Centre for Advanced Analytical Chemistry
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TOXICITY TESTING OF MODIFIED CLAY LEACHATES USING FRESHWATER ORGANISMS

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EXECUTIVE SUMMARY

Background

CSIRO Land and Water (CLW) have developed modified clays that, when added to a water body, will reduce phosphate concentrations and limit the growth of nuisance algae. In order to obtain regulatory authority approval for the use of this material in a range of water bodies, information on its ecotoxicity in freshwater systems was required.

Objective

The objective of this project was to compile initial data on the toxicity of leachates of the modified clay to freshwater organisms. Acute toxicity was determined using the cladoceran *Ceriodaphnia dubia* (48 h survival) and juvenile eastern rainbow fish (*Melanotaenia duboulayi*, 96 h immobilisation). Chronic toxicity was determined using the *Ceriodaphnia dubia* 7-day reproduction test and a 72-h algal growth inhibition test with the green alga *Selenastrum capricornutum*.

Conclusions

Leachates of modified clay were not toxic to juvenile eastern rainbow fish nor to the freshwater alga *Selenastrum capricornutum* in chronic 72-h growth inhibition tests.

The synthetic softwater leachates were of low toxicity to survival of the cladoceran *Ceriodaphnia dubia,* with a 48-h LC50 value (the concentration resulting in a 50% reduction in survival compared with controls) of 49%, equivalent to 24.5 g clay/L. Dilutions of leachate of 1:8 would be sufficient to remove this acute toxicity. The toxicity of the leachate was not due to colloidal material, as removal of this fraction by ultrafiltration through a 0.1 µm membrane filter, did not reduce toxicity. Lanthanum concentrations in the leachate at the 48-h LC50 value were 80 µg/L, much lower than the concentrations reported to be toxic to the cladoceran *Daphnia magna.*

The leachate was also toxic to *Ceriodaphnia* reproduction at all concentrations tested, with a reduced number of young over 7 days compared to controls. The no observable effect concentration was <6.25% i.e <3.1 g clay/L (<126 µg La/L).

Using estimates of clay application rates, estimated environmental concentrations of the clay in the water column were compared to acute effects data based on the toxicity tests. The calculated hazard quotient for freshwaters was <0.1, indicating minimal risk of acute toxicity from clay application. There may be a small risk of chronic toxicity, however this would need to be investigated further in field applications of the modified clay.

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1 INTRODUCTION

CSIRO Land and Water (CLW) have developed modified clays that efficiently remove phosphate from solution. This material can be applied to water bodies to limit the growth of nuisance algae. In order to have this material approved by regulatory authorities for its use, information is required on its potential ecotoxicity in freshwater environments.

For registration of chemicals in Australia under the National Industrial Chemical Notification and Assessment Scheme (NICNAS), the most basic data required to assess environmental impacts are toxicity data for an alga, an invertebrate and a fish. Although it is unlikely that the modified clay would be considered by regulators to be a new chemical, the nature of any potential environmental effects of the material still needed to be established prior to its application to waterways.

The aim of this project was to establish whether freshwater leachates of the modified clay (Phoslock) were toxic to freshwater biota. Acute toxicity was determined using the cladoceran *Ceriodaphnia dubia* (48 h survival) and juvenile eastern rainbow fish (*Melanotaenia duboulayi*, 96 h immobilisation). Chronic toxicity was determined using the *Ceriodaphnia dubia* 7-day reproduction test and a 72-h algal growth inhibition test with the green alga *Selenastrum capricornutum*. Freshwater leachates of the clay in Milli-Q and synthetic softwater were tested for acute toxicity, before and after ultrafiltration, using the water flea *Ceriodaphnia dubia*. This work formed part of a first tier risk assessment for the application of the modified clay to freshwater aquatic systems.

This report summarises all the toxicity testing of the modified clay over the last two years using freshwater organisms. Milli-Q water leachates were originally used for toxicity testing with the freshwater alga and the cladoceran (Vaughan, 1998). Later tests with the cladoceran and fish used synthetic softwater leachates, which would better simulate clay leaching into natural waters (Stauber, 1999; Lim, 1999).

