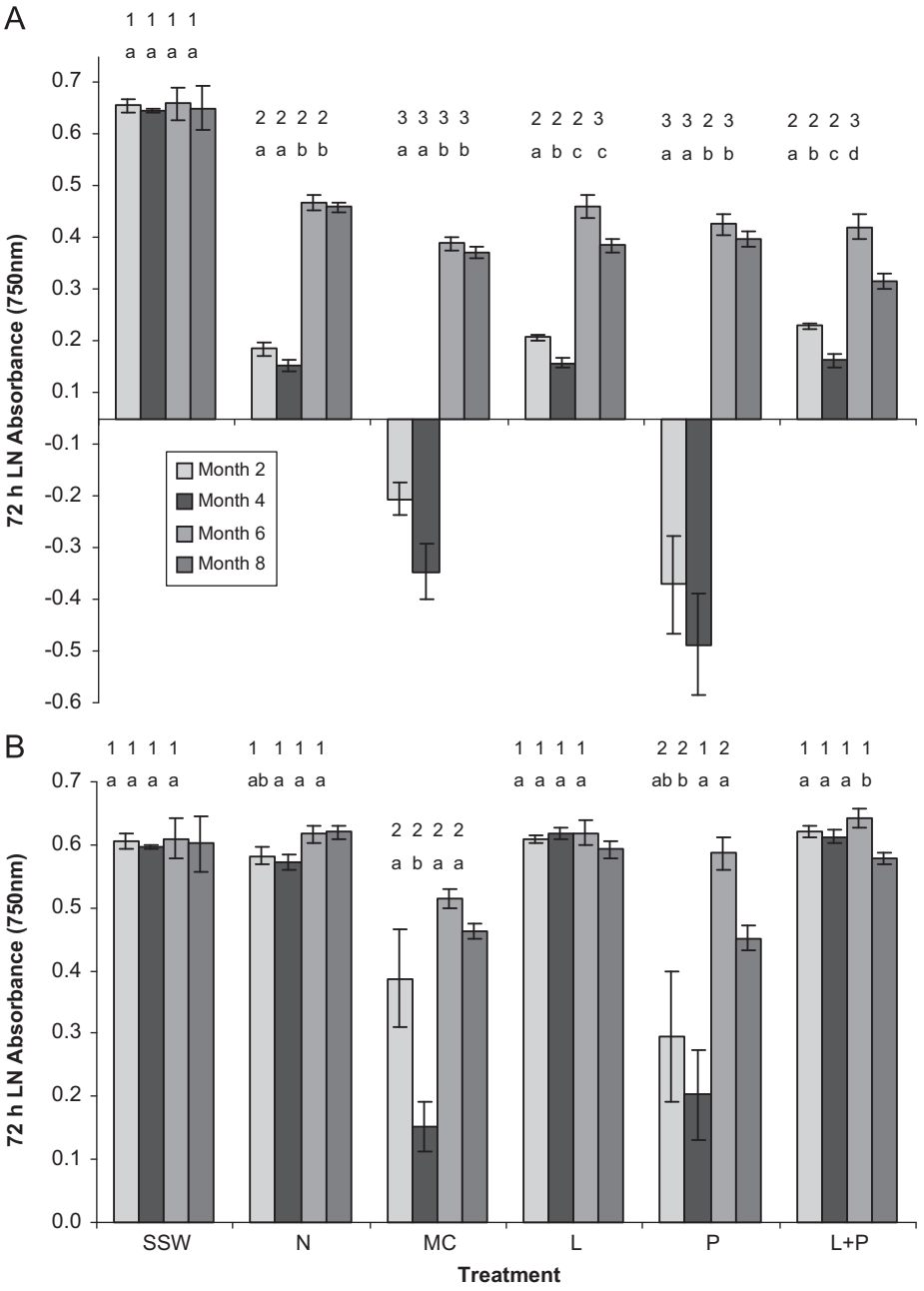

Fig. 5. *Chlorella protothecoides* mean growth rate (slope) ± standard error at 72 h for trial 1 (*sans* nutrients) (A), and trial 2 (nutrients added) (B), for months 2–8. Different lowercase letters indicate statistically significant differences for a control or treatment between sample months. Different numbers indicate statistically significant differences between a control and treatment within a sample month.

