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MOUNT LYELL REMEDIATION

Evaluation of rehabilitation options for Mount Lyell using whole-effluent toxicological tests on freshwater organisms

Christopher Humphrey, Shelley Templeman, Caroline Camilleri & David Klessa

Mount Lyell Remediation Research and Demonstration Program

a Tasmanian and Commonwealth Government initiative

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Department *of* Environment and Land Management

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A joint program between the Supervising Scientist and the Department of Environment and Land Management, Tasmania.

This report describes research that is part of the Mt Lyell Remediation Research and Demonstration Program, a joint program between the Supervising Scientist and the Department of Environment and Land Management, Tasmania.

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Executive summary

A century of continuous copper mining and processing at the Mount Lyell Mine, Queenstown, Tasmania, has resulted in severe environmental impacts both in the mine area and off-site. Amongst the impacts arising from discharge of metal-enriched acid drainage, slag and tailings are the pollution of fresh and marine waters and the deposition of tailings and slag in Queen and King Rivers and Macquarie Harbour.

Part of the objective of the Mount Lyell Remediation Research and Development Program (MLRRDP), established in 1995 as a joint Tasmanian and Federal Government program to develop a strategy for remediating the environmental effects of past mining at Mount Lyell, is to assess the effectiveness of various remediation options in enabling return of life to the polluted Queen and King Rivers. Results of toxicity tests on freshwater organisms may be usefully applied to this problem but proposals for such study were initially discounted as it was anticipated that future concentrations of copper in the receiving waters downstream of the mine would be well above those concentrations for which, based on published guidelines, aquatic life could be sustained and protected (eg ANZECC 1992).

Interim results from other MLRRDP projects and associated studies, however, suggested that these original premises may not have been correct; thus (i) copper concentrations would be reduced substantially in the lower King River (to ~ 20–60 µg/L) if 90% of the acid drainage was treated by neutralisation (DELM, unpub), and (ii) ameliorative effects were observed amongst marine organisms exposed to Macquarie Harbour waters containing 10–42 µg Cu/L — a result, it was suggested, of copper adsorption to colloidal iron, manganese and aluminium oxides/hydroxides (Stauber et al 1996).

The impetus for carrying out an initial investigation of freshwater toxicity was to determine whether the countering of acid mine drainage at source, in association with any possible ameliorative effects present in freshwaters of the Queen and King Rivers downstream of the Mount Lyell mine, would allow biological recovery of the Queen and King Rivers to take place. Results using 'whole-effluent' toxicity testing techniques could then be used to estimate the effectiveness of various remediation options canvassed to reduce acid drainage from mining operations at Mount Lyell, including neutralisation, in allowing the return of aquatic life to the Queen and King Rivers. Specifically, the project was designed to estimate the percentage of acid mine drainage that would be required to be neutralised with lime to produce an effluent mix in which aquatic life could survive.

Two temperate cladoceran species, *Daphnia carinata* and *Ceriodaphnia dubia s. l.*, were tested initially using various ratios of neutralised (to pH 6.5) to raw Mount Lyell mine acid drainage, 65:35, 80:20 and 95:5, each serially diluted with West Queen River water. Both cladoceran species proved intolerant of the soft naturally-acidic diluent water though one partially-successful test using *C. dubia s. l.* established that all concentrations of 65:35 and 80:20 neutralised acid drainage water were toxic to test organisms, resulting in 100% (or near) mortality. From these preliminary results it was concluded that the options of 65:35 and 80:20 neutralisations would be unlikely to support the recovery of aquatic life to the King and Queen Rivers.

Further toxicity tests were conducted using test species that occur naturally in soft acidic stream waters. Two species, a cladoceran, *Moinodaphnia macleayi*, and a freshwater cnidarian, *Hydra viridissima*, were assessed for their ability to survive and breed in West Queen River water. Eventually, *M. macleayi* like the previous cladoceran species used,

proved intolerant of West Queen River water though *H. viridissima* reproduced successfully in this water at 20°C.

On the basis of earlier results, the neutralisation regimes were changed to 95:5 and 99:1 for further testing of *Hydra viridissima.* Four *H. viridissima* population growth tests, including two initial range finding tests, were conducted using a range of concentrations from 95:5 and 99:1 neutralisation regimes. The NOEC, LOEC and EC_{50} for each neutralisation regime were derived from pooled test data with copper concentrations corresponding to these test end-points shown to be very similar. Averaged between the two neutralisation regimes, copper concentrations corresponding to NOEC, LOEC and EC_{50} were ~15, 18 and 28 µg/L respectively.

Using the results of another MLRRDP project, it was shown that, of the constituents present in neutralised mine effluent, projected Cu concentrations would probably be most limiting to biological recovery in the receiving waters downstream of the Mount Lyell mine. The responses of the cladoceran *Ceriodaphnia dubia s. l.* and cnidarian *Hydra viridissima* to copper present in neutralised mine effluent were generally consistent when compared with each other (NOECs of ~12 and 15 µg/L respectively) and with other *C. dubia* data. Equivalent NOEC values for Cu found in this study were generally higher than guideline values from Australia and elsewhere. Nevertheless, the results indicate, in contrast to those reported for the marine environment (Macquarie Harbour), that no comparable ameliorative effects were present in the stream waters around the Mount Lyell mine that would support the viability of any suggested remediation options for assisting recovery of aquatic life.

In a separate study establishing concentration boundary limits for metals, including Cu and Al, within the King and Queen Rivers under a variety of flow conditions and scenarios of mine effluent treatment (principally neutralisation), the greatest potential for biological recovery was shown to be in the King River upstream of the delta and the least, at the confluence of Haulage Creek with the Queen River. The lowest projected concentration of total soluble Cu in the Queen River under various acid neutralisation regimes was an estimate of 366 µg/L (Klessa et al 1997), far exceeding the equivalent NOEC value (~15 µg/L) found in this study. None of the neutralisation options, therefore, would facilitate the return of life to the Queen River.

For the King River, the maximum protection afforded to aquatic organisms was that prevailing under a scenario of 99% neutralisation (to pH 6.5) of acid drainage and maximum dilution with the power station below Lake Burbury operating; under these conditions projected Cu concentration would approach 10 µg/L (Klessa et al 1997). However, with the power station not operating (12 hours in a 24 hour cycle), estimates of Cu concentration in the King River would range between 74 and 235 µg/L. Because the NOEC value is intermediate between these diurnal ranges, the potential may be present for some partial recovery of life in the King River. Additional toxicity tests employing pulsed and episodic exposure of test organisms to neutralised acid drainage would be required to resolve this issue.

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1 Introduction

For a century, the Mount Lyell Mine at Queenstown, Western Tasmania (fig 1.1), was the site of continuous copper mining and processing. Over this period, mining and processing operations resulted in severe environmental impact both in the immediate mine area and well off-site (~50 km^2). Amongst the impacts arising from discharge of acid drainage and tailings are changes in water quality and deposition of tailings and slag in rivers (Queen River and tributaries, particularly Haulage Creek, and King River) and Macquarie Harbour (McQuade et al 1995, Taylor et al 1996). In 1994, a new operator, Copper Mines of Tasmania (CMT), took over the mine. CMT operates in accordance with a modern environmental management plan and to this end has constructed a dam to contain tailings. Acid drainage rich in metals, however, remains a major problem.

In 1995, the Tasmanian and Federal Governments established a joint program to develop a strategy for remediating the environmental effects of past mining at Mount Lyell. The Mount Lyell Remediation Research and Development Program (MLRRDP) has comprised 14 projects to investigate the extent and mechanisms of the environmental impacts which have resulted from mining activities, as well as to set remediation goals for the Queen and King Rivers. Projects have extended across three broad categories:

(i) remediation options to reduce acid-drainage from the Mount Lyell mine lease site;

(ii) studies of the Queen and King rivers, currently polluted with acid drainage and tailings deposits, to provide a basis for assessing effects of various rehabilitation options;

(iii) studies dealing with the tailings deposits in Macquarie Harbour.

One of the MLRRDP projects (9a, SSR 112, Stauber et al 1996) used a range of toxicity tests to determine the concentration and species of copper which could be tolerated in Macquarie Harbour waters without causing detriment to marine life. Well into the MLRRDP, however, no analogous toxicity studies had been instigated for freshwater ecosystems in the vicinity of the Mount Lyell mine. The rationale for the lack of such studies was that anticipated future concentrations of copper in the receiving waters downstream of the mine would be well above those concentrations for which, based on published data, aquatic life could be sustained and protected (eg ANZECC 1992).

Interim results from other MLRRDP projects and associated studies, however, suggested that these original premises may not have been correct and provided a case for pursuing freshwater toxicity studies. Findings from 2 studies were pertinent here:

(i) Preliminary estimates of copper concentrations in receiving waters if 90% of the acid drainage was treated by neutralisation ranged between 20 and 60 µg/L in the lower King River, depending upon river flows and the operation of the hydro-electric power station (operating below Lake Burbury) (DELM, unpub). These values approach concentrations at which aquatic life might be expected to return to streams if water quality alone is the determining factor.

(ii) Interim results from Project 9a (SSR 112, marine toxicity testing) showed that the toxicity of Cu to marine organisms was lower than expected. Thus, few adverse effects were observed amongst microalgae, amphipods and juvenile flounder upon exposure to Macquarie Harbour waters containing 10–42 µg Cu/L and it was suggested (Stauber et al 1996) that this could be the result of copper adsorption to colloidal iron, manganese and aluminium oxyhydroxides.

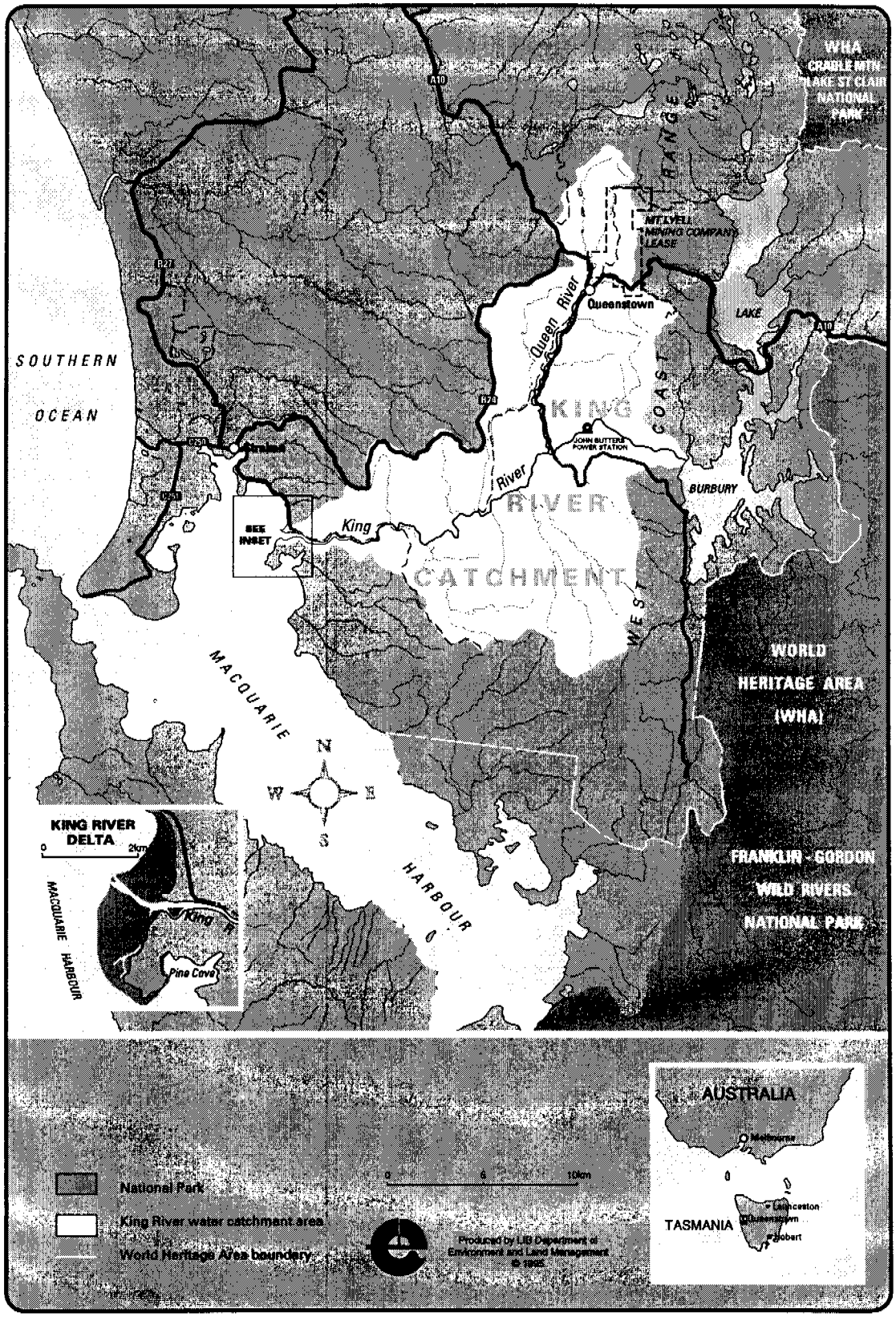

Figure 1.1 Map of the study area around the Mt Lyell mine site in the south-west of Tasmania

On the strength of these results, it was decided to carry out an initial investigation of freshwater toxicity to determine whether the countering of acid mine drainage at source, in association with any possible ameliorative effects present in freshwaters of the Queen and King Rivers downstream of the Mount Lyell mine, would allow biological recovery of the Queen and King Rivers to take place. Results using 'whole-effluent' toxicity testing techniques could then serve to estimate the effectiveness of various remediation options canvassed to reduce acid drainage from mining operations at Mount Lyell (Miedecke et al 1996), including neutralisation, in enabling the return of aquatic life to the Queen and King Rivers. Specifically, the project was designed to determine the percentage of acid mine drainage that would be required to be neutralised (with lime) to produce an effluent mix in which aquatic life could survive.

