NIWA
Taihoro Nukurangi

Didymosphenia geminata experimental control trials: Stage One (screening of biocides and stalk disruption agents) and Stage Two Phase One (biocide testing)

NIWA Client Report: CHC2006-128
December 2006 (revised)

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Executive Summary

- NIWA was contracted by Biosecurity New Zealand to conduct a range of experimental trials on potential *Didymosphenia geminata* control techniques. Our approach was to carry out a structured 3-stage investigation involving product screening (Stage 1), product testing (Stage 2 Phases 1 and 2) and river trials (Stage 3). This report details the results of Stage 1 and Stage 2 Phase 1. This report also includes the results of a parallel work stream in which Michigan Technological University (MTU) was subcontracted to identify agents that degrade the extracellular polymers (stalks) that characterize *D. geminata* mats.

Stage One – Biocide Product Screening

- Trials tested the effectiveness of 10 biocides at controlling *D. geminata*. Three contact times (up to one hour) were used to assess biocide effectiveness for short-term treatments. Longer term efficacy was measured after 15 days for one contact time (one hour).

- Analysis of short-term biocidal effect determined that five biocides significantly reduced *D. geminata* cell viability. Of the five biocides, chelated copper[1] had the greatest effect on *D. geminata* for all contact times. In the longer-term assessment, a treatment effect was observed for four biocides (only two of which were identified from short-term trials).

- We recommend that EDTA, chelated copper[1], Organic Interceptor™ and Hydrothol®191 be tested in Stage 2 trials. A one hour contact time was most effective against *D. geminata* and will allow effective mixing during river application.

Stage One – Screening of Stalk Disrupting Agents

- MTU demonstrated that bases, weak acids, an antiseptic, the biodegrader— Rid-X, detergents, alcohol, hot water, and steam were ineffective degraders of stalk structure (DSS) with minimal disruption of stalk integrity. All mechanical methods tested were ineffective on hydrated mats.

- Chemical agents identified as effective DSS were concentrated hydrochloric and nitric acids, sodium hypochlorite and high concentrations of the chelator EDTA (0.2M or 72 g/L). EDTA and sodium hypochlorite (7.1mM) were effective over long exposure periods. Treatment with acids required high concentrations and elevated temperatures to achieve significant stalk degradation, limiting practical application in natural environments.

- Enzymatic degradation of stalks was highly effective. Crude enzyme extracts from the fungi *Penicillium funiculosum*, and *Aspergillius niger,* and the enzyme-based dietary supplement Omega-zyme showed significant degradation potential. The success of enzymatic degradation of stalk masses has application in future biocontrol initiatives proposed by Biosecurity New Zealand, although feasibility of this approach for in-stream application needs more analysis.

[1] The chelated copper formulation used in these trials is now identified as Gemex™

Stage Two Phase One – Biocide Product Testing

- We assessed the toxicity to *D. geminata* of four selected biocides (chelated copper[1], EDTA, Hydrothol®191, Organic Interceptor™) at three concentrations. Mats of *D. geminata* were grown on artificial substrates and then transferred into experimental channels. Biocides were applied to substrates for one hour. *D. geminata* mats were sampled prior to biocide application, then at 1 hour, 1, 3, 5, 12 and 28 days to assess viability. Fish mortality in response to biocide exposure was also assessed in the channel trials.

- The four biocides differed significantly in their effect on *D. geminata* cell viability. Channels that received Organic Interceptor™ had a lower proportion of viable cells than those that received chelated copper[1]. EDTA or Hydrothol®191 were relatively ineffective at reducing cell viability. Only Organic Interceptor™ at the highest concentration tested achieved 100% mortality of *D. geminata* cells, however, this was also the only biocide to cause fish mortality.

- Laboratory toxicity testing of the non-target alga *Pseudokirchneriella subcapitata* and the invertebrate *Daphnia magna* showed exposure to greater than 0.1 mg Cu/L for longer than 1 h had negative effects on both species. EDTA had no effect on *D. magna* at the concentrations used in field trials. After a 1 h exposure to Hydrothol®191 at the highest concentration used in field trials, *D. magna* were unaffected, however Hydrothol®191 was extremely toxic to the non-target alga. After 1 and 4 h exposures to Organic Interceptor™, 70% of *D. magna* survived at the highest concentration used in field trials. The non-target alga was sensitive to Organic Interceptor™ in the same concentration range as *D. geminata*.

- We recommend conducting further testing on chelated copper[1] and Organic Interceptor™. Hydrothol®191 was relatively ineffective on *D. geminata* at the concentrations trialled. EDTA is not viewed as a biocide warranting further investigation, although it may have application as a DSS Further screening trials are needed to determine the concentration for maximum *D. geminata* mortality with minimal non-target species mortality, particularly for fish.

- A decision-matrix quantifying the characteristics we are seeking in an ideal *D. geminata* control compound ranked chelated copper[1] highest, followed by Organic Interceptor™. With the data available to date, it is considered feasible that an in-river chelated copper[1] treatment can be developed that will have acceptable non-target effects, be cost-effective and achieve >90% control of *D. geminata*. The data indicate biocides could be effective for at least 28 d. Work planned for Stage 2 Phase 2 and Phase 3 (river trials) will refine these findings further, particularly in terms of the difference between controlled environment versus stream application ("real world") effectiveness, and to determine if an even higher rate of *D. geminata* control is possible.

[1] The chelated Cu formulation used in these trials is now identified as Gemex™

1. Introduction

Didymosphenia geminata Schmidt (didymo) is an invasive freshwater diatom that exists in streams, rivers and around some lakeshores. This unwanted algal species was first detected in the lower Waiau and Mararoa River system, Southland in October 2004. The distribution of the alga has now significantly expanded, with the diatom recorded in 15 South Island river systems (and five of New Zealand's eight longest rivers) as of July 2006. The alga has not yet been detected in the North Island.

In New Zealand, this alga forms dense fibrous mats capable of covering the entire stream bed. These mats are composed of extracellular polymers organized into stalks. The capability to secrete large quantities of highly organized extracellular polymer arrays differentiates *D. geminata* from other related benthic diatoms. If the spread of *D. geminata* continues, the following environmental and economic problems may occur: reduced recreation values (including damage to trout fisheries), extinction of native species, reduced tourism expenditure (international and domestic), increased costs for water intakes (industrial and agricultural), impacts on commercial eel fisheries, and potential disruption to community, municipal and domestic drinking water (Campbell 2005, NZIER 2006). The NZIER eight-year economic impact assessment of *D. geminata* estimates potential present value impacts to be $158 million (NZIER 2006).

The impact of large diatom blooms on water chemistry and aquatic flora and fauna also appears to be significant (Kawecka & Sanecki 2003, Kilroy 2004). For example, invertebrate diversity and relative abundance appears to be negatively associated with increasing *D. geminata* populations. Sensitive species such as mayflies, stoneflies and isopods are either absent or present at lower proportions in affected areas (Kilroy et al. 2005).

The potential impacts of *D. geminata* on environmental and economic values have necessitated the need for control or eradication measures to be researched. NIWA was contracted to conduct a range of experimental trials on potential *D. geminata* control techniques to assess their effectiveness, likely impacts on other components of the ecosystem, feasibility, risks, duration of control and cost. To date, there have been no published examples of attempts to contain, control or eradicate blooms of *D. geminata* (Kilroy 2004). Our approach was to conduct this research in natural and controlled stream environments, with additional toxicity work being conducted in the laboratory. We implemented a structured 3-stage process for this investigation (Figure 1). This report merges the results of our screening process for biocides and stalk disrupters (Stage 1), product testing in stream-side channels and non-target toxicity testing (Stage 2 Phase 1). Stage 2 Phase 2 and Stage 3 studies have commenced following an assessment of draft results from this initial part of the project.

Stage 1 involved screening a wide range of potential biocides for their short- and longer term efficacy on *D. geminata*. From these results four products were selected that we believed warranted further testing. Next we assessed the toxicity of the four selected biocides to *D. geminata* across a concentration gradient, as well as the impact of the biocides on non-target organisms (Stage 2 Phase 1). In the future we anticipate refining optimal biocide concentrations and formulations in relation to stream bed hydraulics/contact time, as well as further assessments of non-target organism impacts (Stage 2 Phase 2). If a suitable biocide formulation can be produced, then a full-scale stream trial to measure ecosystem effects and the duration of impact on *D. geminata* is envisaged (Stage 3).

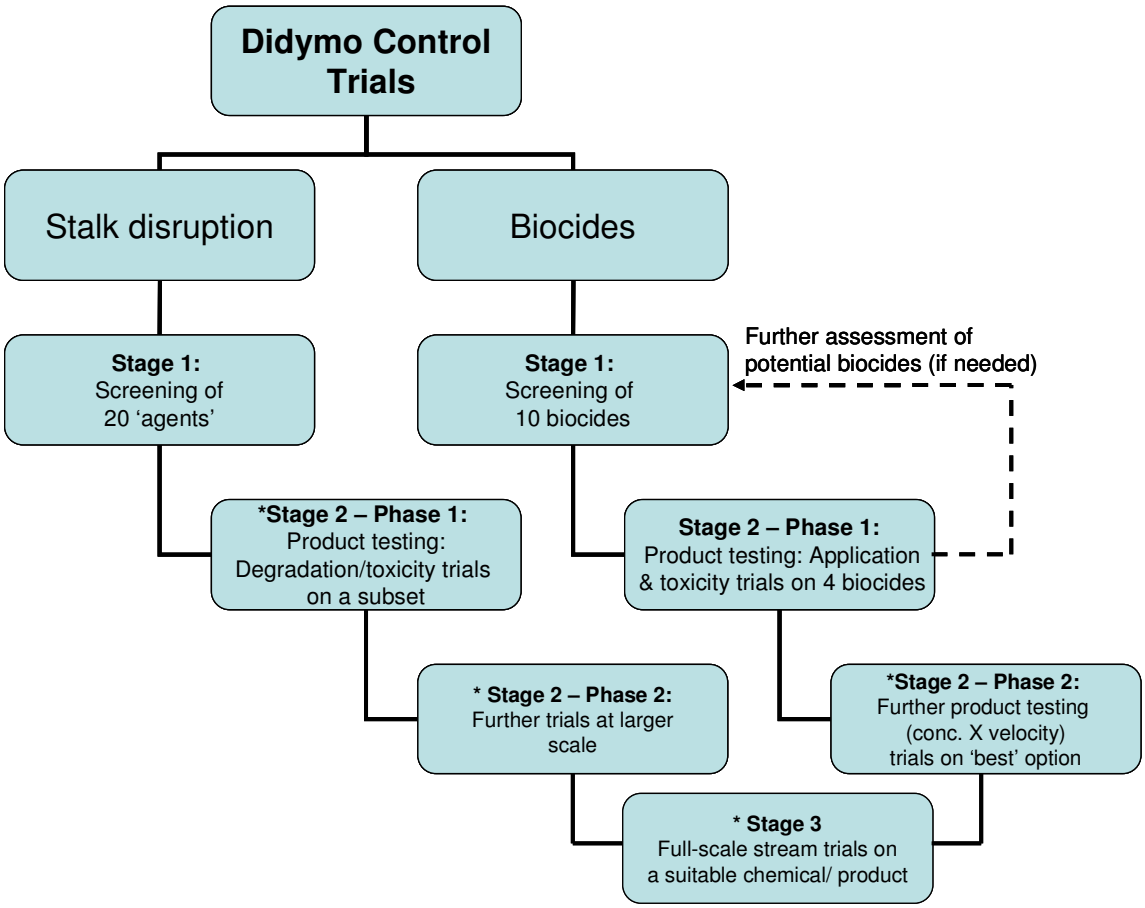


Figure 1: The organization of the 3-stage control study investigation. Asterisk (*) denotes stages that have not yet been completed.

In Stage 1 of the Stalk Disruption studies, Michigan Technological University (MTU) addressed problems related to the stalks produced by *D. geminata*. These stalks are highly resistant to degradation in stream environments in which they are produced and persist after cells have been killed. Difficulties inherent in eradication of *D. geminata* with minimal impact on overall stream ecology may necessitate an approach

encompassing control of the masses of stalk material which are the visible product of *D. geminata* colonization of stream beds. Stage 1 included screening of various degraders of stalk structure (DSS) in a laboratory environment.

Throughout the investigation, the following selection criteria were used to evaluate the effectiveness of each biocide/concentration to assess whether a product progressed onto the next stage:

1. Toxicity to *D. geminata* (% mortality based on a comparison of number of live cells before and after treatment).

2. Defoliation potential for non-living stalk material.

3. Contact time – shorter the better.

4. Ease of application – including human health implications, form of biocide (solid/powder/liquid), whether gels or surfactants are needed, etc.

5. Potential for damage of key non-target species and ecosystem functioning – particularly invertebrate taxa such as mayflies, hydropsychid caddisflies, and juvenile trout.

6. Cost of materials.

7. Consent requirements or restrictions, and any other considerations identified during discussion with regulatory authorities.

The ideal product would have high toxicity to *D. geminata* and minimal toxicity to non-target species. The assessment of the effectiveness, duration, impacts, feasibility risk, and cost of *D. geminata* control options begins in this report and will improve as the project moves into Stage 2 Phase 2 and Stage 3, and more refined information is available. A thorough ecological risk assessment of potential environmental effects will be performed on the lead candidate(s) prior to any major release of a control biocide into an affected waterway.

2. Stage One – Biocide Product Screening

2.1. Methods

2.1.1. Study site

Trials were carried out on land owned by Pioneer Generation Ltd. on the banks of Monowai River, Southland. The Monowai River is a regulated waterway that drains Lake Monowai as part of the hydro-electric power station at Monowai (Map 1). The lower reach of the Monowai River is diverted into a hydro-canal six kilometres downstream of the lake, with a minimum flow of 500 l/s maintained down the lower Monowai River. The regulated residual flow in lower Monowai River provided a reasonably stable flow environment to conduct experimental trials, and the banks of the river had an area of suitable flat surface to perform short-term trials. The lower Monowai River had moderate riparian shading, stable cobble – boulder sized substrate and a relatively uniform channel with a low – moderate gradient (~ 1%). The experimental site was placed *c.* 20 m above what had been determined as the upstream limit of *D. geminata*. This was done to prevent live *D. geminata* cells immigrating into the treatments (potentially confounding evaluation of treatment effectiveness). The water chemistry of the Monowai River was considered suitable for growth of *D. geminata*, as it proliferates throughout the lower reaches (see Appendix 1).

2.1.2. Selected biocides

Based on NIWA's knowledge of existing products used here and overseas for controlling algae and aquatic weeds (and a survey of the international literature – Gee & Wells 2006), we evaluated the effectiveness of the following products against *D. geminata* (a product summary and test concentrations are shown in Table 1) :

Chelated copper[3] - Copper is an established algaecide and has been used extensively for this purpose since the 1960s in USA. It is commonly used in swimming pools and the aquarium trade in chelated copper formulations to control algae and fungi while not harming desirable aquarium organisms (e.g., corals) or fish. Copper has been approved by the U.S. Environmental Protection Agency for use on a repetitive basis in lentic systems (ponds, lakes). Copper-based algaecides are often recommended because they are cheap, efficient, easily obtained and are reasonably specific. *D. geminata* is known to be sensitive to copper with die-back when levels exceed 0.015 $mg.l^{-1}$ Cu, with no conspicuous growth in a polluted Norwegian river system when Cu exceeded 8 $ug.l^{-1}$ (Lindstrom and Rorslett 1991). The formulation of chelated copper

[3] The formulation of chelated copper used in these trials is now identified as Gemex™

used in the *D. geminata* trials was selected because of its stability over a wide range of pH and temperatures[4].

Zinc Sulphate - This was trialled as it is known to be effective in controlling algae and is used in swimming pools for this purpose. Native mayflies are also known to tolerate up to 25 $mg.l^{-1}$ of Zn. Zinc sulphate is used in swimming pools at 7 $mg.l^{-1}$ to control algal black spot.

Organic Interceptor™ – This product is a pine oil-based formulation used to control annual weeds, grasses and brown-off of perennial species. Organic Interceptor™ has been tested in the NIWA ecotoxicology laboratory and is registered by ERMA for use as a herbicide in New Zealand. It has potential for more widespread use as an algaecide.

Germanium dioxide – This is an odourless white powder that is relatively non-toxic and is not listed as a dangerous substance. The Material Safety Data Sheet states that germanium dioxide is of low toxicity to humans both acutely and chronically by all routes of administration including inhalation. Germanium dioxide has moderate solubility in water and is believed to present low environmental risk, with limited bioavailability. Germanium dioxide is known to inhibit or kill diatoms quite specifically at low concentrations (1 – 10 $mg.l^{-1}$) possibly by substituting for silicon, and germanium dioxide is also used in aquariums.

Simazine – Simazine (2-chloro-4,6-bis(ethylamino)-s-triazine) is used as an algaecide and a pre-emergence herbicide, marketed under various trade names. It is available in liquid, tablet or wettable powder form. According to available literature, simazine is effective at controlling unicellular and attached filamentous algae at a concentration of 0.5-1.0 $mg.l^{-1}$. Simazine works by interrupting plant photosynthesis, which then depletes the plant's starch reserve and it subsequently dies. It is most effective against blue-green algae, moderately effective against green algae, while diatoms and flagellates are the least sensitive. CIBA-GEIGY indicate that algae control should occur within 3-14 days after application, depending on the type of algae. Studies performed on fish suggest that simazine is not "toxic" at recommended concentrations to trout, minnows, catfish and bluegill.

Quarternary Ammonium Compounds (QAC's) – QAC's are used extensively in industry for hygiene purposes with effective control against bacteria, fungi and algae. They are used for cleaning buildings, floors, food preparation surfaces and have a long track record of safe use. QAC's are known to suppress plant growth at concentrations of 3-5 $mg.l^{-1}$ (Walker & Evans 1978). A swimming pool treatment formulation known as 303 Clear All (Buckman's) was used for this phase of product screening. This product is a proprietary mix of complex cationic copolymers.

[4] The formulation of chelated copper used in these trials is now identified as Gemex™

Chlorine – Chlorine is an industry standard for sterilisation and is used in many different forms as a bleach and disinfectant, and for the control of biofilms (which include bacteria and algae) in swimming pools and cooling towers. Sodium hypochlorite (NaOCl) was used in this product-screening phase as it is a readily available form of chlorine used in household bleach, and is a strong oxidant that might penetrate the *D. geminata* mats.

EDTA – EDTA or ethylenediaminetetraacetic acid chelates metal ions. It is used to detach marine biofouling diatom cells from their pads/stalks and is thought to act as a chelator of cations (e.g., Ca^{2+}), altering the molecular composition of the extracellular mucilage. The diatom cells separate from their pads after a couple of hours. Urea has a similar mode of action but at higher concentrations. EDTA was included in product screening because of its potential to cause *D. geminata* mat detachment. EDTA is a polyprotic acid containing four carboxylic acid groups (acidic hydrogens) and two amine groups. The unusual property of EDTA is its ability to chelate or complex metal ions in 1:1 metal-to-EDTA complexes. Because of its strong complexing ability for most metal ions, it is used in the food industry as a sequestering agent. The complexing of the metal ion may prevent further reactions, such as binding metals that are cofactors for enzymes. EDTA is found in many natural waters and occurs at higher levels in wastewater effluents, however, EDTA (like other chelating agents) persists for long periods in the environment as it has low biodegradability.

Hydrothol®191 – One of two formulations of endothall used for aquatic weed control Hydrothol®191 has been used to control aquatic vegetation and filamentous algae for over 30 years. Aquathol® K (dipotassium salt of endothall) is used for submerged aquatic weed control and has recently been registered and approved through ERMA for this purpose in NZ. Hydrothol®191 (dimethylalkylamine salt of endothall) is formulated for aquatic algae and at > 1 $mg.l^{-1}$ rapidly inhibits photosynthesis (Axler et al. 1994). Label recommendations are for rates of 0.05-0.3 $mg.l^{-1}$ for most algal control, although dosages may be increased to 1.5 $mg.l^{-1}$ for difficult to control algae or where long-term control is needed. Hydrothol®191 is regarded as moderately toxic to a variety of aquatic biota, particularly fish but was selected over Aquathol K for trials because it is also particularly toxic to diatoms and is recognised as more effective against problem algae.

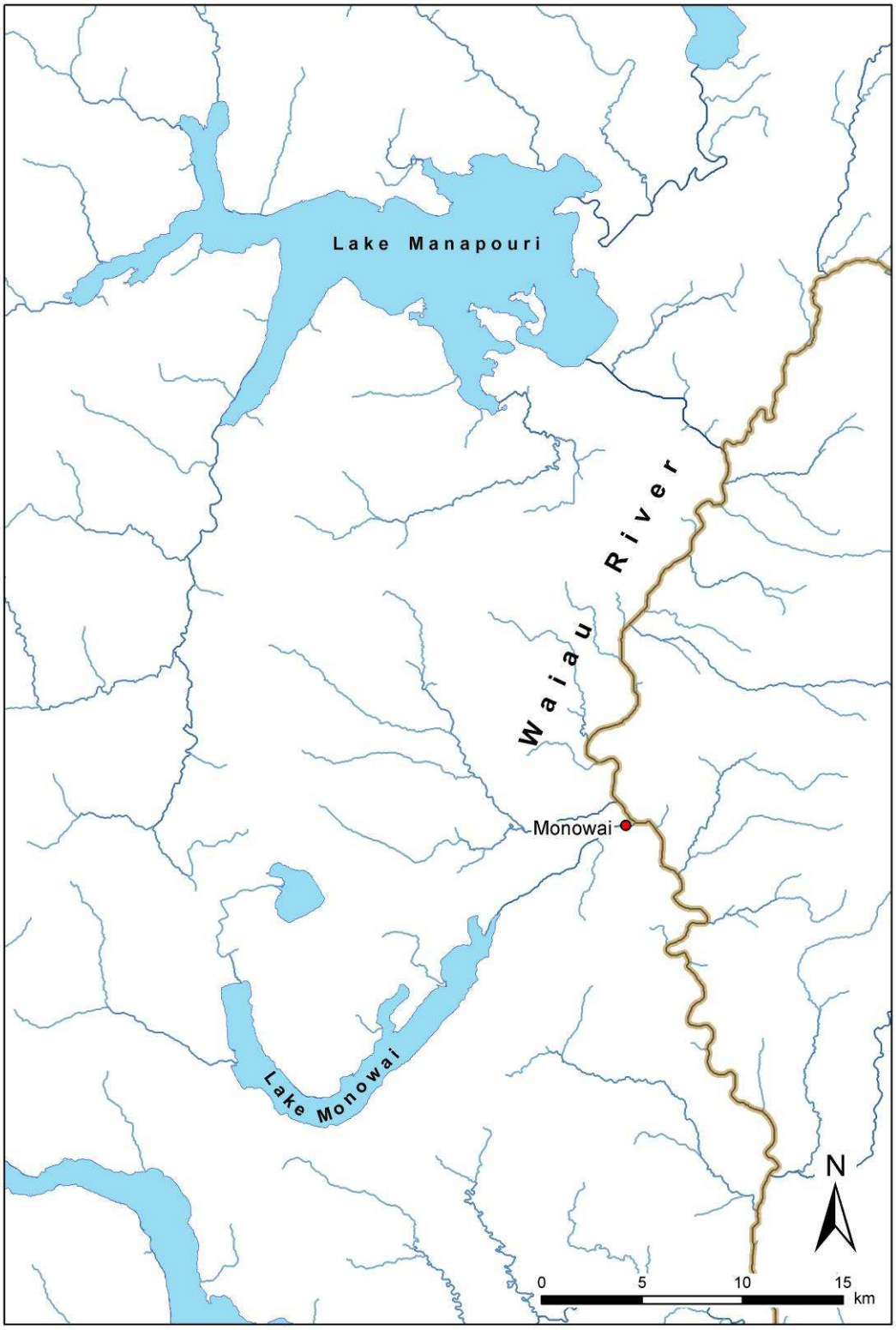
Diquat - Diquat (technical name diquat dibromide) is the active ingredient in Reglone®, a herbicide that has been used in New Zealand for over 40 years for both agricultural operations and lake-weed control. Reglone® contains 20% diquat dibromide and is a selective herbicide that controls most unwanted target weed species in freshwaters (e.g., *Elodea*, *Egeria*, *Lagarosiphon* and hornwort). Many key native plant species are not affected (e.g., *Chara* and *Nitella* species) by diquat. When diquat comes into contact with the green parts of nuisance aquatic weeds (leaves and stems) it is rapidly absorbed producing peroxide, that acts like bleach, desiccating plant tissue

and disrupting cell membranes. Diquat affects the tissue it comes in contact with and is not moved around within the plant, so roots are not killed. Diquat is rapidly removed from the water and deactivated by adsorption onto inorganic and organic compounds in the water and sediments. The affect of diquat on algae is known to be quite variable.

2.1.3. Experimental design

For each treatment, pebble (< 80 mm diameter) and cobble (< 200 mm diameter) sized substrate were selected from a 75 m² area of the Waiau River bed. Substrates were visually assessed (using a divers mask) to select rocks with 'maximum' surface coating and thickness of *D. geminata* (see Kilroy et al. 2005). For each treatment replicate, five pebbles and one cobble were used. Two of the five pebbles were randomly chosen for controls. One of the pebbles was destructively sample before the treatment to assess the initial percentage of viable cells. The other pebble was placed in a bucket of fresh river water for 3600 seconds as a control for the lentic conditions treatment rocks were exposed to during the trial.

Three pebbles and one cobble were placed into the bottom of three replicate, lined 54L fish bins, with 50 L of river water. Each of the fish bins had biocide added to a pre-determined target concentration (based on product label recommendations or literature) to achieve high mortality in a susceptible algal species (Table 1). Biocides were either pipetted (aqueous) or hand delivered (powder) depending on the product (see Appendix 2). All bins were shaded to keep conditions uniform throughout the trials, because changes in water temperature can alter chemical uptake rates.



Map 1: Map showing the location of the Monowai experimental control site. Note the experimental waterway (Monowai River) connects Lake Monowai to the Waiau River. The brown colour/thicker line indicates the stretch of river that is most heavily infested by *D. geminata*.

Fish bins that contained the biocide formulations were aerated over the course of the trial to simulate river turbulence and enhance transfer of the biocide through the mats. Each aerated bin had a single air-line that took air off a manifold connected to a tank of compressed air. A pebble from each of the three bins was withdrawn after 36, 360 and 3600 seconds (0.6, 6, and 60 min respectively) (and placed in separate bins of fresh river water. Pebbles were withdrawn at these contact times to provide a logarithmic time series for statistical analyses. The single cobble was removed after 3600 seconds, and also placed in a separate bucket containing fresh river water. The sets (36, 360 and 3600 seconds) of replicate pebbles were destructively sampled at the conclusion of the final contact time. *D. geminata* mats were removed from the pebbles, and samples taken to the on-site laboratory for live/dead cell enumeration.

Table 1: The mode of action, test concentration and predicted response time of the 10 biocides selected for Stage 1. Test concentrations were selected to correspond with concentrations predicted to cause 100% mortality in responsive algal species.

Biocide	Test Concentration	Mode of action	Response time
Sodium hypochlorite	5 mg $Cl.l^{-1}$	Photosynthetic inhibitor	Immediate
Chelated copper	5 mg $Cu.l^{-1}$	Photosynthetic inhibitor	Immediate
Diquat	5 mg Reglone® l^{-1}	Photosynthetic inhibitor	Immediate
QAC (303 Clear All)	0.02 mL product $.l^{-1}$	Membrane disruption	Immediate
Simazine	1 mg $a.i.l^{-1}$	Metabolic disruption	Immediate
Zinc sulphate	10 mg $Zn.l^{-1}$	Cell toxicant	Immediate
Organic Interceptor™	1000 mg pine $oil.l^{-1}$	Photosynthetic inhibitor	Immediate
Hydrothol®191	1 mg $a.e.l^{-1}$	Photosynthetic inhibitor	Longer term
EDTA	10 mg $EDTA.l^{-1}$	Exfoliation & degradation	Longer term
Germanium dioxide	10 mg $Ge.l^{-1}$	Cell toxicant	Longer term

Cobbles were placed back into the Monowai River after 3600 seconds and left for 15 days (see Appendix 1). A succession of transect lines (3 m wide) were constructed on the river bed; with two chemical treatments placed on each transect line. The position of the cobbles was randomised along the transect line to allow for variation in water velocity. At the completion of the trial, water depth (cm) and water velocity (m/s) were measured at each location of each cobble. An electromagnetic current meter (Marsh-McBirney Flo-Mate™ Model 2000 accuracy ± 2%) was used to measure water velocity at "mat height" to determine the hydraulic stresses under which *D. geminata* mats were exposed. *D. geminata* mats were sampled on-site and taken to the laboratory for live/dead cell enumeration (Kilroy 2006). The condition of each cobble was also photographically documented.