2 EXPERIMENTAL

2.1 Materials

The modified clay was prepared by Dr Grant Douglas from CSIRO Land and Water, Perth, and was sent by courier to Lucas Heights, where laboratory-based toxicity tests were carried out.

2.2 Preparation of Freshwater Leachates

One litre of leachate was prepared according to the standard Toxicity Characteristic Leaching Procedure (TCLP - USEPA, 1986) except that Milli-Q water or synthetic softwater was used as the extraction fluid in place of acetic acid. Synthetic softwater (hardness 40-48 mg $CaCO_3$/L, alkalinity 30-35 mg $CaCO_3$/L) was prepared by dissolving 1.2 g $NaHCO_3$, 0.75 g $CaSO_4.2H_2O$, 0.75 g $MgSO_4$, and 0.05 g KCl in 25 L of Milli-Q water, leaving overnight and filtering through a 0.45 µm membrane filter (USEPA, 1994). The nutrient selenium (2µg/L of Na_2SeO_4) was added to the softwater as dilution water for the *Ceriodaphnia* bioassays.

Modified clay (50 g) was mixed with 1 L of water in 1-L Teflon® bottles and mixed by tumbling end-over-end at 30 ± 2 rpm for 18 h. The leachate was filtered through a 0.45 µm membrane, and split into two subsamples. One subsample was used for toxicity testing without further processing, while the other sample was centrifuged in a Sorvall refrigerated centrifuge for 30 min at 20,000 g. After centrifugation, the leachate was ultrafiltered through a 0.1 µm membrane filter, to remove any colloidal material. Both the filtered and ultrafiltered leachates were stored at 4^oC for three days prior to commencement of the toxicity tests.

For quality assurance purposes, a process blank was carried through the entire procedure. One litre of water (minus clay) was prepared in the same way and split into two subsamples for filtration and centrifugation/ultrafiltration. Toxicity tests were also carried out on the two process blanks to ensure that the leachate manipulations did not introduce toxicity to the sample.

The pH of the leachates and process blanks was adjusted to 7 prior to testing. Subsamples of the leachates and process blanks were acidified and analysed for lanthanum (La) by inductively coupled plasma emission spectroscopy (ICPAES).

The type of leachate used for each of the toxicity tests is shown in Table 1.

Table 1. Leachates Used for Each of the Toxicity Tests

Toxicity Test	Leachate Used		
	Milli-Q	Synthetic Softwater	Ultrafiltered Synthetic Softwater
Acute			
Juvenile rainbow fish		X	
Ceriodaphnia dubia	X	X	X
Chronic			
Ceriodaphnia dubia		X	
Selenastrum capricornutum	X		

2.3 Acute Fish Imbalance Test

The University of Technology, Sydney, conducted a sub-acute fish imbalance toxicity test on the filtered modified clay leachate using juveniles of the eastern rainbow fish, *Melanotaenia duboulayi.* A synthetic softwater leachate (17 L) and a leachate process blank (2 L) were prepared at CSIRO and dispatched to the University of Technology, Sydney. The pH of the leachate was 6.87.

Details of the test procedure are given in Appendix A.

2.4 Acute Cladoceran Toxicity Test

Toxicity tests based on the survival of the cladoceran *Ceriodaphnia dubia* over 48 h were carried out according to the standard USEPA protocols (USEPA, 1993). Four leachates were tested – Milli-Q filtered, Milli-Q ultafiltered, synthetic softwater filtered and synthetic softwater ultrafiltered.

2.4.1 Cladoceran cultures

The initial culture of *Ceriodaphnia dubia* was obtained from the NSW EPA/UTS Centre for Ecotoxicology, Gore Hill, NSW. Mass cultures that were used as the source of brood organisms for individual cultures were maintained in USEPA synthetic soft water (USEPA, 1993). The cultures were fed daily with YCT (yeast-Cerophyll-trout chow) and *Selenastrum* (USEPA, 1993). Cultures were kept at 25 $\pm$ 1°C under ambient laboratory lighting. Neonates for toxicity tests were obtained from individual organisms cultured in 15 mL of synthetic soft water in 50-mL polycarbonate vials. The individual organisms were fed daily with YCT and algae and were transferred to fresh media three times a week.