Table 3
Month 2 *C. protothecoides* growth rate and inhibition for controls and treatments.

Treatment	Trial one (*sans* nutrients)				Trial two (with nutrients)			
	Growth rate mean ± S.E.	CV (%)	Difference to control (%)	Toxicity effect	Growth rate mean ± S.E.	CV (%)	Difference to control (%)	Toxicity effect
SSW	0.61 ± 0.01^{a}	5	N/A	N/A	0.61 ± 0.01^{a}	6	N/A	N/A
N	0.13 ± 0.02^{b}	44	−80	Inhibition	0.58 ± 0.01^{a}	7	−5	None
MC	-0.22 ± 0.04^{c}	54	−136	Algicidal	0.39 ± 0.08^{b}	59	−37	Inhibition
L	0.17 ± 0.00^{b}	6	−72	Inhibition	0.61 ± 0.01^{a}	4	−1	None
P	-0.35 ± 0.1^{c}	91	−156	Algicidal	0.30 ± 0.10^{b}	105	−52	Inhibition
L+P	0.18 ± 0.01^{b}	9	−71	Inhibition	0.62 ± 0.01^{a}	5	1	None

S.E. = standard error. CV = % coefficient of variance (%). Different superscript lowercase letters indicate statistically significant differences between treatment means. N/A = not applicable.

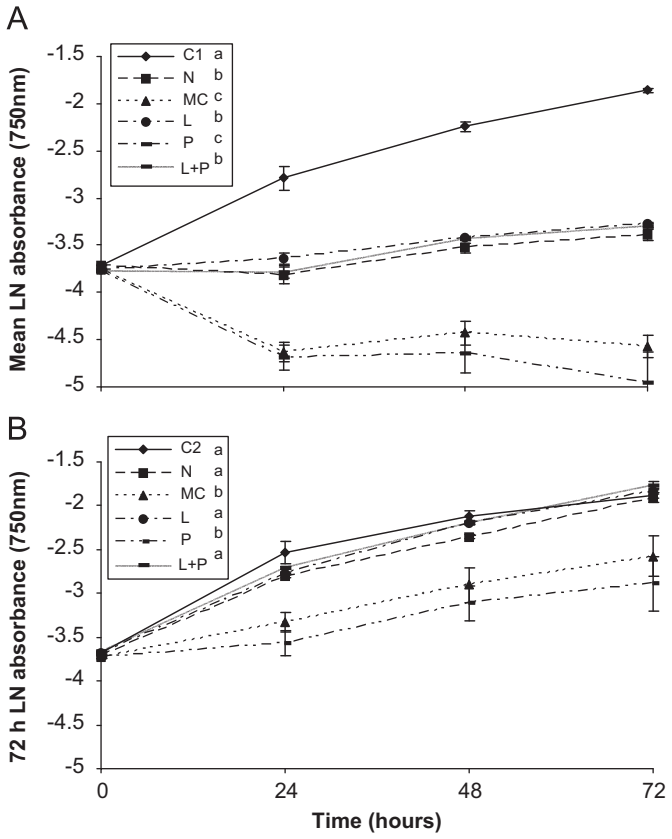

Fig. 6. *Chlorella protothecoides* mean LN absorbance ± standard error for control one trial one (A), and trial two (B), at month 2. C1 is synthetic control 1, C2 is synthetic control 2, N is NaOH neutralised lake water, L is limestone treated lake water, P is phosphorus-treated lake water and L+P is limestone and phosphorus-treated lake water. Different lowercase letters indicate statistically significant differences between treatments at 72 h.

Table 4
Relationship of test species responses at month 4 to physico-chemical parameters X indicates that a water quality variable(s) each bioassay is Spearman rank correlated with a test's response.