2 Project design

2.1 Introduction

2.1.1 Rationale

Options for using toxicity tests to derive water quality remediation goals

Three options were available by which suitable laboratory toxicity tests could be carried out upon freshwater organisms. The first of these was a strictly empirical approach using synthetic waters whilst the remaining two employed 'whole-effluent testing'. These approaches are as follows:

(i) Strict laboratory control of test variables using synthetic waters

This entails making up an artificial water (diluent) and testing in turn single metals and metal mixtures. The advantages of this approach (where metals, pH and organic carbon are controlled and where metal speciation is predicted) can include close mimicking of effects observed using natural waters and isolation of key conditions resulting in toxicity (eg Markich & Camilleri 1996). For Mount Lyell mine effluent, however, this testing approach was regarded as problematic. Apart from the usual problems of replicating a complex natural material, there was the perceived difficulty of incorporating in the laboratory, biotic reactions that would normally be present in the natural setting. In the case of manganese and iron, the most significant oxidation reactions in natural systems are bacterially mediated, particularly at pH less than 7. Mn oxyhydroxides will not precipitate abiotically at pH<7 and therefore would not be expected in synthetic solutions at this pH range. (Microbiologically-induced oxidation was proposed by CSIRO researchers (Project 9a) to ameliorate Cu toxicity to marine organisms—see Stauber et al (1996)). In this respect synthetic-water toxicity tests may give conservative results. A further disadvantage of the approach lies in the potentially large testing matrix (and therefore time and other resources) required to generate useful data.

(ii) Whole-effluent testing conducted in Queenstown

Whole-effluent testing has the advantage of testing actual waters with least ambiguity/equivocation of results. Conducting the tests in Queenstown would have the advantages of minimal delay between water collection and testing, and no major limitation on water availability (and hence provision for rapid turnover of test waters). These factors were regarded as particularly important if it was found that transport and holding of waters over large distances resulted in alteration of water chemistry (especially rises in pH)—see option (iii) below.

(iii) Testing conducted away from Queenstown

Transport and holding of waters over large distances can alter water chemistry in response to the new environment in which the water is stored. For example, the establishment of new equilibria between solids (precipitates, suspended solids or container surfaces), solution and gases may alter the composition of each phase and potentially influence speciation. For this reason whole-effluent testing conducted 'on-site' (in this case, in Queenstown), thereby minimising the time between collection and testing, is often preferred since it leads to the least problems in interpretation of results.

In the study reported here, the chemical composition of future possible mine effluents was approximated. Thus, processes by which Cu and other metals might be removed from, and acidity countered in, acid mine drainage (AMD) were simulated by neutralisation and the implications for recovery of the ecological health of the Queen and King Rivers when mixed proportionally with raw AMD and clean river water (ie diluent) examined.

In this case, possible changes to the chemistry of the waters to be used arising from holding time were considered to be of minor importance. Protocols were established for the preparation of test solutions to minimise, and to counter if necessary, any change, in water chemistry—particularly pH increase for the diluent. Early trials conducted by DELM established, before any testing had commenced, that natural changes in chemistry of test waters for a holding time of about one week (the average period of time from collection to completion of a toxicity test) were negligible. These results appeared later to be vindicated (section 2.4.1, table 2.1) and counter-procedures to alter water chemistry during toxicity tests were never required.

Hence, there were no serious biological or chemical constraints which were thought to demand that toxicity testing took place in Queenstown. Rather, there were no established laboratory facilities in Queenstown and, while temporary facilities could be established, it was deemed time-consuming to ensure that quality assurance needs could be met. Of the other suitable locations in temperate Australia possessing the facilities to undertake this toxicity testing program, the Australian Nuclear Scientific and Technology Organisation (ANSTO, Sydney) was approached and agreed to use of its laboratories.

Time and funding constraints for the freshwater toxicity tests conducted in the project meant that the scope of toxicity testing (described in the following sections) was necessarily limited. It was recognised at the onset, therefore, that the results obtained would only provide a first-pass estimate of the water quality remediation goals for the Queen and King Rivers.

Selection of test protocol

Freshwater toxicity testing in Tasmania has only occasionally been conducted, with previous toxicity work in the King River catchment being limited to an assessment of copper toxicity on trout in Lake Burbury by the Tasmanian Inland Fisheries Commission (IFC). Ninety-six hour LC50 values were determined using rainbow trout acclimated and tested in King River water. The results indicated an LC50 value of 0.09 mg/L copper (IFC 1995).

Though toxicity tests were not conducted in Tasmania, there was more relevance to such testing if it was conducted using test organisms that occurred in freshwaters of Tasmania. At the time of initial planning, there were protocols available for two test organisms both of which were relevant and applicable to temperate southeastern Australia, including freshwaters of Tasmania: acute and/or chronic, lethal and sublethal toxicity tests using rainbow trout and a chronic sublethal toxicity test using the water flea, *Daphnia carinata*. Trout are not native to Tasmania though they have been acclimated in Tasmanian streams for

over a century. *D. carinata* occurs in ephemeral coastal wetlands of northeastern Tasmania (R Walsh, University of Tasmania, pers comm). As a consequence of time and budgetary constraints, it was only possible to conduct tests using one test species, the water flea, *D. carinata*. The rationale behind this selection lay mainly in the minimal infrastructure and equipment required for testing. Serious constraints in conducting tests using rainbow trout lay in the provision of a large laboratory space for tanks and in collections of large volumes of test waters for fish exposure.

Temperature regime for testing

Tests were conducted at ANSTO between June and July 1996. Ambient surface water temperatures in streams of western Tasmania range from about 5°C in winter to 15°C in summer, ie the temperature range within which environmentally-relevant testing would normally be conducted. Toxicity protocols for *Daphnia carinata* have been developed for water temperatures at 18°C or greater and as a consequence survival/ reproductive responses of the organism at water temperatures below this threshold are unknown. Rather than risk possible test failures by conducting tests below 18°C it was decided to proceed with the tests at or slightly above this temperature, ie the lower temperature limit for which viable protocols have been developed.

The consequences to water chemistry and toxicity of such a 10–13°C (at the most) rise to13°C water temperature above ambient were assessed. The most significant change to water chemistry with such a temperature rise was likely to be the faster rate of Fe oxidation. Iron hydroxide is likely to act as an absorbent for metals, particularly Cu, above pH 5 (having the effect of possible reduction in toxicity in these treatments). This was taken into account in the preparation of protocols and, where necessary, in the interpretation of results. As a general rule, toxicity responses of aquatic organisms to acute exposures of metals increase with water temperature. However, there is some evidence from the literature to indicate that temperature may not alter results significantly at chronic exposures (Sprague 1985)—of relevance to much of the testing proposed here.

Details of the treatments to be used in the tests are provided below (section 2.2).

2.1.2 Problems

Sydney testing phase

Stocks of *Daphnia carinata* were successfully acclimated at the Centre for Ecotoxicology (CfE) (University of Technology, Sydney) and ANSTO for eight weeks prior to the commencement of testing using an initial single batch of West Queen River water (diluent). However, this period of acclimation coincided with atypically low rainfall conditions in south-western Tasmania with resultant low flow in the West Queen River. The relatively greater contribution of groundwater inflow to streams under these conditions was sufficient to alter the water quality of the river significantly, raising the pH from 5.5 to 6.0 to approximately pH 7.3. At the time, the effect of the high pH—reflected in the acclimation water collected at this time—was not considered to be a major problem with culturing of the animals.

The commencement of the toxicity testing program at ANSTO coincided with the beginning of winter rains in Tasmania. In response, flow rates and water quality of south-west Tasmanian streams returned to more typical ranges (in particular, lower pH in the range 5.5 to 5.9). Consequently, both cladoceran stocks at CfE and those established at ANSTO concurrently declined shortly after exposure to a second batch of West Queen River collected at this time.

D. carinata was abandoned as a test species after exhibiting stress to the soft, poorly-buffered, low pH waters of the West Queen River whilst an alternative temperate species of water flea, *Ceriodaphnia dubia*, was cultured in the diluent water to determine its suitability. *C. dubia* stock was procurred from the CfE. The identification of this animal is provisional and hence it is presently named *Ceriodaphnia dubia sensu lato.* This species has not been identified in Tasmania but is otherwise reasonably cosmopolitan being found in countries of both world hemispheres, including temperate Australia and New Zealand (M Julli, pers comm, Greenwood et al 1991). In North America, *C. dubia* is used extensively as a toxicity test organism. Copper toxicity tests have been carried out by a number of authors using *Ceriodaphnia dubia* in waters that are slightly acidic but generally better buffered than West Queen River water (Cowgill & Milazzo 1991a,b,c; Winner & Owen 1991) (table 3.4).

The life span of *Ceriodaphnia dubia s. l.* is much shorter than *Daphnia carinata* and reproduction is quite rapid (~ 48 hours elapsing from neonate to production of first brood, compared with 5 days for *D. carinata*, at water temperature of 20°C). A stock of *C. dubia s. l.* was cultured in West Queen River water (of typically low pH) for 10 days, until F_1 generation animals had produced a second brood. Neonates from this brood were subsequently used in 48-hour range-finding toxicity tests (section 2.3.1). However, high control mortality was observed by 48 hours of testing, believed to be the result of stress due to lack of food over the test duration. Additional tests were attempted using *C. dubia s. l.* with and without food supplements but a consistent gradual loss of stock vigour (ie poor brood production and high adult mortality) occurred in each trial. The same physiological stress inferring intolerance of *D. carinata* to soft, low pH water appeared also to be a feature of *C. dubia s. l.* stocks.

Jabiru testing phase

As a result of test failures, the testing program at ANSTO ceased in late July and a decision was made to attempt to carry out a modified testing regime at ***eriss*** (Jabiru) in the Northern Territory. It was thought likely that test species applied in the toxicity testing program at ***eriss*** could successfully be applied to waters from south-western Tasmania because of the similar nature of receiving waters in the two regions—soft, acidic waters. Thus, the NT species used at ***eriss*** are largely pre-adapted to waters otherwise stressful to cladocerans used in toxicity testing programs elsewhere. At ***eriss***, two species of aquatic animals, a tropical-subtropical species of cladoceran, *Moinodaphnia macleayi*, and a freshwater cnidarian, *Hydra viridissima*, were gradually acclimated to a lower temperature regime (20°C as opposed to the normal 27°C). *M. macleayi* has been collected as far south as central NSW (Shiel & Dickson 1995). Little work has been undertaken on the distribution and identification of *Hydra* in Australia, but *Hydra viridissima* is said by Williams (1980) to be widespread. Unidentified *Hydra* species have been collected in many regions in southern Australia including Tasmania (J Bradbury, University of Adelaide, and P Davies, University of Tasmania, pers comm).

After establishing that both test species at ***eriss*** could be adequately cultured at the lower temperature regime and a test duration period determined accordingly, they were slowly acclimated to a new batch of diluent (West Queen River) water.

Moinodaphnia macleayi appeared initially to be a suitable test species in West Queen River water as it acclimated to the water with little mortality or reduced fecundity. F_1 generation animals produced comparable brood sizes to animals cultured in local (Magela Creek) waters. However, the reproductive cycle of *M. macleayi* was significantly longer (4 days to Brood 1 and ~50 hours between subsequent broods at 20°C, cf 2 days to Brood 1 and approximately

26 hours between subsequent broods at 27°C) at the lower temperature in both Magela Creek and West Queen River water. The slower reproductive rate raised concerns about the feasibility of using *M. macleayi* in reproduction tests as the average life span for this species is 10 to 12 days and there was evidence that the life span at the lower water temperature was no different from that at the higher temperature. With a 10 to 12 day life span at 20°C, the last brood in a 3 brood reproduction test would be produced close to the end of an animal's life raising the problem of test invalidity or false responses due to increased (natural) mortality during the test. Ultimately, more serious concerns about the viability of *M. macleayi* for testing were raised when, in subsequent generations, animals began to show similar effects to West Queen River water as was experienced with the previous cladocerans (*Daphnia carinata* and *Ceriodaphnia dubia*), with loss of adult fecundity and increased neonate and adult mortality.

With the loss of vigour in all three cladoceran species exposed to West Queen River water, it was concluded that the group in general were not suitable as test animals using such acidic soft diluents. In an extensive literature review, Havens and Hanazato (1993) showed that cladocerans of large body size, such as *Daphnia* species, are generally intolerant of low pH waters, partly because, it was speculated, the high Ca demand of such animals cannot be met in acidic soft waters. Test failures at ***eriss*** using *Moinodaphnia macleayi* occur occasionally in the Wet season (C Camilleri, unpublished data) and in these cases it is quite likely that intolerance to acidic soft waters at this time of year is the cause. Any future work contemplated using cladocerans as toxicity test species in the south-west Tasmanian region would require use of species found in local streams.

The cnidarian, *Hydra viridissima*, did not exhibit inhibitory effects when exposed to West Queen River water and after 3 weeks exposure, animals were reproducing well in the diluent water. The reproductive rate was slightly lower in diluent water but this was accepted as an effect of the lower water temperature. *H. viridissima* was successfully employed in subsequent tests using Mount Lyell mine waters diluted in West Queen River water and recommendations addressing the project objectives were made on the basis of these test results.

2.2 Test waters and neutralisation regime

2.2.1 Choice of acid drainage source and diluent for toxicity testing

A detailed description of the sources, fluxes and composition of acid drainage entering the Queen River from the Mount Lyell lease site are contained elsewhere (Klessa et al 1997; McQuade et al 1995). In addition, Klessa et al (1997) have collated data on the composition of unpolluted waters of the Queen and King subcatchments.

For the work described in this report, acid drainage was derived from a single source, namely North Lyell Tunnel (fig 2.1), whose total discharge comprises a mixture of Conveyor Tunnel pump discharge and North Lyell Tunnel discharge in the ratio 9:6 (McQuade et al 1995). Using median concentrations (MLMRCL 1995, McQuade et al 1995) the flow-weighted pH and total soluble composition (mg/L) of North Lyell Tunnel discharge is as follows: pH 2.8, total suspended solids 7020, Ca 112, Mg 336, Na 6, K 2, Cl 12, SO_4 3110, Fe 274, Mn 112, Al 157, Cu 104, and Zn 12 (Klessa et al 1997). Discharge from North Lyell Tunnel into Haulage Creek constitutes around 80% of the Cu load from the Mount Lyell Mine lease entering the Queen/King River system. The majority of this Cu is derived from Conveyor Tunnel pump discharge (~65%) with the remainder (~15%) coming from North Lyell Tunnel.