2.1.4. Laboratory procedures

Changes to the viability of *D. geminata* mats were determined microscopically using the staining techniques developed by NIWA to distinguish live and dead cells (Kilroy, 2005). For each sample, at least 100 *D. geminata* cells were enumerated for detection of those that might be live. Samples were macerated prior to the introduction of the stain, to allow uniform stain uptake throughout the mat. Samples were analysed within one hour of staining, with no detectable decline in cell viability over this period. For treated samples (i.e., 36, 360 and 3600 s), the three replicates for the 3600 s contact time were analysed first and if cell viability was > 85%, then the remaining contact times were not examined.

Two 30 mm diameter circles were scribed from the top of each cobble using surgical scissors for chlorophyll *a* and ash-free dry mass (AFDM) analysis. Both samples were placed in individual polyethylene containers and frozen before transportation. Samples for the longer-term trials were analysed for chlorophyll *a* and AFDM using the methods described in Biggs and Kilroy (2000). Each sample was homogenised using a hand blender and made up to a known volume. Duplicate subsamples of known volume were filtered through glass fibre filters. For AFDM, one of the duplicates (a preweighed filter plus filtered sample) was dried at 105°C for 24 h, reweighed, then ashed at 400°C for 4 h and weighed for a final time. Chlorophyll *a* was extracted from the second filter-plus-sample using boiling ethanol, and concentrations of chlorophyll *a* were read spectrophotometrically at 663 nm, including acidification to correct for phaeophytins. In each case, we calculated quantities per m² of stone surface, based on the area of the sampling circle (0.00141 m²).

Note: Chlorophyll *a* (mg/m²) is a measure of the total amount of autotrophic organisms (live algal material) in the sample. AFDM (g/m²) is a measure of the total amount of organic material in the sample, including cell contents, *D. geminata* stalk material, and also any other algae or small organisms trapped within the mat.

In addition to live/dead cell enumeration, cell density was also measured using an inverted microscope. This analysis produced a quantitative estimate of the absolute number of "live" cells in a sample, where live cells were taken to be cells containing intact chloroplasts. One-ml subsamples were extracted from the homogenized solution made up for biomass estimation (see above), and pipetted into the well of an inverted microscope. Entire subsamples were then scanned at a magnification of approx. x 100, and counts were made of all *D. geminata* cells containing chloroplasts in up to 100 fields of view. At least 250 cells were counted per sample. Absolute numbers of cells per sample were calculated from the known areas of the microscope well and the field of view.

2.1.5. Data analysis

Short-term effects (pebbles)

To reduce the number of samples to be processed, a two-stage analysis was adopted. The first stage identified biocides that significantly reduced cell viability at the longest contact time (3600 seconds). The mean proportion of cells alive after 3600 seconds contact with each biocide was compared with a control (the average proportion of viable cells on rocks immersed in water for 3600 seconds = 0 seconds contact time) using a one-tailed one-sample *t*-test for each biocide. The significance level for this test was set at $\alpha = 0.10$ to lower the probability of falsely rejecting the null hypothesis of no effect (i.e., to reduce the risk of accidentally discarding effective biocides). The second stage compared the performance, over a range of contact times (0-3600 seconds), of five biocides identified in stage 1 as being capable of reducing cell viability. The proportion of viable cells was modelled using a Generalised Linear Model (GLM) with a binomial error distribution and a log link function. Preliminary analyses showed no significant variation among replicate buckets for each biocide and so this term was dropped from the model, leaving just two predictors: biocide (a fixed factor with 5 levels: chlorine, chelated Cu, Organic Interceptor^{TM}, QAC and zinc sulphate) and contact time (a fixed factor with 4 levels: 0, 36, 360 and 3600 seconds). Contact time was measured on a continuous scale and $\log_{10}$-transformed. Since the log of 0 is undefined, we approximated this control with a value of 0.36, two $\log_{10}$ units below the lowest "real" treatment. The significance of model terms was evaluated using *F*-ratio tests, which take account of over-dispersion in the data.

Longer-term effects (cobbles)

Ash free dry mass (AFDM), chlorophyll *a*, and the ratio of AFDM to chlorophyll *a* of *D. geminata* on rocks was analysed using one-way analysis of variance (ANOVA). There were 11 levels of the treatment factor – 10 biocides and a control (water). The assumption of homogeneous variances was tested using an F_{max} test. Following a significant main effect, Dunnett's test was used to examine which biocides differed significantly from the control group. Habitat characteristics (water velocity and depth) were analysed using regression analysis.

S-plus v.6.1 (Lucent Technologies, Seattle, WA) was used for all analyses, except Dunnett's test, which was performed by hand.

2.2. Results

2.2.1. Short-term effects (pebbles)

The mean (± S.E.) proportion of viable cells on control rocks (those that had been immersed in water for 3600 seconds) was 0.95 ± 0.01. Five biocides significantly reduced the proportion of viable cells below this level when applied at the longest contact time (3600 seconds): chlorine, chelated Cu, Organic Interceptor™, QAC and zinc sulphate (Table 2, Figure 2). The effectiveness of all five biocides increased with contact time (GLM, $F_{1,47}$ = 127.862, P < 0.001) and their relative performance also varied with contact time (GLM, biocide x contact time interaction: $F_{4,47}$ = 4.956, P = 0.002). Overall, chelated Cu was the most effective, particularly at short contact times; 76.2% of cells died after 36 seconds contact time and 94.3% died after 3600 seconds. Organic Interceptor™, QAC and chlorine performed similarly, achieving >50% mortality only at the longest contact time. Zinc sulphate had only a minor effect on cell viability (Figure 3).

Table 2: One-tailed, one-sample *t*-tests comparing the mean proportion of cells alive after 3600 seconds contact time with each biocide with the average proportion (0.95) of viable cells on control rocks immersed in water for 3600 seconds. Exposure at maximum concentration given in Table 1. n = 3 replicate samples for each biocide. Significant differences are indicated in bold (α = 0.10).

Biocide	Mean ± 1 S.E.	*t*	*P*
Chelated copper	0.06 ± 0.03	-26.65	**0.001**
Chlorine	0.34 ± 0.03	-17.64	**0.002**
QAC	0.39 ± 0.17	-5.60	**0.015**
Organic Interceptor™	0.51 ± 0.15	-5.26	**0.017**
Zinc sulphate	0.74 ± 0.11	-3.12	**0.045**
Germanium	0.74 ± 0.30	-1.24	0.171
Diquat	0.86 ± 0.11	-1.53	0.133
Simazine	0.95 ± 0.01	0.08	0.528
EDTA	0.96 ± 0.01	4.91	0.981
Hydrothol®191	0.96 ± 0.01	4.12	0.973

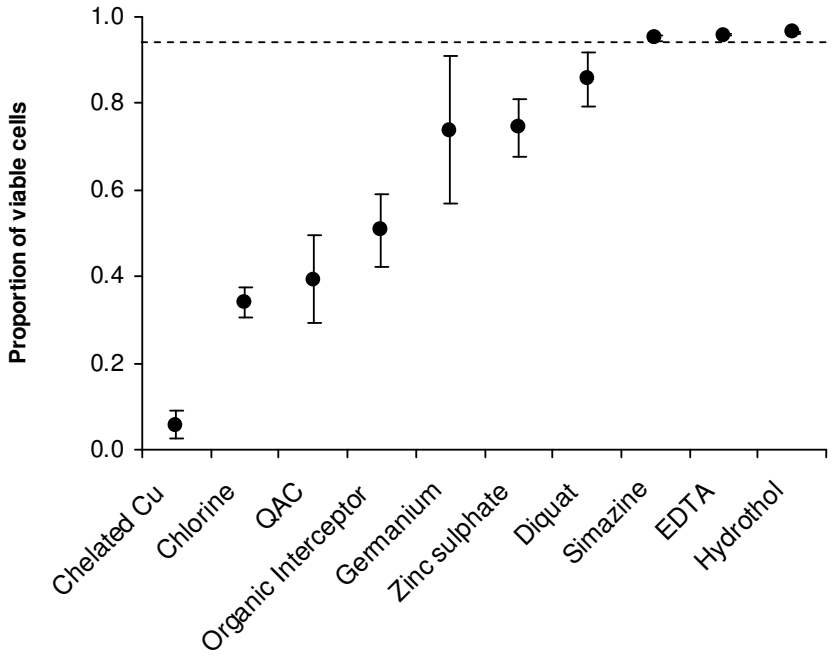


Figure 2: Mean (± 1 S.E.) proportion of viable cells after 3600 seconds contact time with ten biocides. Concentrations provided in Table 1. The dashed line shows the average proportion of viable cells on control rocks immersed in water for 3600 seconds.

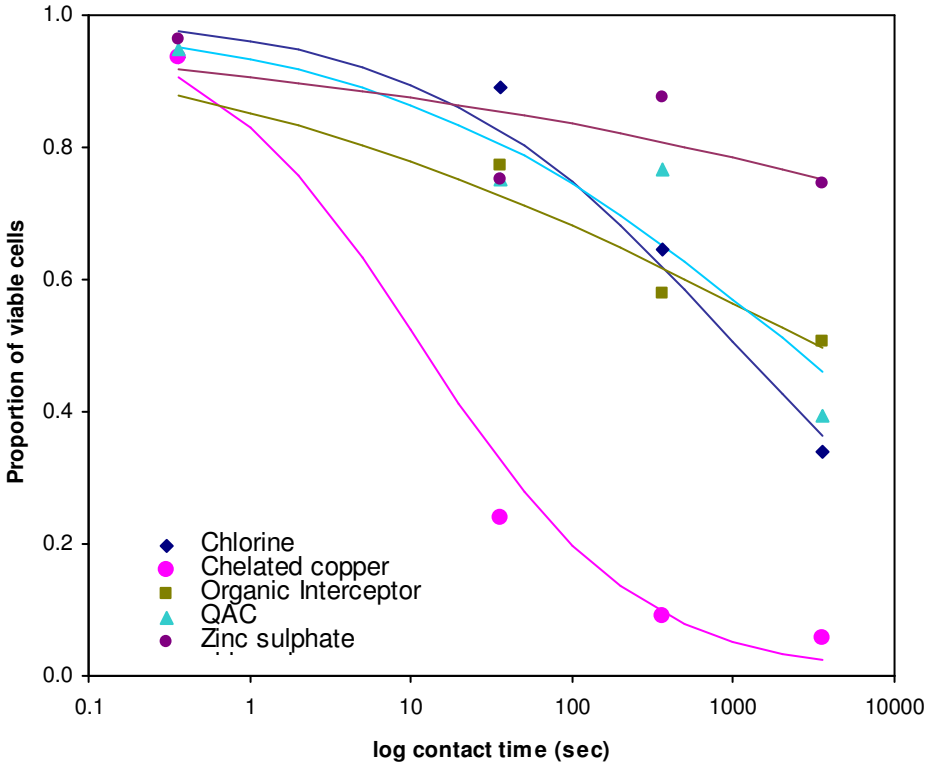


Figure 3: Relationship between cell viability and contact time for five selected biocides. Points are means based on three replicate stones per treatment combination. Lines indicate predictions from a Generalised Linear Model. Concentrations provided in Table 1. Exposure to chelated Cu resulted in the lowest proportion of viable cells, while zinc sulphate exposure resulted in the highest proportion of viable cells.

2.2.2. Longer-term effects (cobbles)

Habitat characteristics (water velocity and depth) had no significant effect ($P > 0.05$) on any of the following responses: cell viability, cell density, chlorophyll *a*, AFDM or the autotrophic index (AI = AFDM: chlorophyll *a*). There was a weakly significant effect of treatment on AFDM (ANOVA: $F_{11,23} = 2.650$, $P = 0.023$). AFDM was significantly higher in the diquat treatment than the control (Dunnett's test: $q = 2.284$, $P < 0.05$; Figure 4). No other biocides had a significant effect on AFDM. We did not observe any increase (for any treatment) in the biomass of *D. geminata* over the course of the 15 day trial. There was no effect of treatment on either chlorophyll *a* biomass (ANOVA: $F_{11,23} = 1.882$, $P = 0.097$) or the ratio of AFDM to chlorophyll *a* (ANOVA: $F_{11,23} = 0.942$, $P = 0.521$).

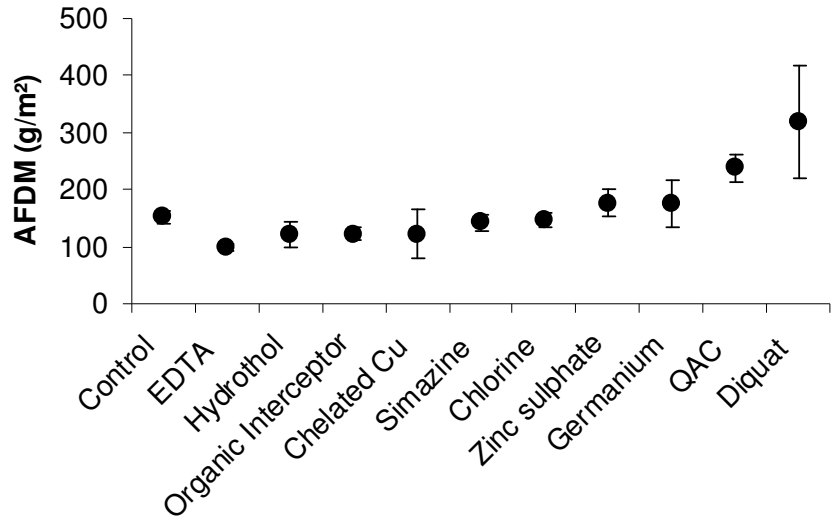


Figure 4: Mean (± 1 S.E.) AFDM of *D. geminata* on rocks 15 days after treatment with 10 biocides and a control (water). $n = 3$ replicate rocks per biocide and 2 replicate rocks for the control treatment.

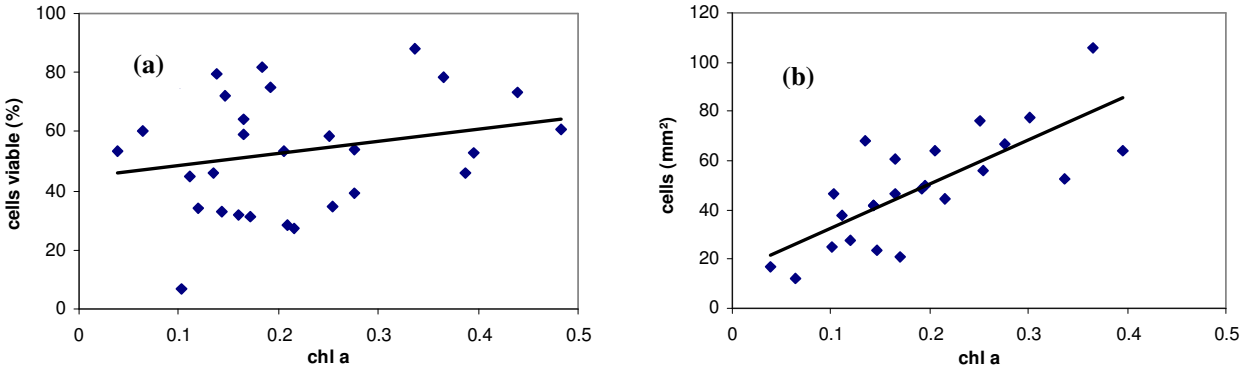


Figure 5: The percentage of viable cells (a) and cell density (b) plotted against chlorophyll *a*. There was no significant relationship between cell viability and chlorophyll *a* for the ten biocides that were analysed (Figure 5a). Cell density was significantly correlated with chlorophyll *a* measurements for the six biocides that were measured using an inverted microscope (Figure 5b).

Longer-term cell viability ratios were highly variable because it was not possible to assess whether cell death was the result of a treatment or environmental conditions. Cell viability showed large variation when measured against chlorophyll *a* ($R^2 = 0.05$, $P = 0.435$), indicating that the amount of live material in the samples was poorly correlated with live cell counts (Figure 5a). However, cell density was highly correlated with chlorophyll *a* ($R^2 = 0.67$, $P < 0.001$) (Figure 5b). Either metric (cell density or chlorophyll *a*) provides a good method for longer-term assessment of biocide effectiveness on *D. geminata*. The significant correlation between cell density and chlorophyll *a* indicates that *D. geminata* cells are the dominant live material in these communities.

Of the four products that had cell densities lower than that of the control, only EDTA had densities significantly different from the control (*t*-test: $t_7 = 3.559$, $P < 0.01$). The four products which had lower mean cell densities than the control were: EDTA, chlorine, Organic Interceptor™ and chelated Cu (Figure 6). Hydrothol®191 contained samples with very low cell densities, but samples were highly variable.

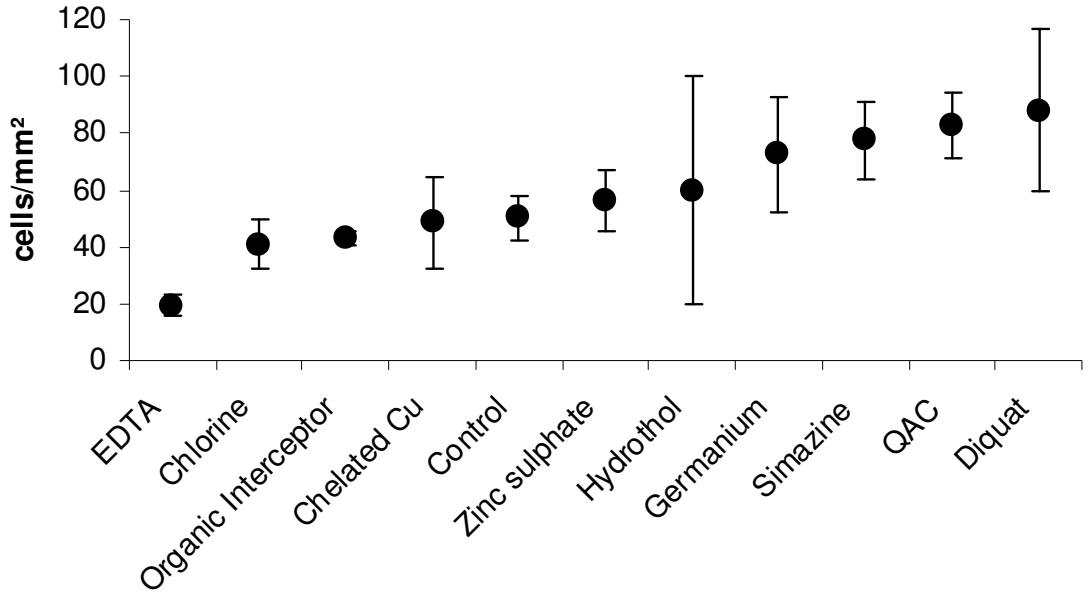


Figure 6: A comparison of mean *D. geminata* cell densities (± 1 S.E.) 15 days after treatment with 10 biocides and a control (water).

There was no effect of treatment on chlorophyll *a* biomass (ANOVA: $F_{11,23} = 1.882$, $P = 0.097$). The four products which had lower mean chlorophyll *a* measurements than the control were: EDTA, chelated Cu, Hydrothol®191 and Organic Interceptor™ (Figure 7). The other biocides had chlorophyll *a* measurements that were similar or higher than the control samples.

There was also no significant effect of treatment on the ratio of AFDM to chlorophyll *a* (ANOVA: $F_{11,23} = 0.942$, $P = 0.521$). Three products had a higher mean autotrophic index than that of the control samples, these products were: chelated Cu, EDTA and Organic Interceptor™ (Figure 8). This indicates that these three products produced more dead *D. geminata* cells per unit biomass than did the other biocides.

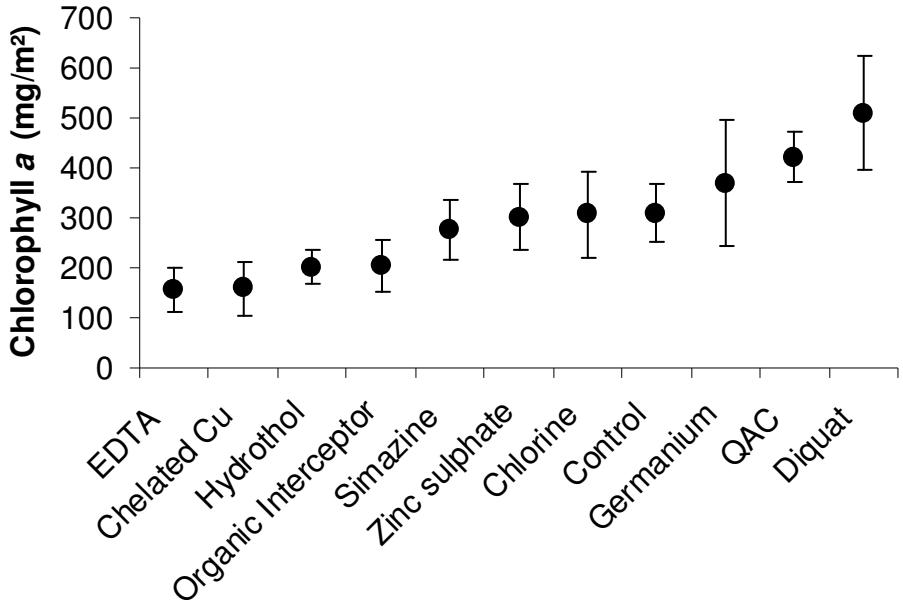


Figure 7: Mean (± 1 S.E.) chlorophyll *a* measurements of *D. geminata* on rocks 15 days after treatment with 10 biocides and a control (water). *n* = 3 replicate rocks per biocide and 6 replicate rocks for the control treatment.

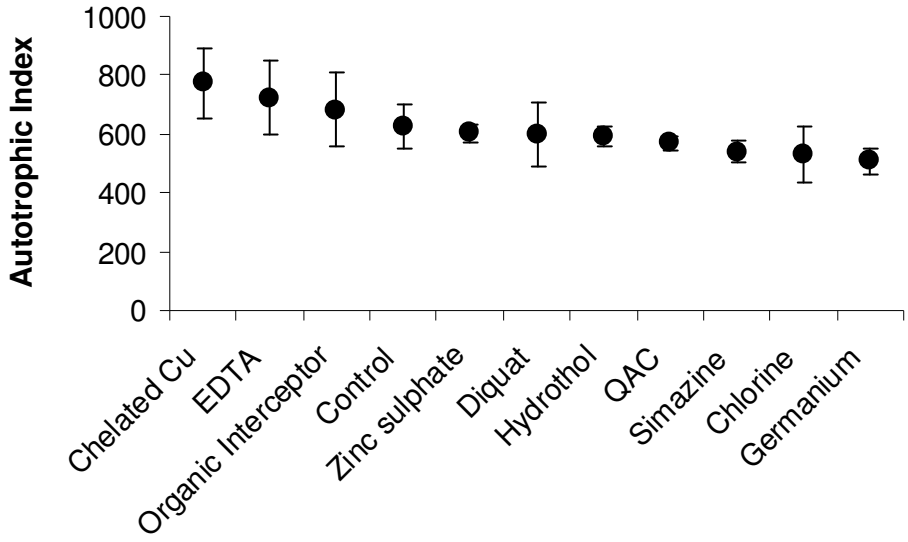


Figure 8: Mean (± 1 S.E.) autotrophic index for the 10 biocides tested in longer-term effects trials.

2.3. Conclusions

The results of these screening trials indicate large differences in the short- and longer-term efficacy of some products. A comparison of all analyses (both short and long-term) consistently showed that germanium, diquat and simazine were the least effective biocides. Short-term trials identified five biocides (chelated Cu, chlorine, QAC, Organic Interceptor™ and zinc sulphate) that significantly reduced the cell viability of *D. geminata* (Table 4). Biocide effectiveness generally increased with contact time, although there was some variability in these results. Subsequent studies will use a one hour contact time because it is effective against *D. geminata* and a one hour river application will allow good mixing and chemical exposure.

Table 4: Summary of the short- and long-term efficacy of the selected biocides.

Biocide	Short-term	Long-term	Reasons for acceptance/rejection
EDTA	X	✓	effective long-term
Chelated copper	✓	✓	highly effective
Organic Interceptor™	✓	✓	highly effective
Hydrothol®191	X	✓	effective long-term
Simazine	X	X	ineffective
Zinc sulphate	✓	X	ineffective long-term
Chlorine	✓	X	highly toxic, non-specific, ineffective long-term
Germanium	X	X	ineffective, expensive
QAC	✓	X	ineffective long-term
Diquat	X	X	ineffective

As initially thought, products identified as having a "longer-term" response time (Table 2) had no effect on cell viability after a short time period. In evaluating the effectiveness of biocides in long-term trials, the most valid analyses are those of cell density and autotrophic index. Without initial measurements to assess changes in chlorophyll *a* and AFDM, these analyses are not as robust or defensible. The autotrophic index indicates the proportion of dead *D. geminata* cells per unit biomass, making it a more valid analysis when evaluating biocide effectiveness than either chlorophyll *a* or AFDM. Analyses of cell density and the autotrophic index consistently showed EDTA, chelated Cu and Organic Interceptor™ as being the most effective biocides when compared to the control samples. EDTA was a product that was identified as having a longer-term response time, and consequently had not been identified in short-term trials. As well as EDTA, Hydrothol®191 also caused detectable changes to *D. geminata* mats in longer-terms. The results of Hydrothol®191 were highly variable but may warrant further investigation.

2.4. Recommendations

We recommend that at least three products should be taken through into Stage 2 for refining optimal concentrations and assessing toxicity to *D. geminata* and non-target organisms. Based on our experimental results these products should be EDTA, chelated Cu and Organic Interceptor™ (Table 4). If a fourth product can be included, we believe Hydrothol®191may warrant further investigation. QAC, chlorine and zinc sulphate have not been totally dismissed, however we think that the recommended products are more likely to be successful, or are a more appropriate choice in accordance with our selection criteria.

None of the biocides at the current concentration caused 100% mortality of *D. geminata* cells. We recommend that biocide concentrations be set higher than those in this report when conducting further trials to determine threshold concentrations. Regeneration of mats was not measured during the longer-term trial, but Stage 2 trials (scheduled to run for 1 month) will assess this factor.

There are products that have not been tested in this trial for various reasons (e.g., malathion which we believe is inappropriate for use with diatoms and has side-effects), but we have developed "small-scale" screening procedures for testing new products we decide merit further investigation in the future. This trial has not tested combinations of treatments. We believe that testing of such combinations may produce "synergistic effects[5]" that may be more effective for both short- and longer-term control of *D. geminata* than a single biocide. It is recommended that suitable

[5] synergistic effects: the interaction of two or more agents so that their combined effect is greater than the sum of their individual effects

options are tested during the monitoring phase of Stage 2 trials. Long-term controls that alter water chemistry (e.g., sodium bisulphate - pH decreaser) also have the potential to control *D. geminata* but it has not been feasible to test such products during our Stage 1 screening trials, but should be considered for further investigation.

3. Stage 1 – Screening of Stalk Disrupting Agents

3.1. Background to stalk research

Preliminary observations of stalks of *D. geminata* indicate a relationship to those of the closely related benthic diatoms *Cymbella* and *Gomphonema*. These stalks are terminated in adhesive pads which attach the stalks to the substrata. From this pad structure the cells propagate stalk material until cells undergo mitosis. Stalks bifurcate at the point of cell division, as each daughter cell continues to produce stalk. This "history" of cell division within a population of cells in a stalk mass can produce estimates of division rates and can be used as an indicator of cell viability. Pads have not been examined in detail in freshwater diatoms, although the pads of the marine *Achnanthes longipes* have been dissected chemically to some degree. Pads are primarily proteoglycan and are related chemically to the material produced during motility (Wustman et al. 1998).

The structure of stalks and pads of benthic diatoms have been examined in previous work in Gretz' lab at MTU. The extracellular polymers of species closely related to *D. geminata*, *Cymbella mexicana* and *Cymbella cistula*, have been reported by Wustman et al. (1997) and papers cited therein. These stalks were similar chemically to those previously reported by Huntsman & Sloneker (1971) for *Gomphonema olivaceum* and the primary polysaccharide component was a sulfated xylogalactan. This sulfated polymer was in some ways structurally similar to agars and carrageenans reported from marine and freshwater red algae (Youngs et al. 1998 and references cited therein). The sulfated xylogalactan from *C. cistula* was shown to be intrinsically hydrophilic and ionic cross bridging with Ca^{2+} was indicated. Based on preliminary results, we expect that *D. geminata* stalks may have similar structure to those of *C. cistula*.

Other aspects of extracellular polymer production important in consideration of *D. geminata* control are cell processes closely related to stalk biogenesis. For the marine diatom *Achnanthes longipes*, a definite sequence of colonisation of substrata and stalk production is observed (Wang et al. 1997, 2000). Adhesion occurs in a predictable sequence: 1) gliding motility; 2) cessation of motility; 3) pad production with permanent attachment; 4) stalk production, elevating the cell from the substratum; 5) cell division; and 6) gliding motility of daughter cells. A variation of this type of sequence may occur in *D. geminata*, with detachment from the stalk being a possible mode of dispersal.