2.4.2 Cladoceran toxicity tests

The 48-h cladoceran survival test was carried out according to the USEPA protocols as summarised in Table 2. The modified clay leachates were tested at 100%, 75%, 50%, 25%, 12.5% and 6.25% dilution in synthetic softwater. Synthetic softwater controls, Milli-Q controls, process blanks and three concentrations of the reference toxicant, copper, were also prepared. Five neonates, less than 24-h old, were added to each 50 mL polycarbonate vial containing 15 mL of sample. Four replicate

containers were used for each sample dilution and controls, giving a total of 20 organisms per concentration. The numbers of immobile *Ceriodaphnia* were recorded at 24 and 48 h. For test acceptability, the survival of control organisms had to exceed 90%. Statistical analysis of the test data was carried out using ToxCalc software. The LC50 (the effective concentration of leachate which gave a 50% reduction in survival compared to the controls) and NOEC (no observable effect concentrations) were calculated after 24 and 48 h exposures.

2.5 Chronic Cladoceran Toxicity Test

This test was carried out by the Sinclair Knight Merz Ecotoxicology Laboratory using a freshly prepared synthetic softwater leachate and leachate blank (see section 2.2). The method (SOP#2-2) was based on the standard method (USEPA, 1994) which determines survival and reproduction of the daphnid over 7 days (three broods) in a static renewal test. A summary of the test method is given in Table 3.

Synthetic softwater controls, a process blank and five concentrations of the reference toxicant, copper, were also prepared. The pH of the leachate was adjusted to pH 7.9 prior to testing. One neonate, less than 24-h old, was added to each of 10 replicates, giving a total of 10 organisms per concentration. Survival and reproduction (the mean number of young per adult female) was determined over 7 days. For test acceptability, the survival of control organisms had to exceed 90% over 7 days, with a mean number of young per female in the controls of greater than 15. Statistical analysis of the test data was carried out using ToxStat software. The 7-day LC50 (the effective concentration of leachate which gave a 50% reduction in survival compared to the controls) and reproductive NOEC and LOEC (lowest observable effect) values were calculated.

Table 2. Summary of Test Conditions for the *Ceriodaphnia dubia* AcuteToxicity Test

1	Test type:	Static non-renewal
2	Test duration:	48 h
3	Temperature:	25 ± 1°C;
4	Light quality:	Ambient 1aboratory illumination
5	Light intensity:	10-20 µE/m^2/s (50-100 ft-c)
		(ambient laboratory levels)
6	Photoperiod:	16 h light, 8 h darkness
7	Test chamber size:	50 mL
8	Test solution volume:	15 mL
9	Age of test organisms:	Less than 24-h old
10	No. organisms per test chamber:	5
11	No. replicate chambers per concentration:	5
12	No. organisms per concentration:	25
13	Feeding regime:	Fed YCT and *Selenastrum* while holding prior to the test; newly-released young had food available a minimum of 2 h prior to use in a test
14	Test chamber aeration:	None
15	Dilution water.	Synthetic soft water
16	Endpoint:	Mortality (LC50 or NOEC)
17	Test acceptability criterion:	90% or greater survival in controls

Table 3. Summary of Test Conditions for the *Ceriodaphnia dubia* Chronic Toxicity Test

1	Test type:	Static non-renewal
2	Test duration:	Usually 7 days (until 60% of surviving control organisms have three broods)
3	Temperature:	25 ± 1°C;
4	Light quality:	Ambient 1aboratory illumination
5	Light intensity:	10-20 µE/m^2/s
		(ambient laboratory levels)
6	Photoperiod:	16 h light, 8 h darkness
7	Test chamber size:	30 mL min
8	Test solution volume	15 mL min
8	Renewal of test solution:	daily
9	Age of test organisms:	Less than 24-h old
10	No. organisms per test chamber:	1
11	No. replicate chambers per concentration:	10
12	No. organisms per concentration:	10
13	Feeding regime:	Daily feeding with YCT and algal suspension
14	Test chamber aeration:	None
15	Dilution water:	Synthetic soft water
16	Endpoint:	Survival and reproduction
17	Test acceptability criterion:	90% or greater survival in controls and a mean of >15 young per surviving female in controls