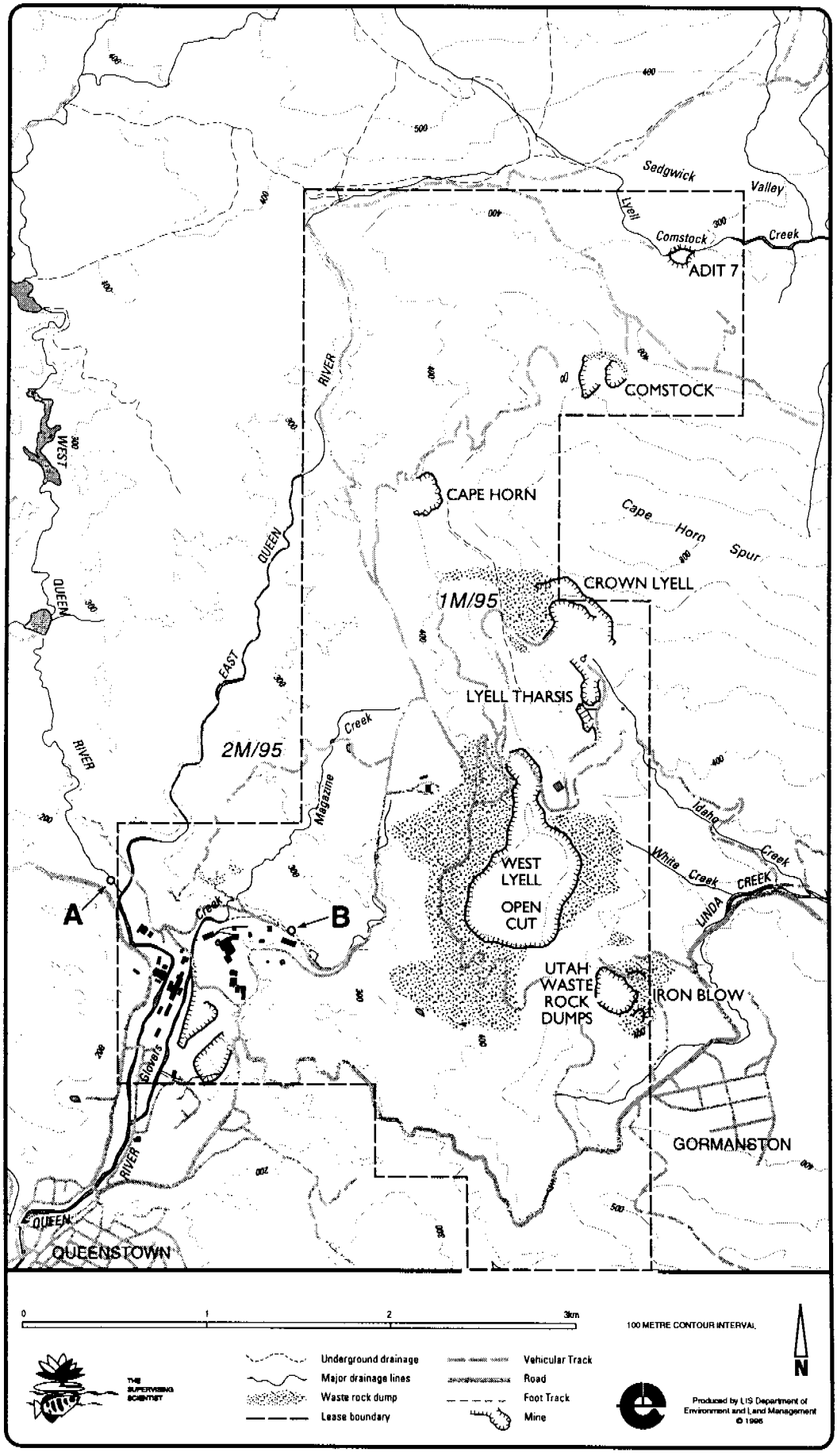

Figure 2.1 Sites in the Mt Lyell mine lease area and adjacent streams from which diluent (site A) and mine effluent (site B) waters were collected

The other principal source of Cu entering Haulage Creek is from the West Lyell waste rock area (~19%). In summary, therefore, 99% of the Cu load entering Haulage Creek comes from three sources (Conveyor Tunnel, North Lyell Tunnel and West Lyell waste rock) and collectively this constitutes 99% of all the Cu entering the Queen River. For the purposes of toxicity testing, mixing of acid drainage from the three main sources in Haulage Creek has been simplified by choosing North Lyell Tunnel discharge as representative of metal composition and acidity.

The diluent used in the toxicity tests was taken from the West Queen River (fig 2.1), an unpolluted tributary of the Queen River, whose confluence with the latter is upsteam of Haulage Creek. There is a deficiency of information on the composition of uncontaminated waters of the Queen and King catchments but for the purposes of speciation modelling, Klessa et al (1997) derived the following median total soluble composition (mg/L) based on available data; pH 5.5, Ca 1, Mg 1, Na 4, K 0.5, SO_4 3, Fe 0.03, Mn 0.01, Al 0.075, Cu 0.005, organic C 10.

2.2.2 Neutralisation of acid drainage

Because the constituents of acid drainage pose a potential limitation to the biological recovery of the Queen and King Rivers, mainly through the toxicity of pH, Cu and Al but in addition to possible constraints from F, Fe and Mn (Klessa et al 1997), remediation of the rivers will likely depend on the countering of the acid drainage by liming (Miedecke et al 1996). Since a progressive approach to neutralising specific sources of acid drainage on the Mount Lyell lease was envisaged as the key to a remediation strategy, toxicity experiments were designed accordingly. In this way, test solutions were prepared by mixing varying proportions of raw and neutralised acid drainage (pH 6.5) with diluent. A description and discussion of the products of neutralisation and the effects on the speciation of the components of acid drainage and river water once mixed and at chemical equilibrium are contained in Klessa et al (1997). Initially, the design of the toxicity experiments was based on 65, 80 and 99% of the acid drainage (expressed as Cu load) from the lease site being treated by liming to pH 6.5. These percentages represent the principal options being considered for rehabilitation by neutralisation of the various sources of acid drainage, namely:

- Conveyor Tunnel (65%),
- Conveyor Tunnel plus North Lyell Tunnel (80%),
- Conveyor and North Lyell tunnels plus the West Lyell waste rock area (99%).

Hereafter, the notation and terminology 65:35, 80:20, 99:1 (etc) neutralisation, refer to percentage proportional mixtures of neutralised to raw acid drainage.

2.3 Design of toxicity tests

2.3.1 Cladoceran tests

Daphnia carinata

OECD test protocols were adopted for the testing of *Daphnia carinata* (OECD 1984). The effect of test waters on (i) neonate survival (where neonates refer to cladocerans that are less than 24 hours old) and (ii) the reproductive output of adult water fleas, was to be measured and assessed. For (i), termed an 'acute immobilisation test', the OECD protocol covers an exposure period of 48 hours (though there may be greater sensitivity in the endpoint if this is modified to a 96-hour exposure, D Baird, Stirling University, pers comm). For (ii), reproductive output, test duration is normally 21 days and covers production of 4 to 5 broods of offspring. The measured end-point is the total number of living offspring produced per

parental animal at the end of the test. However, following recommendations from staff of the CfE (Sydney), the OECD protocol was modified by shortening the test to approximately 14 days, measuring the offspring from only three broods.

It was anticipated that the following work would be carried out at ANSTO:

1 Initial work would use the 48-hour neonate survival test to determine the appropriate range of dilutions to be used in subsequent tests (ie range finding);

2 Following from (i), 3 consecutive 48-hour neonate survival tests would be carried out, one or more of which could be modified to a 96-hour exposure test depending upon the results of previous tests and availability of time; and

3 The survival test would be followed by a 14-day reproduction test using relevant treatments as determined by the previous tests.

Though some limited 48-hour neonate survival tests were completed, no reproduction tests were carried out on any of the cladoceran species tested because of the general intolerance of the species to West Queen River water (section 2.1.2). The protocol used for the 48-hour neonate survival test is presented in Appendix A, general principles of which may be described as follows:

- The objective of the 48-hour neonate survival tests is to determine the maximum concentration at which neutralised AMD waste water has no statistically significant effect on cladoceran survival over a 48-hour period of exposure. Toxicity tests based on the 48-hour survival test, were to span a geometric range of dilutions 0, 0.3, 1, 3, 10 and 30% stock solution. The three different stock solutions treatments were to be tested concurrently with exposure waters held at 18°C.
- Asexually-reproducing (female) test cladocerans less than 24 hours old (ie neonates) are exposed at the commencement of the test to a range of concentrations of the waste water to be assessed under 'static-renewal' conditions. Observations are made after 24 and 48 hours. Each female is accounted for as alive, dead or missing, rather than assuming that all missing animals are dead. The test is terminated after 48 hours. The quantitative response is statistically analysed and the maximum no effect or no-observed-effect concentration (NOEC) (ie concentration showing no statistical difference ($P<0.05$) between effluent-exposed and control animals) and lowest-observed-effect concentration (LOEC) for survival determined.

As described above (section 2.1.2), stocks of *Daphnia carinata* declined when exposed to diluent West Queen River water and in practice, no testing of this species was carried out.

Ceriodaphnia dubia

One 48-hour neonate survival test was conducted using this species at dilutions from each of 65:35, 80:20 and 99:1 neutralisation regimes, before a reduction of adult vigour and high neonate mortality, similar to that experienced with *D. carinata*, precluded its further use. The test protocol used for neonate survival was the same as that described for *D. carinata* (above).

Moinodaphnia macleayi

No successful testing was completed for this species.

2.3.2 Hydra tests

An ***eriss*** test protocol for green hydra (*Hydra viridissima*), designed to determine the effect of a toxicant on hydra population growth (Markich & Camilleri 1996), was used, with

modifications, to test Mount Lyell mine effluents. *H. viridissima* is referred to as green hydra due to the presence of symbiotic green algal cells in the gastrodermal cells. Precise distribution has not been mapped, but hydra have been found in a variety of aquatic habitats in northern Australia. The test protocol is presented in Appendix B, general principles of which may be described as follows:

- The objective of the test is to determine the maximum concentration at which neutralised AMD water has no statistically significant effect on hydra population growth over a 120-hour (5 d) period of exposure. Toxicity tests were based on hydra population growth, and spanned a geometric range of dilutions of 95:5 and 99:1 neutralisation stock solutions.
- Asexually-reproducing (budding) test hydra, each with one developing bud, are selected to initiate the test. The hydra are exposed to a range of concentrations of the waste water to be assessed under 24-hour 'static-renewal' conditions. Observations upon changes in the number of intact hydroids (one hydroid equals one animal plus any attached buds) are recorded at 24-hour intervals until test completion at 120 hours. Comments on physical appearance of the hydra (eg appearance of clubbed tentacles or contraction, both indicative of hydra in sub-optimal conditions), are also recorded and used for qualitative interpretation of the results. Population growth data over the test period are expressed as growth rate, K, defined in Appendix B. The quantitative response (K) is statistically analysed and the maximum no-effect or no-observed-effect-concentration (NOEC) (ie concentration showing no statistical difference ($P<0.05$) between effluent-exposed and control animals) and lowest-observed-effect concentration (LOEC) for population number determined.

The decision to alter the original three neutralisation regimes proposed for cladoceran tests to 95:5 and 99:1 for *Hydra viridissima*, was based on knowledge that the 65:35 and 80:20 were toxic to *C. dubia s. l.* at all test concentrations (section 3.1). Moreover, tests using hydra spanned a geometric range of dilutions up to but not exceeding 3% neutralisation stock solution for the 95:5 regime and 10% stock solution for the 99:1 regime, with higher concentrations in the respective neutralisations previously having been shown to result in 100% mortality of cladocerans (section 3.1). Four consecutive toxicity tests were completed using *H. viridissima.* The first two of these tests were range-finding tests in which the two neutralisation treatments were tested concurrently, using a geometric range of dilutions of 0, 0.1, 0.3, 1, 3 and (for 99:1 neutralisation only) 10% stock solution. Results from these tests were used to refine dilution ranges for the subsequent two tests. The 99:1 neutralisation treatment was used in the third test, with a dilution range of 0, 0.3, 0.5, 0.7, 0.9, 1.1, 1.4 and 2% while the 95:5 treatment was used in the fourth test, with a dilution range of 0, 0.07, 0.1, 0.13, 0.15, 0.2, 0.25 and 0.3%. All tests were conducted with exposure waters held at 20°C.

2.3.3 Statistical analysis

Cladoceran test

A modified Dunnett procedure, based on Brown (1992) and as described in Hyne et al (1996), was used to derive NOEC and LOEC values for the *Ceriodaphnia dubia s. l.* test. A concentration resulting in a reduction in survival in 50% of cladocerans (EC_{50}) was also calculated, using the same procedure as described below for 'hydra tests'.

Hydra tests

For each neutralisation, data for the three replicates of each test concentration (population growth, K) were pooled for the three tests and a one-way analysis of variance with Dunnett's

multiple comparison test used to determine NOEC and LOEC values from the combined data. The MINITAB software package was used for this analysis (MINITAB 1995). To validate the pooling of data in this way, it was necessary to establish, using Analysis of Covariance (ANCOVA) testing, that the three derived concentration-response regressions of each neutralisation treatment were not significantly different. The ANCOVA module in the STATISTICA software package was used for this analysis (StatSoft 1995), with results described in section 3.2.

Using mean response data (K), logistic regression models were derived to describe the concentration-response curves for each neutralisation regime. The function is described as:

$$Y = \frac{a - d}{1 + (X / c)^{b+d}}$$

where Y is the population growth response; X, the arithmetic treatment concentration; a, the minimum calculated response; d, the maximum calculated response; c, the concentration whose response is midway between a and d — equivalent to the EC_{50} value; and b, the slope of the curve around c. Model parameters were estimated using the ORIGIN software package (MICROCAL 1995).