3.2. Trial goals and research plan

The overall goal of Stage One stalk disruption trials was to identify agents that function as degraders of stalk structure (DSS). We have performed small-scale *in situ* experiments to test the ability of a range of potential DSS, from which the most promising formulations will be selected for Stage Two trials.

3.3. Experimental approach

3.3.1. Experimental methodology

Chemical DSS treatment

Source of chemicals: Hydrochloric acid (Sigma-Aldrich), nitric acid (Sigma-Aldrich), acetic acid (Sigma-Aldrich), trifluoroacetic acid (Sigma-Aldrich), sodium hypochlorite (The Clorox Company), ethanol (Sigma-Aldrich), sodium hydroxide (Sigma-Aldrich), ammonium hydroxide (Sigma-Aldrich), RBS-35 (Fisher), Rid-X (Reckitt Benckiser, Inc.), and Crest ® Pro-Health mouthwash (Procter & Gamble) were purchased from companies listed.

Steam Treatment Preparation

For steam treatment, samples were placed in a steam container (WAHMANN MFG, Co.) directly attached to a steam line with a temperature of 97^{o}C.

EDTA / Hot Water Extraction Protocol

The hot water and EDTA extraction was a sequential protocol described in Wustman et al. (1997) for *C. cistula* stalk material. Prior to this extraction, the sample was defatted with 90% ethanol (which has no detectable effect on *D. geminata* stalks).

Enzyme Treatments

Activity of crude enzyme extracts from *P. funiculosum* (Sigma Co., Inc.) and *A. niger* (Worthington-BioChemical) was routinely assayed at 40°C in 0.2M acetate buffer, pH 5.0.

The dietary supplement Omega-zyme and was utilized at 40°C in 0.2M acetate buffer, pH 5.0. Crude enzyme extract from *Clostridium histolyticum* was purchased from Worthington-BioChemical and degradation was performed at 37°C in phosphate

buffer (0.1M), pH 7.5. *E. coli* (Worthington-BioChemical) enzyme assays were performed in water at 40°C, pH 7.

Crude extract preparation of the enzyme from *T. reesei* (Sigma-Aldrich) and degradation was performed at 40°C in acetate buffer (0.2M) with pH adjusted to 5.0 before the enzyme was added.

3.3.2. Literature search

Literature searches identified several candidate DSS agents that have been shown to degrade polymers related to those previously described in stalks and pads of *Cymbella* and *Gomphonema* species. The literature databases are stored in a folder in the didymo Refworks database (www.refworks.com).

3.3.3. Proposed agents for screening

Based on our knowledge of the chemistry and organisation of diatom stalks and of existing degradation products, we selected several agents for efficacy against *D. geminata* stalks, including:

Enzymes - These agents included various cell wall degrading enzymes to which we have access and have utilized in previous studies of algal cell walls. In addition, we have investigated various common deglycosylation agents that we have utilized in our structural studies of *Achnanthes longipes* proteoglycans (Wustman et al. 1998).

Chelators - We have shown that chelators can disrupt the structure of *Cymbella cistula* stalks (Wustman et al. 1997) and have tested these and similar chemical agents against *D. geminata* stalks.

Hot aqueous extractants – We have utilized a variety of hot aqueous extractants to degrade stalks of related diatoms.

Acids and bases – High and low pH have been shown to be effective disruptors of the integrity of extracellular matrices of diatoms.

3.3.4. Source of stalks and trial protocol

Stalk mats were collected from Boulder Creek, Colorado, purified by a combination of squeezing, filtration, picking, brief sonication and gentle washing of mats and stored at 5°C maximum two days. Alternatively, stalks were frozen or freeze-dried prior to trials. Laboratory tests were conducted on a minimum of three replicate samples and

scored using microscopy, turbidometric and/or gravimetric methods. The primary emphasis was stalk disruption and/or dissolution, although other effects were also documented. Effects of contact time were also documented.

3.3.5. Criteria for ranking treatment potential of agents

For Stage One stalk disruption trials, the selection criteria for determining the relative success of each of the degradation agents were:

1. Degree of degradation of the stalks – agents were deemed effective if they caused dissolution to the point that stalk integrity was compromised in a significant way.

2. Contact time – shorter the better

3.3.6. Analytical methods

Microscopic determination

Stalk degradation was primarily measured by direct observation coupled with staining. Stalk degradation was determined microscopically in conjunction with staining techniques (Wustman et al. 1997). Digital photomicrographs were taken to document microscopic observations. For each sample, at least 20 *D. geminata* stalks were enumerated for detection of those that were altered by agents.

Turbidometric analysis

Stalk structural modifications in response to agents were also measured by turbidometry. Degraded stalks showed increased light scattering when analyzed at 560 nm.

3.4. Results

3.4.1. Identification of Potential DSS from Literature Search

Published descriptions of DSS directed specifically against *D. geminata* stalks or diatom stalks in general were not found. Previous investigations of the chemistry of *D. geminata* stalks (Riccio et al. unpublished) suggested that polymers similar to those found in algal and higher plant cell walls were present. Search results for agents that degrade these polysaccharides and related polymers are summarized in Table 5.

Table 5: Literature survey of potential DSS.

Type	Treatment	Rationale	Author
Antiseptic	Mouthwash	Adhesion of the stalk in marine mussels (*Mytilus edulis*) to the substrate involves a plaque substrate	Vreeland et al. 1998
Chemical	Acetic Acid	Degrades bacterial capsules; partially extracts the stalk of *Gomphonema*	Morgan & Partridge 1940; Huntsman 1966
Chemical	Aqueous dioxane	Degrades unbleached hardwood sulfate pulp, destroys lignin in cell wall	Udoratina & Demin 2005; Fry 1988
Chemical	Ozone	Shown to degrade lignin into smaller compounds through secondary reactions with lignin oxidation	Nakamura et al. 1997
Chemical	Sodium Hydroxide with steam	Aids lignin destruction of *Eucalyptus globulus* in streams of paper industry	Assada et al. 2005
Chemical	Alkaline degradation	Extracts the capsule of *Navicula pelliculose* and *Porphyra* mannan, phlorotannnins, lignin, and hemicellulose from the cell wall	Lewin 1995; Jones 1950; Koivikko et al. 2005; Fry 1988
Chemical	Ethanol	Extracts lipids and leucosin from the cell	Fry 1988; Beatie et al. 1961
Chemical	Guanidinidinium thiocyanate	Removes mannose-rich hemicellulose	Fry 1988
Chemical	Hydrochloric Acid	Removes laminaran of *Dilsea edulis* mucilage	Barry & Dillon 1945; Black 1965
Chemical	Nitric Acid	Degrades stalk organic compounds	Fry 1988
Chemical	Trifluoroacetic acid	Hydrolyzes the non-cellulosic polysaccharides and destabilize the glycosidic linkages of the uronic acids	Fry 1988
Enzymatic	Cellulase from *Penicillium funiculosum*	Hydrolyzes 1,4-beta-glucan linkages in polysaccharides such as cellulose, yielding beta-dextrins	FNP 2000

Type	Treatment	Rationale	Author
Enzymatic	Enzymes from *Trametes villosus*	Contains lignin peroxidase, laccase, aryl-glucosidase and xylanase.	Medvedeva et al. 1995
Enzymatic	Laccase from *Coriolus veriscolor*	Depolymerization of polymeric lignin	Kawai et al. 1999
Enzymatic	Ring-cleavage dioxyngenase enzyme from bacterial genus, *Saguttila*	Involves lignin destruction	Gonzalez et al. 1997
Enzymatic	Enzyme from *Penicillium purpurogenum*	Lysis of hyphae and spores of pathogens of the plants *Monilinia laxa* and *Fusarium oxysporum*	Larena & Melgarejo 1996
Enzymatic	Enzyme from *Penicillium janthinellum*	Activity for the production of cellulose and xylanase enzymes enhanced from bagasse polysaccharides	Adsul et al. 2004
Enzymatic	Enzyme from *Penicillium chrysogenum*	Oxidizes 2, 6-dimethoxyphenol but not involved in lignin mineralization	Rodriguez et al. 1996
Enzymatic	Cellulase from *Penicillium funiculosum*	Hydrolyzes cellulose of wastepaper into glucose for biodegradable products	van Wyk 2001

Table 5 represents selected potential DSS agents; the complete citation index has been uploaded to the Biosecurity New Zealand website. DSS identified from literature searches were prioritised for testing and included: hydrochloric acid (12M), nitric acid (16M), acetic acid (17M), trifluoroacetic acid (TFA) (13M), ethanol (0.15 or 0.22M), sodium hydroxide (1 or 10M), ammonium hydroxide (14M), sodium hypochlorite (7.1mM), RBS-35 (detergent +) (0.045M), *Penicillium* crude enzyme extracts, *Aspergillius niger* crude enzyme extracts, *Escherichia coli* crude enzyme extracts, *Clostridium histolyticum* crude enzyme extracts, *Trichoderma reesei* crude enzyme extracts, crude enzyme extracts from the dietary supplement Omega-zyme, EDTA (0.2M), hot water, steam, Rid-X, and a commercially available antiseptic solution (Crest ® Pro-Health Mouthwash) (0.021mM).

3.4.2. Results for potential DSS

Strong acids, sodium hypochlorite (bleach), chelators, *P. funiculosum, , A. niger, E. coli, T. reesei, C. histolyticum* crude enzyme extracts, and the crude enzyme preparation from the dietary supplement *Omega-zyme* were effective agents for dissolution of the *D. geminata* stalk (Appendix 6). Other agents tested which had minimal effect on stalk integrity included: bases, weak acids, detergents, antiseptics, biodegradation agents, alcohol, hot water, and steam.

Development of a Standard Assay for Stalk Degradation

Turbidometric methods for determination of the effectiveness of DSS were not found to be time effective. The requirement to utilize a stabilizing agent (e.g., agarose) negated any advantage over other methods and without a stabilizer spectrophotometric measurements of stalk degradation were not reproducible.

During preliminary testing, a standard protocol was developed to assay probable DSS utilizing microscopic (Figures 9, 10 & 11) and gravimetric methods. As we were concerned about penetration of DSS into freeze-dried stalk material, we initially treated dried stalk material with distilled water (5:1) and incubated at 22°C for 12 h prior to the addition of DSS agents. This "prehydration" step was eliminated after testing revealed that adding DSS solutions directly to freeze-dried stalk mats yielded results equivalent to those with prehydration (Figure 13). Following addition of 1 ml of DSS solution to stalk mats (with vortexing to homogenize the solution), samples were incubated at 22°C for 10mins, 1 h, 5 h, 24 h, 96 h and then centrifuged at 1100 x g, the supernatant discarded, and 20 µl of the pellet was removed and examined microscopically. Five random fields of view and three replicates per treatment were examined with a Zeiss Axioskop equipped with differential interference contrast

optics (DIC). In replicate experiments, stalk masses remaining following treatments described above were washed extensively with water and loss was determined gravimetrically (Figure 12).

Figure 9: Stalk material collected from Boulder Creek, Colorado. A) Fresh samples from the stream, filled with the macroinvertebrates and organic debris. B) Cleaned stalks following filtration, sonication, and hand picking to remove macro- invertebrates.

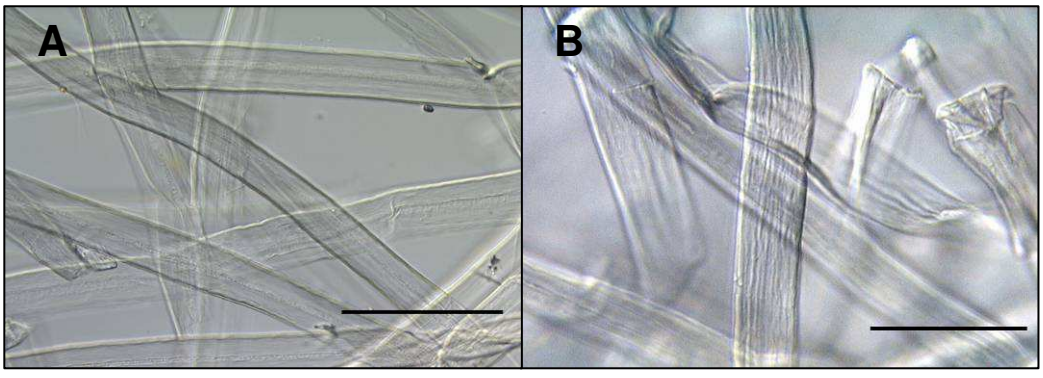


Figure 10: DIC images of clean *Didymosphenia geminata* stalks without stream debris. A) Fresh stalk B) Freeze-dried stalk. Scale bar A = 20µm, B = 30 µm.

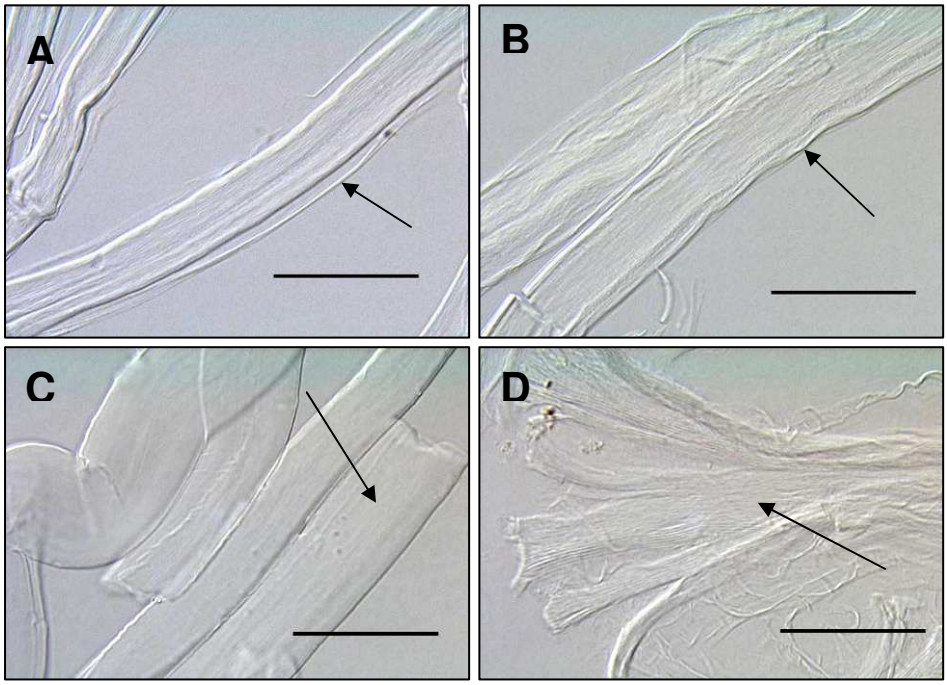


Figure 11: DIC microscopy of degraded *D. geminata* stalks. A) Sloughing = separation of fibrous materials from the periphery of the stalk. B) Scalloping = a series of semi-circular carvings on stalk edges. C) Swelling = increase in stalk diameter. D) Unravelling = comparable to unravelling of a multistrand rope. Scale bar= 20µm.

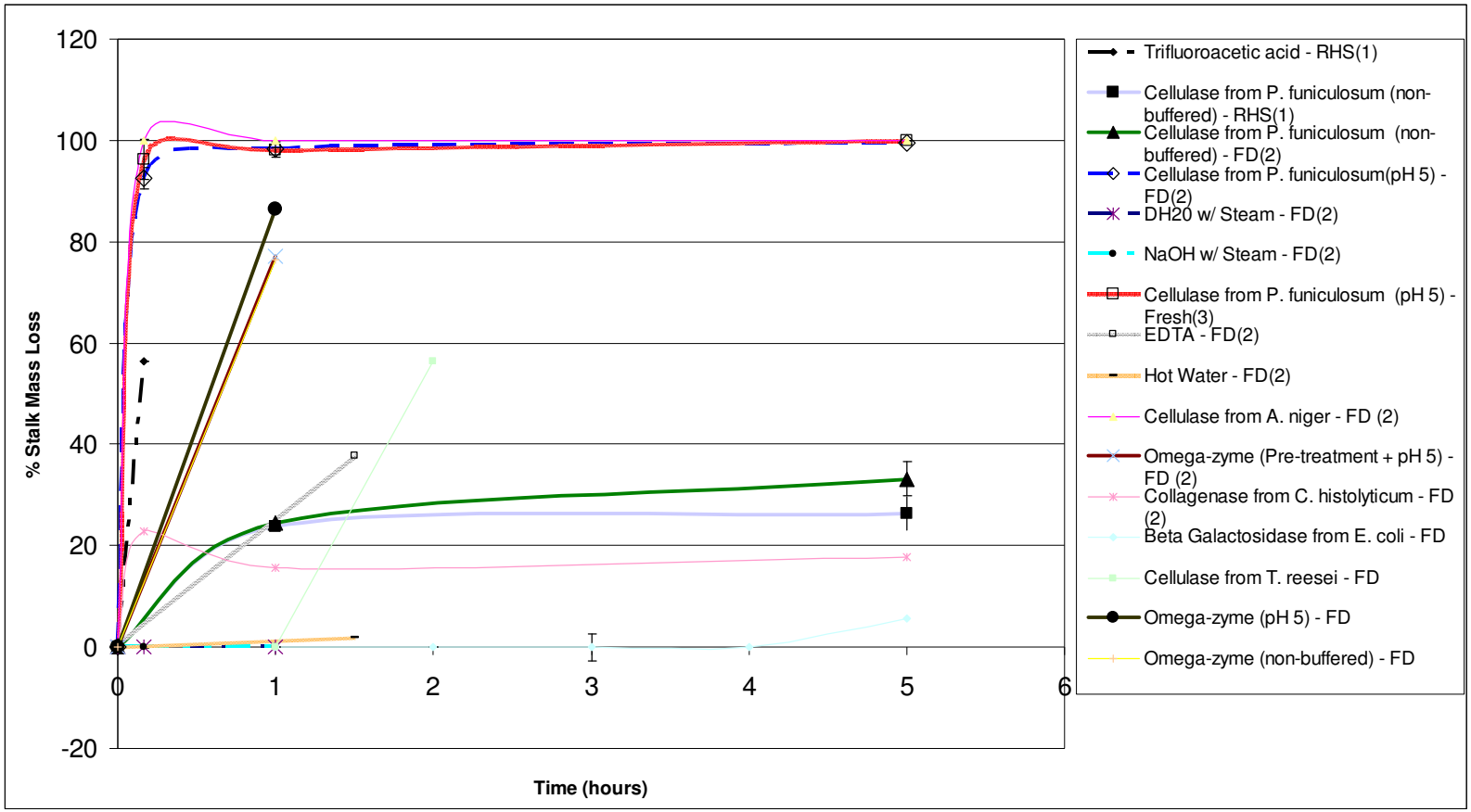


Figure 12: Percent stalk mass loss due to treatments with selected degrading agents. (RHS) Rehydrated stalks - freeze dried and rehydrated before DSS treatment. (FD) Freeze dried stalks - cleaned and freeze dried, but not rehydrated. (Fresh) Fresh stalks - cleaned and stored at 5°C.

The degree of stalk degradation was categorized using DIC microscopy. Observed structural changes were classified as follows (Figure 11):

1) Intact - the integrity of stalk material not altered.

2) Dissolution - no defined stalk or organised fibrous materials observed.

3) Scalloping - a series of semi-circular carvings at the stalk periphery.

4) Sloughing - separation of fibrous materials from the stalk periphery.

5) Unravelling – comparable to unravelling at the end of a multi-strand rope.

6) Swelling - stalk diameter increases.

7) Combined effect – loss of structural integrity due to a combination of scalloping, sloughing or unravelling.

Although significant degradation of individual stalks was observed following treatment with several DSS agents, the integrity of the stalk mass as a whole was only affected with agents that caused total dissolution or unravelling. Agents such as Rid-X, antiseptic mouthwash, bases, ethanol and RBS-35 detergent yielded no obvious physical changes to the integrity of the stalk mats, in that the mats appeared stringy in texture and cohesive after treatment. Conversely, strong acids and enzymes completely disrupted mat integrity. Some agents, such as TFA, produced mats with different physical characteristics (e.g., more slimy upon swelling), but these mats remained relatively cohesive. These results point to a potential benefit of performing sequential treatments to completely disrupt stalk mat integrity.

Mechanical approaches to stalk degradation were also tested. Blending wet stalk material resulted in further tangling and knotting of the mats, and was therefore an ineffective method to disrupt mat integrity. However, grinding or chopping dry material was successful in breaking up stalk mats, although the structure of individual stalks within the chopped material was not affected.

3.4.3. Effectiveness of DSS

Acids

As predicted from knowledge of diatom stalk chemistry (Riccio et al. unpublished, Wustman et al. 1997), strong acids had an effect on the integrity of the stalk material. Hydrochloric and nitric acid have been shown to effectively hydrolyze cell wall polymers from higher plants and algae (Fry 1988). Treatment with 12 M hydrochloric acid and 16 M nitric acid completely dissolved 5 mg of stalk mat in 10 mins (Appendix 6). Glacial acetic acid (pH 4) had no effect on the stalk material (Appendix 6). 13M TFA (Appendix 6) had a unique effect in that it caused the stalks to swell. Concentrated TFA has been shown to hydrolyze polysaccharides to oligosaccharides and to preferentially degrade non-cellulosic polysaccharides which contributed to stalk swelling (Fry 1988). Stalk diameter in the native state was 14 µm and TFA caused swelling to 20 µm (1.4x)(Figure 11). Upon swelling the internal ridges present in controls were not visible (Figure 11).

Bases

Strong bases had no major effect on *D. geminata* stalks. There were no morphological or gravimetric changes following 1M or 10M sodium hydroxide treatment (Appendix 6). Sodium hydroxide has been used to solublize the hemicelluloses present in plant cell walls (Fry 1988). Heating the basic solution with steam did not have any apparent effect on stalk structure, as was the case for dH_2O and steam treatments (Appendix 7, Figure 12). This was somewhat unexpected as steam treatment has been reported as an effective treatment for removing pulp remnants in streambeds and for lignin destruction of *Eucalyptus globulus* residue downstream from paper mills (Asada et al. 2005). Ammonium hydroxide (14M) had no effect until the 24 h time point when stalks showed signs of scalloping (Appendix 6, Figure 11).

Alcohols

Alcohol-based solvents produced no observable effects on the integrity of the stalks (Appendix 6). Ethanol has been shown to solublise lipoidal components associated with the plant cell wall (Fry 1988). The alcohol containing antiseptic, Crest ® Mouthwash, had no effect on stalk material during exposures of 10 mins, 1 h, and 5 h (Appendix 6). Crest ® Mouthwash functions as an anti-plaque (biofilm) agent, which breaks down oral biofilms and associated muco-polysaccharides.

Chelators/Hot Aqueous Extractions

Treatment with the chelator EDTA produced marked effects on the integrity of the stalks. Sloughing and unravelling were observed after the 1.5 h at 22°C (Appendix 6). EDTA dissolved approximately 40% of the stalk mass after 1.5 h. These types of observed stalk changes suggest that EDTA could be an effective DSS, especially if used in a sequential extraction procedure. EDTA has been used to remove pectins from cell walls (Fry 1988, Wustman et al. 1997). Hot aqueous extraction was not an effective DSS. Some sloughing was observed after 1.5 h at 95°C, but only 4% of the stalk material was dissolved. Hot water has been commonly used to remove loosely associated polysaccharides of plant cell walls (Fry 1988).

Detergents/Bleaching Agents

The detergent RBS-35 and bleach (sodium hypochlorite) caused stalk scalloping after 5 h (Appendix 6), while sloughing was noted after 24 h (Appendix 8). Fresh (never dried) stalks showed significantly more scalloping than rehydrated stalks. This result may suggest that fresh stalks are more accessible to bleach than rehydrated stalks. Dissolution occurred with bleach but not with RBS-35 after 96 h (Appendix 6). RBS-35 is a basic detergent with a pH of 13 and a very low amount of the active ingredient sodium hydroxide. Consumer bleach contains 5.25% sodium hypochlorite (NaOCl), a strong oxidizer used as a bleaching agent, oxidant, sterilizer, decoloring agent, deodorant, and in water treatment.

Biodegraders

RID-X ® Septic System Treatment did not have any effect on stalk material (Appendices 6 & 7). The manufacturer claims RID-X contains billions of natural active bacteria and enzymes (cellulase, lipase, protease, and amylase) used to break down household waste. Other active ingredients of RID-X are calcium carbonate and subtilisin (proteolytic enzyme).

Enzymes

The crude enzyme preparation from *Penicillium funiculosum* degraded approximately 30-40% of stalk mass under suboptimal conditions of unbuffered water at pH 7 (Figure 12). The *P. funiculosum* enzyme was most effective on material pre-treated with heat and low pH. Digestion of stalk mats was very much more efficient at pH 5 in acetate buffer at elevated temperature where 90% of stalk material was dissolved in 10 minutes, with remaining stalks both unravelling and sloughing (Appendices 6 & 7).

Complete degradation (100%) was observed at 5 h (Figures 12 & 13), indicating the efficacy of an enzyme approach to degrading *D. geminata* stalks and providing a clear indication that certain components of this crude enzyme preparation must be effective DSS. The *P. funiculosum* enzyme mixture includes cellulase (endo-1,4-beta-glucanase), endo-1,3(4)-beta-glucanase, endo-1,4-beta-xylanase, and the secondary enzyme activities of: alpha-N-arabinofuranosidase, cellulose 1,4-beta-cellobiosidase, beta-glucosidase and xylan 1,4-beta-xylosidase.

As shown in Figure 12, the crude enzyme preparation from *A. niger* degraded 100% of the stalk material in as little as 10 mins. These results were observed with pre-treatment and buffering (0.2M acetate buffer, pH 5). Of all the enzymatic agents tested, the *A. niger* mixture had the greatest degradation potential. The *A. niger* enzyme mixture contains the following: 1,4-beta-D-xylanase, xylan 1,4-beta-xylosidase, alpha-L-Arabinofuranosidase, Cellulase, Galactomannanase, with secondary enzyme activities: 1,4-alpha-D-Glucan glucohydrase, alpha-D-Glucosidase glucohydrolase, beta-galactosidase, and Saccharase (Worthington-Biochemical). Riccio, et al. (unpublished) discovered that *D. geminata* stalks are primarily compromised of galactose, xylose, glucose and mannose. The *A. niger* mixture contains galactomannase and beta-galactosidase, which are not present in the *P. funiculosum* extract and may have produced the extensive degradation of stalks.

The crude enzyme preparation from the dietary supplement Omega-zyme (supplied by Garden of Life) showed much promise as a potential DSS as it degraded approximately 80% of the stalk material within 1 h (Figure 12) and caused scalloping, sloughing and unravelling (Appendix 7). These results were observed with and without pre-treatments and pH adjustments. Since this product is a dietary supplement and not regulated by the U.S. Food and Drug Administration (FDA), the constituents are unknown, although the manufacturer claims that this product contains the following crude enzymes: protease blend, amylase, lipase, glucoamylase, maltase, sucrase, alpha-galactosidase, lactase, cellulase, xylanase, pectinase, hemicellulase, mannanase, phytase, beta-glucanase, and arabinosidase.

The crude collagenase enzyme extract from *Clostridium histolyticum* displayed some degradation potential, but was not as effective as enzyme preparations discussed above (Figure 12). After 10 mins, approximately 23% of the stalk material was degraded. When viewing with DIC microscopy, a combination of sloughing and unravelling was noted in less that 5% of stalks, but the majority were intact. The *C. histolyticum* extract could be useful in a sequential treatment. Collagenase targets collagen, a component of animal connective tissue, and contains the following enzymatic activities: sulfhydryl protease, clostripain, aminopeptidase, and a collagenase that

degrades at the Y-Gly bond in the peptide sequence Pro-Y-Gly-Pro, where Y denotes a neutral amino acid.

The partially purified B-Galactosidase from *E.coli* produced only 5% degradation after 1 h (Figure 12). Although this enzyme has optimal activity in the pH range found in *D.geminata* environments, the lack of substantial activity precludes this enzyme as an effective DSS. The crude cellulase extract from *T. reesei* displayed limited degradation potential. After 1 h, approximately 56% of the stalk material was degraded (Figure 13). The enzymatic activity for this enzyme extract is 1, 4-(1,3:1,4)-beta-D-Glucan 4-glucano-hydrolase (Sigma-Aldrich) which acts primarily on microcrystalline cellulose.