2.6 Chronic Algal Growth Inhibition Test

2.6.1 Algal stock cultures

The freshwater green alga, *Selenastrum capricornutum* Printz, was imported from the American Type Culture Collection (ATCC 22662). This alga was renamed *Raphidocellus subcapitata,* however, the species is commonly known as *Selenastrum capricornutum* in the literature (Environment Canada, 1992). Stock algal cultures were maintained axenically in EPA medium (USEPA. 1994) at 24 ± 2° C under continuous "cool white" fluorescent light with an intensity of 4000 ± 10% lux (light quantal flux approximately 60 to 80 µE/m^2.s). Cells in exponential phase growth were used in the algal bioassays, after washing to remove culture medium.

2.6.2 Algal bioassay

The algal bioassay was based on the OECD Test Guideline 201(1984) and the USEPA protocol (USEPA, 1994) and is summarised in Table 4. For the test, six concentrations of the Milli-Q leachate of the modified clay, each in triplicate, were prepared in 20 mL silanised glass scintillation vials containing 6 mL algal culture medium (with EDTA). Triplicate controls in algal culture medium were also prepared.

Each vial was inoculated with 1-2 x 10^4 cells/mL of a *Selenastrum* suspension. Vials were incubated at 24 ± 2^o C under continuous light at 4000 ± 10% lux on an orbital shaker (100 rpm). Cell density in each treatment was determined daily for 3 days by counting cells using a Coulter Multisizer IIE particle analyser with 70 µm aperture. The pH at each concentration of the leachates was determined at the beginning and end of the test.

2.6.3 Statistical analyses

A regression line was fitted to a plot of $\log_{10}$ (cell density) versus time (h) for each vial and the cell division rate (µ) determined from the slope. Cell division rates per day (3.32 x µ x 24) were calculated. For the *Selenastrum* bioassay, the test was acceptable if the final cell densities in the controls were greater than 4 x 10^5 cells/mL with variability of less than 20%.

The 72-h EC50 value (the effective concentration of leachate which gave a 50% reduction in cell division rate compared to the controls) was calculated using ToxCalc Version 5.0.14 (Tidepool Software). After testing the data for normality and homogeneity of variance, Dunnett's Multiple Comparison Test was used to determine which treatment concentrations were significantly different to the controls in order to estimate NOEC values.

Table 4. Summary of Toxicity Test Conditions for the Freshwater Algal *Selenastrum capricornutum* Growth Inhibition Test

1	Test type:	Static
2	Temperature:	24 ± 2°C
3	Light quality:	"Cool white" fluorescent lighting
4	Light intensity:	4000 ± 10% lux
5	Photoperiod:	Continuous illumination
6	Test chamber size:	20 mL
7	Test solution volume:	6 mL
8	Renewal of test solutions:	None
9	Age of test organisms:	4 - 7 days (in exponential phase of growth)
10	Initial cell density:	10,000 cells/mL
11	No. replicate chambers /concentration:	3
12	Shaking rate:	100 rpm
13	Dilution water:	Algal culture medium (with EDTA)
14	pH range:	≥ 5 but ≤ 10
15	Dilution factor:	0.5 or 0.3
16	Test duration:	72 h
17	Effect measured:	Growth (cell division) inhibition
18	Test acceptability:	At least $4x10^5$ cells/mL in the controls after 72 h. Variability in the controls not to exceed 20%.

[redacted]

3 RESULTS AND DISCUSSION

3.1 Chronic Toxicity of Milli-Q Freshwater Leachate to Selenastrum capricornutum

The effect of the 0.22 µm-filtered leachate on the growth of the green alga *Selenastrum capricornutum* is shown in Table 5.

3.1.1 Chemical analyses

The Milli-Q water leachate of the modified clay had a pH of 7.8 and a conductivity of 3 µmhos/cm.

3.1.2 Quality assurance

The mean cell yield in the controls was in excess of 10^6 cells/mL, which met the test acceptability criteria. The pH of the controls throughout the 72-h test ranged from 7.2 to 9.0.