2.4 Test solutions

2.4.1 Collection of acid drainage and diluent

Acid drainage and diluent, required for the preparation of toxicity test solutions, were collected in either 25 L or 50 L acid-washed polypropylene containers, rinsed twice with the water being collected prior to filling. Diluent was obtained from the West Queen River, immediately above its confluence with the East Queen River and pH noted at the time of sampling. The pH of West Queen River water at the time of sampling was inversely related to flow rate and ranged from 5.8 to 6.1. The apparent small decrease in pH of West Queen River water from time of collection, to transit and finally to completion of toxicity testing (table 2.1) may possibly have been either a calibration artefact or the result of some precipitation of Fe and/or Al. (Transport of poorly-buffered waters would normally be associated with *increases* in pH as the result of CO_2 loss.) Acid drainage was taken from North Lyell Tunnell into which Conveyor Tunnel water has been routed since April 1995. Concentrations of metals in acid drainage found in this study (tables C.2 and D.2) were lower than median values for combined Conveyor and North Lyell Tunnel waters as reported by Klessa et al (1997) reflecting wet weather which had preceded sampling (J Johnston pers comm).

Table 2.1 pH of unfiltered West Queen River water at time of collection, at arrival to testing facilities and at test completion

Date	Time		
	At collection	At arrival	At test completion
14/6/96	6.2	5.85	–
28/6/96	6.50	6.10	6.16
12/7/96	6.10	5.99	–

Diluent waters were freighted to ANSTO or ***eriss*** prior to each test, each batch of water sufficing for tests and maintenance conducted in the ensuing test. A single large sample of AMD was collected at the onset of each of the ANSTO and ***eriss*** testing phases (to suffice for all subsequent tests at the respective laboratories).

2.4.2 Neutralisation of acid drainage

Test solutions were composed of varying ratios of acid drainage, comprising raw AMD and AMD partially neutralised to pH 6.5 (ie stock solution), and diluent water. Neutralised acid drainage was freshly prepared prior to the start of each toxicity test. A description of the buffering characteristics of acid drainage and the products of neutralisation is contained in Klessa et al (1997).

Neutralisation of acid drainage to pH 6.5 was achieved as follows. Dried calcium hydroxide, $Ca(OH)_2$, was added at the rate of 0.0077 moles/L to 5 L of constantly stirred acid drainage contained in a plastic beaker. After 8 h stirring, a further 0.0077 moles/L dried $Ca(OH)_2$ was added and the mixture further stirred for 16 h. A subsample was then taken for pH, electrical conductivity and dissolved oxygen determinations. The remainder was filtered through a prepared (by acid washing and rinsing with deionised water) Whatman GF/C paper. A subsample of the filtrate was stored at 4°C in the dark until required for analyses.

2.4.3 Preparation of stock solutions

Stock solutions comprising neutralised and raw acid drainage in the ratios of either 65:35, 80:20, 95:5 or 99:1 were prepared fresh before each toxicity test by taking an appropriate volume of each to constitute 1 L total volume. Raw acid drainage was added with constant stirring to neutralised acid drainage contained in a 2 L beaker. A subsample was then taken and filtered as described above (section 2.4.2). pH, electric conductivity and dissolved oxygen were determined on a portion of this subsample and the remainder stored at 4°C until required for chemical analyses.

2.4.4 Preparation of test solutions

Stock solutions were diluted in varying ratios with diluent water by subsampling a stirred stock solution and making up to volume with unfiltered diluent. After 2 h standing time, the test solutions were filtered as described above (section 2.4.2) and stored at 4°C until required. Before being used in the toxicity tests, test solutions were placed in an incubator to raise them to the test temperature. A subsample of each test solution was then used to determine pH, electrical conductivity and dissolved oxygen while chemical analyses were performed on another subsample.

2.4.5 Chemical analyses

Each time a toxicity test was conducted, subsamples of filtered raw acid drainage, neutralised acid drainage, stock solutions, and diluent used in the preparation of test solutions were analysed, together with the test solutions themselves. Basic cations (ie Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}), sulphate and chloride and were analysed using ion chromatography (Noller & Currey 1990, leGras 1993, ERISS 1996). Copper, aluminium and iron were measured by inductively coupled plasma atomic emission spectroscopy after first acidifying samples to 1% v/v with BDH Aristar HNO_3.

3 Results and discussion

3.1 Cladoceran tests

One 48-hour 'range-finder' acute toxicity test was conducted using *Ceriodaphnia dubia s. l.*. Neonates of *C. dubia s. l.* were exposed to dilutions of mine waters treated to each of the neutralisation regimes, 99:1, 80:20, and 65:35. Control survival in all three tests was valid at 24 hours, but at 48 hours control survival had fallen below the acceptable 80% (see section A1.11). Percentage survival after 24 hours for the three replicates of each test concentration is shown in table C.1 for the three neutralisation regimes, with corresponding water quality data shown in table C.2. Results show 100% (or near) mortality at all concentrations of 65:35 and 80:20 neutralisation test waters (table C.1). In the 99:1 neutralisation, provisional NOEC, LOEC and EC_{50} based on survival after 24 h were calculated at 1%, 3% and 2.4% respectively. Total soluble copper concentrations, corresponding to the NOEC, LOEC and EC_{50}, were 15, 30 and 26 µg/L respectively (table C.2). (The Cu value derived for the EC_{50} was interpolated from a plot of Cu against test water concentration.)

Continued culturing of *Ceriodaphnia dubia s. l.* in West Queen River water was unsuccessful (section 2.1.2). Nevertheless, on the basis of the preliminary results showing the severity of effects upon *C. dubia s. l.*, it was concluded that the options of 65:35 and 80:20 neutralisations would be unlikely to support the return of life to the King and Queen Rivers.

3.2 Hydra tests

Four *H. viridissima* population growth tests, including two initial range finding tests, were conducted using a range of concentrations from 95:5 and 99:1 neutralisation regimes, as described in section 2.3.2. Throughout the 5-day test period of each test, water quality parameters of the test solutions remained within an acceptable range (as described in Appendix B1.11).

Summary population growth data for the concentrations of each test are shown in table 3.1. (Raw data are provided in table D.1.) Corresponding water quality data are provided in table D.2. Plots of the concentration-response data from table 3.1 showed that the regression relationships for each test of a given neutralisation regime could be adequately described by linear models for the purpose of ANCOVA testing (plots not provided here). From ANCOVA, it was shown for each neutralisation treatment that population growth constants amongst the 3 tests did not differ when concentration of test waters was kept constant ($F = 1.99$, $P > 0.05$ for 95:5 neutralisation; $F = 3.05$, $P > 0.05$ for 99:1 neutralisation). These results validated use of mean, pooled data for derivation of NOEC and LOEC values, and estimation of parameters for logistic regression models.

NOEC, LOEC and EC_{50} values for the two neutralisation regimes, together with corresponding summary water quality data, are shown in table 3.2. Water quality values that corresponded to the NOEC, LOEC and EC_{50} values were estimated as means of data from the three tests conducted for each neutralisation regime; data used to derive mean water quality values were interpolated where necessary from plots of each water quality variable against test water concentration (plots not shown here). Averaged between the two neutralisation regimes, copper concentrations corresponding to the NOEC, LOEC and EC_{50} were approximately 15, 18 and 28 µg/L respectively (table 3.2). The concentration-response relationships with fitted logistic curves for the *H. viridissima* tests are shown in figs 3.1 (95:5 neutralisation) and 3.2 (99:1 neutralisation). Estimated parameters for the logistic regression functions are given in table 3.3.

Table 3.1 Mean population growth constant (K, average of 3 replicates) with SD, after 5-day exposure of *Hydra viridissima* to two neutralisation regimes

Concentration of test water (%)	Test				
	1	2	3	4	Mean (all tests)
95:5 Neutralisation					
Control	0.245 (0.012)	0.215 (0.01)	–	0.279 (0.016)	0.246 (0.013)
0.03	–	0.236 (0.013)	–	–	0.236 (0.013)
0.07	–	–	–	0.238 (0.016)	0.238 (0.016)
0.1	0.222 (0.004)	0.21 (0.018)	–	0.252 (0.017)	0.228 (0.013)
0.13	–	–	–	0.226 (0.006)	0.226 (0.006)
0.15	–	–	–	0.254 (0.013)	0.254 (0.013)
0.2	–	–	–	0.214 (0.021	0.214 (0.021)
0.25	–	–	–	0.211 (0.008)	0.211 (0.008)
0.3	0.139 (0)	0.163 (0.018)	–	0.18 (0.019)	0.161 (0.012)
1.0	0	0	–	–	0
3.0	0	0	–	–	0
99:1 Neutralisation					
Control	0.245 (0.012)	0.215 (0.01)	0.252 (0.014)	–	0.237 (0.02)
0.1	–	0.245 (0.009)	–	–	0.235 (0.009)
0.3	0.233 (8.2E–11)	0.22 (0.007)	0.239 (0.029)	–	0.231 (0.017)
0.5	–	–	0.237 (0.009)	–	0.237 (0.009)
0.7	–	–	0.232 (0.016)	–	0.232 (0.016)
0.9	–	–	0.226 (0.017)	–	0.226 (0.018)
1.0	0.203 (0.018)	0.193 (0.004)	–	–	0.198 (0.013)
1.1	–	–	0.22 (0.007)	–	0.22 (0.007)
1.4	–	–	0.217 (0.014)	–	0.217 (0.014)
2.0	–	–	0.177 (0.026)	–	0.177 (0.026)
3.0	0.11 (0.007)	0.101 (0.024)	–	–	0.106 (0.017)
10.0	–	0	–	–	0

Table 3.2 NOEC and LOEC calculated after exposure of *Hydra viridissima* to two neutralisation regimes, in relation to water chemistry. NOEC and LOEC calculated from mean response values for *H. viridissima* for 3 successive tests. Analytes are mean values for the 3 tests (and SD, range for pH) with metal concentrations in µg/L.

	Neutralisation ratio and toxicological endpoint					
	95:5			**99:1**		
	NOEC	LOEC	EC_{50}	NOEC	LOEC	EC_{50}
Treatment concentration (%)	0.25	0.3	0.35	1.4	2.0	2.96
Chemical analyte						
pH	6.45 (6.42–6.54)	6.03 (5.83–6.09)	5.89 (5.83–5.96)	6.28 (6.17–6.28)	6.21 (6.14–6.27)	5.68 (5.54–5.89)
Copper	14.8 (1.8)	15.2 (2.3)	26.0 (2.1)	15.5 (2.2)	20.5 (1.8)	29.0 (2.8)
Aluminium	154 (25)	157 (26)	190 (16)	169 (13)	172 (15)	188 (3)
Manganese	130 (26)	150 (50)	295 (162)	877 (31)	1353 (129)	1995 (18)

Table 3.3 Parameters of logistic regression equations used to describe concentration-response curves for *Hydra viridissima* tests under two neutralisation regimes (see figs 3.1 and 3.2). Form of the equation is $Y = (a - d)/(1 + [X/c]^{b+d})$ where Y is the population growth response; X, the arithmetic treatment concentration; a, the minimum calculated response; d, the maximum calculated response; c, the concentration whose response is midway between a and d (EC_{50}); and b, the slope of the curve around c.

Neutralisation ratio	Parameters of equation (with SE)			
	a	b	c	d
95:5	0.235 (0.006)	5.168 (1.997)	0.346 (0.028)	0.002 (0.008)
99:1	0.232 (0.005)	2.868 (0.547)	2.957 (0.210)	-0.008 (0.013)

3.3 Implications of test results for assessing rehabilitation options

Based upon published water quality guidelines for protection of aquatic ecosystems, Klessa et al (1997) concluded that the principal limiting chemical factors to biological recovery within the Queen and King Rivers were likely to be Cu and Al. In the present study, Al concentrations corresponding to the NOEC, LOEC and EC_{50} for *Hydra viridissima*, were 154–169, 157–172 and 190–192 µg/L respectively (table 3.2). The Al values corresponding to these end-point responses are above those cited in water quality guidelines in Australia and elsewhere as being detrimental to aquatic life for similar pH (ANZECC 1992, Klessa et al 1997, table 6.9). This discrepancy is most likely due to: (i) the use of application factors in guidelines (eg a factor of 10 applied to LOEC data for the Australian guidelines); and (ii) the presence in the test waters of this study of colloidal or other Al species of low toxicity in the < 0.45 µm filtrates.

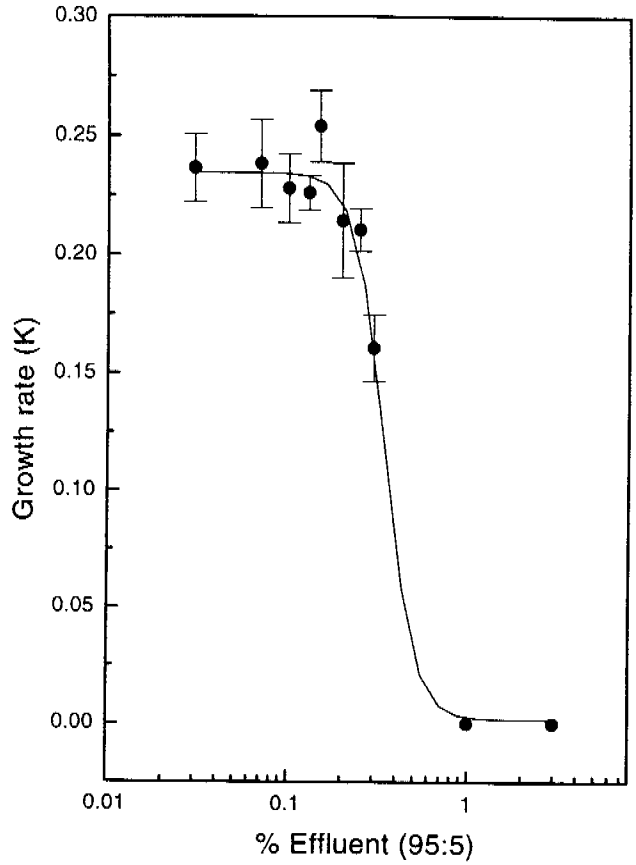

Figure 3.1 Concentration—response curve for data pooled from 3 separate hydra tests and conducted for different concentrations of 95:5 neutralisation regime. Data points are means (and 95% CIs) of 3 replicates per test. Parameters of fitted logistic regression model are shown in table 3.3.