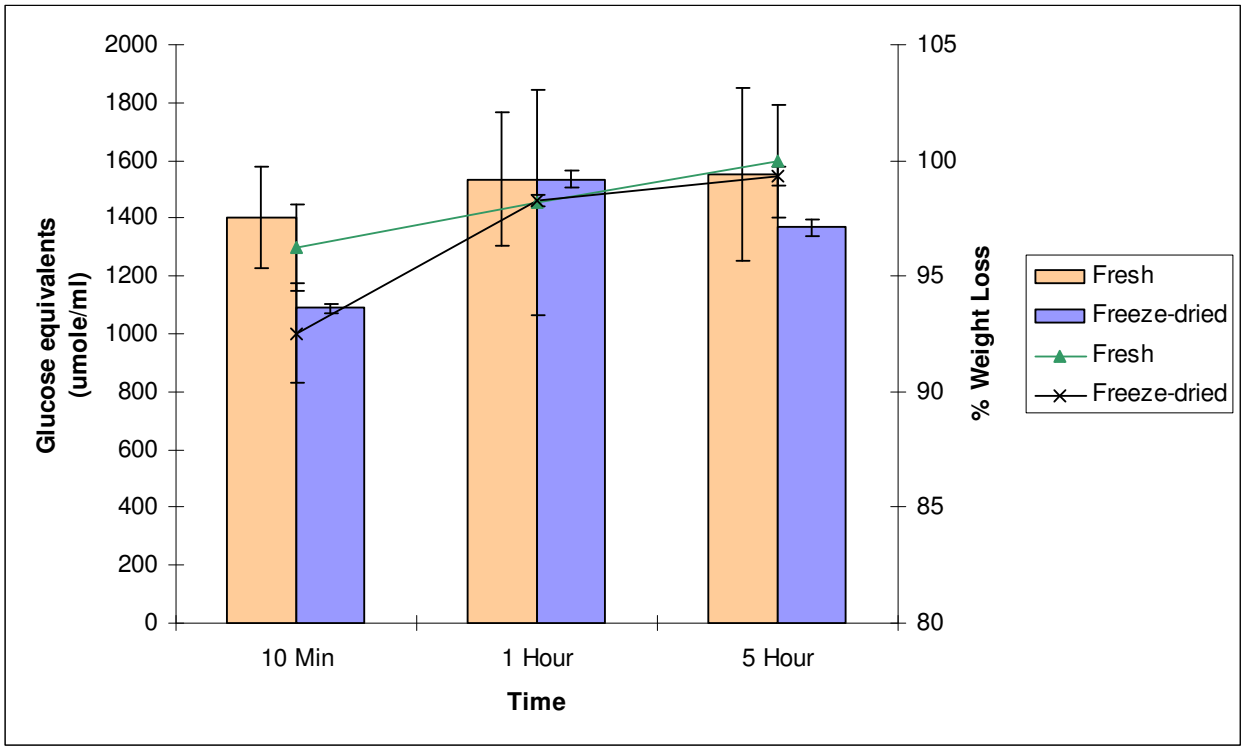


Figure 13: Reducing sugar (as glucose equivalents) liberated and percent weight loss of *D. geminata* stalk treated with *P. funiculosum* crude enzyme extract. Freeze dried stalks - cleaned and freeze dried, but not rehydrated. Fresh stalks - cleaned and stored at 5°C.

4. Stage Two Phase One – Biocide Product Testing

4.1. Methods

4.1.1. Field trials

Study site – Monowai experimental facility

The trial was carried out at the purpose-built experimental streamside channel facility located on land owned by Pioneer Generation Ltd. at the Monowai Power Station, Southland (5478740 N, 2091405 E). The hydro-electric power station at Monowai is supplied by a 1.2 km pipeline which is sourced from the Monowai River (a regulated waterway that drains Lake Monowai, see Map 1). The water used at the Monowai experimental facility (MEF) has very low nutrient concentrations (see Appendix 1), but is suitable for growth of *D. geminata*, which proliferates throughout the lower reaches. The water that supplies the facility is sourced from the upper reaches of the river which contains no *D. geminata* cells at present. Infestation of the upper reaches might result in recolonisation of the test substrates following biocide application (which, in-turn, would confound the experimental results).

The MEF is supplied by an underground pipeline which is an off-take from the bottom of the penstocks (this is the discharge pipe for flushing out the penstocks inside the generation plant). A 200 mm polyethylene pipeline connected to the underground steel pipeline supplies two 3000 litre tanks on a level concrete pad. Each tank supplies 24 channels (48 channels total), which are set up in a radial configuration (Figure 14). The 48 channels are standard PVC storm drains, with a bottom width of 100 mm, height of 100 mm, and length of 1.5 m. Each channel is isolated as it has an individual connection and ball valve. Having individually isolated channels allows experiments to be conducted across a wide range of flow rates. When a number of channels are turned off at once the excess water is diverted to the over flows, and has little impact on the hydraulics of the channels remaining open. When running at full capacity, the facility can extract *c.*125 L/s from the penstocks, with each channel discharging around 2.5 L/s. Additional pictures of the MEF and the channels are shown in Appendix 2.

Selected biocides

Based on knowledge gained during Stage 1 product testing, we assessed the toxicity of four selected biocides to *D. geminata* across a concentration gradient, as well as the impact on non-target organisms in Stage 2 (Table 6).

Figure 14: The Monowai Experimental Facility (MEF) used for Stage 2 trials.

Table 6: Chemical concentrations used in Stage 2 Phase 1 trials in 1 h exposures in simulated streams. Refer to sections on individual chemicals for further explanation of units of measurement, and refer to Appendix 3 for derivation of concentrations.

Chemical	Rate	Units	Concentration
Chelated copper	1	mg Cu/L	1
	2	mg Cu/L	2
	3	mg Cu/L	4
EDTA	1	mg EDTA/L	11
	2	mg EDTA/L	23
	3	mg EDTA/L	45
Hydrothol®191	1	mg a.e./L	0.5
	2	mg a.e./L	1
	3	mg a.e./L	2
Organic Interceptor™[6]	1	mg pine oil/L	68
	2	mg pine oil/L	135
	3	mg pine oil/L	271

[6] The exact chemicals that comprise the biocide Organic Interceptor™ are trade-marked and there is no certainty that the active ingredient that affects *D. geminata* is pine oil as is specified.

Chelated copper[7]

The chelated copper (Cu) formulation (20.36 g Cu/L) used in these trials includes Cu as copper sulphate[8] ($CuSO_4.5H_2O$, CAS Number 7758-99-8), and other non-toxic ingredients. The active ingredient is copper which is toxic to aquatic organisms and has been used in many formulations as an algaecide and antifouling agent. Copper is an established algaecide and has been used extensively for this purpose since the 1960s in the U.S.A. It is commonly used in chelated copper formulations in swimming pools or the aquarium trade to control algae at low dose rates while not harming desirable aquarium organisms (e.g., corals) or fish. Copper sulphate has been approved by the U.S. Environmental Protection Agency for use on a repetitive basis in lentic systems (ponds, lakes). Copper based algaecides are often recommended because they are cheap, efficient, easily obtained and are specific. Copper has the disadvantage of being a cumulative element. Dissolved copper toxicity is significantly affected by water quality: pH, alkalinity, hardness and dissolved organic matter (DOM) will all influence dissolved copper toxicity. In general copper toxicity will be high in low hardness, low pH waters typical of South Island Rivers. High concentrations of humic and fluvic acids (DOM) leaching from vegetation will tend to decrease copper toxicity. *D. geminata* is known to be sensitive to copper with die-back when levels exceed 0.015 $mg.l^{-1}$ Cu, with no conspicuous growth in a Norwegian river system when Cu exceeded 8 $\mu g.l^{-1}$ (Lindstrom and Rorslett 1991).

The formulation of copper used in these trials, results in the chelation of copper in compounds that remain in solution over a wide range of pH and temperatures. Chelation refers to the presence of bonds between the copper ion and multiple atoms of another compound. The presence of multiple bonds results in a stable copper compound that is less reactive with living organisms or organic matter in an aquatic environment and is also less likely to precipitate than a non-chelated compound (e.g., copper carbonate). This is in contrast to a solution of copper sulphate where most of the copper would be present as the cupric ion (Cu^{2+}) which is highly toxic to aquatic organisms. When the copper is present in a chelated compound it binds more slowly (if at all) with biological membranes than the Cu^{2+} ion. It is likely that chelated copper is toxic to *D. geminata* because the cell membranes are actively transporting the chelating compounds and the copper is either transported incidentally (i.e., as a "hijacker") through the membrane where it causes toxicity intracellularly or, the copper is disassociated from the chelated compound right at the cell surface during the process of transport leaving it present as either the Cu^{2+} or cuprous ion (Cu^{+}) where it reacts with the cell membrane and causes toxicity. The chelated compound is probably not as readily transported by fish gill membranes, hence the much lower toxicity of chelated copper compounds to fish (Meyer et al. *in prep*).

[7] The formulation of chelated copper used in these trials is now known as Gemex™
[8] Copper sulphate anhydrous = CAS Number 7758-98-7

EDTA (Titriplex III)

Titriplex III is also known as EDTA (Ethylene Diamine Tetraacetic Acid) and in this study the disodium, dihydrate salt of EDTA was used (MWt 372.24 g/mol) (CAS Number 6381-92-6 for the dihydrate disodium salt)($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)[9]. EDTA was included as a possible *D. geminata* control agent because it has been found to detach some diatoms from their pads (*email 9/5/06, A. Dugdale, University of Melbourne*). EDTA is thought to act on diatoms by chelating cations and altering the molecular composition of the extracellular mucilage, with the diatom cells separating from their pads after a couple of hours. It is envisioned that EDTA might be used in conjunction with other *D. geminata* treatments, perhaps once the thick mats have been at least partially removed. We expect this product may cause a large decrease in AFDM if it is causing a breakdown in the stalk material of *D. geminata*.

Although EDTA is widely used as a chelating agent there is not much toxicity data available on this compound alone in natural waters (much of the data refers to modified nutrient solutions). The majority of toxicity testing to date has EDTA associated with at least one or two other transition metals (e.g., Cd, Cu etc.), because it is a strong chelant. EDTA is often used in conjunction with metals, and is thought to increase metal toxicity to fish by destabilizing the gill membranes (Cascella and Kindelspire 1980 *cited in USEPA ECOTOX database*), though low EDTA concentrations may decrease metal bioavailability and toxicity to fish.

Hydrothol®191

Hydrothol®191 is a formulation based on the dimethylalkylamine salt of endothall. Hydrothol®191 has been used to control aquatic vegetation and filamentous algae for over 30 years. Hydrothol®191 is not currently registered for aquatic use in New Zealand, however the same formulation labelled as DES-I-CATE II is used as a harvest aid for potatoes (supplied by Elliot Chemicals Ltd., Brian Smith, 09 237 0431). Endothall is a dicarboxylic acid, and is a selective contact herbicide that acts as a dessicant and defoliant. Endothall is thought to act by disrupting solute transport in plants as well as protein and lipid synthesis. Endothall is available as the endothall acid (technical grade), a dipotassium salt, a disodium salt, a dimethylalkylamine salt, and is formulated in liquid and granular forms. The three chemical forms of endothall have different toxicities with the dimethylalkylamine salt ranking as the most toxic to aquatic organisms. The dipotassium salt of endothall is used in the aquatic herbicide Aquathol® K (a liquid formulation) and Aquathol® Super K (a granular formulation) that was recently registered for use in New Zealand by ERMA

[9] CAS Number 139-33-3 for anhydrous EDTA, MWt = 292 g/mol.

Much of the information on Hydrothol®191 toxicity is based upon toxicity testing of endothall acid (CAS Number 145-73-3), and the concentration of the product is expressed as acid equivalents (e.g., mg acid equivalents/L or mg a.e./L). Hydrothol®191 contains 53% active ingredient (dimethylalkylamine salt of endothall[10]), or 23.36% of acid equivalents or 240 g a.e./L.

Hydrothol®191 is used as an aquatic herbicide in the USA but with more limitations than Aquathol® K due to its greater toxicity to aquatic organisms. Information on Hydrothol®191 was obtained from the Supplemental Environmental Impact Statement (SEIS) provided to Washington State Department of Ecology for an application to use endothall formulations as aquatic herbicides (WSDE 2001). The WSDE (2001) recommended that Hydrothol®191 is applied at <0.2 mg a.e./L to prevent toxicity to fish (in particular) and other non-target species, as sufficient data exist to conclude that concentrations greater than 0.2-0.5 mg a.e./L are likely to kill fish and possibly free-swimming and benthic biota (WSDE 2001). However, Hydrothol®191 is usually applied to lakes or channels with relatively low-flows resulting in extended contact times. Different application rates might be appropriate where short contact times (1-4 h) will occur in the high flow environments where *D. geminata* infestations are located.

Organic Interceptor™ - Certified Organics compound

Organic Interceptor™ is a herbicide used in the control of annual weeds, grasses and brown-off of perennial species. Organic Interceptor™ (OI) is a contact weed killer made from pine oil and sold by Certified Organics Ltd (supplied by H. Frith Ph. 09 525 3432). The formulation used in these exposures is described on the label as 680 g pine oil/L (emusifiable concentrate) and is registered for terrestrial use with ACVM and ERMA. The biocides in the formulation are not described or identified any further (e.g., with CAS Numbers[11]), as pine oil is a complex mixture of compounds. Organic Interceptor™ contains other compounds such as surfactants and carriers to increase product effectiveness. Certified Organics Ltd. has the ability to produce different formulations of this product (e.g., gel, or other slow-release delivery system) to aid effective treatment of *D. geminata* in an aquatic environment if this is required. Organic Interceptor™'s mode of toxicity against plants is thought to be via dessication.

[10]The dimethylalkylamine salt of endothall CAS Number 66330-89-9 listed in the Bayer Crop Science 2005 DESICATE II MSDS could not be independently verified (e.g., Merck Index, US EPA Ecotoxs database, web searches). The other salts of endothall are listed as, dipotassium salt of endothall (CAS Number 2164-07-0), disodium salt of endothall (CAS Number 129-67-9, CAS Number 6385-60-0).

[11] CAS Numbers (Chemical Abstracts Registry) are used as identifiers of chemicals and can be found in the Merck Index (1989).

Experimental design

Before the commencement of trials at the MEF, testing was undertaken to assess the survival of *D. geminata* in channels. In addition to testing for any changes to the viability of *D. geminata*, daily fluctuations in water temperature and light levels were also measured. It was found that there was a moderate effect of shading by the tanks on some sections of selected channels; however, this effect could be significantly reduced by placing the algae in the bottom section of the channels.

Fifty artificial substrates were deployed in the Waiau River (22/02/06) in preparation for the biocide trials. A further 50 substrates were placed in a side channel of the Clutha River (11/03/06) as a 'backup' to those in the Waiau River in-case events such as floods/vandals disrupted the incubations. Artificial substrates consisted of sheets of plastic (50 cm x 9 cm) with a tomentose upper surface, blow moulded to approximate the roughness of a gravel bed stream (Figure 15). These sheets were then glued onto acrylic strips (55 cm x 9.5 cm x 0.6 cm) and bolted at both ends to concrete pavers to keep them in place. Artificial substrates provide a uniform sampling surface, and allow for easier and more reproducible sampling because they reduce heterogeneity in microhabitats (Biggs & Kilroy 2000).

Figure 15: Partially colonized artificial substrate from the Clutha River. See Appendix 2 for fully colonized substrates secured in channels.

Initially, artificial substrates were removed from the Waiau River (12/04/06) after accruing a substantial mat of *D. geminata* and reattached to untreated timber sections (55 cm x 9.5 cm x 5 cm) in the channels. The timber was used to both raise the algae off the channel bottom to minimise channel shading and increase the water velocity over the *D. geminata*. Substrates were left to acclimatize for two weeks. However, a major rainfall event in the Monowai catchment created a large amount of runoff, with high suspended sediment concentrations for a number of days. As a result, >95% of

the *D. geminata* cells on the substrates were dead. A visual assessment of the mats indicated that a large quantity of sediment was trapped amongst the stalk material, and may have been contributing to the decline in cell viability. The viability of the backup artificial substrates in the Clutha River were then assessed, and flown by fixed wing aircraft to Monowai (2/05/06). Substrates were flown because minimising the time spent out of water is critical to ensuring high rates of *D. geminata* survival during transportation. Only 39 substrates were transported to Monowai because of retrieval problems associated with increased flows at the Clutha incubation site (i.e., substrates had to be retrieved by diving in swift two-metre deep water). The viability of *D. geminata* at Monowai was equivalent to the viability of substrates the day prior to transportation, indicating no loss of viability during the transport process. Substrates were reattached at the MEF and given a 10-day acclimation period in the channels. Due to the flooding in the Waiau catchment (and other Southland and Otago catchments), appropriate macroinvertebrate species for toxicity testing in channels could not be sourced. Although disappointing, similar testing on macroinvertebrates will be conducted in the future at the NIWA ecotoxicology laboratory in Hamilton.

For the two days prior to the start of the experiment, fish were sourced for the channels. The fish species that were selected for the trials were rainbow trout (*Oncorhynchus mykiss*) and common bully (*Gobiomorphus cotidianus*). These species are commonly used in laboratory toxicity testing, with rainbow trout known to be particularly sensitive to toxicants (US EPA 1996, Hickey 2000). Rainbow trout were caught via electrofishing from the Pamela Burn (5470965 N, 2071760 E) and transported back to the MEF**.** Rainbow trout used in the channels weighed (live) on average 4.2 g (range: 2.0 – 12.6 g) and were Year 1 age class. Common bullies were sourced from the Waiau River around the Monowai Power Station. Common bullies were captured using a series of baited gee-minnow traps set in the slack water around macrophyte beds. Common bullies used in the experiments had an average live weight of 5.8 g (range: 2.0 – 18.2 g). Fish were placed into experimental channels with the common bullies upstream of the rainbow trout. The areas in which fish were contained were *c.* 30 cm x 10 cm with stainless steel mesh screens (mesh size 750µm) dividing each section. Each fish section was lined with 2 – 3 cm of coarse gravel, with a cobble *c.* 200 cm^3 (7 x 7 x 4cm) placed in each section for refuge. Fish sections were covered in shade cloth to stop fish jumping between sections and prevent bird predation. Fish were fed on a mixed diet of either grade 3 salmon pellets or frozen bloodworms (standard aquarium variety). The amount of food being fed to fish during an experiment is normally proportional to their biomass (i.e., fish are often fed 5% of their body weight per day to maintain normal growth). It could not be established whether either fish species were feeding in the experimental channels (due to the shade cloth cover). As a precaution, fish were overfed and given a minimum of 1.5 g/day of food.

At the start of the experiment, there were 36 channels allocated for biocide application and 6 channels for fish controls (3 of these controls did not have colonized substrates present). Fish status was noted after the application of a biocide. All fish were left in their sections for 24 hours after biocide application in case chemical effects were only temporary. After 24 hours, dead fish were removed off the screens. Fish were re-checked after 2, 6 and 12 days**.** A small proportion (17%) of the fish escaped over the 12 day period; however, dead fish were always present on the mesh screens and did not wash away. Fish that escaped were assumed to be alive but were excluded from statistical analyses. The fish survival component of the trial was ended after 12 days. It was thought that after this duration, without having seen fish actively feeding, any further fish mortality could not be identified as purely the result of biocide application.

All biocides were prepared from stock solutions to the concentrations shown in Table 6. Each channel was calibrated to flow at 1.25 L/s. This meant that the velocities colonized substrates were exposed too were comparable to those of natural waterways. The amount of biocide used in each channel was calculated according the above flow. Each concentration of a biocide was transferred into a modified garden spray pack that allowed it to fill clean intravenous (IV) bags. A "giving set" was then inserted into the IV bag that had been pre-calibrated to drain from a two metre rack over *c.* 1 hour (see Appendix 1). At the higher concentrations, some biocides required two IV bags (e.g., EDTA) so that the biocide did not precipitate out and 'clog' the giving sets. Due to the viscosity of the Organic Interceptor^{TM} biocide, it was made up to 10 litres (in 20 litre bins) and applied using two giving sets per 20 litre bin. Biocides were applied at a rate of six channels per hour. Giving sets were attached to the inlet pipes, with the nozzles positioned in the centre of the flow. Positioning the nozzles in this way allows the turbulence associated with the incoming flow to mix and evenly disperse the biocides across the channels. This mixing was checked visually using food colouring. Biocides and application rates were randomised across all channels, to account for any differences in channel position.

All concentrations of biocides used in these screening trials were nominal concentrations based on product specifications provided on the proprietary product.

The viability of *D. geminata* for all artificial substrates was sampled prior to biocide application (Day 0). After biocide application, all substrates were sampled at 1 hour, 1 day, 3 days, 5 days, 12 days and 28 days. *D. geminata* mats were progressively sampled upstream towards the 'tank end' of the mat, to avoid re-sampling the same area. Three to four small pieces of the mat were snipped off and placed into sample containers. The viability of the mats were analysed using the procedures below.

Laboratory procedures

Changes to the viability of *D. geminata* mats were determined microscopically using the staining techniques used by NIWA for other *D. geminata* studies to distinguish live and dead cells (Kilroy 2005). For each sample, at least 100 *D. geminata* cells were enumerated for detection of those that might be live. Samples were blended prior to the introduction of the stain, to allow uniform stain uptake throughout the mat. Samples were analysed within one hour of staining, with no detectable decline in cell viability over this period.

A 30 mm diameter circle was scribed from the artificial substrate using surgical scissors for chlorophyll *a* and ash-free dry mass (AFDM) analysis at the start and conclusion of the trial. All samples were placed in individual polyethylene containers and frozen before transportation. Samples were analysed for chlorophyll *a* and AFDM using the methods described in Biggs and Kilroy (2000). Each sample was freeze dried, and then weighed. Samples (a preweighed aluminium foil dish plus sample) then had a small portion removed (*c.* 50mg for chlorophyll *a* analysis), reweighed, then ashed at 400°C for 4 h and weighed for a final time. Each chlorophyll *a* sample was homogenized and made up to a known volume. Duplicate subsamples of known volume were filtered through glass fibre filters. Chlorophyll *a* was extracted from the second filter-plus-sample using boiling ethanol, and concentrations of chlorophyll *a* were read spectrophotometrically at 663 nm, including acidification to correct for phaeophytins. In each case, we calculated quantities per m² of substrate surface, based on the area of the sampling circle (0.000707 m²).

Note: Chlorophyll *a* (mg/m²) is a measure of the total amount of autotrophic organisms (live algal material) in the sample. AFDM (g/m²) is a measure of the total amount of organic material in the sample, including cell contents, *D. geminata* stalk material, and also any other algae or small organisms trapped within the mat.

In addition to live/dead cell enumeration, cell density was also measured with an inverted microscope. Cell density analysis produced a quantitative estimate of the absolute number of "live" cells in a sample, where live cells were taken to be cells containing intact chloroplasts. A 30 mm diameter circle was scribed from the artificial substrate using surgical scissors for cell density analysis. One-ml subsamples were extracted from the homogenized solution, and pipetted into the well of an inverted microscope. Entire subsamples were then scanned at a magnification of *c.* x 100, and counts were made of all *D. geminata* cells containing chloroplasts in the fields of view. Either 200 cells were counted per sample or 100 fields of view. Absolute numbers of cells per square millimetre were calculated from the known areas of the microscope well and the field of view.

Data analysis – field trials

D. geminata mortality

The proportion of viable cells was analysed using Generalised Linear Models (GLM) with a binomial error distribution and a logit link function. Individual cells were classified as alive (1) or dead (0). Preliminary analyses showed that cell viability was not a linear function of time (log x + 1 transformed); cell viability decreased within 1 hour of biocide application and thereafter showed no significant change over time. Since there was little variation among treatments in the proportion of viable *D. geminata* cells at the start of the experiment, time was dropped from the model and observations collected from each channel between 1 and 672 hours were pooled to give a single measure of *D. geminata* survival in each channel after application of treatments.

Cell viability was first compared among the 13 treatments (control, plus four biocides each applied at three different rates). Four orthogonal contrasts were then used to explore the factors contributing to mortality: the first contrasted the control with all four biocides combined, the second compared the four biocides, the third tested the effect of application rate (measured on a continuous, geometric scale), and the fourth tested for an interaction between biocide and application rate. The significance of model terms was tested using F-ratio tests, which take account of over-dispersion in the data, and evaluated at $\alpha = 0.05$. Where a significant effect of biocide was detected, Tukey's HSD test was used to identify significant pairwise comparisons.

D. geminata abundance

The effect of biocide treatment and application rate (measured on a continuous geometric scale) on *D. geminata* abundance was analysed using analysis of variance (ANOVA). Four response variables were used to quantify *D. geminata* abundance: chlorophyll *a*, ash-free dry mass (AFDM), autotrophic index (AI) and cell density. AFDM, AI and cell density were log-transformed to fulfil assumptions of normality and homogeneity of variances. Each response variable was first compared across all 13 treatments (control, plus four biocides each applied at three different rates), and four orthogonal contrasts (planned a priori) were then used to explore the factors contributing to observed differences. The first contrasted the control with all four biocides combined, the second compared the four biocides, the third tested the effect of application rate, and the fourth tested for an interaction between biocide and application rate. F-ratio tests were as used to evaluate the significance of factors and significance evaluated at $\alpha = 0.05$. Where a significant effect of biocide was detected, Tukey's HSD test was used to identify significant pairwise comparisons.

Note: the four biocides were not compared with the control separately because in many cases a high variance among control channels resulted in a series of non-significant results, which obscured important differences among the biocides.

Fish mortality

The effect of biocides upon common bully and rainbow trout survival was analysed using generalised linear models (GLM). For each species, the survival (1) or death (0) of individual fish was first compared among the 13 different treatments (control, plus four biocides each applied at three different rates) using a binomial error distribution and a logit link function. Four orthogonal contrasts were then used to explore the factors contributing to mortality: the first contrasted the control with all four biocides combined, the second compared the four biocides, the third tested the effect of application rate (measured on a continuous, geometric scale), and the fourth tested for an interaction between biocide and application rate. A chi-square test was used to evaluate the significance of model terms and significance evaluated at $\alpha = 0.05$.

4.1.2. Laboratory toxicity testing

Non-target toxicity testing rationale

A series of rangefinder toxicity tests were conducted on the green alga *Pseudokirchneriella subcapitata*[12] and the cladoceran *Daphnia magna* (also known as the waterflea) to assess the potential non-target toxicity of the four biocides being considered for use as control agents. These species were chosen because they are standard toxicity testing species that will provide a good first assessment of the likely effect of the control compounds on non-target algae, plants and invertebrates. In addition, standard toxicity tests using these species are routinely performed at NIWA, and a large amount of data on their sensitivity to the control compounds and other chemicals is readily available.

The effect of the biocides on the alga, *D. magna* (invertebrates) and fish will be compared to the 3 one hour treatment rates in the Stage 2 Phase 1 trials conducted at the Monowai experimental facility (listed in Table 6) and their effectiveness against *D. geminata*. Most standard toxicity tests on these and other species are conducted using 72 h (3 day) and 96 h (4 day) exposures; however in this situation the biocide is likely to be applied as a "pulse" of biocide through a flowing water system. The Stage 2 research used 1 h applications to test toxicity to *D. geminata,* however it is possible that longer exposures (i.e., >1 h) will be delivered in a large river system to ensure

[12] Formerly known as *Selenastrum capricornutum.*

penetration of the biocides into the thick *D. geminata* mats, therefore the non-target rangefinder tests examined the effect on *D. magna* of 1 h, 4 h and 24 h exposures to the biocides. One hour and 24 h exposures were tested on the green alga *P. subcapitata*. In both cases the organisms were exposed to the biocide (e.g., for 1 h) then removed to clean culture water and observed for effects (e.g., growth, behaviour, or survival) for the remainder of the normal testing period (48 h for *D. magna*, 72 h for *P. subcapitata*). These test methods mimicked the likely effect of a pulse of biocide through the river, and measured the short-term effects on the exposed organisms (i.e., chronic effects and reproductive toxicity should be examined later). In some cases immediate toxicity and death was observed, in others (e.g., after chelated copper exposure) organisms died a day or two after the initial exposure. In still other cases the organisms were initially negatively affected by the biocide, but recovered after transfer to clean water. This report will focus on the survival/growth of the organisms 48 or 72 h after exposure to the biocide, however we also provide information on the initial response of the organisms to the biocide (at the time of transfer), particularly if they were immobilised by the initial exposure, as this could significantly affect the recovery of the species after a *D. geminata* treatment. For example, immobilised invertebrates are likely to be washed out of the treatment area.

Very little information was available on the effect of such short-term exposures to the biocides, so these rangefinder tests examined a wide range of concentrations in order to ensure that we could identify the concentration at which toxicity would occur. Only two replicates at each concentration were used, as the intention is to continue the testing with additional replication over a narrower range of concentrations for definitive results. In addition, all readily available information was gathered from toxicity testing databases and previous research regarding the likely effect of these biocides on fish.

All toxicity testing was completed during the period 19/6/06-7/7/06 using samples of test biocides provided as detailed in Section 2.1.2 and each relevant section below. Physico-chemistry (temperature, pH, dissolved oxygen (DO), salinity and conductivity) was measured in solutions prepared for *D. magna* only. To obtain a comprehensive toxicity profile of each of the biocides, a much wider range of biocide concentrations was used than applied in the MEF trials. Testing the effect of a short-term exposure to the biocide required modification of the standard toxicity testing protocols and suitable controls were included in all the tests to take into account the effects of these modifications. Results were analysed using the ToxCalc™ program (Tidepool Scientific Software) as recommended by the US Environmental Protection Agency (Tidepool 1994).