Bioassay variables	ρ	pH	Cr	B	Zn	NO_x	Ca	Cu	Mn/Co	Ni	EC	Mg
C. cf. *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. cf. *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					

MC displayed higher adverse responses across all the bioassays when compared with 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.

4. Discussion

4.1. Water quality changes

pH at the commencement of the mesocosm trial was lower (ca. 4.8) 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 naturally acidic fresh surface waters (Johnson and Hallberg, 2005; Lychie-Solheim et al., 2001) 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 and 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 pH may not directly reduce toxicity, but rather increase the non-bioavailable proportion of the metal (Fig. 8).

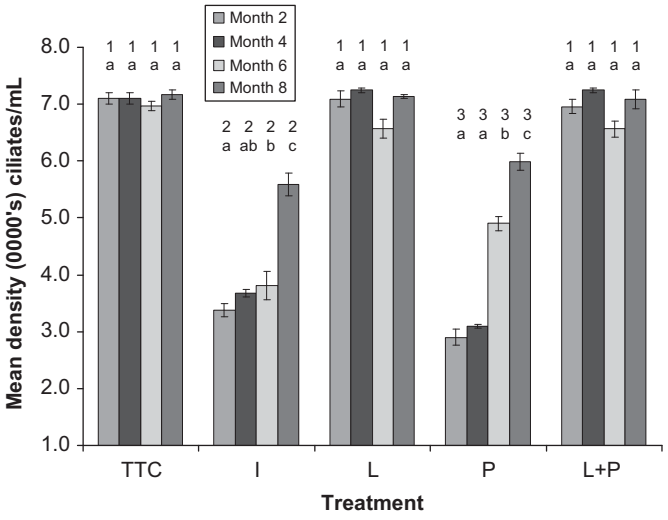

Fig. 7. Final mean density of *T. thermophila* after 24 h of incubation. Different lowercase letters indicate significant differences within treatments and between sample months. Different numbers indicate statistically significant differences between treatments within a particular sample month.

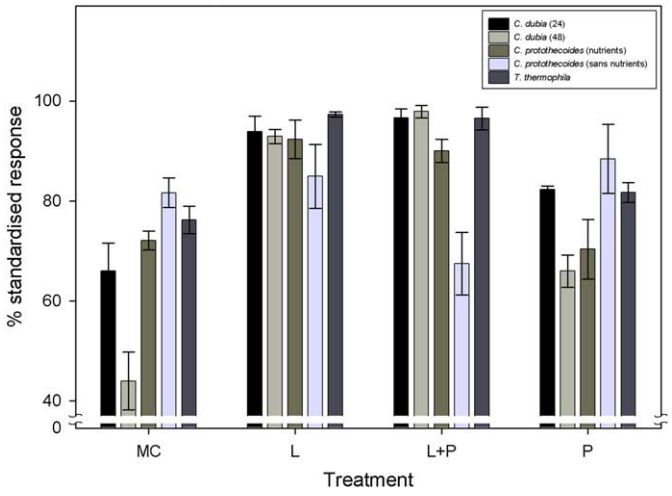

Fig. 8. Standardised responses for toxicity bioassays after 8 months of the different treatments and the control.

Month 0 Al, Mn and Zn concentrations exceeded Australasian aquaculture guidelines and Zn exceeded Australasian aquatic ecosystem protection guidelines in the untreated MC (ANZECC/ARMCANZ, 2000). 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. Of the three elevated metals HMGV were only available for Zn with the HMGV for aquaculture and for aquatic ecosystems, 11 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.

Uptake rate and toxicity of aluminium in freshwater organisms generally decreases with increasing water hardness under acidic, neutral and alkaline conditions (Gundersen and Steinnes, 2003). Dissolved Al concentration decreased significantly in the treatments and MC by month 2, but was still above the guidelines in the mesocosm control and P. 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} is likely 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 Al concentration. Inorganic single unit $Al(OH)_2^+$ 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 P in months 4, 6 and 8. Unexpected toxicity of Al at pH 6.4 has been seen even with pH close to neutral if concentrations of Al are high (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 (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 by an unknown mechanism.