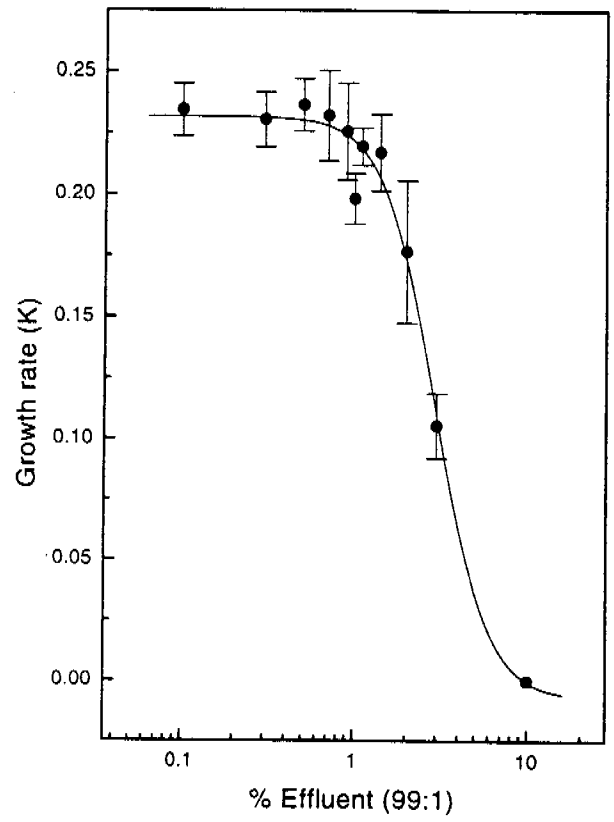

Figure 3.2 Concentration—response curve for data pooled from 3 separate hydra tests and conducted for different concentrations of 99:1 neutralisation regime. Data points are means (and 95% CIs) of 3 replicates per test. Parameters of fitted logistic regression model are shown in table 3.3.

In their study, Klessa et al (1997) suggested that for the receiving waters downstream of the Mount Lyell mine under various remediation options and flow conditions, predicted soluble Cu rather than Al concentrations would probably be more limiting to biological recovery. Thus, Cu in the neutralised Mount Lyell mine drainage waters is likely to be more detrimental to biological recovery in receiving waters than Al. Subsequent discussion, therefore, is focused on the relevance of test results to copper toxicity reported in the literature and to concentrations of Cu predicted by Klessa et al (1997) in the receiving waters under various scenarios for remediation. (Nevertheless, because of the complexity of the effluent mixture tested here, the possibility that other constituents in the waters (including Al) may be contributing to toxicity, is not ruled out.)

3.3.1 Comparison of test results with literature values for copper toxicity

Neutralised Mount Lyell acid drainage, like many 'whole-effluents', is a complex mixture. Therefore, caution is required when comparing concentrations of individual constituents from a whole-effluent where toxicity is observed, with literature single-element toxicity values. This is because synergistic and antagonistic interactions may be present. Nevertheless, the responses of the cladoceran *Ceriodaphnia dubia s. l.* and cnidarian *Hydra viridissima* to copper present in neutralised mine effluent are consistent when compared with each other (common NOEC, LOEC and EC_{50} of ~ 15, 18 and 28 µg/L respectively) and reasonably consistent when compared with other *C. dubia* data (table 3.4).

The Cu concentration at which an EC_{50} was observed in the *C. dubia s. l.* test, though higher than that reported elsewhere for the pH and hardness range of waters tested (table 3.4), could partly reflect the fact that exposure was limited to 24 hours, compared with 48-hour exposures conducted in other studies. Of particular relevance to this study were the findings of the Tasmanian Inland Fisheries Commission (IFC 1995) who reported a 96-h LC50 value after exposure of rainbow trout to King River water dosed with copper, of 90 µg/L Cu. Thus, the invertebrates tested in the present study (EC_{50} of ~ 26–28 µg/L Cu) appear more sensitive than trout. Although Markich and Camilleri (1996) reported a NOEC for *H. viridissima* exposure at Cu concentrations of 1.8 µg/L, their tests were conducted using an extremely soft, synthetic water, free of organic complexing agents.

Klessa et al (1997) summarised guideline values for total Cu required for protection of aquatic ecosystems in Australia and elsewhere. Guideline values ranged from 0.5 to 40 µg/L, with values >10 µg/L generally reflecting acute toxicity data at high water hardness (>100 mg/L $CaCO_3$). Given that these reported values typically incorporate an application factor (eg a factor of 10 to LOEC data in the Australian guidelines (ANZECC 1992)), the values of Cu for the toxicological end-points measured in this study are consistent with guideline values. Nevertheless, the results indicate, in contrast to those reported by Stauber et al (1996) for the marine environment, that no comparable ameliorative effects were present in the stream waters around the Mount Lyell mine that would support the viability of any proposed remediation options for assisting recovery of aquatic life.

3.3.2 Comparison of test results with modelled scenarios for water chemistry in the Queen and King Rivers

Klessa et al (1997) established concentration boundary limits for metals, including Cu and Al, within the King and Queen Rivers under a variety of flow conditions and scenarios of mine effluent treatment (principally neutralisation). The greatest potential for biological recovery would be shown in the King River upstream of the delta and the least, at the confluence of Haulage Creek with the Queen River. The lowest projected concentration of total soluble Cu in the Queen River under various acid neutralisation regimes was an estimate

of 366 µg/L (Klessa et al 1997), far exceeding the equivalent NOEC value (~15 µg/L) found in this study. None of the neutralisation options, therefore, would facilitate the return of life to the Queen River.

For the King River, the maximum protection afforded to aquatic organisms was that prevailing under a scenario of 99% neutralisation (to pH 6.5) of acid drainage and maximum dilution with the power station below Lake Burbury operating; under these conditions projected total soluble Cu concentration would approach 10 µg/L (Klessa et al 1997). However, with the power station not operating and minimum dilution (12 hours in a 24 hour cycle), estimates of total soluble Cu concentration in the King River would range between 74 and 235 µg/L. Because the NOEC value is intermediate between these diurnal ranges, the potential may be present for some partial recovery of life at least, in the King River. Additional toxicity tests employing pulsed and episodic exposure of test organisms to neutralised acid drainage would be required to resolve this issue.

3.3.3 Growth rate of *H. viridissima* in relation to predicted Cu^{2+}_{aq}

The hexaquo cupric ion (Cu^{2+}_{aq}) is commonly regarded as the principal form of biologically-available Cu in well-oxidised aquatic systems (Deighton & Goodman 1995) and as such determines toxicological response. Hydroxy-Cu complexes may also be toxic but their relative importance is less clear (Klessa et al 1997). Consequently, the relationship between the growth rate of *H. viridissima* and Cu availability, expressed in terms of the activity of Cu^{2+}_{aq}, was investigated to determine whether variance in growth rate could be explained by Cu speciation. A background to speciation modelling using MINTEQA2 based on remediation strategies for the Queen and King Rivers is contained elsewhere (Klessa et al 1997) and will not be expanded upon here suffice that with the exception of modelling at 19°C, at a fixed pH and with test solutions having compositions as given in table D2 (Appendix), all other parameters and conditions as described in Klessa et al (1997) were the same. In the absence of analytical data on F, Cl, Zn and dissolved organic matter (DOM) in either test solutions, diluent or mine drainage, estimates were made using median composition data for river and mine waters contained in Klessa et al (1997).

Mean population growth rate as a percentage of the growth rate in the controls was regressed separately against total Cu and Cu^{2+}_{aq}, the latter being derived from speciation modelling. Zero growth rate data were not used in the regressions and normality checks were undertaken first on data sets. Total Cu and Cu^{2+}_{aq} data were normalised by taking logarithms. Results are shown in fig 3.3.

Both regression relationships (fig 3.3) were highly significant ($p<0.001$) but importantly, more variance in growth rate was explained when total Cu was used as the predictor. The reason for this is unclear. Other than artefacts of the data set, the results could suggest that (an)other chemical constituent(s) of the waters were also responsible for limiting growth. Alternatively, on the basis that Cu^{2+} activity was the primary chemical factor limiting growth, it is possible that certain assumptions used in speciation modelling did not hold. For example, if DOM concentration was overestimated, then the majority of soluble Cu would be present as Cu^{2+} and, hence, total soluble Cu concentration would be almost as good a predictor of growth rate as an estimate of Cu^{2+} concentration. The current data, however, are insufficient to elucidate this further.

Table 3.4 Summary of literature values for copper toxicity to *Ceriodaphnia dubia* (water quality variables are ranges across test solutions and controls)

LC50 (ppb)	NOEL (ppb)	NOEC (ppb)	pH	Conductivity ($\mu S.cm^{-1}$)	Hardness ($CaCO_3$, mg/L)	Temp (°C)	Water type (diluent)	Test type	Reference
26	–	15	6.27	99	~25	20±2	River water	24-hr Survival	Present study
9.5	–	–	6–6.5	–	280–300	25	Reconstituted	48-hr Survival	Schubauer-Berigan, Dierkes, Monson & Ankley (1993)
28	–	–	7–7.5	–	280–300	25	Reconstituted	48-hr Survival	Schubauer-Berigan et al (1993)
200	–	–	8–8.5	–	280–300	25	Reconstituted	48-hr Survival	Schubauer-Berigan et al (1993)
13.4	–	–	8.18±0.04	–	57±4.14	25±1	Reconstituted	48-hr Survival	Oris, Winner & Moore (1991)
97 [1]	2.7 [1]	–	8.2–8.6	350–650	84–140	25±2	Reconstituted	7-Day Survival	Cowgill & Milazzo (1991c)
249 [2]	79 [2]	–	8.2–8.6	350–650	84–140	25±2	Reconstituted	7-Day Survival	Cowgill & Milazzo (1991c)
-	–	41–106 [3]	–	–	70–140	25±1	Pond Water	7-Day Reproduction	Winner & Owen (1991)

1 as $Cu(NO_3)_2.3H_2O$

2 as Cu (metallic)

3 results from 17 tests.

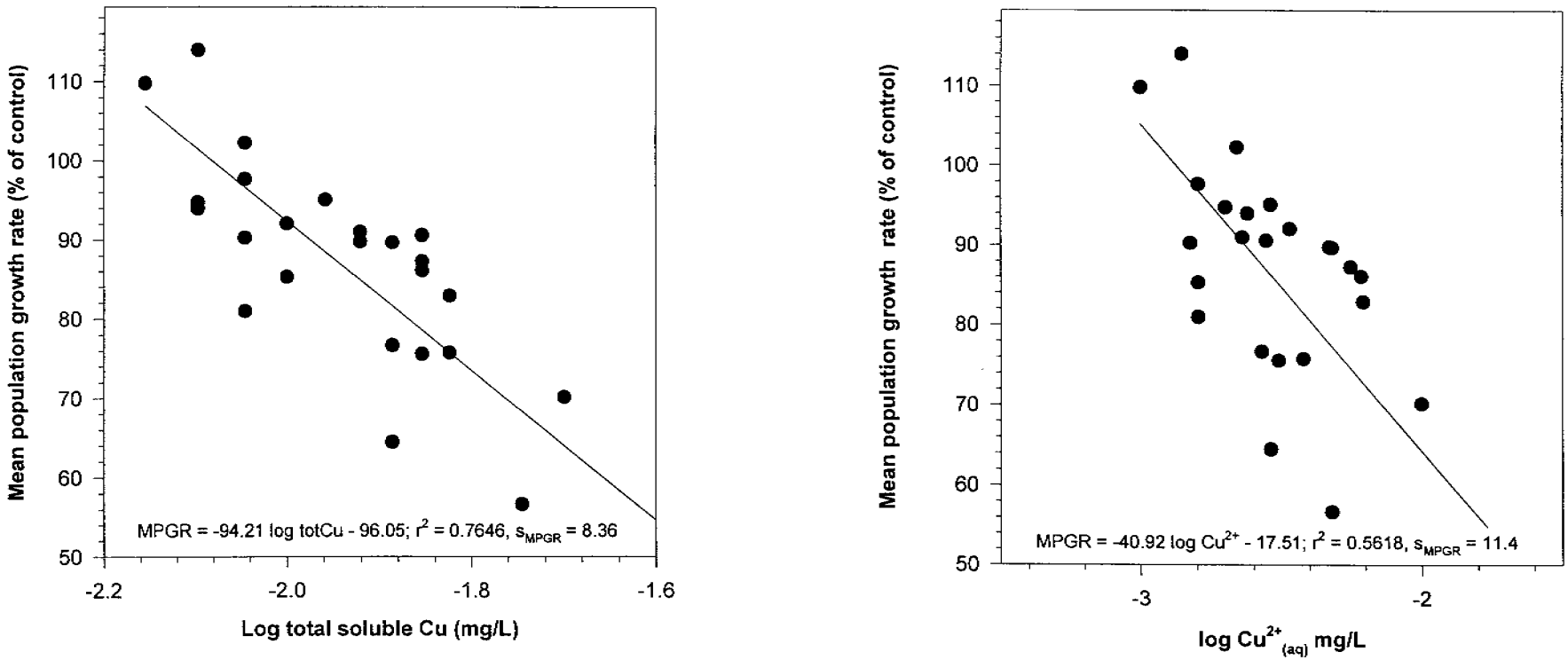

Figure 3.3 The effect of total soluble Cu (left) and predicted Cu^{2+}_{aq} (right) in test solutions on the mean population growth of *H. viridissima* relative to the controls (n = 26)

3.4 Appropriateness of the test procedures for addressing the project objectives

The objective of the project was to carry out single-species 'whole-effluent' toxicity tests to estimate the effectiveness of various remediation options canvased to reduce acid drainage from mining operations at Mount Lyell, including neutralisation, in enabling the return of aquatic life to the Queen and King Rivers. In selecting single-species tests for this work, there are the usual limitations that such an investigation might not accurately reflect effects that can occur at the ecosystem level. This discrepancy can arise because the sensitivity of the selected test species is unrepresentative of the wider assemblage of organisms in the field and because test conditions may not simulate actual environmental conditions.

In terms of the representativeness of test species selected for this toxicity work, results of limited *C. dubia s. l.* study and those of more extensive *H. viridissima* tests are consistent and are in general agreement with results from Lake Burbury and elsewhere (IFC 1995, references cited in table 3.4). Though *H. viridissima* occurs in tropical Australia, it is probably ubiquitous elsewhere in Australia and in a literature review in which toxicological responses of temperate and tropical freshwater fish and crustacea to Cu and U were compared, Markich and Camilleri (1996) found no apparent latitudinal difference in sensitivity. All tests in the current study were conducted at 19–20°C, a standard water temperature regime used in test protocols developed for temperate aquatic organisms (see table 3.4). Whilst this temperature is above that occurring in streams of south-western Tasmania, experience elsewhere (eg Sprague 1985) suggests that conclusions drawn from results of chronic exposures of the type used for *H. viridissima* would not be expected to differ significantly.