Daphnia magna (invertebrate, waterflea) toxicity tests

All exposure solutions were prepared in *D. magna* culture water (hardness ~90 mg/L as $CaCO_3$) from the toxicant formulations on the day of testing, except for the 1.08 g Cu/L and the 3355 mg EDTA/L stock solutions, that were prepared at the start of the week and used for all subsequent dilutions.

Juvenile *D. magna* (<24 h) were transferred in groups of 10 to ~20 mL of exposure solution for the specified exposure period (i.e., 1, 4 or 24 h). Each exposure at each biocide concentration was replicated twice except for controls which were replicated 3-8 times depending on the availability of test organisms. At the end of the exposures, the organisms were sieved from the solutions, rinsed in clean culture water, transferred to 40 mL clean culture water, counted and kept for continued observation (i.e., at 24 and 48 h). A standard 48 h zinc (Zn) toxicity test was also conducted for analytical quality control. Testing methods are summarized in Appendix 4.

Pseudokirchneriella subcapitata (green alga) toxicity tests

All exposure solutions were prepared in sterile *P. subcapitata* culture solution (pH 7.5) from the toxicant formulations on the day of testing, except for the 100 mg Cu/L chelated copper and the 3355 mg EDTA/L stock solutions that were prepared at the start of the week and used for all subsequent dilutions.

The algal testing required modification of the initial steps of the standard microplate protocol (NIWA SOP 15.2, Golding 2004). Algal cell density was measured and 44,000 cells were transferred to exposure solutions in 10 mL centrifuge tubes, giving a final concentration of 10,000 cells/mL. During the exposure period (1 h or 24 h), the capped vials were laid horizontally and exposed to culture lights on a shaker table to ensure thorough mixing of the cells with the biocides and continued algal growth. Afterwards the vials were centrifuged, then the supernatant was removed (x3) and replaced with sterile culture solution in order to rinse the toxicants from the algae. Next, microplates were prepared as normal and algal cell growth (i.e., final cell density in each replicate) was compared between control treatments and biocide treatments 72 h after initiation of the exposures. Testing methods are summarized in Appendix 5. Like the *D. magna* tests, these exposures were conducted as rangefinders only and initial exposures were conducted in only one replicate of each biocide concentration. Once the cells were transferred to microplates, they were spread across replicates. A Zn toxicity test was included as an analytical quality control (AQC).

Toxicity testing - Chelated copper

Chelated Cu was tested on *D. magna* at up to 7 concentrations for 3 exposure times and on *P. subcapitata* at 5 concentrations for 2 exposure times (Tables 7 and 8). Reported copper concentrations are nominal, not measured.

Table 7: Chelated Cu concentrations (mg Cu/L) and exposure times (h) tested on *D. magna.*

	Exposure Time (h)		
	1	**4**	**24**
Concentration	0.001	0.001	0.001
(mg Cu/L)	0.01	0.01	0.01
	0.10	0.10	0.10
	1.00	1.00	1.00
	10.00	10.00	10.00
	50.00	-	-
	100.00	-	-

Table 8: Chelated Cu concentrations (mg Cu/L) and exposure times (h) tested on *P. subcapitata.*

	Exposure Time (h)	
	1	**24**
Concentration	0.01	0.0001
(mg Cu /L)	0.10	0.001
	1.00	0.010
	10.00	0.100
	100.00	1.000

Toxicity testing - EDTA

It was expected that a solution of EDTA would have minimal toxicity to algae or *D. magna*, therefore a wide range of concentrations was tested at 2-3 different exposure times for each species (Tables 9-10).

Table 9: EDTA concentrations (as the disodium dihydrate salt) (mg EDTA/L) and exposure times (h) tested on *D. magna.*

	Exposure Time (h)		
	1	**4**	**24**
Concentration	32	32	32
(mg EDTA/L)	102	102	102
	328	328	328
	1049	1049	1049
	3355	3355	3355

Table 10: EDTA concentrations (as the disodium dihydrate salt) (mg EDTA/L) and exposure times (h) tested on *P. subcapitata.*

	Exposure Time (h)	
	1	**24**
Concentration (mg EDTA/L)	32	10
	102	32
	328	102
	1049	328
	3355	1049

Toxicity testing - Hydrothol®191

Hydrothol®191 was tested on *D. magna* at 5-8 concentrations for 3 different exposure times (Table 11) and on *P. subcapitata* at 5 concentrations for 2 exposure times (Table 12). Previously Hydrothol®191 was tested in one hour exposures of *D. geminata* at 0.5, 1.0 and 2.0 mg a.e./L (Rates 1, 2, and 3 respectively).

Table 11: Hydrothol®191 concentrations (mg a.e./L) and exposure times (h) tested on *D. magna.* Note, the concentration of the product is expressed as mg acid equivalents/L.

	Exposure Time (h)		
	1	**4**	**24**
Concentration (mg a.e./L)	0.16	1.28	0.16
	0.32	2.56	0.32
	0.64	5.1	0.64
	1.28	10.2	1.28
	2.56	20.4	2.56
	5.20	-	-
	10.40	-	-
	20.50	-	-

Table 12: Hydrothol®191 concentrations (mg a.e./L) and exposure times (h) tested on *P. subcapitata.*

	Exposure Time (h)	
	1	**24**
Concentration (mg a.e. /L)	0.01	0.001
	0.10	0.01
	1.00	0.10
	10.00	1.00
	100.00	10.00

Toxicity testing - Organic Interceptor™

Organic Interceptor™ was tested on *D. magna* at 5 concentrations for 3 different exposure times (Table 13) and OI was tested on *P. subcapitata* at 10 concentrations for 2 exposure times in two sets of experiments (Table 14). The initial set of 1 h exposures was repeated as the *D. magna* were not transferred until 85 min, however very little variation in the results was observed.

Table 13: Organic Interceptor™ concentrations (mg pine oil/L) and exposure times (h) tested on *D. magna*.

	Exposure Time (h)		
	1	4	24
Concentration	5	1	1
(mg pine oil/L)	25	5	5
	125	25	25
	625	125	125
	3125	625	625

Table 14: Organic Interceptor™ concentrations (mg pine oil/L) and exposure times (h) tested on *P. subcapitata* in two sets of experiments (19/6/06 or 4/7/06).

Experiment		Exposure Time (h)	
Date		1	24
19/6/06	Concentration	0.001	0.001
19/6/06	(mg pine oil/L)	0.01	0.01
19/6/06		0.10	0.10
19/6/06		1.00	1.00
19/6/06		10.00	10.00
4/7/06		0.68	0.07
4/7/06		6.80	0.68
4/7/06		68.00	6.80
4/7/06		680.00	68.00
4/7/06		6800.00	680.00

4.2. Results

4.2.1. Field trials at the Monowai experimental facility

D. geminata mortality

The average viability of the starting material for all trial substrates was constant at 91% ± 0.66. The decline in the cell viability of control substrates over the 28-day trial was linear, with a final mean viability of 74% ± 4.77 (Figure 16).

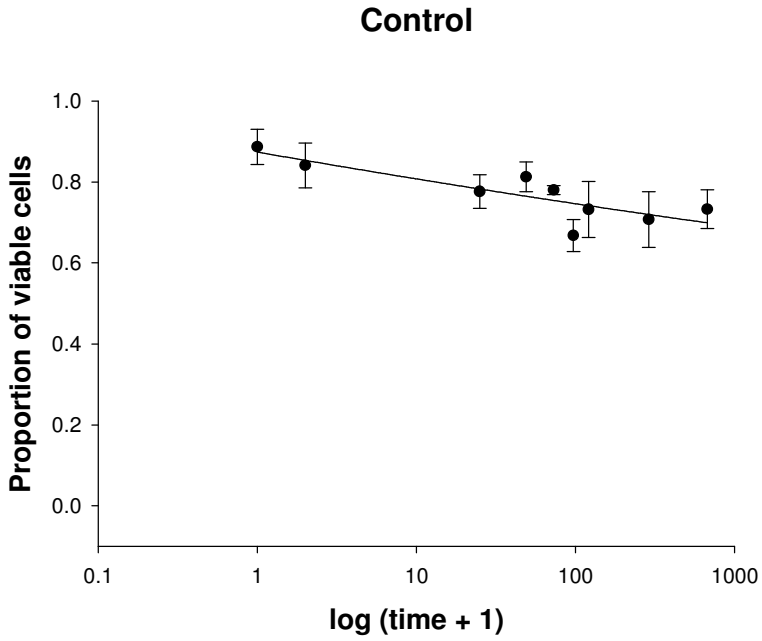


Figure 16: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time (hours) for the control substrates.

For chelated Cu, the largest decrease in cell viability for all rates was recorded after 1 h. As expected, the weakest concentration had the least initial cell mortality and the strongest concentration the highest cell mortality. For two of the three application rates, cell viability remained relatively constant after the initial chelated Cu dose (Figure 17). There was no viable material entering the MEF, so the apparent increase in cell viability at the strongest concentration between days 3 – 5 cannot easily be explained.

EDTA resulted in a minor initial decrease in cell viability, which was more pronounced at the higher concentrations. Overall, there was little variation between the three application rates, with cell viability around 65% for the majority of the trial (for all rates) (Figure 18).

Hydrothol®191 achieved an initial decrease in cell viability to 41% at its highest concentration (2 mg a.e./L). Average cell viability ranged from 63% at the lowest concentration to 53% at the highest concentration, over the 28 days (Figure 19).

Organic Interceptor™ was relatively toxic at all application rates, especially at the two higher concentrations. At rate 2 (135 mg pine oil/L) cell viability never got above 12%. At the highest concentration (271 mg pine oil/L), no viable cells were recorded after 3 days (Figure 20).

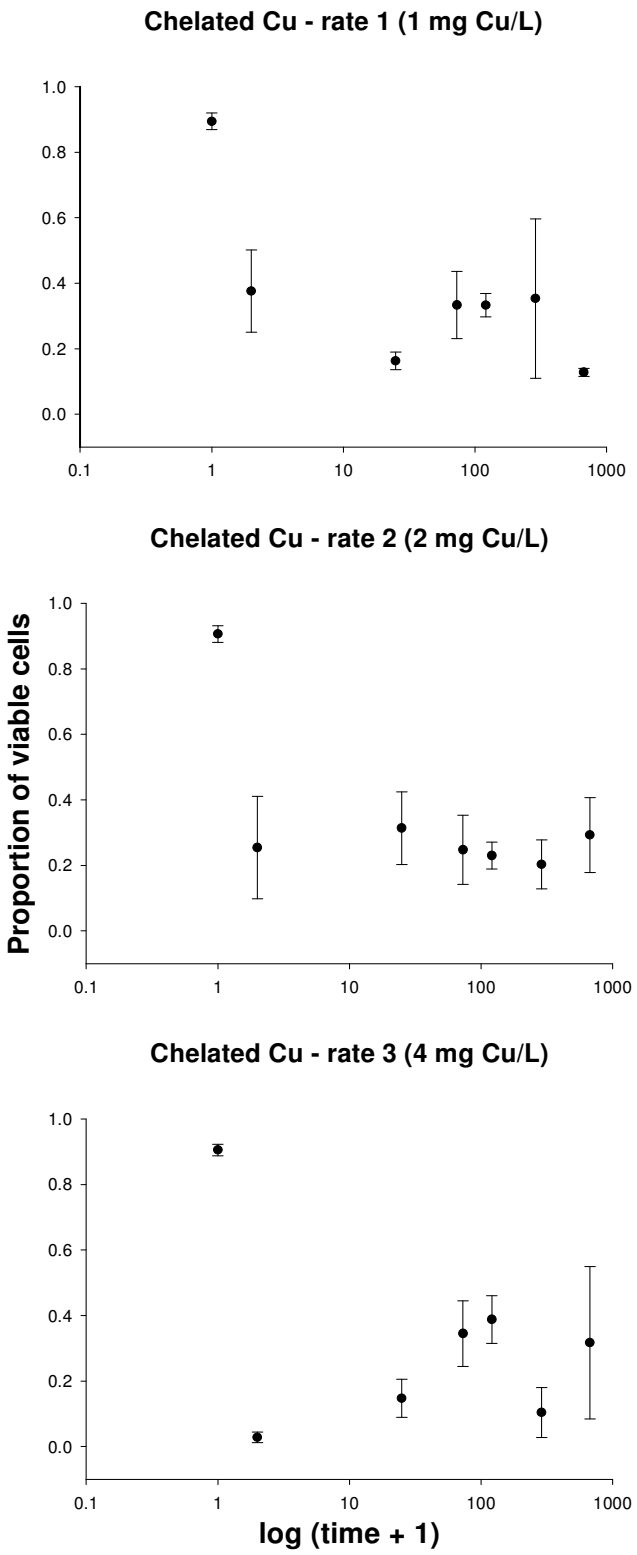


Figure 17: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time (hours) for each rate of the biocide chelated Cu. (Note: Time 1 value is control value prior to biocide addition).

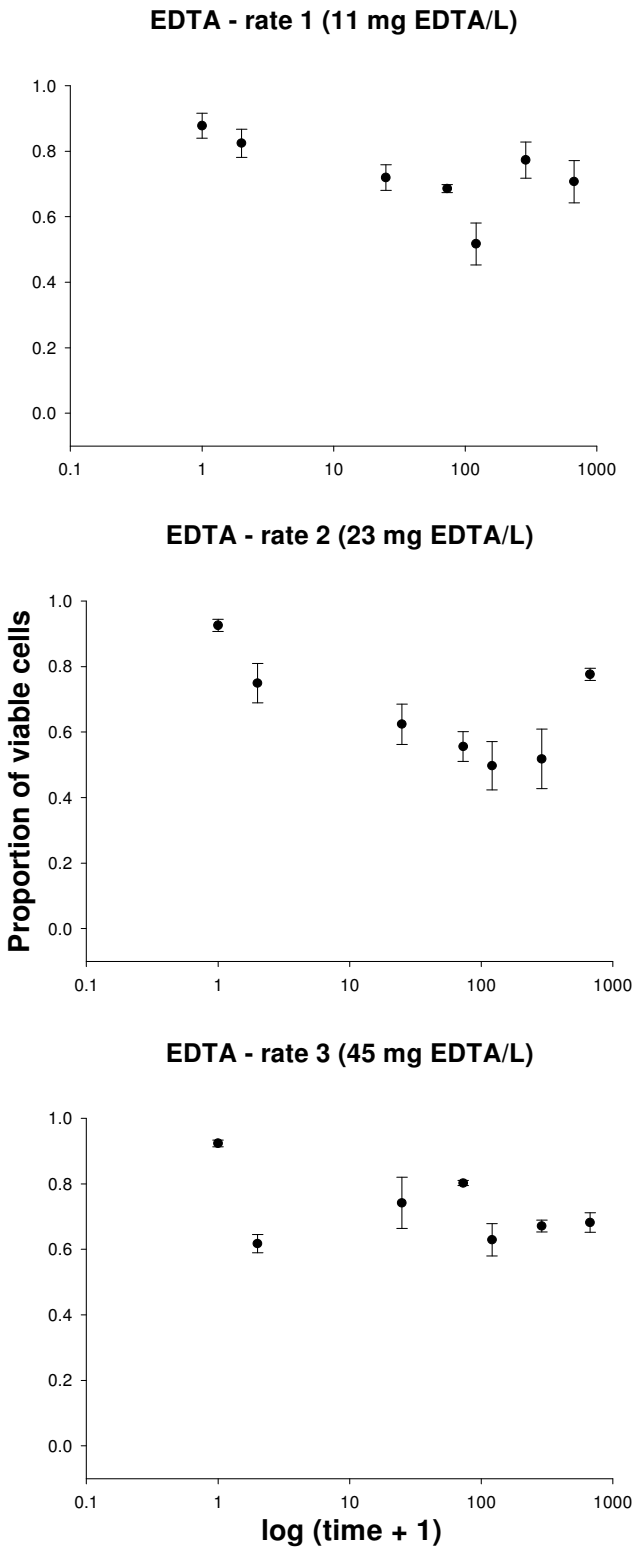


Figure 18: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time (hours) for each rate of the biocide EDTA. (Note: Time 1 value is control value prior to biocide addition).

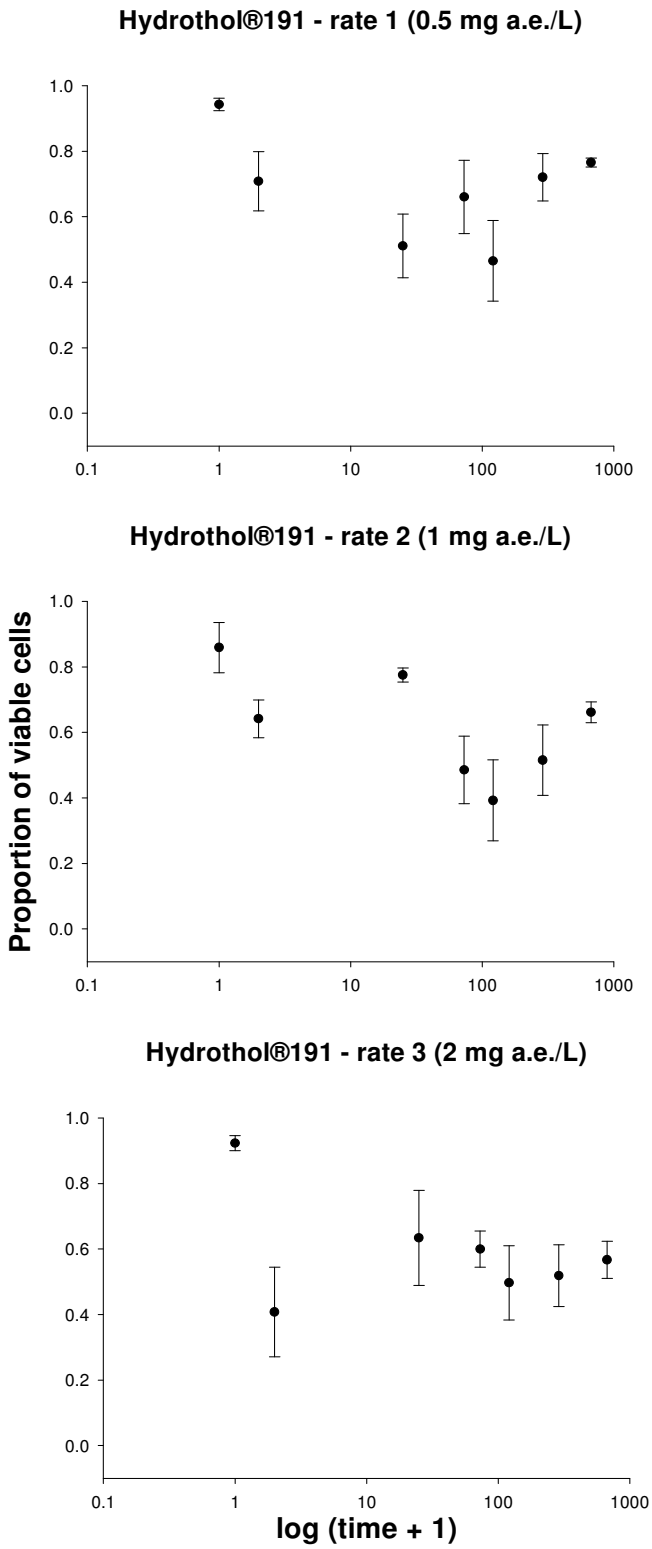


Figure 19: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time (hours) for each rate of the biocide Hydrothol®191. (Note: Time 1 value is control value prior to biocide addition).

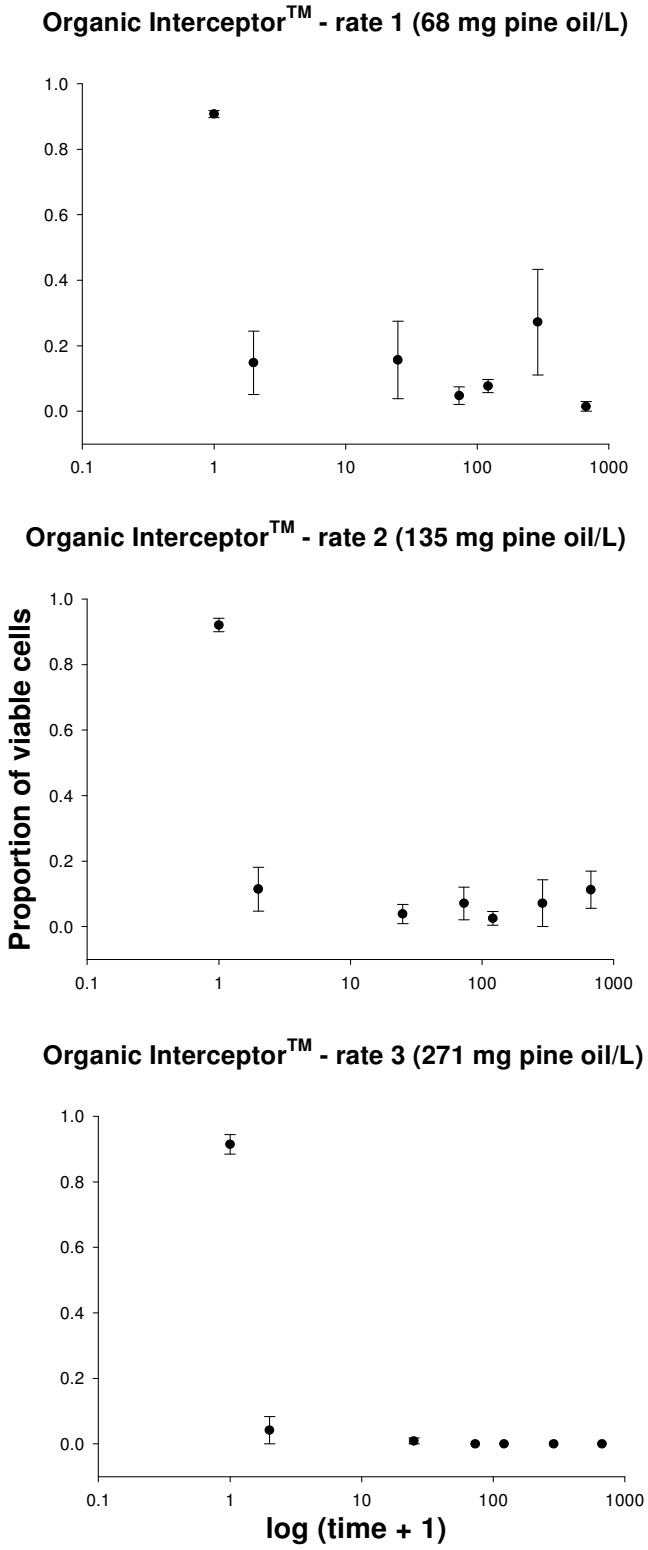


Figure 20: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time (hours) for each rate of the biocide Organic Interceptor™. (Note: Time 1 value is control value prior to biocide addition).

Preliminary analyses showed that cell viability was not a linear function of time following cessation of contact with the biocide; cell viability decreased within 1 hour of biocide application and thereafter showed no significant change over time (Figure 21). Using a single measure of *D. geminata* survival in each channel after application of treatments, cell viability was lower in biocide channels than in control channels (GLM: $F_{1,37} = 5.35$, $P = 0.026$) (Figure 22). The four biocides differed significantly in their effect on cell viability (GLM: $F_{3,28} = 116.6$, $P < 0.001$): channels that received Organic Interceptor™ had a lower proportion of viable cells than those that received chelated Cu, which in turn had a lower proportion of viable cells than those that received EDTA or Hydrothol®191 (Figure 22). Cell viability decreased with increasing application rate (GLM: $F_{1,28} = 4.74$, $P = 0.038$) and the effect of application rate differed among biocides (GLM: $F_{3,28} = 3.27$, $P = 0.036$).

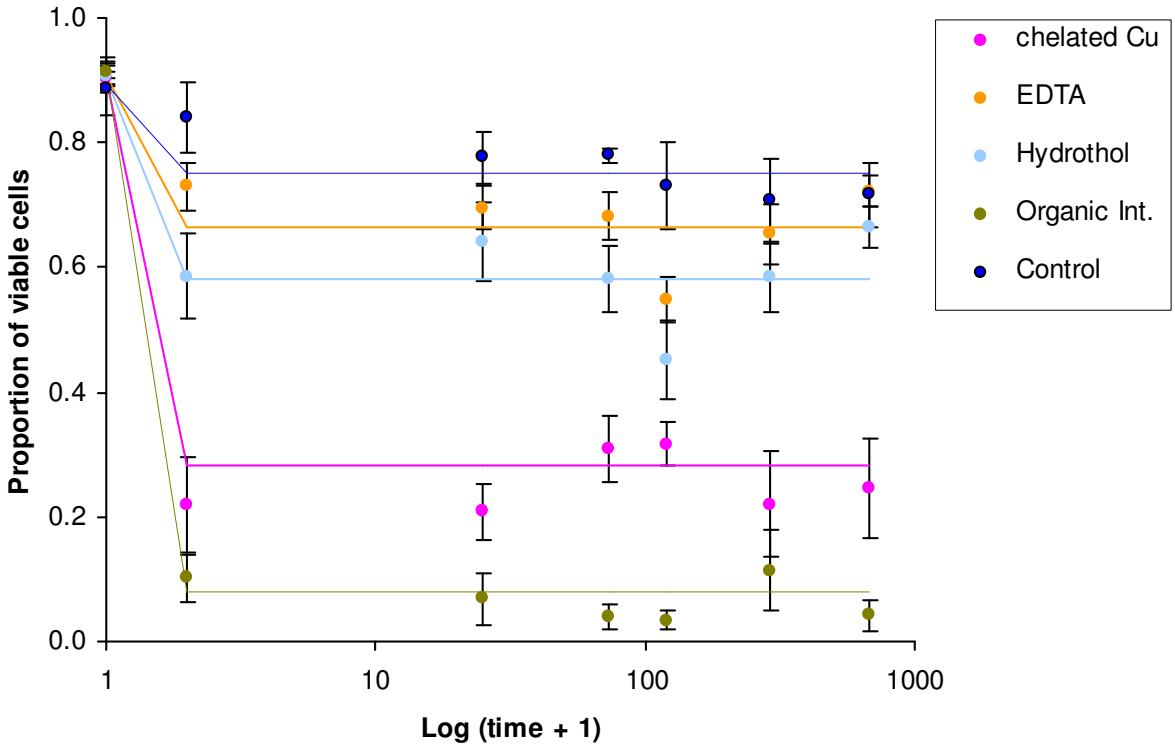


Figure 21: Mean (± 1 S.E.) proportion of viable *D. geminata* cells as a function of time since application (hours) and biocide. Fitted lines from Generalised Linear Model with time as a binary factor (before and after biocide application). For clarity, application rates have been averaged; S.E.s are therefore based on three (control) or nine (biocides) replicate channels per treatment.

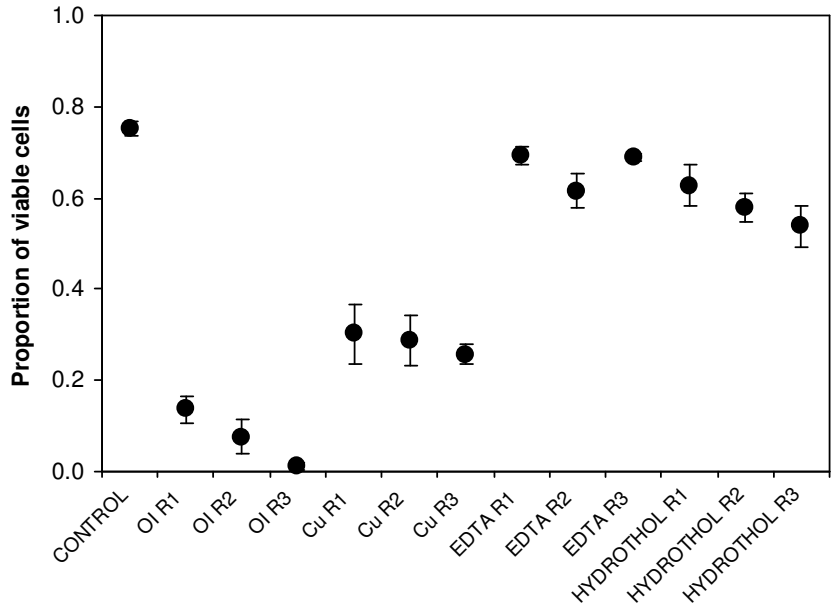


Figure 22: Mean (± 1 S.E.) proportion of viable *D. geminata* cells after application of treatments as a function of biocide and application rate. Repeated measurements of cell viability taken from each channel between 1 and 672 hours after treatment were pooled for each channel and S.E.s calculated for three replicate channels per treatment.

D. geminata abundance

Although the four biocides had no overall effect on chlorophyll *a* relative to the control (ANOVA: $F_{1,37}$ = 0.06, P = 0.81) (Figure 23), the biocides did differ significantly in their effect on chlorophyll *a* (ANOVA: $F_{3,28}$ = 7.06, P = 0.001).