3.1.3 Leachate toxicity

The effect of the 0.22 µm-filtered leachate on the growth of the green alga *Selenastrum capricornutum* is shown in Table 5. The leachate was not toxic to algal growth over 72 h. The mean growth of algae in all dilutions of the Milli-Q water leachate was significantly higher than the controls. The maximum growth, which occurred at a 50% dilution of the leachate, was 2.3 times the growth of the controls.

Table 5. Toxicity of the Milli-Q Water Leachate of Modified Clay to the Green Alga *Selenastrum capricornutum*

Sample dilution (%)	Mean Algal Cell Yield		CV (%)
	(cells/mL x 10^4)	(% of Control)	
Control	109	100	17.4
6.25	199[a]	183	15.2
12.5	207[a]	190	4.6
25	227[a]	210	4.5
50	247[a]	228	5.3
100	245[a]	225	1.7

[a] Significantly stimulated compared to controls

3.2 Acute Toxicity of Milli-Q Freshwater Leachates to Ceriodaphnia dubia

The filtered and ultrafiltered leachates of the modified clays were assessed for toxicity to the cladoceran *Ceriodaphnia dubia* . Results are presented in Table 6, with raw data and Toxcalc statistics reports in Appendix B.

3.2.1 Chemical analyses

The pH of the filtered and ultrafiltered Milli-Q leachates was 7.13 and 4.25 respectively, with a conductivity of 101 µS and 94% dissolved oxygen. Both leachates were adjusted to pH 7.0 prior to commencement of the toxicity testing.

The filtered leachate contained 396 µg La/L, while the ultrafiltered leachate contained 83 µg La/L. Both process blanks contained 5 µg La/L.

3.2.2 Quality assurance

The pH in the controls ranged from 7.85-7.99, the conductivity ranged from 138-146 µS, dissolved oxygen from 99-103% and the temperature was 25±1^{o}C.

The mean survival in the synthetic softwater controls after 48 h was 90% in the filtered leachate and 100% in the ultrafiltered leachate, indicating test acceptability. However, survival in the Milli-Q water controls was 0%, possibly due to the water's low conductivity (3.7-13 µS).

A copper reference toxicant test was conducted using copper concentrations of 3.5, 7 and 14 µg/L. The 48-h EC50 for copper was 10 µg/L (95% confidence limits of 8-14%), within the normal range of 5.7 ± 4.3 µg/L, indicating test acceptability.

3.2.3 Leachate toxicity

Ceriodaphnia dubia did not survive in the Milli-Q water process blanks nor in Milli-Q water controls, suggesting that waters with conductivity lower than 100 µS were not suitable for maintaining this cladoceran. The pH was also lower in the Milli-Q controls and process blank (7.0) than in the synthetic softwater controls (7.9).

Poor survival in the Milli-Q water controls makes interpretation of the toxicity of the filtered leachate difficult. At low leachate concentrations (6.25 and 12.5%), in which the conductivity was 133-140 µS, the water fleas' survival rate was >90%. At concentrations of leachate of 75-100%, no water fleas survived over 48 h, despite the fact that the conductivity was close to 100 µS. This suggests that some chemical component of the leachate was toxic at these higher concentrations. The 48-h LC50 was 10% leachate with 95 % confidence limits of 8-14%. A similar trend was found after a 24-h exposure, except that toxicity of the leachate was reduced at shorter exposure times (see Table 6).

The ultrafiltered leachate was even more toxic. *Ceriodaphnia* survival was reduced at all leachate concentrations and in the Milli-Q water process blanks. Reliable LC50 values with 95% confidence limits could not be determined due to the non-dose-response relationship.

Table 6. Toxicity of Filtered and Ultrafiltered Milli-Q Clay Leachate to *Ceriodaphnia dubia*

Sample (%)	No. Surviving		Survival (% of Control)	
	24 h	48 h	24 h	48 h
Filtered Leachate				
Control	19[a]	18	100	100
Milli-Q Control	0	0	0	0
Process Blank	3	0	6	0
6.3	19	19	100	106
12.5	20	18	105	100
25	19	14	105	78
50	17	2	89	11
75	13	0	68	0
100	0	0	0	0
Ultrafiltered Leachate				
Control	20	20	100	100
Milli-Q Control	1	0	5	100
Process Blank	2	0	10	0
6.3	15	6	75	30
12.5	14	5	75	25
25	15	9	75	45
50	18	13	90	65
75	12	2	60	10
100	13	1	65	5

[a] The total number of organisms was 20 for each treatment.