3.5 Future directions

Experimental assessment of the toxicity of actual or simulated mine waste waters to aquatic organisms will remain the most feasible approach to estimating the effectiveness of remediation options being considered to reduce acid drainage from mining operations at Mount Lyell. Single-species toxicity tests, measuring lethal and sublethal responses of organisms exposed to actual or simulated effluents, should be considered in parallel with field mesocosms and experimental manipulations of the type recommended by Davies et al (1996).

For further single-species toxicity tests, development of protocols for lethal and sublethal toxicity using local test organims should be a priority of future research. The present study served to highlight the difficulty in applying standard test organisms such as cladocerans to surface waters of south-west Tasmania where such organisms respond unfavourably to the local acidic soft waters. Development of locally-based protocols using local stream organisms should provide the most cost-effective results for any biological assessment of water quality.

4 Conclusions

Regardless of the difficulties in applying toxicity tests to surface waters of the Mount Lyell mine region, it was concluded that representative biological responses to whole effluents were derived. Thus, nothing in the acid drainage neutralisation nor receiving waters is likely to significantly ameliorate toxicity, particularly of Cu. Even at near total neutralisation,

concentrations of Cu in mine effluent were predicted to be still too high in receiving waters for any chance of anything other than a partial recovery of aquatic life in streams.

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Appendix A

Protocol for cladoceran test

A1 Cladoceran 48-hr acute immobilisation test

The test is based on an OECD protocol (see below) with a number of modifications made to reflect the specificity of the whole effluent testing used in this study. These modifications are included in the following description of the test protocol. All aspects of the protocol are described other than data analysis (see section 2.3.3 of main text).

A1.1 Objective

The objective of the test is to determine the concentration of neutralised acid mine drainage that has no statistically significant effect on survival of juvenile cladocerans, *Daphnia carinata, Ceriodaphnia dubia s. l.* and *Moinodaphnia macleayi* over a 48-hour period of exposure.

A1.2 Principle of the test

Animals less than 24 hours old are exposed to a range of concentrations of neutralised acid mine drainage for a period of 48 hours. Observations are made of the number of animals that show no movement after 15 seconds of gentle agitation at 24 and 48 hours. The statistical endpoint is determined as the NOEC and LOEC after 48 hours exposure. The method is based on the OECD Guidelines for Testing Chemicals 202 Part 1 (OECD 1984).

A1.3 Test organism

The species is *Daphnia carinata* (Crustacea, Cladocera). This species is found in many temperate locations in Australia including the ephemeral wetlands of north-eastern Tasmania (R Walsh pers comm). Stock cultures were procured from the Centre for Ecotoxicology (University of Technology, Sydney); methods for subsequent laboratory culture are described in section A2.1. Reproduction is asexual (parthenogenetic).

An alternative temperate climate species, *Ceriodaphnia dubia s. l.*, was also cultured for testing purposes. This species is widespread in temperate Australia and this culture arose from Parramatta Lake (Sydney) which has an urban catchment. Culture methods for this species are described in section A2.2.

A third species, *Moinodaphnia macleayi,* routinely cultured at ***eriss***, was also identified as a possible test animal, but not used for testing due to poor survival in stock cultures of Queen River diluent water.

Test Cladocera are selected as second brood neonates less than 24-hrs old from parental stock that itself was second or third brood stock. All animals selected for testing should be free of overt disease and gross morphological deformity.

A1.4 Dilution water

The dilution water is taken from upstream of any source of contamination, in this case an uncontaminated tributary stream of the Queen River (fig 2.1). Water is collected in acid washed polyethylene containers as close as is practical to the commencement of the test (ie on a weekly or fortnightly basis depending on requirements), and air freighted to test facilities. A representative batch of the water is required and is filtered through coarse glass fibre (GF/C) filter paper capable of removing resident zooplankton. The water should be stored in acid washed, sealed polyethylene containers and refrigerated (4°C) until use.

A1.5 Stock solutions

Acid Mine Drainage (AMD) water was collected from the North Lyell Tunnel in a single batch at the start of the project and stored at 4°C until required. Stock solutions are prepared as different percentages of neutralised AMD (section 2.4). The stock solutions are prepared and stored in acid washed, sealed polyethylene containers at 4°C until required. Source and details of preparation of stock solutions should be recorded on data sheets.

A1.6 Test solutions

Test solutions are prepared by serially diluting appropriate volumes of stock solution with the filtered diluent water to provide required concentrations. Test solutions are prepared in bulk at the start of a test in 2 L polyethylene screw-topped containers and refrigerated (4°C) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies appreciably when stored during the period of the test.

A1.7 Apparatus and test equipment

All materials used for solution preparation, storage or animal holding should be chemically inert.

(a) Container preparation

All containers (ie vials, bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

1. Undergo an analytical grade dish washer (eg Gallay Laboratory 999) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using reverse osmosis (RO) grade water for two rinse cycles;
2. rinse with deionised (DI) water (<1 $\mu S\ cm^{-1}$); and
3. allow to air dry.

OR

- immerse in a 1 to 3% detergent solution (eg Decon Neutracon) for up to 24 h;
- scrub to remove extraneous material, then rinse thoroughly in tap water;
- immediately immerse in a 5% HNO_3 solution for up to 24 h;
- thoroughly rinse at least 3 times with DI water; and
- allow to air dry.

Immediately before use, the containers should be rinsed with diluent water. Other equipment should be rinsed thoroughly with DI water before use.

(b) Temperature control

Tests are conducted at 18±1°C using a constant temperature incubator. The temperature of the test containers is maintained at 18±1°C (eg by lowering the room temperature of the testing laboratory to 18±1°C, and placing on the microscope bench) when removed from the incubator for observation.

(c) Photoperiod

Tests are conducted with a 12 h light:dark photoperiod, where the mid point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie 10-50 $\mu E\ m^{-2}\ s^{-1}$ Photosynthetic Active Radiation).

(d) Equipment

- six 2 L polyethylene containers;
- refrigerator for storage of test and stock solutions;
- twenty-four 60 mL plastic vials with screw-capped lids and 2 x 2 mm diameter holes drilled in lids;
- twelve 100 mL disposable plastic vials with screw-capped lids (for measurement of water quality variables);
- maximum-minimum thermometers (to monitor daily incubator temperature range);
- calibrated mercury thermometer (to monitor test solution temperatures);
- pH meter, pH probe, and pH buffer solutions of 7.00 and 4.00;
- conductivity meter and probe;
- dissolved oxygen meter fitted with a micro-oxygen electrode;
- Maggy lamp;
- automatic 0–50 mL dispenser;
- clear plastic trays capable of holding 24 vials, with position numbers 1 to 24 marked;
- random number table or generator;
- Pasteur pipettes with internal tip diameter > 2 mm;

A1.8 Test environment

The preparation and storage of test solutions, the culturing of Cladocera to be used in the tests, and all manipulations and tests should be carried out in premises free from harmful vapours and dusts, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

A1.9 Recording of data

Test animals are observed and data recorded at 24-hour intervals after commencement of the test. The commencement of the test is designated Day 0. Observations made after 24 hours are designated Day 1 observations.

Water quality variables are measured at the start (Day 0) and at the end of the test.

A1.10 Test procedure

All equipment is prepared and labelled prior to each test.

Twenty-four hours prior to commencement of each test, animals bearing eyed Brood 2 or 3 young are isolated.

Day 1

1 Test waters are prepared according to Sections 2.4 and A1.6, and then left at room temperature.

2 Suitable female neonates <24 h old are isolated.

3 Dispense 50 mL aliquots of each test concentration (six in this test) into four appropriately labelled replicate vials (ie 4 x 50 mL for each test solution), and arrange in

replicate groups on clear perspex trays (Control replicate 1 → X% neutralised AMD replicate 1 on Tray 1 etc).

4 Dispense also 90 mL of each test solution into 100 mL vials for measurement of pH, conductivity and dissolved oxygen, and a further 50 mL of each acidified test solution into acid washed 100 mL polyethylene bottles for analysis of chemical analytes.

5 Using a Maggy lamp and Pasteur pipette, select one cladoceran from isolated neonates and place in Control replicate 1.

6 Repeat for remaining 5 concentrations of replicate 1, working from the lowest to highest concentration.

7 Discard pipette, and select a new one.

8 Repeat steps 5 to 7 until all vials in that replicate contain 5 animals each.

9 Check to ensure there are 5 Cladocera in each vial.

10 Repeat steps 5 to 7 for remaining replicates.

11 Place lids on vials, randomise on trays according to random number sheet prepared for that day and place trays in incubator. This constitutes the start time of the test (T = 0 h).

12 Repeat the above steps for concurrent tests.

Day 2

13 24 hours after commencement of test, sort vials into original replicate groups.

14 For each vial, observe under Maggy lamp for immobilisation[1] of animals. Record number of active, mobile animals and number immobilised for each replicate and each treatment. Remove any animals that are immobile from container.

15 Replace vials in incubator.

Day 3

16. Sort vials into replicate groups as per Day 2.

17. Observe under Maggy lamp for immobilisation of animals. Record number of active, mobile animals and number immobilised for each replicate and each treatment.

18. Place all surviving animals in a single container and humanely euthanase.

19. Collect all treatment waters and measure pH, conductivity and dissolved oxygen of each.

A1.11 Test validity

The test is considered valid if the following criteria are met:

- not more than 20% of animals in the Control are immobilised or trapped at the surface of the water;
- The recorded temperature of the incubator remains within the prescribed limits of 18±1°C;
- pH of test solutions does not vary more than 0.5 unit from Day 1 results; and

[1] Immobilisation refers to no movement of an animal after 15 seconds of gentle agitation.

- dissolved oxygen concentration is >70% air saturation value in the test solutions at test completion.

Immobilisation refers to no movement of an animal after 15 seconds of gentle agitation.

A2 Culturing of cladocerans

A2.1 Culturing conditions for *Daphnia carinata*

Animals of this species used for testing were sourced from the Centre for Ecotoxicology, University of Technology, Sydney.

The animals are acclimated to the diluent water for 6 weeks prior to commencement of the test. Fourteen days prior to test commencement, 7 animals (less than 24 hr old) are selected and each animal is placed in 200 mL of filtered diluent water, into which 1.0×10^7 *Selanastrum capricornatum* cells have been added, then placed in an incubator set at 18 ± 1°C and 12 hr photoperiod.

This procedure is repeated a further 3 times until there are 4 cultures of 7 animals each. This number of individual cultures should allow sufficient animals from brood 2 or 3 for commencement of 3 concurrent tests of 6 treatments, 4 replicates and 5 animals per replicate. (For *D. carinata* cultured at 18°C, there are approximately 48 hr between each successive brood.)

Culture animals are fed daily and their culture water renewed four times per week, ie Monday, Wednesday, Friday and one day on the weekend.

Twenty-four hours prior to test commencement, adults with eyed young in their brood pouch are placed in 2 x 1L diluent water with 5×10^7 *Selanastrum capricornatum* cells. The neonates from these adults will constitute the test animals.

A2.2 Culturing conditions for *Ceriodaphnia dubia s. l.*

Animals of this species used for testing were sourced from the Centre for Ecotoxicology, University of Technology, Sydney. The original culture arose from specimens collected at Lake Parramatta in Sydney.

The animals are acclimated to the diluent water for 14 days prior to commencement of the test. Seven days prior to test commencement, approximately 150 animals (less than 24 hr old) are selected and approximately 75 of these are placed in 2000 mL of filtered diluent water to which has been added 50 000 cells/mL of *Selanastrum capricornatum.* The stock is then placed in an incubator set at 18±1°C and 12 hr photoperiod.

This is repeated for the remaining 75 animals. This number of animals should allow sufficient animals from brood 2 or 3 for commencement of 3 concurrent tests of 6 treatments, 4 replicates and 5 animals per replicate.

Culture animals are fed daily and their culture water renewed four times per week, ie Monday, Wednesday, Friday and one day on the weekend.

Twenty-four hours prior to commencement of a test, 5 adults with eyed young in their brood pouch are placed in 200 mL of filtered diluent water to which has been added 1.0×10^7 *Selanastrum capricornatum* cells. Twenty 200 mL vials are set up in this manner with remaining adults used as back up stocks should the 200 mL cultures fail to produce sufficient neonates. The neonates from these adults will constitute the test animals.

Appendix B

Protocol for Hydra test

B1 Green hydra (*H. viridissima*) population growth test

This toxicity test protocol is based on an existing ***eriss*** protocol (see below) with a number of modifications made to reflect the specificity of the whole effluent testing used in this study. These modifications are included in the following description of the test protocol. All aspects of the protocol are described other than data analysis (see section 2.3.3 of main text).

B1.1 Objective

The objective of the test is to determine the concentration of neutralised acid mine drainage that has no statistically significant effect on the population growth of *Hydra viridissima* (green hydra) over a 120-hour (5 d) period of exposure.

B1.2 Principle of the test

Asexually reproducing (budding) test hydra are exposed to a range of concentrations of neutralised acid mine drainage for a period of 120 hours. Observations of any changes to the hydra population (ie changes in the number of intact hydroids; one hydroid equals one animal plus any attached buds) are recorded at 24 h intervals. The method is based on the Hydra Population Growth Test described by Hyne et al (1996) and modified by Markich and Camilleri (1996).

B1.3 Test organism

The species is *Hydra viridissima* (Cnidaria, Hydrozoa). *H. viridissima* is referred to as 'green' hydra because of its green colouration resulting from the presence of a symbiotic green alga in the gastrodermal cells of the animal. Although the precise distribution of this species has not been mapped, it has been found in a variety of aquatic habitats in northern Australia. Test hydra were obtained from laboratory cultures, as described in section B2 below. Hydra used for testing are selected only if budding, with one bud just showing signs of becoming tentacled. Asexual budding is a characteristic of hydra in optimal environmental conditions. The physical features of tentacle clubbing and contraction are used as qualitative test endpoints—indicators of hydra in sub-optimal conditions. Hydra selected for testing must be free of overt disease and gross morphological deformity (ie show no signs of clubbing or contraction).