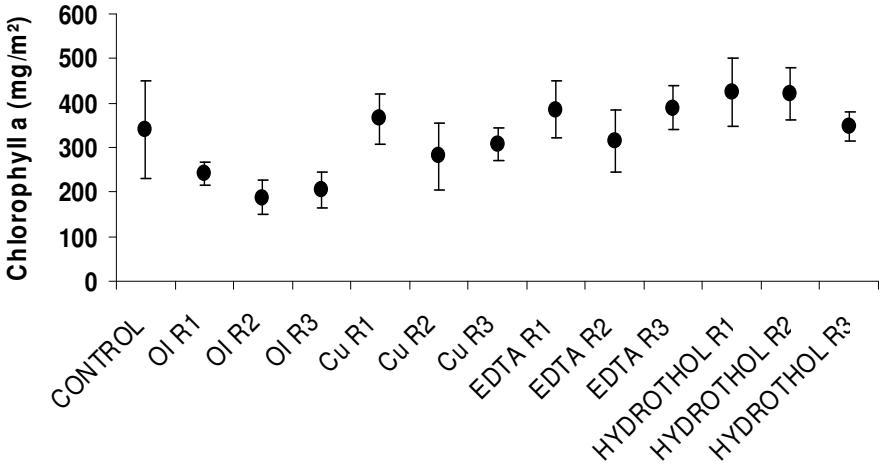


Figure 23: Mean (± 1 S.E.) chlorophyll *a* measurements of *D. geminata* for each rate of biocide at the conclusion of the field trial. n = 3 for all treatments.

Channels that received Organic Interceptor™ had lower chlorophyll *a* than those that received EDTA or Hydrothol®191. Application rate (ANOVA: $F_{1,28} = 1.27$, $P = 0.27$) and the interaction of application rate with biocide treatment (ANOVA: $F_{3,28} = 0.23$, $P = 0.88$) had no effect on chlorophyll *a*.

AFDM was lower in biocide channels than in control channels (ANOVA: $F_{1,37} = 4.84$, $P = 0.034$) (Figure 24). The four biocides differed significantly in their effect on AFDM (ANOVA: $F_{3,28} = 3.03$, $P = 0.046$) but Tukey's HSD test failed to detect any significant pairwise comparisons at a family-wise error rate of $\alpha = 0.05$. Using a less conservative value for α of 0.20, channels receiving chelated Cu had a significantly lower AFDM than those receiving EDTA and Hydrothol®191. Application rate (ANOVA: $F_{1,28} = 2.84$, $P = 0.10$) and the interaction of application rate with biocide treatment (ANOVA: $F_{3,28} = 1.02$, $P = 0.40$) had no effect on AFDM.

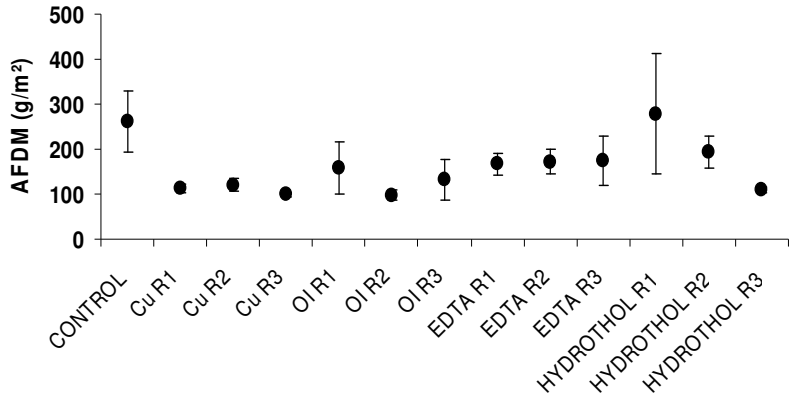


Figure 24: Mean (± 1 S.E.) AFDM of *D. geminata* for each rate of biocide at the conclusion of the field trial. $n = 3$ for all treatments.

The four biocides had no overall effect on AI relative to the control (ANOVA: $F_{1,37} = 2.91$, $P = 0.10$) (Figure 25), in part because there was a high variation among the control channels. There was no effect of biocide (ANOVA: $F_{3,28} = 1.29$, $P = 0.30$), application rate (ANOVA: $F_{1,28} = 0.45$, $P = 0.51$) or their interaction (ANOVA: $F_{3,28} = 0.45$, $P = 0.72$) on AI.

Although the four biocides had no overall effect on cell density relative to the control (ANOVA: $F_{1,37} = 2.47$, $P = 0.12$), the biocides did differ significantly in their effect on cell density (ANOVA: $F_{3,28} = 52.5$, $P < 0.001$) (Figure 26). Channels that received Organic Interceptor™ had lower cell densities than those that received chelated Cu, which in turn had lower cell densities than those that received EDTA or Hydrothol®191. Cell density decreased with increasing application rate (ANOVA: $F_{1,28} = 24.9$, $P < 0.001$) and the effect of application rate differed significantly among biocides (ANOVA: $F_{3,28} = 10.4$, $P < 0.001$).

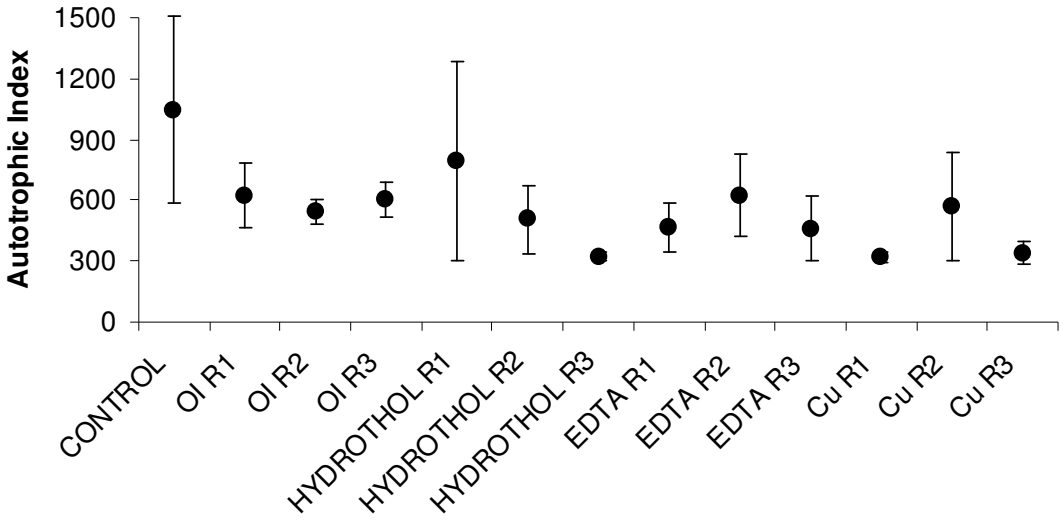


Figure 25: Mean (± 1 S.E.) autotrophic index for each rate of four biocides at the conclusion of the field trial. n = 3 for all treatments.

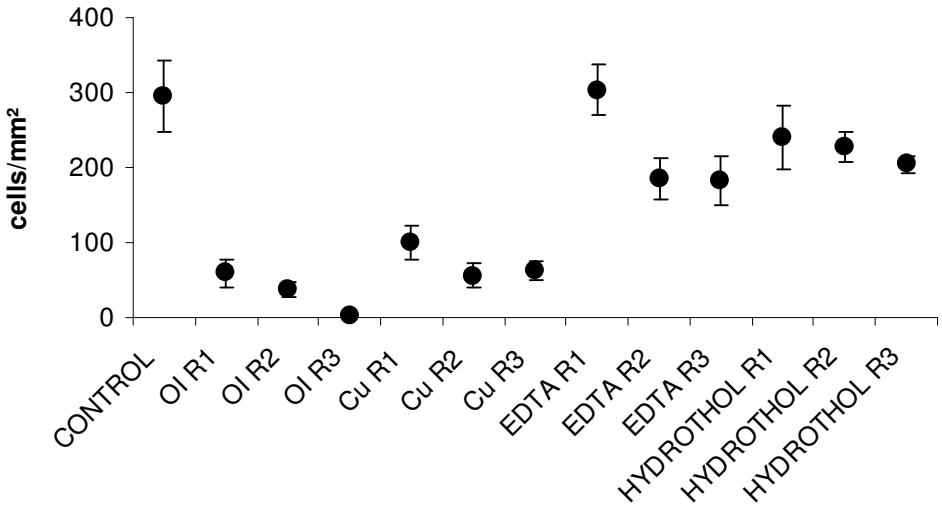


Figure 26: Mean (± 1 S.E.) *D. geminata* cell densities for each rate of four biocides at the conclusion of the field trial. n = 3 for all treatments.

Cell density was highly correlated with the proportion of viable cells (ANOVA: $F_{1,38}$ = 58.07, R^2 = 0.61, $P < 0.001$) (Figure 27). As the proportion of viable cells increases, so does the density of cells per mm². This indicates that either metric (cell density or cell viability) provides useful information about biocide effectiveness on *D. geminata*.

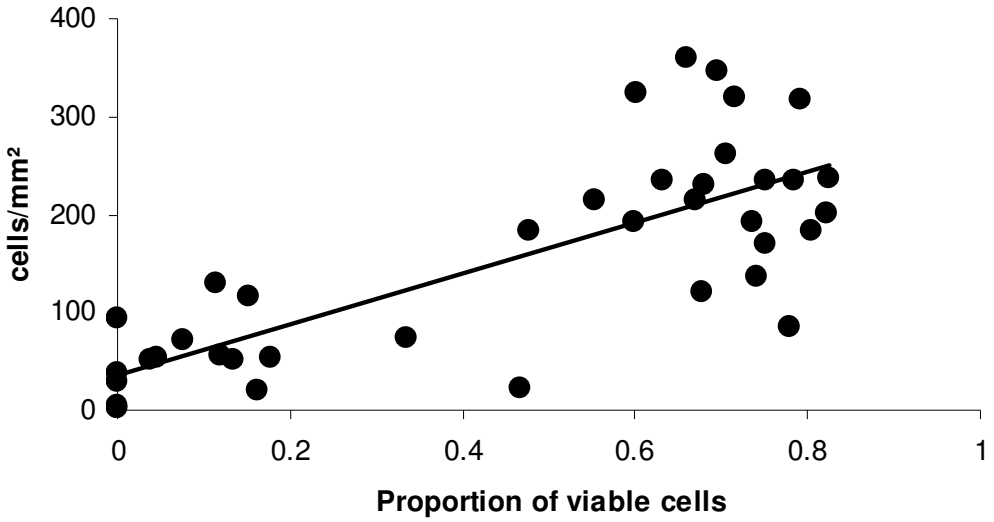


Figure 27: A comparison of *D. geminata* cell densities plotted against the proportion of viable cells for each substrate.

Fish mortality

For each rate of a particular biocide, fish mortality rates were assessed for three common bullies and three rainbow trout. A small proportion (17%) of these fish escaped during the 12-day trial, and the following analyses does not include escaped fish (assumed to be live – see methods). No fish mortality was observed in any of the treatments except Organic Interceptor™. All rainbow trout died after exposure to the lowest concentration of OI, whereas bully survival was 66%, 100% and 33% in 68, 135 and 271 mg pine oil/L respectively (Table 15). The low number of fish tested meant that these results are indicative only.

Although the four biocides had no overall effect on survival of common bullies relative to the control (GLM: χ^2 = 1.12, d.f. = 1, P = 0.29), however, survival was significantly lower in the Organic Interceptor™ treatment than in the other three biocide treatments (GLM: χ^2 = 7.90, d.f. = 3, P = 0.048). Application rate (GLM: χ^2 = 0.84, d.f. = 1, P = 0.36) and the interaction of application rate with biocide treatment (GLM: χ^2 = 0.00, d.f. = 3, P = 1.00) had no effect on survival.

Although the four biocides had no overall effect on survival of rainbow trout relative to the control (GLM: χ^2 = 2.64, d.f. = 1, P = 0.10), survival was significantly lower in the organic interceptor treatment than in the other three biocide treatments (GLM: χ^2 = 35.99, d.f. = 3, P < 0.001). Application rate (GLM: χ^2 = 0.00, d.f. = 1, P = 1.00) and the interaction of application rate with biocide treatment (GLM: χ^2 = 0.00, d.f. = 3, P = 1.00) had no effect on survival.

Table 15: Fish survival from 12-day field trials for each rate of the four biocides. The counts (n) indicate the number of fish left in the channels at the conclusion of the experiment. Note: CB = common bully and RT = rainbow trout.

Treatment	Rate	n	CB live (%)	n	RT live (%)
Control		5	100	5	100
Chelated Cu	1	3	100	3	100
Chelated Cu	2	3	100	3	100
Chelated Cu	3	2	100	3	100
EDTA	1	1	100	2	100
EDTA	2	2	100	3	100
EDTA	3	2	100	3	100
Hydrothol®191	1	3	100	3	100
Hydrothol®191	2	1	100	2	100
Hydrothol®191	3	2	100	3	100
Organic Interceptor™	1	3	66	3	0
Organic Interceptor™	2	2	100	2	0
Organic Interceptor™	3	3	33	3	0

4.2.2. Laboratory toxicity testing

Chelated copper

Daphnia magna exposed to 1.0 mg Cu/L for 1 h did not appear to be negatively affected as they were transferred to freshwater, however by 48 h survival was only 14% (Figure 28a). A 1 h exposure to 10 mg Cu/L immobilised the *D. magna*, with apparent recovery upon transfer to freshwater, however by 24 h 100% were dead.

As expected, a 4 h exposure to Cu was even more toxic with 100% mortality by 48 h after exposure to 1 mg Cu/L and decreased (variable) survival in both 0.10 and 0.01 mg Cu/L (Figure 28b). A 24 h exposure to ≥0.1 mg Cu/L was highly toxic to *D. magna* (no recovery when transferred to freshwater) (Figure 28c). The 48 h survival was 90% after a 24 h exposure to 0.01 mg Cu/L.

The mechanism of toxicity for 10 mg Cu/L is likely to be the combined effects of Cu and the low pH (pH=3.8), however in ≤1 mg Cu/L the pH was 6.8-7.4, so Cu was probably the direct cause of toxicity. The temperature (19-20°C), DO (9.1-9.2 mg O_2/L), salinity (0 ppt) and conductivity (310- 347 µS/cm for ≤1 mg Cu/L, and 632 µS/cm for 10 mg Cu/L) were also within expected ranges and were unlikely to have caused reduced survival.

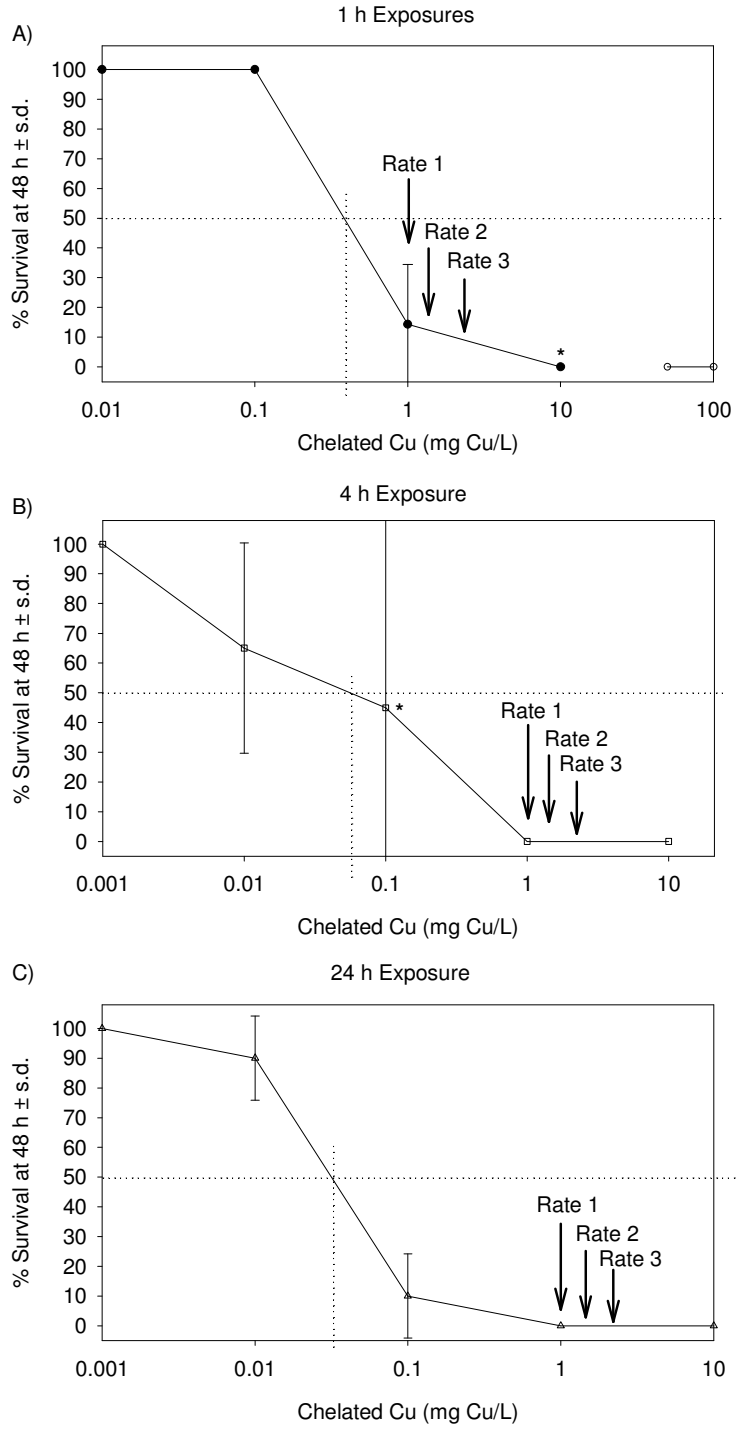


Figure 28: Percentage 48 h survival of *D. magna* after a) 1 hour, b) 4 hour or c) 24 hour exposure to different chelated Cu concentrations. Note the change in scale on the x-axis. The rates tested on *D. geminata* for 1 h at Stage 2 are noted on the x-axis for comparison. Asterisk indicates *D. magna* negatively affected at the time of transfer from chelated Cu to freshwater but later recovered. Dashed lines give an estimate of the LC_{50} for each exposure but could be an over- or underestimate if the response is non-linear.

A 1 h exposure to 0.01-100 mg Cu/L negatively affected subsequent 72 h algal growth in a more-or-less dose-dependent fashion (Figure 29a). Growth was 27% and 46% less than controls in algae exposed to 0.01 and 100 mg Cu/L respectively. The response to a 24 h Cu exposure was much more obvious; algal growth was not significantly affected by exposure to up to 0.01 mg Cu/L, but decreased to only 16% of the controls after 24 h exposure to 0.10 mg Cu/L (Figure 29b). The copper concentrations are nominal rather than measured.

EDTA

A 1 h exposure to 1049 or 3355 mg EDTA/L reduced subsequent 48 h survival of *D. magna* to 85 and 57% respectively (Figure 30a). A slight decrease in survival to 95% occurred after 1 h exposure to 328 mg EDTA/L. The pH of these solutions was 5.9, 5.3 and 5.0 for 328, 1049 and 3355 mg EDTA/L respectively. The pH of 32-102 mg EDTA/L was 8.2.

Both a 4 h and 24 h exposure to ≥328 mg EDTA/L were toxic to *D. magna*, probably due to the acidic pH of these solutions (Figures 30b,c). The temperature (19-20°C), DO (9.4-9.6 mg O_2/L), and salinity (0-3 ppt) were within the expected ranges and probably did not contribute to toxicity. The conductivity of 32-1049 mg EDTA/L was 180-393 µS/cm and was 794 µS/cm for 3355 mg EDTA/L.

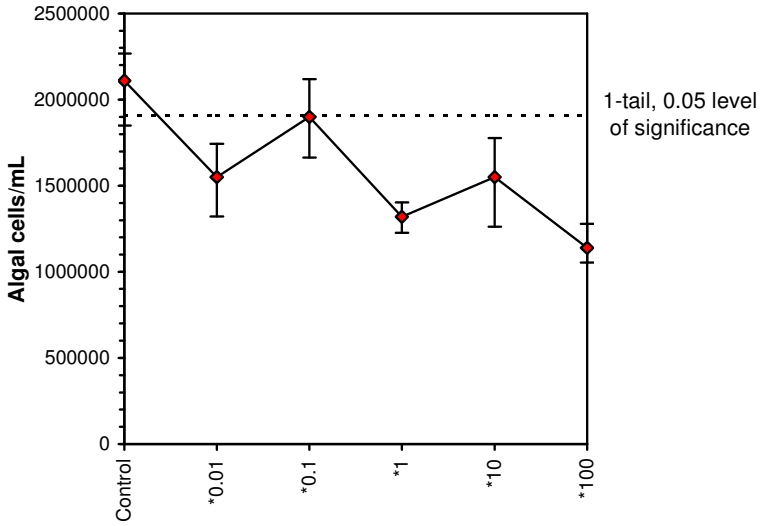
A 1 h exposure to 32-3355 mg EDTA/L tended to decrease subsequent algal growth to a minimum of 56% of the controls, although the response was non-linear (Figure 31a). A 24 h exposure to EDTA produced a dose-dependent decrease in algal growth. Only 10 mg EDTA/L caused a 37% decrease in algal growth (Figure 31b). These data suggest that the EDTA alone was toxic to the alga, as the pH only decreased in solutions of ≥328 mg EDTA/L. It is possible that the EDTA was interacting with some component of the algal culturing solution and altering the bioavailability or toxicity of (for example) trace amounts of metals.

Hydrothol®191

A 1 h exposure to ≥0.64 mg a.e./L Hydrothol®191 immobilised *D. magna*, however transfer into freshwater caused slow recovery of swimming activity in organisms exposed to ≤5 mg a.e./L (Figure 32a). Ultimately by 48 h, only 25% survived the 1h exposure to 10 mg a.e./L, and 90% survived 1 h exposure to 5 mg a.e./L. A 4 h exposure caused 100% mortality at 5 mg a.e./L and similarly a 24 h exposure to only 2.6 mg a.e./L caused 100% mortality (Figures 32b,c). Survival after 24 h exposure to 1.3 mg a.e./L was highly variable between the two replicates with 90% survival in one

and 0% survival in the other. The LC_{50} for each exposure time would be approximately 8, 4 and 1.2 mg a.e./L for a 1 h, 4 h and 24 h exposure respectively.

A)



B)

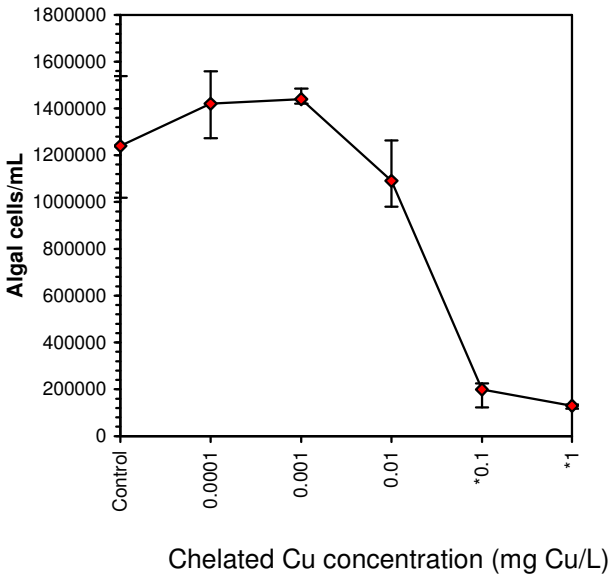


Figure 29: Algal cell density at 72 h after an a) 1 h or b) 24 h exposure to chelated Cu. Asterisk (*) beside numbers on the x-axis indicate significantly different from control treatment.

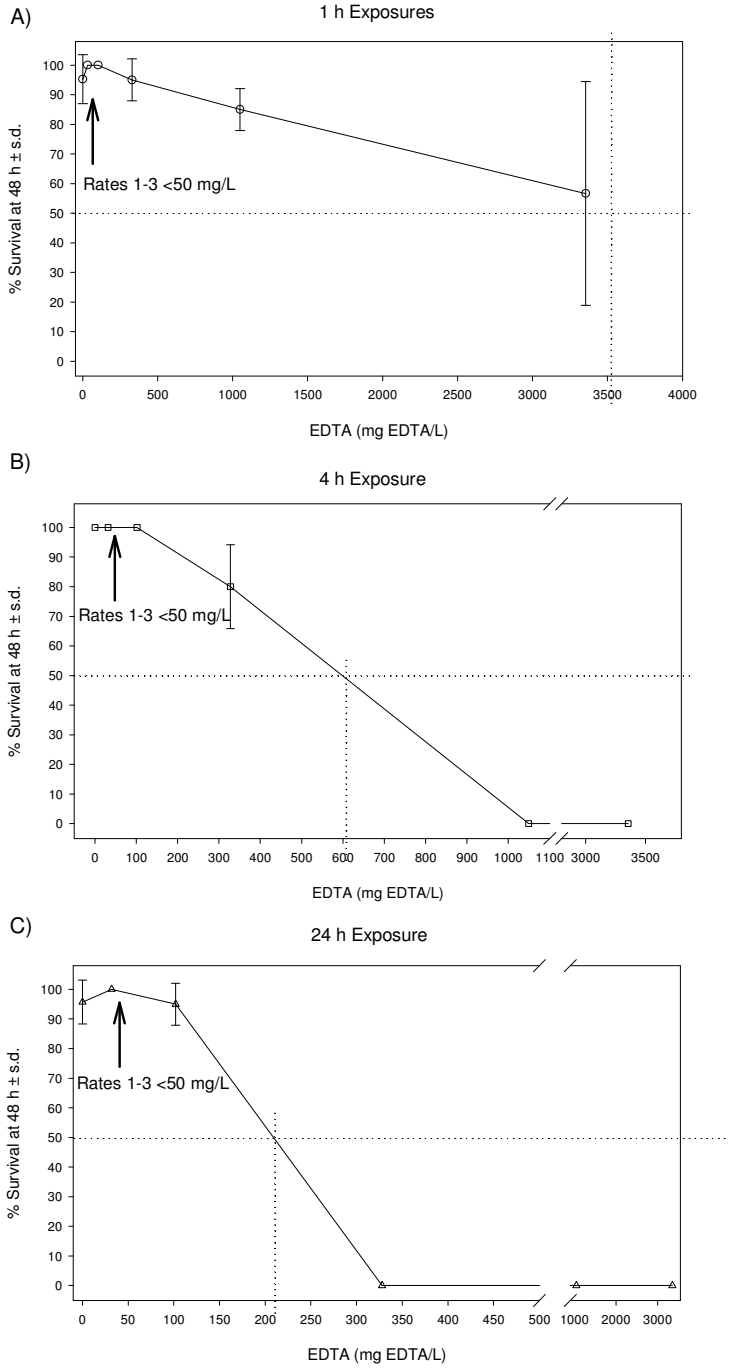
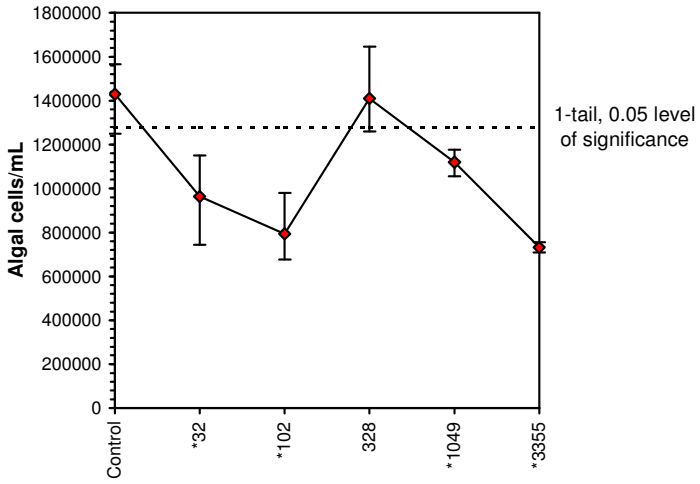


Figure 30: Percentage 48 h survival of *D. magna* after a) 1 hour, b) 4 hour or c) 24 hour exposure to different EDTA concentrations. Note the change in scale on the x-axis. The rates tested on *D. geminata* for 1 h at Stage 2 are noted on the x-axis for comparison. Asterisk (*) indicates *D. magna* negatively affected at the time of transfer from EDTA to freshwater but later recovered. Dashed lines give an estimate of the LC_{50} for each exposure but could be an over- or underestimate if the response is non-linear.