Limestone remediation of the Lake Kepwari water was the only treatment to quickly reduce toxicity for all three test species and is known to decrease acidity and reduce metal bioavailability (Kalin et al., 2005; Koschorreck et al., 2007). Increased calcium concentration (true water hardness) has been shown to reduce toxicity and bioavailability through competition with other divalent metals (Gensemer et al., 2002). Additions of nutrients (e.g., P) can stimulate primary productivity, which can also produce alkalinity and biogenic metal scavenging (Davison et al., 1995a). P addition was used to stimulate primary productivity resulting in passive alkalinity production. Nevertheless, P treatment had less impact on reducing toxicity to the test species than the limestone treatments. Adding P to the limed treatment also failed to reduce toxicity more than liming alone.

Toxicity results from the *C.* cf. *dubia* bioassay showed mean mortality over the 8-month trial in treatments L (10.7%) and L+P (7.0%) was similar. Furthermore, mean mortalities were not significantly different from the bioassay controls CRW (5.0%) and HHC (8.5%) indicating toxicity to *C.* cf. *dubia* was removed in these treatments. Toxicity to *C.* cf. *dubia* 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.

Mesocosm control and P demonstrated equally high but variable mortality ($> 40\%$) of *C.* cf. *dubia* from month to month showing poor removal of toxicity to *C.* cf. *dubia* until month 6. Toxicity from the mesocosm control from months 2 to 6 corresponded with the high proportion of $Al(OH)_2^+$ indicating that this complex was the main toxicant. 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, P displayed lower mean mortality than previous months and lower mortality than the MC, indicating that P had decreased toxicity to *C.* cf. *dubia* from the pit lake water. The reduced toxicity from the P 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.* cf. *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). *C.* cf. *dubia* mortality varied between MC and P and was probably due to small metal concentration differences between replicates of MC and P.

4.2. *C. protothecoides* 72 h chronic bioassay

C. protothecoides was effective in differentiating the toxicity from the three remediation treatments. 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 affect to toxicity that may eventuate from the addition of nutrients. Another way of removing error from nutrient addition has been suggested through utilising the luxury

uptake of P exhibited by algae, allowing continued algal growth without extra addition of P to experiments (Parent and Campbell, 1994). However, the use of trial one gives the extra advantage of also not adding nitrate.

C. 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 therefore identified availability or deficiency of pit lake nutrients, 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 with mesocosm control and P treatment showing a growth rate decrease 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 P corresponded with an increases in $Al(OH)_2^+$ indicating that this Al form was causing toxicity. A significant difference occurred between the mesocosm control and P at month 6, which was correlated with the increase of $Al(OH)_4^-$ and the decrease of $Al(OH)_2^+$ in P allowing a higher growth rate. Trial one growth rate in MC at month 8 was similar to that of 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 always similar for the control C and treatments L and L+P 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 $Al(OH)_4^-$ indicating limestone addition alone to be sufficient for toxicity removal. The *C. protothecoides* result was also comparable to the result from the *C.* cf. *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 P additions had very little influence in the earlier months of 2 and 4.

The growth rate from P 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. Phosphorus amendment as P and L+P did not provide long-term toxicity reductions probably due to elevated Zn concentrations. Furthermore, the concentration of $Al(OH)_2^+$ at month 8 may have also contributed to the reduced growth rate. Therefore, P addition may need to be continued to be effective over a long term.

The main removal mechanism for SRP is thought to be adsorption by aluminium species (Kopacek et al., 2000; Ulrich and Pöthig, 2000). Water column nitrate concentrations are thought to be reduced by ammonification (mineralisation) and ammonia volatilisation, denitrification and mineral sorption. 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 and Campbell, 1994).