B1.4 Dilution water

The dilution water is taken from upstream of any source of contamination, in this case an uncontaminated tributary stream of the Queen River (fig 2.1). Water is collected in acid washed polyethylene containers as close as is practical to the commencement of the test (ie on a weekly or fortnightly basis depending on requirements), and air freighted to ***eriss***. A representative batch of the water is required and is filtered through coarse glass fibre (GF/C) filter paper capable of removing resident zooplankton. The water should be stored in acid washed, sealed polyethylene containers and refrigerated (4°C) until use.

B1.5 Stock solutions

Acid Mine Drainage (AMD) water was collected from the North Lyell Tunnel (section 2.4) in a single batch at the start of the project and stored at 4°C until required.

Stock solutions are prepared as different percentages of neutralised AMD (section 2.4). The stock solutions are prepared and stored in acid washed, sealed polyethylene containers at 4°C until required. Source and details of preparation of stock solutions should be recorded on data sheets.

B1.6 Test solutions

Test solutions are prepared by serially diluting appropriate volumes of stock solution with the filtered diluent water to provide required concentrations. Test solutions are prepared in bulk at the start of a test in 5 L polyethylene screw-topped containers and refrigerated (4°C) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies appreciably when stored during the period of the test.

B1.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the hydra are placed or the hydra themselves, should be chemically inert.

(a) Container preparation

All containers (ie vials, bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using reverse osmosis (RO) grade water for two rinse cycles;
- rinse with deionised (DI) water (<1 µS cm-1); and
- allow to air dry.

OR

- immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- scrub to remove extraneous material, then rinse thoroughly in tap water;
- immediately immerse in a 5% HNO_3 solution for up to 24 h;
- thoroughly rinse at least 3 times with DI water; and
- allow to air dry.

Immediately before use, the containers should be rinsed with diluent water. Other equipment should be rinsed thoroughly with DI water before use.

(b) Temperature control

Tests are conducted at 20±1°C using a constant temperature incubator. The temperature of the test containers is maintained at 20±1°C (eg by lowering the room temperature of the testing laboratory to 20±1°C, and placing on the microscope bench) when removed from the incubator for observation.

(c) Photoperiod

Tests are conducted with a 12 h light:dark photoperiod, where the mid point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie 10–50 µE m^{-2} s^{-1} Photosynthetic Active Radiation).

(d) Equipment

- seven 5 L polyethylene containers with screw-top lids;
- refrigerator for storage of test and stock solutions;
- twenty-one 45 mL disposable plastic vials with screw-capped lids;
- twenty-one 90 mm diameter disposable plastic Petri dishes with lids;
- fourteen 100 mL disposable plastic vials with screw-capped lids (for measurement of water quality variables);
- maximum-minimum thermometers (to monitor daily incubator temperature range);
- calibrated mercury thermometer (to monitor test solution temperatures);
- pH meter, pH probe, and pH buffer solutions of 6.87 and 4.01;
- conductivity meter and probe;
- dissolved oxygen meter fitted with a micro-oxygen electrode;
- binocular dissecting microscope with bright field/dark field illumination;
- automatic 0–50 mL dispenser;
- clear plastic trays capable of holding 21 Petri dishes, with position numbers 1 to 21 marked;
- random number tables or generator;
- two perspex trays, each capable of holding 10 vials;
- Pasteur pipettes, with internal tip diameter >2 mm;

B1.8 Test environment

The preparation and storage of test solutions, culturing of hydra to be used in the tests, and all manipulations and tests should be carried out in premises free from harmful vapours and dusts, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

B1.9 Data recording

Test animals are observed and data recorded at 24-hour intervals after commencement of the test. The commencement of the test is designated Day 0. Observations made at the end of the first 24-h period are designated Day 1 observations; at the end of the second 24-h period, Day 2 observations etc.

Water quality variables are measured and recorded at the beginning and end of each 24-h period, these time periods being designated Fresh Water Day 1, 24-h-old Water Day 1, respectively, and so forth during the test. Where necessary (see section B1.11), adjustments to water quality should be made.

B1.10 Test procedure

Day 1

1 Prepare the test solutions (as outlined in section 2.4) and leave at room temperature.

2 Isolate approximately 220 suitable hydra and place in 3 petri dishes containing diluent water held at room temperature. A 'suitable test hydra' is a hydra with one bud. The bud

must not be fully developed (ie tentacles are present only as 'bumps', and the bud must not appear ready to detach from the main stem of the hydra).

3 Dispense 30 mL aliquots of each test concentration (normally 7) into three appropriately labelled replicate Petri dishes (ie 3 x 30 mL for each test solution), and arrange in three replicate groups on clear plastic trays (Control replicate 1 → X% neutralised AMD replicate 1 on Tray 1 etc).

4 Dispense at least 60–70 mL of each test solution into 100 mL vials for measurement of pH, conductivity and dissolved oxygen. Dispense 50 mL of each test solution into acid washed 50 mL bottles for analysis of chemical analytes, and acidify solutions to 1% with HNO_3.

5 Using a microscope and Pasteur pipette, select one hydra from the isolated stock and place into Control replicate 1.

6 Repeat for remaining test concentrations of replicate 1, working from the lowest to highest concentration.

7 Discard pipette and select a new one.

8 Repeat steps 4 to 6 until all test dishes for that replicate group contain 10 hydra each.

9 Observe each dish under the microscope to ensure that there are 10 hydra in each dish, replacing immediately any hydra that are damaged in any way (eg all buds must be attached) with 'suitable test hydra', using a new pipette.

10 Repeat steps 4 to 8 for the remaining two replicate groups.

More than one person can distribute test hydra simultaneously, ensuring each person is responsible for separate and entire replicate groups.

11 Cover the dishes and place them in the random sequence determined for that day (see below), in the positions 1 to 21 (ie 7 x 3 replicates).

12 Place trays in the incubator.

Completion of this stage constitutes the start of the test (T = 0 h).

Note: Whenever test dishes are removed from the incubator, they must be maintained at 20°C (eg by placing them on the bench at pre-set room temperature).

13 Observe each Petri dish under the microscope at T = 2 h after commencement of the test. Do not change positions of the dishes on the tray and return dishes immediately to the incubator following:

a) counting and recording the number of individual hydra (ie with or without buds);

b) noting of whether tentacles appear clubbed or contracted;

c) noting of any other observations that suggest the hydra are not behaving or developing normally.

Observations are recorded at T = 2 h on the data sheets. These observations constitute results for Day 1 of the test. To avoid observer or temporal biases, select a different replicate to commence observations each day. Further, commence observations with the next highest chemical concentration to that observed on the previous day (see below).

Note: Water movement will cause temporary tentacle contraction; allow the water to settle before recording observations.

Day 2

14 Dispense fresh test solutions into appropriately labelled 45 mL vials (3 reps x 35 mL of each solution, for daily water change). Also dispense a 50–100 mL sample of each test solution for measurement of pH, conductivity and DO.

15 Twenty hours after the commencement of the test, remove the trays from the incubator, sort the test dishes into replicate groups (ie 3 replicate groups of each treatment concentration), observe under the microscope and record as per Day 1 observations. By working through the water changes from a lower to a higher chemical concentration, the need to continually replace glass pipettes is avoided.

16 After recording observations for each dish (as in step 15), feed each hydra in the dish, as follows: hydra are fed individually with at least 3 to 4 live brine shrimp nauplii (*Artemia salina*, see section B2 below). The nauplii are rinsed with diluent water, then placed in each dish using a glass Pasteur pipette. Feeding is allowed to proceed *ad libitum* for at least 30 minutes, but is generally best left for 2 to 3 h.

17 After all hydra in the dishes have been observed and fed, place the test dishes onto trays in the random order determined for the day (see below), and return the trays to the appropriate position in the incubator.

18 Twenty-four hours after the commencement of the test, test solutions are renewed as follows:

a) the test solution is swirled around the Petri dish to dislodge any uneaten brine shrimp and regurgitated food;

b) the solution is then tipped carefully into a second Petri dish (or cleaning dish) so as not to dislodge adhering hydra;

c) an aliquot of the fresh test solution (5 mL) is immediately added to cover the bottom of the test dish, the swirling process is repeated, and the solution tipped into the cleaning dish;

d) the remaining fresh solution (30 mL) is immediately added to the test dish;

e) any hydra that are dislodged into the cleaning dish are carefully picked up with a little water using a clean pipette and returned to the test dish;

f) any remaining brine shrimp, or other debris, in the test dish are removed by pipette, with care taken to minimise removal of test solution;

g) the cleaning dish is checked again for hydra, with any found being returned to the test dish; and

h) the solution in the cleaning dish is collected for the measurement of water quality variables in each treatment after 24 h.

Note: Ensure that cross-contamination does not occur by obtaining a new pipette and cleaning dish whenever a dish of lower chemical concentration is cleaned after that containing a higher concentration.

19 Measure water quality variables (ie pH, conductivity, dissolved oxygen) at the end of 24 h using solutions obtained from step 18(h).

Days 3 to 5

20 Repeat steps 12 to 19 (ie count and record observations for the relevant day, feed at 24 h, clean and renew test solutions, measure and record water quality variables).

On each day, a new set of random numbers must be used for the position of each Petri dish in the incubator for the ensuing 24 h period (see below).

Day 6

21 Count and record observations made on each test dish 96 hours (4 x 24 h) after the commencement of the test. Do not feed hydra and do not renew test solutions.

22 Measure and record the water quality variables.

Test is complete.

Further notes on test procedures

On each day of the test, a new set of random numbers must be used for the position of each Petri dish on the trays (and, therefore, in the incubator) for the ensuing 24 h period. Randomness is an important component of the experimental design. Random numbers are obtained from a random number table or generator.

To avoid observer bias, there should be at least two observers. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Each observer observes from lowest to highest test concentration within each replicate. Occasional checks should be made on the incubator performance (ie constant temperature and light intensity and their variation) by observing the performance of hydra in different incubators; this procedure is described in Hyne et al (1996).

B1.11 Acceptability of test data

The test data are considered acceptable if:

1 The recorded temperature of the incubator remains within the prescribed limits of 20 ± 1°C;

2 The mean mortality across the three replicate Controls does not exceed 20%;

3 Greater than 80% of the surviving hydra in the combined Controls are healthy on completion of the test;

4 The recorded pH does not vary more than 0.5 unit from Day 1 results;

5 The dissolved oxygen concentration is greater than 70% air saturation value throughout the test at 20°C;

6 The conductivity for each test solution is within ±10% of the values obtained on Day 1; and

7 The presence of fungus on hydra does not exceed 20% of individuals from combined treatment replicates.

B2 Culturing of hydra and food source

B2.1 Culturing of hydra

Green hydra (*Hydra viridissima*) are cultured in the laboratory in bubble-aerated water held in 2 L glass bowls (primary stock). The bowls are loosely covered with clear polyethylene

food film (eg 'Gladwrap') so as to allow ventilation around the sides. The culture water is taken from the same batch of diluent water that is used to commence the test. The water movement caused by the gentle aeration results in most hydra attaching to the sides of the bowl via the basal disc, thus reducing time taken to perform water changes. Reserve (backup) stock hydra are maintained in tap water in back-up aquaria at a separate location, as a precaution against unknown chemicals or accidents occurring with the diluent water. The backup aquaria are maintained as 'community' tanks, with 3 to 4 small fish (eg *Ambassis* spp, *Pseudomugil* sp) and freshwater snails present.

Primary stock hydra are fed three times a week. One week prior to commencement of a test, they are fed daily to achieve maximum budding rates. Prior to commencement of this intensive feeding, hydra are observed and notes on culture health and density recorded in the primary hydra stock log book. A sample of water is then taken and the dissolved oxygen (DO) concentration measured and recorded as a water quality check. Hydra are then fed with newly-hatched brine shrimp nauplii (*Artemia salina*—see section B2.2). Prior to being fed to hydra, the brine shrimp are thoroughly washed in a suspension of diluent water. They are then pipetted into each primary stock bowl so that they are evenly distributed over the hydra. The hydra are allowed to feed for at least 30 minutes, and up to 4 to 5 hours when possible. Six hours later, any uneaten brine shrimp and regurgitated food pellets are removed by swirling the water around each bowl and emptying it into a second cleaning dish (eg 4 L plastic container). More synthetic water is added and the procedure repeated until each bowl is free of brine shrimp. The bowls are then re-filled with approximately 1.5 L of clean water. Any hydra removed by the process are pipetted back into the glass bowl containing the fresh water. This process is referred to as a 'rinse' clean.

Stock bowls are cleaned at least twice weekly by performing a 'scrub' clean. After observations are made and recorded, and samples for DO measurement taken, excess water is carefully decanted away, ensuring that minimal hydra are lost. If necessary, the old water can be decanted into a cleaning dish so that enough hydra can be retained during cleaning. The bowls are then cleaned by gently pushing with the fingers the attached hydra away from the sides of the bowl, and into a cleaning dish. Clean hands, or hands covered by gloves can be used to carry out this procedure. The detached hydra are allowed to settle into a corner of the cleaning dish by slightly tipping the dish. Using a glass Pasteur pipette, the hydra can then be transferred to a clean glass bowl containing fresh water. Backup hydra stock are fed daily with brine shrimp, and the aquaria cleaned at least once a week. Excess hydra are gently pushed away from the sides of the aquaria and siphoned out, with a one-third water replacement. Bowls are washed by analytical-grade dishwasher (eg Gallay Laboratory 999). Immediately prior to use, the bowls are rinsed with fresh synthetic water.

Periodically, hydra are observed to reproduce sexually, making it difficult to maintain an isogenic population. This could be related to fouling of the holding water and fungal growth on the uneaten brine shrimp. The frequency with which sexual reproduction occurs can sometimes be reduced by increasing the rate of feeding and cleaning of the primary cultures. If fungal contamination is observed at any time, the bowls can be given a 'rinse' clean. Cladocera (*Moinodaphnia macleayi*) are fed at least once a week to the primary and backup hydra cultures as a natural diet supplement.