A)



B)

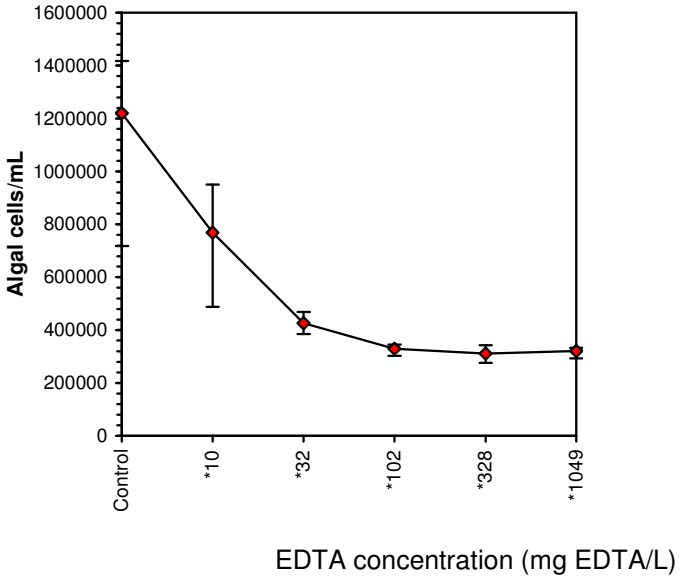


Figure 31: Algal cell density at 72 h after an a) 1 h or b) 24 h exposure to EDTA. Asterisk (*) beside numbers on the x-axis indicate significantly different from control treatment. The non-linear response to 1 h EDTA exposures could be caused by changes in pH from 8.2 in 32-102 mg EDTA/L to pH 5.9 at 328 mg EDTA/L. Alterations in pH might change the speciation, bioavailability and toxicity of different components of the algal culturing solution (e.g., metals). As EDTA concentrations increased to 328-1049 mg EDTA/L pH decreased to 5.0-5.3, and acidity might have caused toxicity.

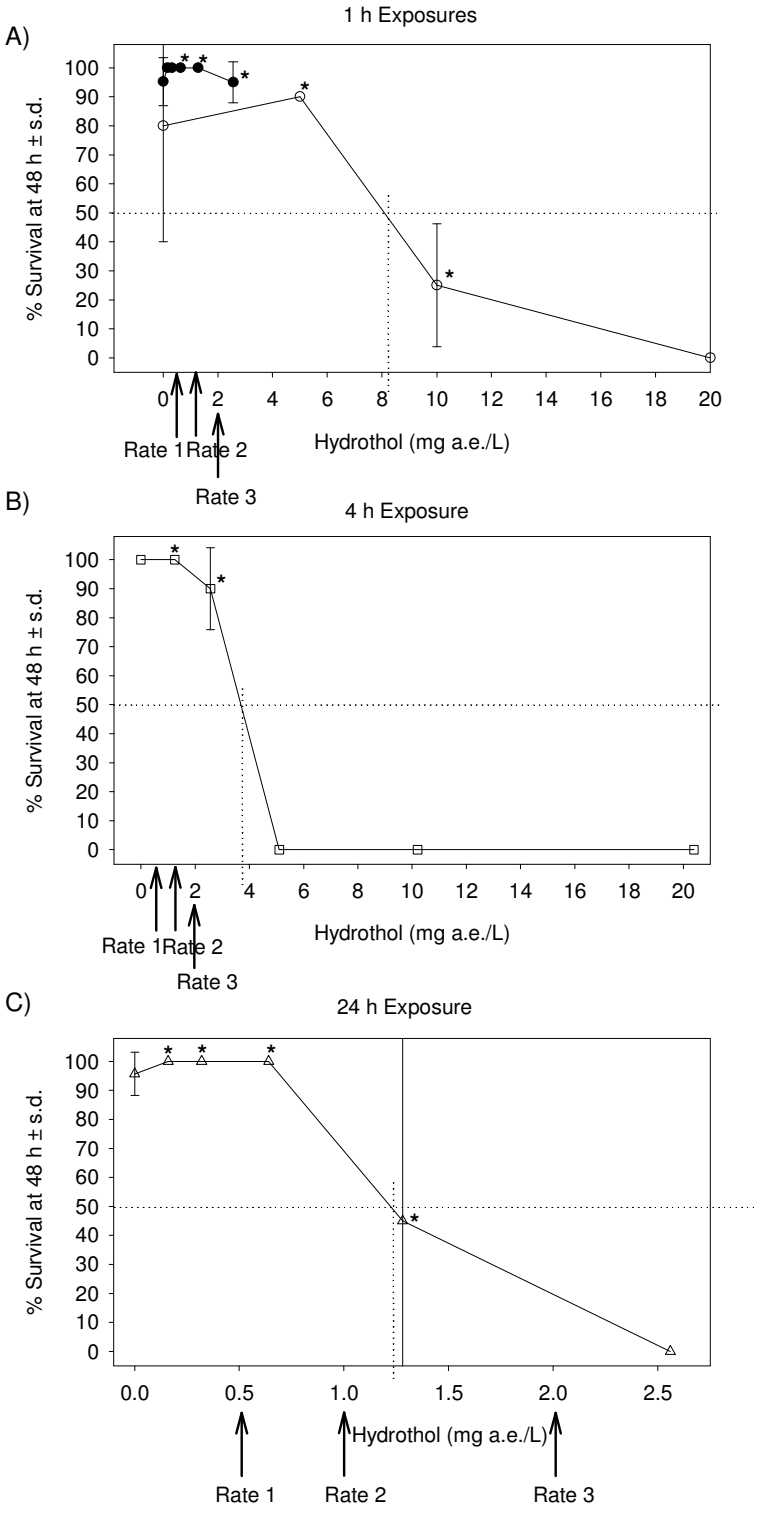


Figure 32: Percentage 48 h survival of D. magna after a) 1 hour, b) 4 hour or c) 24 hour exposure to different Hydrothol®191 concentrations. The rates tested on *D. geminata* in the channel trials at Monowai for 1 h at Stage 2 are noted on the x-axis for comparison. Asterisk (*) indicates *D. magna* negatively affected at the time of transfer from Hydrothol®191 to freshwater but later recovered. Dashed lines give an estimate of the LC50 for each exposure, but could be an over- or underestimate if the response is non-linear.

Growth of the green alga (at 72 h) was negatively affected by a 1 h exposure to ≥0.01 mg a.e./L (Figure 33). The EC_{50} for a 1 h exposure was estimated at 0.05 mg a.e./L. A 24 h exposure caused decreased growth at ≥1 mg a.e./L and the EC_{50} was estimated at 0.58 mg a.e./L. The higher sensitivity estimated for a 1 h exposure could be due to good growth in the controls, however there was a clear suppression of growth after exposure to only 0.1 mg a.e./L for 1 h.

The temperature (19-20°C), DO (9.3-9.5 mg O_2/L), pH (8.0-8.2), salinity (0 ppt) and conductivity (344-370 µS/cm) of the 1-20 mg a.e./L Hydrothol®191 solutions prepared for the *D. magna* were within limits that would not be expected to directly cause toxicity.

Organic Interceptor™

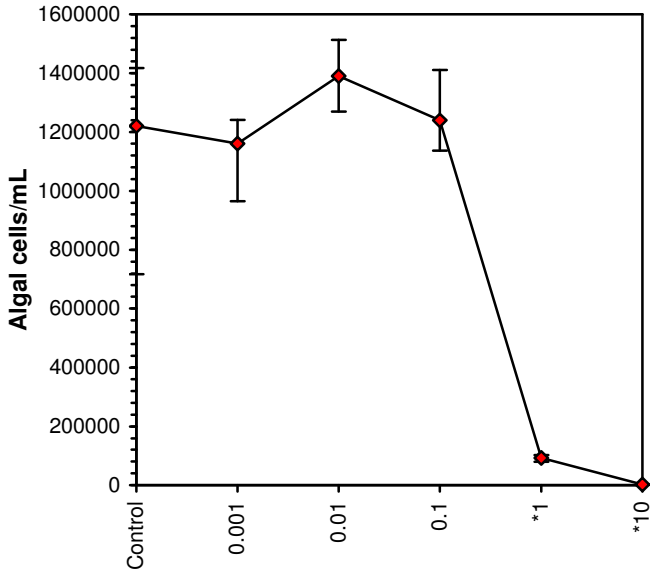
Although a 1 h or a 4 h exposure to ≥125 mg pine oil/L immobilised the *D. magna* they recovered after transfer to freshwater and 95-100% were alive 2 d later (Figure 34). In contrast a 1 h or 4 h exposure to 625 mg pine oil/L caused 100% mortality of *D. magna*. A 24 h exposure to ≥125 mg pine oil/L was 100% lethal. The temperature (19-20°C), DO (8.9-9.5 mg O_2/L), pH (8.1-8.6), salinity (0-2 ppt) and conductivity (247-412 µS/cm) of the 1-3125 mg pine oil/L OI solutions were within limits that would not be expected to directly cause toxicity.

A 1 h exposure of the alga *P. subcapitata* to 680 mg pine oil/L caused a 67% decrease in growth, while 6800 mg pine oil/L inhibited growth by 97%. A 24 h exposure to 68 mg pine oil/L caused a 59% decrease in growth relative to controls, while 680 mg pine oil/L inhibited growth by 98%. One hour exposures to 6.8-68 mg pine oil/L tended to increase algal growth relative to the controls, this did not occur in the 24 h exposures (Figure 35).

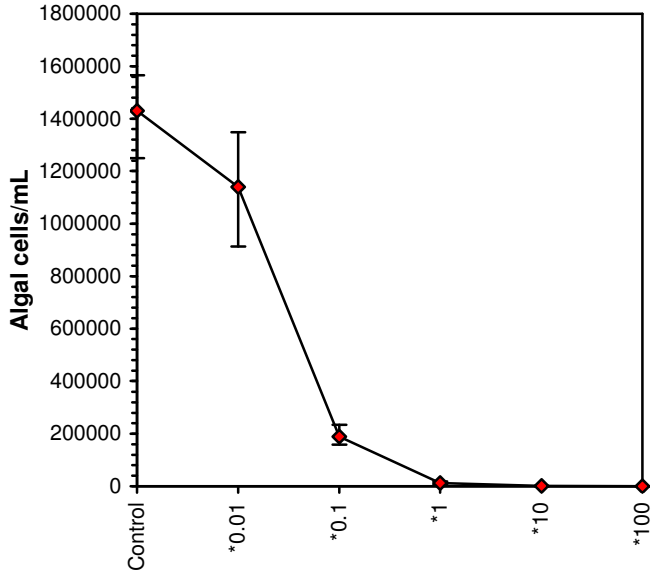
Standard zinc toxicity tests results for Analytical Quality Control (AQC)

The *D. magna* Zn toxicity test 48 h LC_{50} (0.55 mg Zn/L) was within acceptable limits (±2 s.d.) of the long term average for the NIWA Ecotoxicity database for this AQC test. All *D. magna* tests passed the acceptability criteria of <10% mortality in controls except for one control replicate of the 1h repeat trial of Organic Interceptor™ (0-3125 mg pine oil/L) and Hydrothol®191 (0-20 mg a.e./L). The *P. subcapitata* Zn toxicity test 72 h EC_{50} values (0.0050 and 0.0092 mg Zn/L) were within acceptable limits (±2 s.d.) of the long term average for the NIWA Ecotoxicity database for this AQC test. All algal tests passed the acceptability criteria (>16x growth increase in controls and <20% coefficient of variation in controls).

A)



B)



Hydrothol®191 concentration (mg a.e./L)

Figure 33: Algal cell density at 72 h after an a) 1 h or b) 24 h exposure to Hydrothol®191. Asterisk (*) beside numbers on the x-axis indicate significantly different from control treatment.

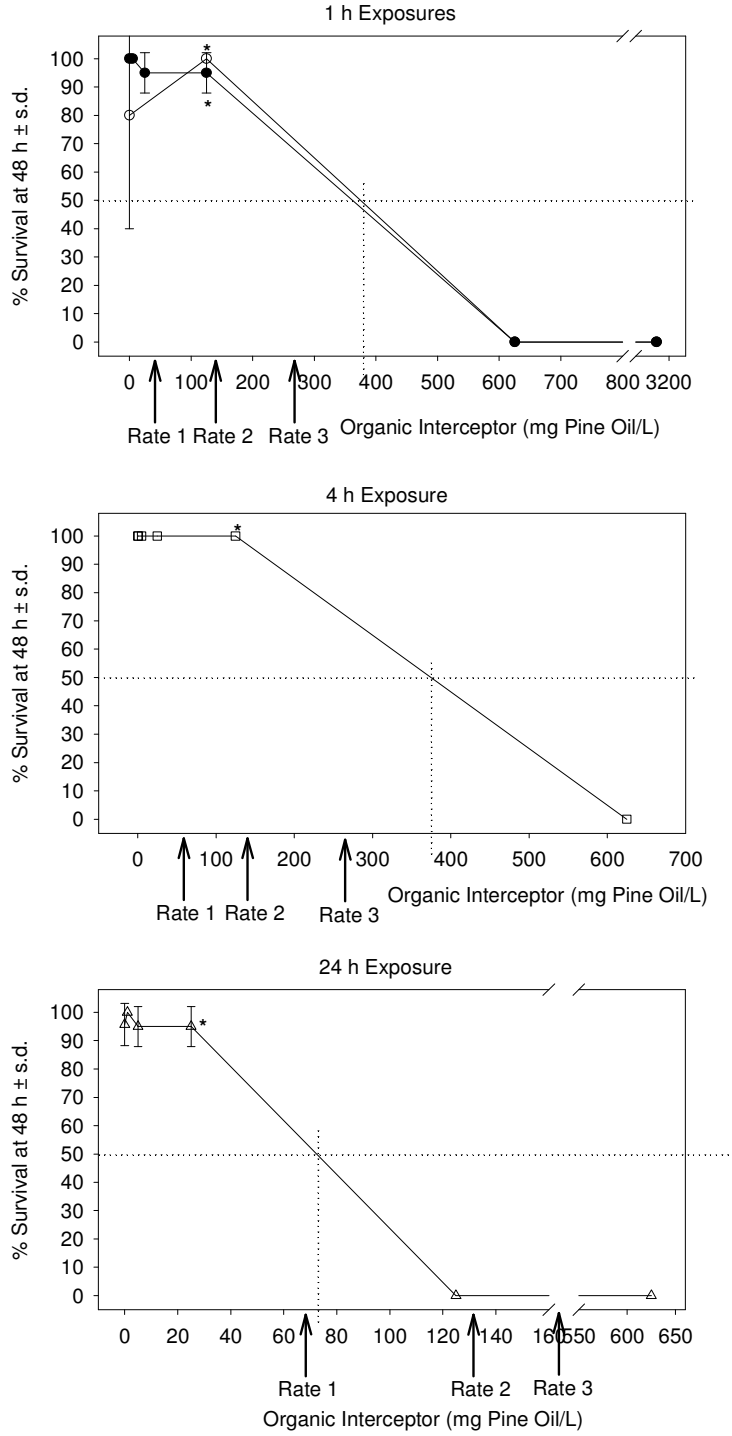
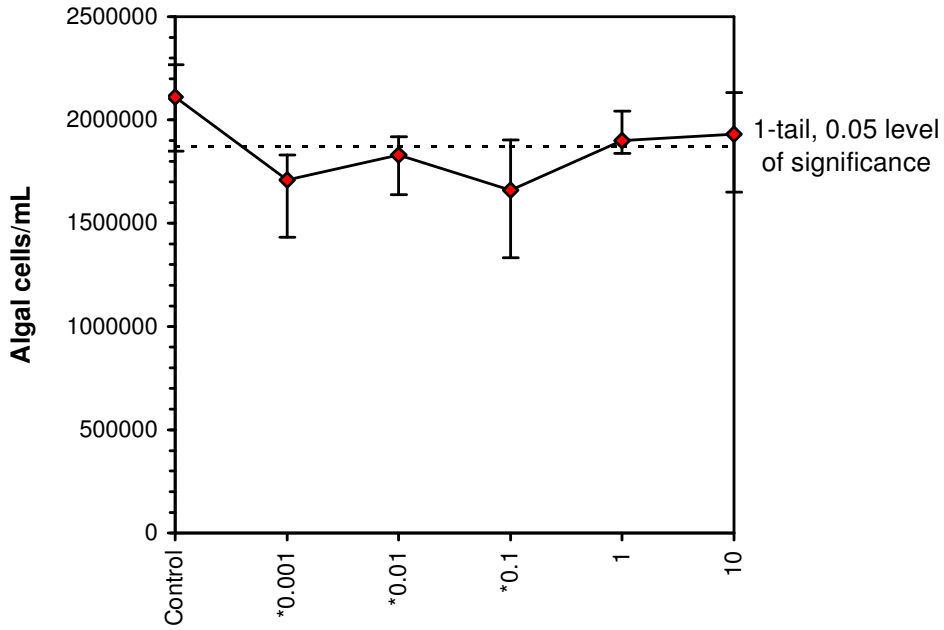
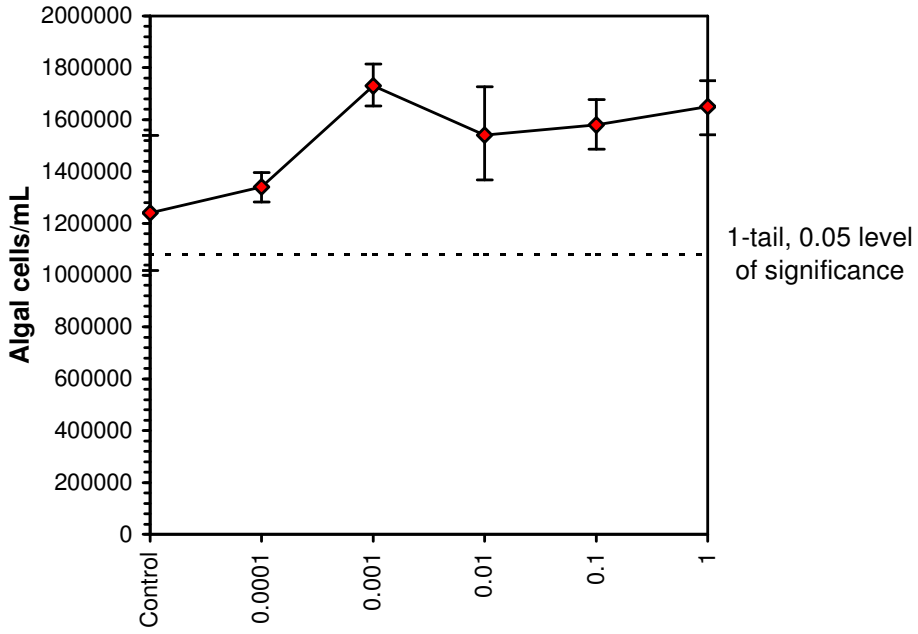


Figure 34: Percentage 48 h survival of *D. magna* after 1, 4 or 24 h exposure to different concentrations of Organic Interceptor™. The rates tested on *D. geminata* in the channel trials at Monowai for 1 h are noted on the x-axis for comparison. Asterisk (*) indicates *D. magna* negatively affected at the time of transfer from OI to freshwater. Dashed lines give an estimate of the LC_{50} for each exposure but could be an over- or underestimate if the response is non-linear.

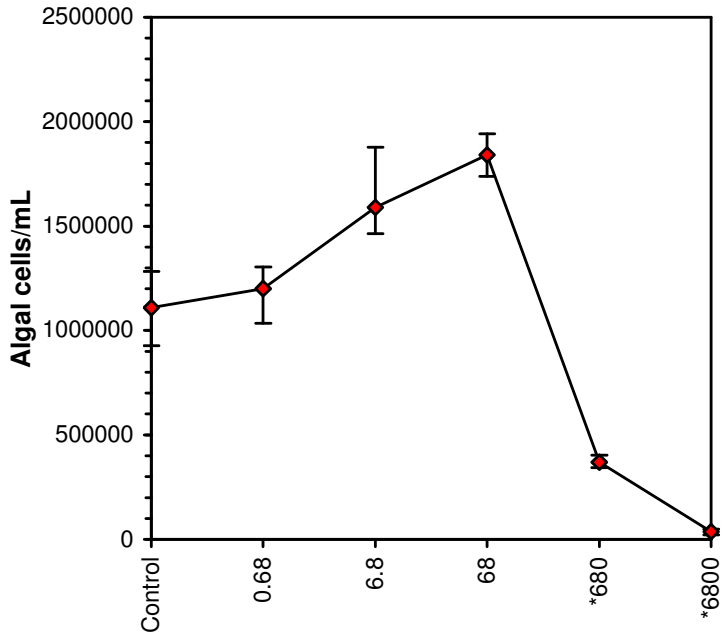
A)


2500000
2000000
1500000
1000000
500000
0
Algal cells/mL
Control
*0.001
*0.01
*0.1
1
10
1-tail, 0.05 level
of significance

B)


2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Algal cells/mL
Control
0.0001
0.001
0.01
0.1
1
1-tail, 0.05 level
of significance
Organic Interceptor™ concentration (mg a.e./L)

C)



D)

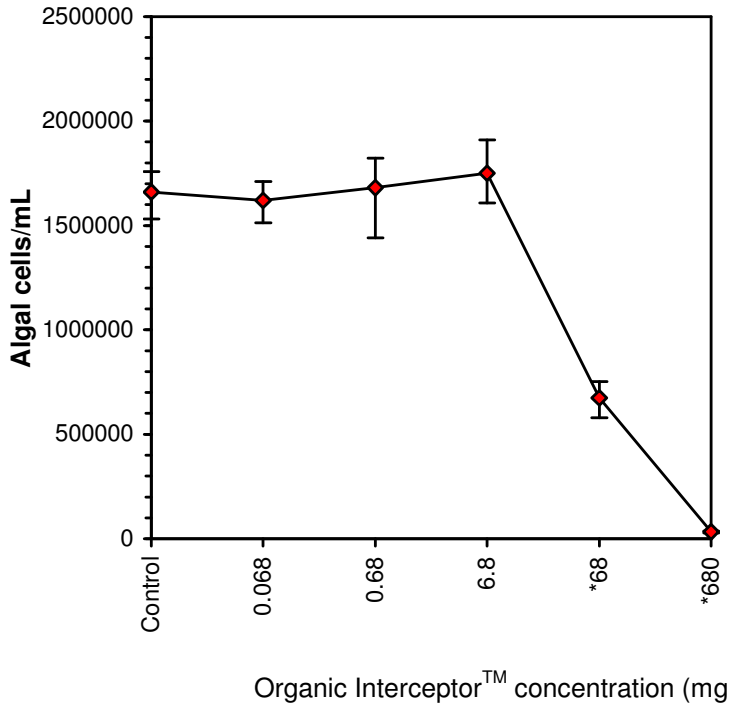


Figure 35: Algal cell density at 72 h after an a) 1 h or b) 24 h exposure to a range of Organic Interceptor™ concentrations. Asterisk beside numbers on the x-axis indicate significantly different from control treatment (0.068 – 680 mg a.e./L).

4.3. Conclusions

D. geminata control agents will be selected by a comparison of their effectiveness against *D. geminata* balanced against their relative toxicity to non-target species (Tables 16-18). This summary mainly refers to the relative toxicity of the biocides to *D. geminata* and the toxicity endpoints (final cell density for *P. subcapitata*, mortality for *D. magna*) examined in the non-target species investigated in this study. As a decision tool we compare the observed toxicity of a 1 h exposure to *D. geminata*, the green alga, *D. magna* and fish (Table 16) and then predict toxicity of 4 h and 24 h *D. geminata* exposures along with algae, *D. magna* and fish data (Tables 17-18).

4.3.1. Chelated copper (Gemex™)

Laboratory and channel toxicity data (nominal Cu concentrations) indicate that if chelated Cu was used in 1 h, 4 h or 24 h treatments at the rates used in the field trials (1, 2 and 4 mg Cu/L), non-target algae, and invertebrates would be immediately negatively affected but fish survival would be 100% (Table 16). The U.S. Environmental Protection Agency (US EPA) ECOTOX database (http://mountain.epa.gov/ecotox, accessed June 2006) has a large amount of toxicity data on copper sulphate (CAS 7758987). However, the toxicity of the chelated copper formulation used on *D. geminata* is different due to the presence of the chelating agents. The database showed copper sulphate studies have found 100% mortality of *Daphnia pulex* exposed to 0.3-2.0 mg Cu/L for 0.8-2.6 h and reduced survival after 8.5 h exposure to 0.1 mg Cu/L. The database also lists numerous green algae and flagellate euglenoid species that are negatively affected by 0.17-0.5 h exposure to 1-100 mg Cu/L as $CuSO_4$. Shaw (1979) found that exposure to 1 or 2 mg Cu/L (as $CuSO_4$) caused 50% mortality of rainbow trout in 69 and 62 min respectively. In the same experiment 10% of the fish lost their ability to swim upright in 41-58 min. Exposure to 0.5 mg Cu/L caused 50% mortality in 8.3 h and 10% of the fish were unable to swim upright after 59 min.

Table 16: Comparison of the observed toxicity to *D. geminata* of one hour exposures to 3 concentrations of 4 toxicants used in Stage 2 Phase 1 trials with the toxicity of one hour exposures to the alga *Pseudokirchneriella subcapitata*, and *D. magna* (invertebrate) in lab trials. The predicted/observed toxicity to fish also included. Y = yes likely to be toxic, P = probably toxic, N = unlikely to be toxic. no toxicity (clear), temporary toxicity (green), <50% mortality (blue), ≥50% mortality (grey).

Compound	Rate	Concentration (mg a.i./L)	Toxic to Didymo?	Toxic to Algae?	Toxic to Daphnia?	Toxic to Fish?
Organic Interceptor™	1	68	Y	N	N	Y[1]
	2	135	Y	Y	Temporary	Y[1]
	3	271	Y	Y	Y	Y[1]
Hydrothol®191	1	0.5 mg a.e./L	~40%	Y	N	N[2]
	2	1.0 mg a.e./L	~45%	Y	Temporary	N[2]
	3	2.0 mg a.e./L	~50%	Y	Temporary	N[2]
Chelated Cu	1	1	Y	Y	Y	N[3]
	2	2	Y	Y	Y	N[3]
	3	4	Y	Y	Y	N[3]
EDTA	1	11	Minimal 30 % kill	P	N	N[4]
	2	23	Minimal 40% kill	Y	N	N[4]
	3	45	Minimal 40% kill	Y	N	N[4]

[1]100% rainbow trout mortality and 33-67% dead bullies in the Stage 2 Phase 1 field trials (indicative only).

[2]No mortalities observed in Stage 2 Phase 1 field trials (indicative only), 96 h LC_{50} for fish 0.08-0.8 mg a.e./L (WSDE 2001).

[3]Stage 2 Phase 1 field trials observed no mortality with chelated Cu (indicative only).

[4]No mortalities observed in Stage 2 Phase 1 field trials (indicative only).

Table 17: Comparison of predicted toxicity to *D. geminata* and the alga *Pseudokirchneriella subcapitata* of 4 hour exposures to 3 concentrations of 4 toxicants with the toxicity of 4 h exposures to *D. magna* (invertebrate) in lab trials. The predicted/observed toxicity to fish is also included. Y = yes likely to be toxic, P = probably toxic, N = unlikely to be toxic, ND? = no data. no toxicity (clear), temporary toxicity (green), <50% mortality (blue), ≥50% mortality (grey).

Compound	Rate	Concentration (mg a.i./L)	Toxic to Didymo?	Toxic to Algae?	Toxic to Daphnia?	Toxic to Fish?
Organic Interceptor™	1	68	Y	Y	Temporary	Y[1]
	2	135	Y	Y	Temporary	Y[1]
	3	271	Y	Y	Y	Y[1]
Hydrothol®191	1	0.5 mg a.e./L	Y	Y	Temporary	N[2]
	2	1.0 mg a.e./L	Y	Y	Temporary	N[2]
	3	2.0 mg a.e./L	Y	Y	Y 10% mortality	N[2]
Chelated Cu	1	1	Y	Y	Y	Y[3]
	2	2	Y	Y	Y	Y[3]
	3	4	Y	Y	Y	Y[3]
EDTA	1	11	P	Y	N	ND?
	2	23	P	Y	N	ND?
	3	45	P	Y	N	ND?

[1]100% rainbow trout mortality and 33-67% dead bullies in the Stage 2 Phase 1 field trials (indicative only).

[2]No mortalities observed in Stage 2 Phase 1 field trials (indicative only), 96 h LC_{50} for fish 0.08-0.8 mg a.e./L (WSDE 2001).

[3]These concentrations were tested on rainbow trout by Shaw (1979) and caused 50% mortality in 1h. However, these tests were performed using $CuSO_4$ not chelated Cu.

Table 18: Comparison of predicted toxicity to *D. geminata* of 24 hour exposures to 3 concentrations of 4 toxicants with the toxicity of 24 h exposures to the alga *Pseudokirchneriella subcapitata*, and *D. magna* (invertebrate) in lab trials. The predicted/observed toxicity to fish also included. Y = yes likely to be toxic, P = probably toxic, N = unlikely to be toxic, ND? = no data. no toxicity (clear), temporary toxicity (green), <50% mortality (blue), ≥50% mortality (grey).