Addition of K_2HPO_4 as a nutrient will therefore only increase phytoplankton growth if added in excess to P sorption and consequent precipitation with Al. Toxicity of Al should, therefore, decrease by amendment with P as Al is removed from the water column to the sediment (Lund et al., 2006). Alkalinity increases from phytoplankton will then only occur with additional amendment of P (Fyson et al., 1998). 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., 1995b). 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). Addition of P 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.* cf. *dubia* bioassay by month 8, suggesting a higher sensitivity for this species to this remediation regime.

4.3. *T. thermophila* 24 h chronic bioassay

The *T. thermophila* toxicity control bioassay results was consistent with that of the previous two bioassays presenting a consistent FTD over the 8-month trial similar to the limestone treatments. The MC and P 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 MC 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 mass-balance significant contributions from acid-producing sources around the pit lake are unquantified.

The FTD from MC increased 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 causing the toxicity to *T. thermophila*. FTD from P was slightly lower but significantly lower than that of MC at months 2 and 4. Toxicity from P 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 P treatment at months 2 and 4 was not seen in the other species bioassays. Nevertheless, from months 6 to 8 the FTD from P treatment was significantly higher than that of MC. FTD increase in P treatment corresponded to an increase in $Al(OH)_4^-$ and a decrease in $Al(OH)_2^+$. Therefore, showing that toxicity was caused by the $Al(OH)_2^+$ complex at the pH range 5–6.5.

The slow effect of P treatment in removing toxicity could be due to low ambient temperature and light levels present in early spring when the mesocosm experiment began. These physical factors could easily have slowed phytoplankton, bacterial and algal population toxicity removal processes. Delayed amelioration of toxicity may also have been due to the removal of the limiting nutrient phosphorus by Al, in-turn, restricting growth of phytoplankton and benthic algae populations (Parent and Campbell, 1994).

4.4. Comparison of bioassay responses to physiochemical parameters

The main physico-chemical parameter combinations influencing the *C.* cf. *dubia* bioassay at both 24 and 48 h were negative correlation to NO_x 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.* cf. *dubia* bioassays with a strong negative correlation of $\rho > 0.85$ to NO_x and positive correlation to Ca, but with pH and concentrations of Zn found in four and three computed results, respectively. The correlation with NO_x indicates a possible relationship with alkalinity forming reactions through

phytoplankton assimilation of NO_x, which may not be pronounced in the *C. protothecoides* bioassay trial two (nutrients added) (Lychie-Solheim et al., 2001; Parent and Campbell, 1994).

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. 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.

4.5. Comparison of bioassay responses to mesocosm treatments

The standardised test species response provided a way of determining a possible overall mode of ecological response to remediation treatment (McCullough, 2009). The mesocosm control displayed a lower (but still positive) response than treatments when comparing the bioassay responses to the water quality improvement. However, all treatments on the pit lake water had a positive response to improving water quality of the acidic pit lake water. The *C.* cf. *dubia* 48 h 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 regime.

Bioassay responses indicated that amendment of P to acid pit lake water was not as effective in removing toxicity to *C.* cf. *dubia* as it was in other bioassays, suggesting this species was more sensitive to this treatment of acid pit lake water. 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 and 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.

4.6. Conclusion and recommendations

All remediation treatments reduced toxicity to test organisms in the 8 months 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.* cf. *dubia, C. protothecoides* and *T. thermophila* from the Kepwari pit lake water, but did not remove toxicity as quickly as limestone neutralisation alone. Nevertheless, although toxicity is not always pronounced from bioassays in these treatments, bioaccumulation of metals (Barron and 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 corresponded the proportion of the $Al(OH)_2^+$ complex. As $Al(OH)_4^-$ increased, toxicity reduced indicating that $Al(OH)_4^-$ was not toxic, and may in fact have competed for organism binding sites when $Al(OH)_2^+$ was present. 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, use of this test species' battery gave an understanding of toxicity that may remain in an acid pit lake with and without remediation with limestone neutralisation and P-amendment. 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 field scale to allow for a natural ecosystem to develop.

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Ethical statement

There were no human subjects or animals requiring ethics approval used in this research.

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