3.3 Acute Toxicity of Synthetic Softwater Leachates to *Ceriodaphnia dubia*

The filtered and ultrafiltered leachates of the modified clays were assessed for toxicity to the cladoceran *Ceriodaphnia dubia* (Table 7). Raw data and Toxcalc statistics reports are given in Appendix C.

3.3.1 Chemical analyses

The pH of the filtered and ultrafiltered softwater leachates was 4.56 and 4.76 respectively, with a conductivity of 179 µS and 94% dissolved oxygen. Both leachates were adjusted to pH 7.0 ± 0.1 prior to commencement of the toxicity testing.

The filtered leachate contained 163 µg La/L, while the ultrafiltered leachate contained 156 µg La/L. Both process blanks contained <1µg La/L.

3.3.2 Quality assurance

The pH in the controls ranged from 7.48-7.99, the conductivity ranged from 152-160 µS, dissolved oxygen from 96-99% and the temperature was 25±1^{o}C.

The mean survival in the synthetic softwater controls after 48 h was 93%, indicating test acceptability.

A copper reference toxicant test was conducted using copper concentrations of 3.5, 7 and 14 µg/L. The 48-h EC50 for copper was 7 µg/L (95% confidence limits of 6-9%), within the normal range of 5.7 ± 4.3 µg/L, indicating test acceptability.

3.3.3 Leachates

The filtered leachate process blank was not toxic to *Ceriodaphnia*, with 95% survival after 48 h, similar to the controls. Leachate concentrations of up to 25% had no detrimental effect on *Ceriodaphnia* survival over 48 h. Concentrations of leachate of 50-100% were toxic over 48 h, with a 48 h LC50 of 49% leachate (95 % confidence limits of 41-58%). The filtered leachate was less toxic over 24 h, with an LC50 of >100% leachate.

The ultrafiltered leachate was more toxic than the filtered leachate. Some toxicity may have been introduced from the ultrafiltration procedure as *Ceriodaphnia* survival in the process blank after 48 h was reduced to 59% of the controls. It is also possible that ultrafiltration removed colloidal material necessary for optimal *Ceriodaphnia* survival. Low concentrations of leachate (6.3-12.5%) were not toxic after a 48-h exposure, whereas no cladocerans survived at concentrations of 75% and above. The 48 h LC50 was 36%, with 95% confidence limits of 31-42%. The ultrafiltered leachate was less toxic over shorter exposure times, with a 24 h LC50 of 100%.

3.3.4 Summary

The softwater leachate was of low acute toxicity to the cladoceran *Ceriodaphnia dubia.* Dilutions of leachate of 1:8 would be sufficient to remove this acute toxicity. Toxicity of the leachate was not due to colloidal material, as removal of this fraction by ultrafiltration through a 0.1 µm membrane filter, did not reduce toxicity.

Table 7. Toxicity of Filtered and Ultrafiltered Softwater Clay Leachate to *Ceriodaphnia dubia*

Sample (%)	No. Surviving		Survival (% of Control)	
	24 h	48 h	24 h	48 h
Control	39[a]	37[a]	100	100
Filtered Leachate				
Process Blank	19	19	97	103
6.3	20	20	103	108
12.5	20	20	103	108
25	20	19	103	103
50	20	9	103	49
75	18	4	92	22
100	17	2	87	11
Ultrafiltered Leachate				
Process Blank	18	11	92	59
6.3	19	20	97	108
12.5	20	19	103	103
25	19	16	97	86
50	18	4	92	22
75	13	0	67	0
100	10	0	51	0

[a] Total number of organisms was 40. For all other treatments, the total number of organisms was 20.

3.4 Chronic Toxicity of Softwater Leachate to *Ceriodaphnia dubia*

A summary of the toxicity of the filtered synthetic softwater leachate to survival and reproduction of the cladoceran over 7 days is given in Table 8 and Appendix D.