B2.2 Culturing of live brine shrimp larvae

Brine shrimp (*Artemia salina*) are used as food for many types of aquatic organisms, including larval fish and hydra. Brine shrimp can be cultured in a variety of containers to give an uninterrupted supply of nauplii (juvenile brine shrimp). The most appropriate type of

culture containers are conical flasks (conical 1 L separation funnels are ideal) which, when inverted with the neck downwards, can be bubble-aerated from the bottom with oil-free compressed air. A 1 L salt solution is made by dissolving 30 g of coarse rock salt, or sea salt, in 1 L of warm water (30°C). After the salt is fully dissolved, one teaspoon (approximately 5 g) of commercially harvested, dried brine shrimp cysts is added. Vigorous bubbling from the bottom of the container prevents eggs from settling.

Brine shrimp eggs will hatch in 18 to 24 h at an incubation temperature of 28°C and in an outside shaded position. At lower temperatures, hatching is delayed. On cloudy days the culture may need to be directly illuminated by a fluorescent lamp, since hatching is light dependent. To harvest the newly-hatched nauplii, the compressed air is turned off 24 h after addition of the eggs (average water temperature of about 28°C) to allow the nauplii to settle and the empty egg shells to float. After 5 mins, the nauplii are strained through a fine nylon mesh net which is able to retain the nauplii, and then washed with the test dilution water. The washed nauplii are then suspended in a small volume of dilution water (about 5 mL) and placed in a small beaker or Petri dish which is inclined at an angle of approximately 45° towards the light. Live nauplii will concentrate in the upper layer, while the unhatched cysts will remain on the bottom of the container. The upper layer, containing live nauplii, is then collected for feeding. A Pasteur pipette or syringe is used to collect and distribute the nauplii.

Appendix C

Results of *Ceriodaphnia dubia s. l.* test

C1 Biological data

Table C.1 *Ceriodaphnia dubia* test results presented as mean survival after 24 hours exposure

Neutralisation	Concentration	24 Hour Survival (%)			
		Rep 1	Rep 2	Rep 3	mean (SD)
99:1	Control	80	80	100	86.6 (11.5)
	0.3%	80	100	80	86.6 (11.5)
	1.0%	100	60	60	73.3 (23.1)
	3.0%	40	20	40	33.3 (11.5)
	10%	0	0	0	0 (0)
	30%	0	0	0	0 (0)
80:20	Control	100	100	100	100 (0)
	0.3%	20	0	0	20 (11.5)
	1.0–30%	0	0	0	0 (0)
65:35	Control	100	100	100	100 (0)
	0.3–30%	0	0	0	0 (0)

C2 Water chemistry data

Table C.2 Chemical analysis (total soluble) of waters for *Ceriodaphnia dubia s. l.* testing program. All results (except pH) expressed in ppm.

Water type	pH	Na	K	Ca	Mg	Al	Mn	Cu	Fe	F	Cl	SO_4
Blank	–	0.01	<0.03	0.006	<0.001	0.005	0.0001	0.002	0.002	<0.5	<0.2	<0.5
West Queen River (unfilt)	6.10	6.6	0.45	0.78	0.82	0.094	0.009	0.008	0.149	<0.5	12.3	2.87
AMD raw filtered	2.61	50	20	87	218	114	90	63	21	<0.5	8.07	8370
AMD raw unfiltered	6.41	30	30	88	218	117	90	63	275			
AMD neutralised	6.38	5	5	640	214	0.4	71	0.6	0.7	0.67	11	3300
65:35 Stock Solution	2.95	7	2	450	215	41.2	79	22.3	18.3	<0.5	11.3	6460
80:20 Stock Solution	3.09	7	5	540	216	24	76	13.1	13.5	<0.5	11.2	4830
99:1 Stock Solution	4.47	7	1	640	215	1.3	72	0.9	0.2	0.56	11.1	3270
West Queen River	6.16	–	–	–	–	–	–	0.008	–	–	–	–
99:1 0.3%	6.22	–	–	–	–	–	–	0.01	–	–	–	–
99:1 1.0%	6.27	–	–	–	–	–	–	0.015	–	–	–	–
99:1 3.0%	6.35	–	–	–	–	–	–	0.03	–	–	–	–
80:20 0.3%	6.07	–	–	–	–	–	–	0.04	–	–	–	–

Appendix D

Results of Hydra viridissima tests

D1 Biological data

Table D.1a Daily population size of *H. viridissima* in Test 1 (three replicates per treatment)

	Test concentration (%)														
DAY	Control			0.1			0.3			1.0			3.0		
95:5 Neutralisation															
2hr	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1	10	10	10	10	10	10	10	10	10	10	10	10	0	0	0
2	20	13	14	16	19	17	12	13	13	10	10	10	0	0	0
3	20	20	19	20	20	18	14	19	17	10	10	11	0	0	0
4	22	22	22	24	23	23	18	20	19	9	8	7	0	0	0
5	32	36	34	31	30	30	20	20	20	3	3	2	0	0	0
	Control			0.3			1.0			3.0			–		
99:1 Neutralisation															
2hr	10	10	10	10	10	10	10	10	10	10	10	10	–	–	–
1	10	10	10	10	10	10	10	10	10	10	10	10	–	–	–
2	20	13	14	18	13	18	12	14	12	10	10	10	–	–	–
3	20	20	19	21	19	20	21	20	20	11	10	10	–	–	–
4	22	22	22	24	24	21	21	21	20	14	14	12	–	–	–
5	32	36	34	32	32	32	28	30	25	17	17	18	–	–	–

Table D.1b Daily population size of *H. viridissima* in Test 2 (three replicates per treatment)

	Test concentration (%)																	
DAY	Control			0.03			0.1			0.3			1.0			3.0		
95:5 Neutralisation																		
2hr	10	10	10	10	10	10	10	10	10	10	11	10	10	10	10	10	10	10
1	10	10	10	10	10	10	10	10	10	0	0	0	10	10	10	0	0	0
2	19	18	17	17	17	16	19	18	18	10	14	16	10	10	10	0	0	0
3	20	20	19	20	21	21	20	19	20	19	20	19	10	11	10	0	0	0
4	23	23	18	22	24	22	23	20	21	19	20	19	10	9	9	0	0	0
5	29	31	28	32	35	31	31	26	29	25	22	21	8	6	7	0	0	0
	Control			0.1			0.3			1.0			3.0			10.0		
99:1 Neutralisation																		
2hr	10	10	10	10	10	10	10	10	10	10	11	10	10	10	10	10	10	10
1	10	10	10	10	10	10	10	10	10	10	11	10	10	10	10	0	0	0
2	19	18	17	18	19	18	17	16	17	16	16	15	10	12	10	0	0	0
3	20	20	19	21	21	21	19	20	21	21	20	20	13	16	10	0	0	0
4	23	23	18	22	24	24	22	21	22	22	21	21	13	18	12	0	0	0
5	29	31	28	31	32	34	31	30	29	26	27	26	16	19	15	0	0	0

Table D.1c Daily population size of *H. viridissima* in Test 3, 99:1 neutralisation regime (three replicates per treatment)

	Test concentration (%)																							
DAY	Control			0.3			0.5			0.7			0.9			1.1			1.4			2.0		
2hr	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1	11	10	11	11	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	11	10	10	10
2	17	18	18	19	18	14	20	17	18	19	19	18	16	19	18	19	18	18	16	16	17	17	13	16
3	21	20	21	20	20	20	20	21	20	20	19	21	20	21	20	20	20	18	20	21	21	19	20	20
4	27	25	25	26	23	21	24	24	23	24	22	23	20	22	25	21	22	20	20	22	24	20	21	21
5	38	33	35	39	31	30	34	31	33	31	30	35	28	32	33	29	31	30	28	29	32	21	27	25

Table D.1d Daily population size of *H. viridissima* in Test 4, 95:5 neutralisation regime (three replicates per treatment)

	Test concentration (%)																							
DAY	Control			0.07			0.1			0.13			0.15			0.2			0.25			0.3		
2hr	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1	10	10	10	10	10	10	10	11	10	10	12	10	10	12	10	10	12	10	10	13	10	10	11	10
2	21	19	19	18	17	19	18	20	20	17	19	20	19	20	20	15	19	17	20	16	18	16	16	15
3	24	22	25	20	23	24	25	21	24	22	27	20	26	27	23	25	24	21	22	21	22	21	19	19
4	32	31	34	27	27	28	26	27	32	25	27	26	26	30	25	24	28	25	23	25	25	22	21	25
5	38	39	44	30	34	35	32	37	37	31	32	30	37	37	33	27	33	28	28	30	28	22	26	26

D2 Water chemistry data

Table D.2a Chemical analysis (total soluble) of waters for Tests 1and 2, *H. viridissima* testing program (West Queen River and control water refer to unfiltered and filtered diluent water respectively)

Water type	pH	Na (mg/L)	K (mg/L)	Mg (mg/L)	Ca (mg/L)	Cu (µg/L)	Fe (µg/L)	Mn (µg/L)	Al (µg/L)
Test 1									
West Queen River	5.94	4.8	0.3	0.68	0.51	10	130	7	160
AMD raw	2.58	<20	<200	220.0	80.0	59000	200000	85000	100000
AMD neutralised	6.4	7.00	<20	220.0	630.0	<200	<500	65000	100
AMD neutralised (duplicate)	–	5.00	<20	220.0	630.0	400	<500	66000	300
99:1 Stock	4.82	5.00	<20	220.0	640.0	700	2400	68000	1100
95:5 Stock	3.55	6.00	<20	220.0	610.0	3200	11000	68000	5300
Control	5.95	4.7	0.4	0.68	0.50	10	130	7	170
0.3% diluted 99:1	5.82	4.8	0.3	1.3	2.4	11	130	200	170
1.0% diluted 99:1	5.66	4.8	0.4	2.8	6.7	15	140	660	170
3.0% diluted 99:1	5.54	4.8	0.4	6.9	19	30	140	2000	190
0.1% diluted 95:5	5.84	4.7	0.3	0.92	1.2	14	140	80	170
0.3% diluted 95:5	5.84	4.8	0.4	1.3	2.4	18	150	210	180
1.0% diluted 95:5	5.82	4.7	0.4	2.9	6.8	39	190	700	220
3.0% diluted 95:5	5.09	4.8	0.3	7.1	18	100	270	2000	320
Test 2									
AMD raw	2.56	30.58	<200	230.0	80.0	62000	220000	88000	100000
AMD neutralised	6.42	8.81	<20	220.0	630.0	<200	<500	67000	<100
99:1 Stock	4.45	7.09	<20	220.0	630.0	700	2200	68000	700
95:5 Stock	3.54	7.86	<20	220.0	600.0	3100	11000	69000	4600
West Queen River	6.03	5.0	0.3	0.74	0.57	5	110	3	150
Control	6.09	5.0	0.3	0.74	0.57	5	110	3	150
0.1% diluted 99:1	6.12	5.1	0.3	1.0	1.2	8	110	69	150
0.3% diluted 99:1	6.1	5.0	0.3	1.4	2.4	9	110	200	150
1.0% diluted 99:1	5.99	5.1	0.3	2.9	6.7	12	120	670	160
3.0% diluted 99:1	5.89	5.1	0.3	7.1	18	26	110	2000	170
10.0% diluted 99:1	5.51	5.2	0.4	22	62	76	100	6700	230
0.03% diluted 95:5	6.28	5.1	0.2	0.83	0.78	7	120	26	150
0.1% diluted 95:5	6.18	5.1	0.3	1.0	1.2	9	120	71	150
0.3% diluted 95:5	6.07	5.0	0.3	1.4	2.4	15	130	210	160
1.0% diluted 95:5	5.84	5.0	0.3	2.9	6.5	35	160	680	200
3.0% diluted 95:5	5.3	5.1	0.3	7.1	18	100	250	2000	280

Table D.2b Chemical analysis (total soluble) of waters for Tests 3 and 4, *H. viridissima* testing program (West Queen River and control water refer to unfiltered and filtered diluent water respectively)

Water type	pH	Na (mg/L)	K (mg/L)	Mg (mg/L)	Ca (mg/L)	Cu (µg/L)	Fe (µg/L)	Mn (µg/L)	Al (µg/L)
Test 3									
AMD raw	2.56	<20	<200	230.0	0.09	61000	250000	87000	110000
AMD neutralised	6.48	12.00	<20	22.0	640.0	<200	<500	68000	<100
99:1 Stock	4.54	8.00	<20	230.0	650.0	500	1900	69000	1000
99:1 Stock	–	6.00	<20	230.0	650.0	600	2000	69000	1000
West Queen River	6.18	5.4	0.3	0.79	0.61	8	110	6	150
Control	6.16	5.3	0.3	0.78	0.61	7	110	5	150
0.3% diluted 99:1	6.12	5.4	0.3	1.5	2.6	8	110	210	150
0.5% diluted 99:1	6.14	5.4	0.3	1.9	3.8	8	110	350	150
0.7% diluted 99:1	6.17	5.4	0.3	2.3	5.0	10	110	470	150
0.9% diluted 99:1	6.17	5.4	0.3	2.8	6.4	13	110	630	150
1.1% diluted 99:1	6.16	5.4	0.3	3.3	7.7	14	110	770	160
1.4% diluted 99:1	6.18	5.7	0.3	3.9	9.6	14	110	970	160
2.0% diluted 99:1	6.14	5.4	0.3	5.3	14	20	110	1400	160
Test 4									
AMD raw	–	<200	<200	200.0	80.0	62000	270000	81000	100000
AMD neutralised	–	12.0	<20	230.0	660.0	<200	<500	72000	200
95:5 Stock	–	12.0	<20	230.0	630.0	3300	3600	73000	5100
95:5 Stock	–	11.0	<20	230.0	630.0	3300	3500	72000	4900
Control	6.42	5.5	0.4	0.79	0.62	6	130	7	110
Control duplicate	–	5.6	0.4	0.78	0.61	7	130	7	120
0.07% diluted 95:5	6.49	5.7	0.4	0.95	1.1	10	130	57	120
0.10% diluted 95:5	6.48	5.6	0.4	1.0	1.3	9	130	82	120
0.13% diluted 95:5	6.51	5.6	0.4	1.1	1.5	9	130	100	120
0.15% diluted 95:5	6.48	5.6	0.3	1.1	1.6	12	130	110	120
0.20% diluted 95:5	6.51	5.6	0.3	1.3	1.9	13	130	150	130
0.25% diluted 95:5	6.51	5.7	0.3	1.4	2.2	14	130	190	130
0.30% diluted 95:5	6.48	5.6	0.4	1.4	2.2	13	130	190	130