3.4.1 Chemical analyses

A separate batch of softwater leachate was prepared on 11/11/99 for the chronic *Ceriodaphnia* tests, using a new sample of modified clay. The concentration of La in this filtered leachate was much higher (2.01 mg/L). The process blanks contained 30 µg La /L.

3.4.2 Quality assurance

Water quality parameters including dissolved oxygen, pH and temperature were within acceptable limits for the test (see appendix D). Control survival was 100% and mean young produced per female in controls was 19.9 ± 3.2 over 7 days, indicating test acceptability. Using potassium chloride as a reference toxicant, the 7-day LC50 was 182 mg/L, within the acceptable range of 132-264 mg/L for the test.

3.4.3 Leachate toxicity

Survival and reproduction in the process blank (method blank) was the same as the controls indicating that no toxicity was due to the leachate preparation procedure.

Survival of the daphnids was significantly reduced over 7 days at test concentrations of 50% and 100% leachate. The 7-day LC50 was 41% leachate, with 95% confidence limits of 32-52%, similar to the acute toxicity over 48 h. The higher lanthanum concentration in this leachate compared to the leachate used in the acute test, did not affect the observed acute toxicity. There was a significant reduction in the mean number of young produced at all concentrations tested, with a LOEC of 6.25% leachate. The NOEC was <6.25% (the lowest concentration tested).

Table 8. Chronic toxicity of softwater clay leachate to *Ceriodaphnia* survival and reproduction over 7 days

Test Treatment (% Leachate)	% Survival over 7 Days	Mean (±SD) Young Produced
0 (Control)	100	19.9 ± 1.6
0 (Method Blank)	100	19.5 ± 3.2
6.25	100	14.9 ± 3.9[a]
12.5	100	10.9 ± 4.1[a]
25	90	6.4 ± 3.4[a]
50	30[a]	0.4 ± 0.8[a]
100	0[a]	0[a]

[a] significantly different to the control at alpha =0.05 by Dunnett's test

3.5 Acute Toxicity of Softwater Lachates to Juvenile Eastern Rainbow Fish

Details of the toxicity test results are given in Appendix E.

3.5.1 Chemical analyses

A separate batch of softwater leachate was prepared for the acute rainbow fish. The concentration of lanthanum in this filtered leachate was 127 µg/L. The process blank contained less than 10 µg La /L.

3.5.2 Quality assurance

The physico-chemical measurements of the test solutions including pH, conductivity, dissolved oxygen and temperature were within acceptable limits for the test (see Appendix A). Imbalance in the control fish was 0% over 96 h, indicating test acceptability.

3.5.3 Leachate toxicity

The filtered leachate was not toxic to juvenile eastern rainbow fish. Imbalance in 25% of juvenile fish was observed in the 100% leachate concentration over the 96-h test period (Table 9), however this was not significantly different to the control (t-test, $p>0.05$). The EC50 value was greater than 100% leachate, with a NOEC of 100%.

Table 9. Cumulative Percent of Imbalanced Fish Exposed to the Test Solutions and Controls at 24-h Intervals over the 96 h Test Period.

Test Concentration	Cumulative % Imbalanced Fish			
(% Leachate)	24 h	48 h	72 h	96 h
Control	0	0	0	0
Process Blank	0	0	0	0
12.5	0	0	0	0
25	0	0	0	0
50	0	0	0	0
75	0	0	0	0
100	0	0	0	25

3.6 Implications for Application of the Modified Clays to Aquatic Systems

Modified clay leachates were not acutely toxic to juvenile eastern rainbow fish. Clay leachates were also not toxic to *Selenastrum capricornutum* in chronic 72-h growth inhibition tests. Significant stimulation of algal growth compared to media controls was observed.

Freshwater leachates of the modified clay showed acute and chronic toxicity to *Ceriodaphnia dubia*. Compounds in the leachate responsible for toxicity were not identified. Ultrafiltration of the leachate, however, showed that toxicity was not associated with colloidal material leached from the clays.

Lanthanum is a rare earth element that is leached in small amounts from the clay in freshwater. The toxicity of lanthanum is due to its ability to displace calcium in various cell processes and due to its high affinity for phosphate groups of biological macromolecules. Calcium uptake and transport are reversibly inhibited in a range of organisms including human red cells, algae and yeasts (Sarkadi *et al*, 1977, Hader 1982). Lanthanum can also block the release of serotonin in the branchial nerve of the mussel *Mytilus edulis* (Das *et al*., 1988).

The lanthanum concentration at the leachate acute 48-h LC50 value (49%) for *Ceriodaphnia* was 80 µg/L, much lower than the concentrations of 2.8 and 1.6 mg/L of lanthanum chloride reported to immobilise the cladoceran *Daphnia magna* after a 72-h or 96-h exposure respectively (Peterson *et al*, 1974). These concentrations are orders of magnitude higher than that found in the softwater clay leachate prepared under standard batch conditions. Unless *Ceriodaphnia* dubia is particularly sensitive compared to *Daphnia magna*, it is unlikely that lanthanum is the cause of the leachate's toxicity. Lanthanum, however, appears to be more toxic to coldwater fish species including rainbow trout and salmon, after long exposure durations e.g. 28-day LC50 of 20 µg/L lanthanum chloride (Birge, 1978).

Typical clay application rates to waterways would be around 1.3 kg/m^2/year, resulting in approximately a 0.5 mm-1 mm layer of clay on bottom sediments (Douglas, pers comm). Assuming a 1% uptake capacity of the clay for phosphorus, for every 10 µg P/L in the water column, 1 mg/L of modified clay would be added. Phosphorus concentrations in typical freshwaters range from <10µg/L to 100 µg/L, with severely eutrophic systems up to 1000 µg P/L. Using this worst case scenario, a predicted

maximum estimated environmental concentration (EEC) of the clay in the water column would be 100 mg/L.

A preliminary estimate of acute risk can be made using the hazard quotient (Q) method where:

$$Q = \frac{\text{estimated environmental concentration}}{\text{effect concentration}}$$

where the effect concentration is the 48-h LC50 for the most sensitive freshwater test species (in this case the cladoceran).

Thus $Q = 0.1\ g/24.5\ g = 0.004$

Because Q is less than 0.1, there is little risk of acute toxicity to freshwater organisms. Even if a safety factor of 2-20 is applied to the LC50 value (Sydney Water, 1995), the hazard quotient is still less than 0.1.

To calculate potential chronic risk, the maximum EEC of 0.1 g/L is divided by the chronic NOEC from the *Ceriodaphnia* test. The NOEC must be estimated as the LOEC (3.1 g/L) divided by an extrapolation factor of 10. The resulting hazard quotient is 0.3. However, the EEC represents a maximum value and for chronic exposures may be substantially less. Nevertheless, there may be some risk of chronic toxicity to freshwater invertebrates but this would have to be examined in more detail in field trials of the modified clay.

[redacted]

4 RECOMMENDATIONS FOR FUTURE WORK

1. Ecotoxicity tests should be carried out in conjunction with field trials of clay application to provide more data on the potential acute and chronic risk to freshwater biota.

2. The clays may also cause physical irritation e.g to fish gills. A toxicity test with juveniles or adult fish species in which the clay is sprinkled over the surface of overlying water may test for these physical effects. The settling rates of the clay could also be determined.

3. Apart from toxicity, the clays have the potential to cause physical disturbances to sediment dwelling biota. For example, a reduction in microbial activity could deplete sediments of food sources for burrowing animals and for benthic organisms at the sediment water interface. Appropriate sediment toxicity tests may include whole sediment tests with the burrowing amphipod Corophium sp, polychaete worms or freshwater mayflies.

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Appendix A

Toxicity of Modified Clay Leachate to Juvenile Eastern Rainbow Fish

APPENDIX B

Acute Toxicity of Milli-Q Leachates to *Ceriodaphnia dubia* – Raw Data and Toxcalc Reports

APPENDIX C

Acute Toxicity of Softwater Leachates to *Ceriodaphnia dubia* – Raw Data and Toxcalc Reports

APPENDIX D

Chronic Toxicity of Softwater Leachate to *Ceriodaphnia dubia*