Compound	Rate	Concentration (mg a.i./L)	Toxic to Didymo?	Toxic to Algae?	Toxic to Daphnia?	Toxic to Fish?
Organic Interceptor™	1	68	Y	Y	Y	Y[1]
	2	135	Y	Y	Y	Y[1]
	3	271	Y	Y	Y	Y[1]
Hydrothol®191	1	0.5 mg a.e./L	P	Y	Temporary	N[2]
	2	1.0 mg a.e./L	P	Y	Y	P[2]
	3	2.0 mg a.e./L	P	Y	Y	P[2]
Chelated Cu	1	1	Y	Y	Y	Y[3]
	2	2	Y	Y	Y	Y[3]
	3	4	Y	Y	Y	Y[3]
EDTA	1	11	P	Y	N	ND?
	2	23	P	Y	N	ND?
	3	45	P	Y	N	ND?

[1]100% rainbow trout mortality and 33-67% dead bullies in Stage 2 Phase 1 field trials (indicative only).

[2]No mortalities observed in Stage 2 Phase 1 field trials (indicative only), 96 h LC_{50} for fish 0.08-0.8 mg a.e./L (WSDE 2001).

[3]These concentrations were tested on rainbow trout by Shaw (1979) and caused 50% mortality in 1h. However, these tests were performed using $CuSO_4$ not chelated Cu.

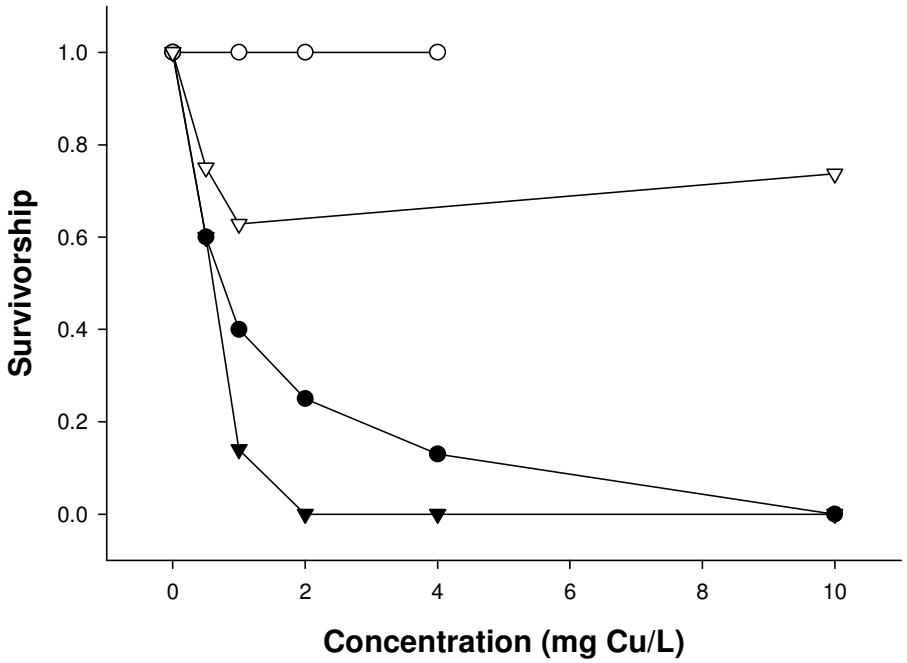


Figure 36: The combined responses of all species to a range of chelated Cu concentrations. All responses shown are based on an exposure time of 1 h. Note: Data point for *D. geminata* survivorship at 10 m.g. Cu/L has been extrapolated. *D. geminata*=filled circles, fish=open circles, *D. magna* (pond invertebrates)=filled triangles, alga=open triangles.

These US EPA data suggest that a 1 h treatment of *D. geminata* with 1, 2 or 4 mg Cu/L (Rates 1, 2 and 3 respectively used in the field trials) would be acutely toxic to invertebrates and fish in the river system, and likely result in reduced algal growth. However, field trials of chelated Cu resulted in no mortality at any Cu concentration in common bullies or rainbow trout (Figure 36) almost certainly because the chelating agents reduced the bioavailability and toxicity of Cu to the fish in this short-term exposure; the Cu-chelate complexes could possibly be taken up in longer exposures. Slow transport of complexed Cu would explain the increase in toxicity to *D. magna* after 4 h compared to 1 h. Preliminary data from our follow-up studies indicate 100% survival of juvenile rainbow trout after 1 h exposure to 16 mg Cu/L as chelated Cu. In contrast metal toxicity studies show that algae readily take up some metal-chelate complexes, thus explaining the selective toxicity of chelated copper to *D. geminata* and other algae (Errecalde et al. 1998, Meyer et al. *in prep*).

Chelated Cu complexes are specifically designed to decrease the bioavailability of the copper ion in the water column and hence decrease the toxicity to non-target organisms such as fish (Ross & Lembi 1985). We believe that because chelated Cu is not toxic to fish at the concentrations trialled it is the most suitable product to continue

testing. A major disadvantage of using Cu as a control agent is its persistence in the environment. Copper sulphate has been used for 30 years in North America to control unwanted algae associated with power station operations, but now the USEPA is phasing out its use because of the ecological consequences of its accumulation in aquatic ecosystems (Nicole Seltzer, Northern Colorado Water Conservancy District, pers. comm., June 2006). Chelated copper therefore could not be considered a long-term treatment for ongoing *D. geminata* control, however if it proves effective it will be appropriate to use it in the short-term as an eradication tool because of the massive dilution factors in a river system treated with a 1 hour pulse-dose. The name Gemex™ now uniquely identifies the particular chelated Cu formulation used in these trials.

4.3.2. EDTA

A one hour exposure of EDTA was relatively ineffective at decreasing the viability of *D. geminata* cells, (for all three concentrations) and there were no observed effects on stalk detachment (expressed as a decrease in AFDM). However, a one hour exposure to the two higher concentrations of EDTA (23 and 45 mg EDTA/L) would probably be toxic to non-target algal species. Otherwise 1 h exposures to 11-45 mg EDTA/L would probably have minimal effect on resident invertebrates. Data from the field trials showed no observed fish mortality for any channels treated with EDTA. The *D. magna* and alga data suggest that these species would be unaffected by the 1 h exposure to 11-45 mg EDTA/L (Rates 1-3 used in the trials), however EDTA concentrations >102 mg EDTA/L and longer exposures should be used with caution. Longer exposures 4-24 h might improve the effectiveness of EDTA against *D. geminata* with potentially minimal effects on invertebrates. However, toxicity to non-target algae increases with exposure duration.

The aquatic ecotoxicity of a tetrasodium salt of EDTA (Na_4-EDTA) and the acid of EDTA (H_4-EDTA) has been reviewed by the European Union (2004) whereas the disodium dihydrate salt of EDTA (Na_2-EDTA) was used in the current study. Water quality (e.g., hardness, pH), the ratio of EDTA concentrations to toxic and essential cations in the exposure media, and which EDTA compound is used, all affect the outcome of the EDTA exposures (European Union 2004). For example, the 24 h EC_{50} (immobilisation) to *D. magna* for Na_4-EDTA was 625-1033 mg/L (high hardness waters) compared to a 24 h LC_{50} of approximately 210 mg/L for Na_2-EDTA (low hardness, natural waters) in the current study. Toxicity to *D. magna* in the current study was probably caused by the low pH of the exposure solution.

Several EDTA studies were cited in the USEPA ECOTOX database, however these were exposures in combination with metals. If EDTA was used in conjunction with other chemicals, particularly copper, it could alter the toxicity of these biocides to non-

target organisms. EDTA doses >102 mg EDTA/L should be used with caution as pH decreases significantly and toxicity to invertebrates increases markedly.

EDTA was included in the Stage 2 trials as a potential growth regulator of *D. geminata* that might cause stalk detachment, rather than as a direct biocide. As a potential biocide for the control of *D. geminata*, EDTA performed poorly (Figure 37). It had little effect on cell viability, cell density or chlorophyll *a*. It is evident that EDTA would need to be applied at a very high concentration or for an extensive period of time to cause a significant reduction in the viability of *D. geminata*. However, this would significantly affect the pH and have considerable impacts to non-target organisms. As a result, EDTA scored poorly on six of the seven selection criteria previously outlined (Page 2). EDTA is not viewed as a biocide warranting further investigation; however its effect on stalk detachment could potentially be investigated further in the laboratory in conjunction with other biocides. The stalk degradation studies showed very high concentrations of EDTA (0.2 M or ~72 g EDTA/L) was an effective DSS, but these application rates are not practical for a river application. Lower concentrations will be trialled in screening studies.

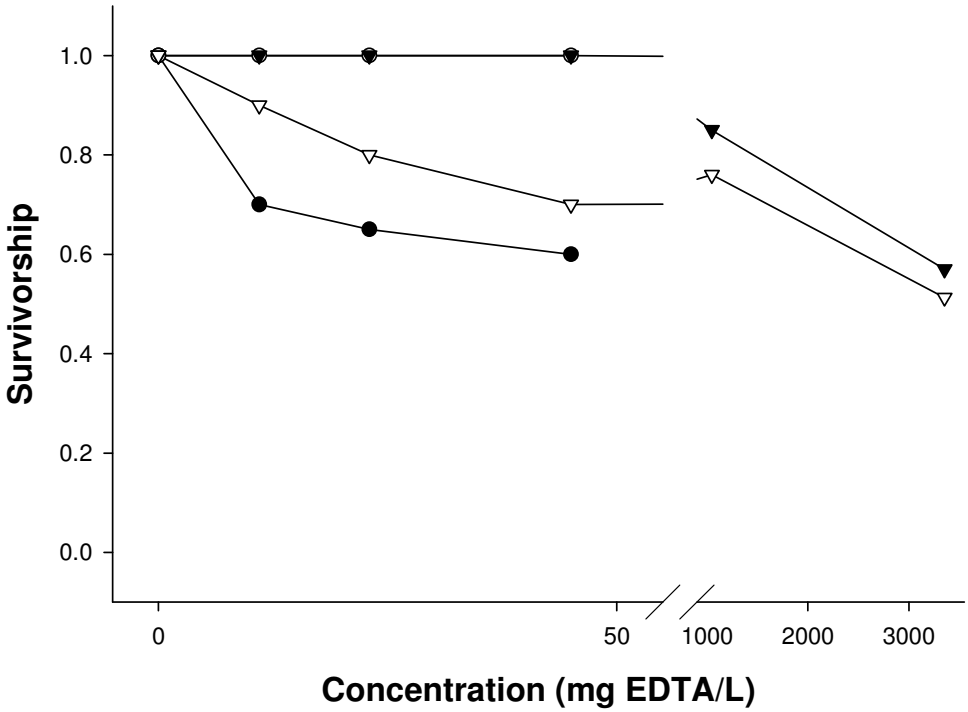


Figure 37: The combined responses of all species to a range of EDTA concentrations. All responses shown are based on an exposure time of 1 h. *D. geminata*=filled circles, fish=open circles, *D. magna* (pond invertebrates)=filled triangles, alga=open triangles.

4.3.3. Hydrothol®191

Available toxicity data indicate that a 1 h exposure to 0.5-2.0 mg a.e./L Hydrothol®191 would be acutely toxic to non-target algal species. A 1 h exposure to the lowest rate (0.5 mg a.e./L) would probably have little effect on resident invertebrates or fish species, but 1.0-2.0 mg a.e./L would temporarily immobilise resident invertebrates, potentially leading to their wash-out from the treated areas. The highest rate of treatment (2.0 mg a.e./L) would probably negatively affect resident fish species however minimal data is available for short exposures. Four hour exposures to 0.5-1.0 mg a.e./L would probably be effective against *D. geminata*, temporarily affect resident invertebrates and have minimal affect on fish. Twenty-four hour exposures would be toxic to invertebrates, and potentially begin to affect fish. All applications would negatively affect non-target algae. Laboratory fish toxicity trials for 1-4 h exposures at 0.5-2.0 mg a.e./L should be investigated as Hydrothol®191 use is generally restricted to areas where fish kill is not considered objectionable (compared to the treatment benefits).

Other studies have estimated 96 h LC_{50} for fish between 0.08-0.8 mg a.e./L (WSDE 2001). The most sensitive fish species was the cutthroat trout, whereas rainbow trout were moderately sensitive (96 h LC_{50} 0.2 mg a.e./L) and the least sensitive species was the sheepshead minnow (96 h LC_{50} 0.8 mg a.e./L)(WSDE 2001). Other studies have found 48 h EC_{50} measurements for *D. magna* range between 0.09-0.63 mg a.e./L compared to 1.4 mg a.e./L for a stonefly. A 24 h EC_{50} for a rotifer was measured at 0.4 mg a.e./L and 96 h EC_{50} of between 0.1-1.6 mg a.e./L for a variety of invertebrate species including scud, mayflies, amphipods, oligochaetes, midges and crayfish (WSDE 2001).

When considered together these data suggest that 1 h exposures to 2 mg a.e./L (i.e., Rate 3 tested on *D. geminata* in field trials) might have short-term negative effects on invertebrates and fish but recovery might be expected, whereas 24 h exposures to 2 mg a.e./L would be expected to cause lethality to both invertebrates and fish. Both rainbow trout and common bullies survived all three 1 h treatment rates (0.5, 1 and 2 mg a.e./L) in the field trials, however only a small number of fish were tested (3 fish/treatment) therefore these results are indicative only. Live invertebrates were also observed in the highest rate of treatment.

At 2.0 mg a.e./L, the effect of Hydrothol®191 on *D. geminata* biomass was comparable to chelated Cu. However, in contrast to Cu there were no significant negative effects on non-target biota at this concentration (for 1 h exposure). Although Hydrothol®191 was relatively ineffective on *D. geminata* at the concentrations trialled (Figure 38); toxicity testing indicated that concentrations could be significantly increased with only modest non-target impacts. The LC_{50} for *D. magna* at a 1 h

exposure was estimated at 8.0 mg a.e./L (Fig 18a). It can argued that a 50% reduction in invertebrate density does not constitute a severe impact, as a standard flood through a South Island River would result in a far greater decrease in invertebrate abundance (e.g., Scrimgeour et al. 1985). Hydrothol®191 warrants a testing at higher concentrations (4.0-8.0 mg a.e./L) for 1 h exposures, and longer exposures and ≤4.0 mg a.e./L to assess *D. geminata* mortality and non-target impacts. This could be done in a small-scale field experiment, not requiring the use of the MEF.

4.3.4. Organic Interceptor™

Available toxicity data indicate that a 1 h exposure of *D. geminata* to even the lowest rate of OI (68 mg pine oil/L[13]) would significantly affect resident fish, whereas local invertebrates and non-target algal species might not be significantly negatively affected (Table 16). The higher rates of treatment (135-271 mg pine oil/L) would almost certainly be toxic to fish, would temporarily immobilise invertebrates or be immediately toxic, but would probably only slightly affect non-target algal growth.

Longer exposures (4-24 h) would probably be more effective against *D. geminata* but would be toxic to fish and at higher rates would also be acutely toxic to invertebrates. Non-target algal species would probably be only slightly affected by a 4 h exposure at the lowest treatment concentration, but higher concentrations or longer treatment times would probably cause >50% decrease in algal growth. These data suggest that to avoid significant non-target species effects, treatment rates of <68 mg pine oil/L and exposure times of 1-4 h should be tested for effectiveness against *D. geminata*.

[13] Organic Interceptor™ is expressed in mg pine oil/ L. However, the exact chemicals that comprise the biocide are trade-marked and there is no certainty as to exactly what the active ingredient is that affects *D. geminata*.

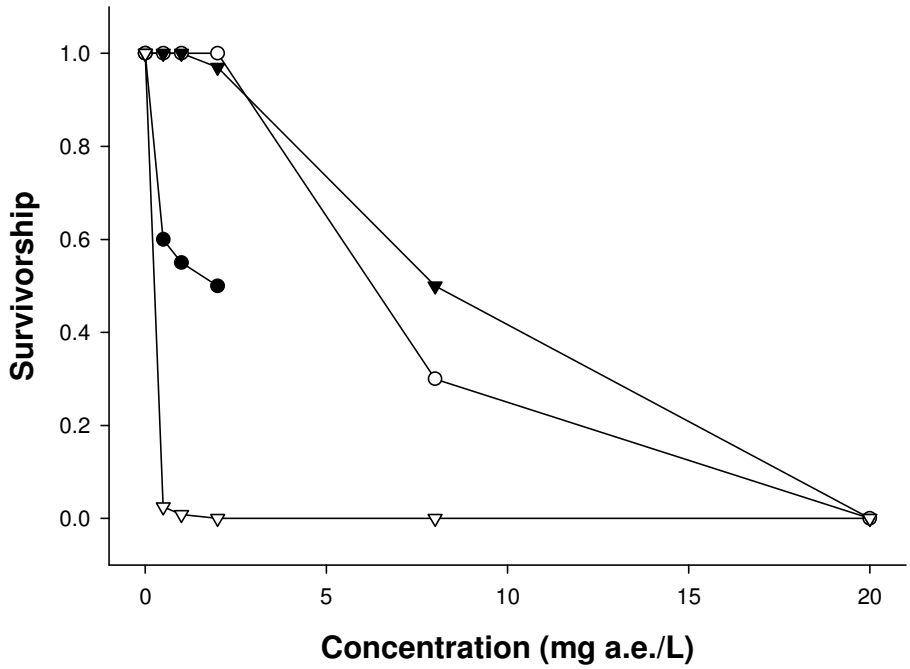


Figure 38: The combined responses of all species to a range of Hydrothol®191 concentrations. All responses shown are based on an exposure time of 1 h. The data points for fish survivorship at 8 and 20 mg a.e./L are predicted values. *D. geminata*=filled circles, fish=open circles, *D. magna* (pond invertebrates)=filled triangles, alga=open triangles.

Organic Interceptor™ was the only biocide (at a concentration of 271 mg pine oil/L) that achieved 100% *D. geminata* mortality (Figure 39), and warrants further testing. However, it also had the most severe non-target impacts during the field trials, being the only product to result in fish mortality. We can conclusively state that if this product is used at a concentration needed to ensure 100% *D. geminata* mortality it will result in severe juvenile salmonid deaths. Its effect on other fish species is less certain, as is its effects on fish of different size classes. It is important to refine the concentration needed to achieve 100% *D. geminata* mortality to reduce non-target impacts. We would suggest that non-migratory galaxiid fishes also be tested to assess their mortality rates to the product. This group of fishes often has 'threatened' populations, existing only in isolated areas (e.g., upper Taieri River catchment). These populations would be particularly vulnerable to extinction if *D. geminata* was transferred there (Larned et al. 2006), and would greatly benefit from a biocide that achieved 100% *D. geminata* mortality without killing a significant proportion of these fish.

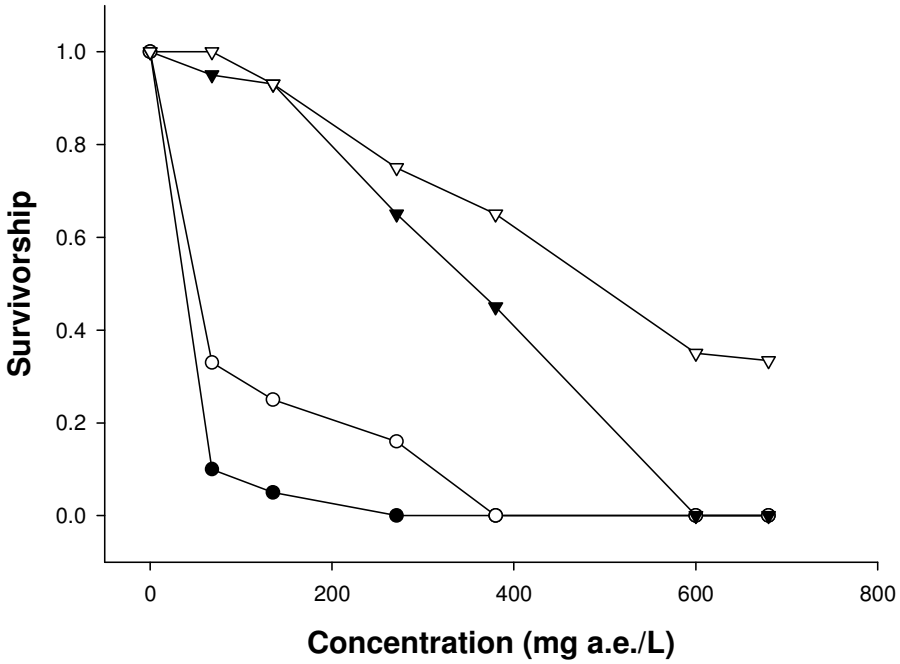


Figure 39: The combined responses of all species to a range of Organic Interceptor™ concentrations. All responses shown are based on an exposure time of 1 h. *D. geminata*=filled circles, fish=open circles, *D. magna* (pond invertebrates)=filled triangles, alga=open triangles.

Organic Interceptor™ is a complex mixture of compounds that includes surfactants and carriers. The ecotoxicological profile of the formulated product (not just the active ingredients) should be reviewed with consideration of its effects upon direct application to water.

4.4. Decision Support Matrix

The decision support matrix is used to quantify the characteristics we are seeking in an ideal *D. geminata* control compound, and to then rank each biocide against these idealized characteristics (Table 19). Overall, the management aim behind these criteria is to prevent the long-term decrease in abundance and diversity of resident aquatic species by controlling the pest alga *D. geminata*. A short-term decrease in resident algal and invertebrate species, is acceptable (although not ideal) to allow the long-term control of *D. geminata* because in most locations these species will readily re-establish by downstream migration. In contrast, control methods will be selected that avoid significant fish mortality during and after *D .geminata* treatment, because the recovery time for these species (years) would be an unacceptable environmental impact.

4.4.1. Decision Support Matrix Criteria and Ranks

Effectiveness: Demonstrated effectiveness against *D. geminata* without consideration of non-target effects:

Rank 4 >95% *D. geminata* mortality
Rank 3 >90% *D. geminata* mortality
Rank 2 >80% *D. geminata* mortality
Rank 1 >50% *D. geminata* mortality
Rank 0 <50% *D. geminata* mortality

Specificity: A measure of biocide effects on non-target species at an application rate resulting in >95% *D. geminata* mortality. The ideal biocide would kill only *D. geminata*, however as achieving this degree of specificity appears unlikely; a practical management goal is to find a biocide that will not cause significant fish mortality. Some degree of toxicity towards non-target algae and invertebrates is acceptable (although not desirable), as these species will rapidly re-establish by migration from upstream sites. Significant fish mortality during *D. geminata* treatment is considered an unacceptable outcome as *D. geminata* has infested locations inhabited by moderately rare native galaxiid fish and commercially valuable trout populations.

Rank 5 0-2% fish mortality, 0-2 % invertebrate mortality, 0-50% non-target algae

Rank 4 0-2% fish mortality, 0-50% invertebrate mortality, 0-100% non-target algae

Rank 3 0-2% fish mortality, 0-100% invertebrate mortality, 0-100% non-target algae

Rank 2 <50% fish mortality, 0-100% invertebrate mortality, 0-100% non-target algae

Rank 1 >50% fish mortality, 0-100% invertebrate mortality, 0-100% non-target algae

Rank 0 100% fish mortality, 0-100% invertebrate mortality, 0-100% non-target algae.

Table 19: Decision Support Matrix for selection of biocides for *D. geminata* control. Prices are not available for EDTA. Biocides are ranked for each characteristic with criteria and rankings described in the preceding section. Ranks calculated either as a sum of all ranks with equal weight, or as a weighted rank with 80% for effectiveness and specificity combined and 20% for the remaining criteria. A higher rank indicates greater suitability as a *D. geminata*-specific algaecide. ? indicates this is a preliminary assessment only.

Biocide	Effectiveness	Specificity	Disrupts Stalk Material[3]	Degradation Profile	Health & Safety	Application Ease	Available & Registered	Neutralization Possible?	Cost				Sum Rank	Weight Rank
									$/L	Application rate	Model cost ($)[1]	Didymo (% M)		
Chelated Cu	3	3	0	1	2	3	3	1	1.15	9 mg Cu/L	9200	92	16	7
Organic Interceptor™	4	0	0	1?	2?	2	1	0	6.00	50 mg pine oil/L	8000	75	10	4
Hydrothol®191	0	0	0	2	2?	3	1	0	17.50	3 mg a.e./L	4000	50	8	2
EDTA	0	0[2]	0[3]	2?	3?	1	3	1	-	45 mg EDTA/L	-	40	10	2

[1]The model to calculate cost of a treatment is for a 5 m^3s^{-1} river flow using an application rate that will result in ~0-5% trout mortality, % *D. geminata* mortality is estimated.

[2]Extrapolation suggests that EDTA will not achieve >40% *D. geminata* mortality, at least until the pH <5, at which point most aquatic organisms are likely to be negatively affected.

[3]Stalk degradation was not assessed in detail for this part of the study, but observations of mat integrity were made throughout the *D. geminata* channel exposures. The maximum EDTA concentration tested in the channel trials was 45 mg EDTA/L, whereas EDTA was effective as a stalk degradation agent at 0.2M (74 g/L) in the DSS trials.

Disrupts Stalk Material: A major ecological impact of *D. geminata* is the establishment of large mats of extracellular stalk material that physically smother and disrupt the normal water flow in cobbled river environments. The mats prevent the complete penetration of biocides and 100% kill of *D. geminata* cells. In addition it has been observed that the mats remain intact in the short-term (e.g., a week) after most biocide treatments even if most cells are dead. It is thought that dead mats would eventually be sloughed off the substrate during high flow events. The ability to disrupt or penetrate the mats would be a favourable characteristic of a biocide, with minimal side-effects on living organisms and other substances in the river environment. This category focuses on stalk disruption rather than mat penetration. Effective mat penetration by the biocide will be taken into account in the "effectiveness" rank.

Rank 3 Significantly disrupts stalk material and promotes mat removal with minimal side-effects on river ecology

Rank 2 Significantly disrupts stalk material, and promotes mat removal with "acceptable" side-effects on river ecology (e.g., short-term invertebrate toxicity)

Rank 1 Moderately disrupts stalk material and promotes mat removal with "acceptable" side-effects on river ecology (e.g., short-term invertebrate toxicity).

Rank 0 Does not disrupt stalk material.

Degradation Profile: The ideal biocide will rapidly (0-5 d) degrade to non-toxic by-products. In practice, some toxic degradation products are expected to be produced by most biocides in minimal quantities that will be rapidly diluted and detoxified (e.g., copper binding to organic matter) as the product is transported downstream. A biocide that produced significant quantities of toxic by-product that could cause a measurable effect on river ecology after the treatment (i.e., separate to the removal of *D. geminata*) would not be acceptable for use against *D. geminata*.

Rank 2 Rapid (0-5 d) degradation of biocide to non-toxic by-products.

Rank 1 Slow (5-30 d) degradation of biocide to non-toxic by-product or degradation to toxic by-product in quantities that will have no measurable effect on river ecology

Rank 0 Degrades to toxic by products that could have a measurable effect on river ecology.

Human Health and Safety: Use of the ideal biocide will cause minimal risk to operators applying the product when basic safety precautions are in place (e.g., protective clothing including waterproof gloves, glasses, overalls and footwear), and direct contact with the undiluted stock solution is avoided. The product should be safe to transport (in terms of human health and safety) in quantities of ≤10,000 L. Ideally in addition, normal water-associated activities (i.e., swimming, water abstraction for drinking water, fishing, removal of fish for human consumption) should be possible within hours of the application in order to reduce the health and safety management required post-treatment. It is considered impractical to require these conditions for water-related activities to be met during the *D. geminata* treatment, which is likely to take place as a one hour pulse of chemical through the river.

Rank 3 Minimal health and safety risk to operators during product application after basic precautions in place. Product is safe to transport in quantities of ≤10,000 L. Normal water-related activities can resume a day after product application.

Rank 2 Minimal health and safety risk to operators during product application after moderate precautions place (e.g., face masks for applicators). Product is safe to transport in quantities of ≤10,000 L. Normal water-related activities can resume a day after product application.

Rank 1 Minimal health and safety risk to operators during product application after moderate precautions place (e.g., face masks for applicators). Product requires specialized transport in quantities of ≤10,000 L (e.g., isolation from other compounds). Normal water-related activities can resume a day after product application.

Rank 0 Either product requires significant health and safety precautions during application, or it requires specialized transport, or normal water-related activities are restricted for more than a day after product application.

Ease of application: The ideal biocide will be a liquid that mixes readily with river water from the stock solution (without premixing) and readily penetrates thick *D. geminata* mats. Alternatively a slow-release or solid formulation would be considered if it could be applied in a manner that achieved even river coverage.

Rank 3 Readily mixes with river water and penetrates well established *D. geminata* mats. Can be applied with standard aquatic herbicide equipment.

Rank 2 Requires pre-mixing with river water and penetrates well established *D. geminata* mats. Can be applied with standard aquatic herbicide equipment.

Rank 1 Requires pre-mixing with river water OR only penetrates thin to moderate *D. geminata* mats OR requires significant modification of standard aquatic herbicide equipment.

Rank 0 Product is difficult to apply and achieve even treatment of the *D. geminata* infestation.

Available and Registered in New Zealand: The control compound should be readily available in New Zealand and registered (or not requiring registration) for use in waterways. A resource consent will be required regardless of the product's registration status.

Rank 3 Readily available and product is either registered or not requiring registration.

Rank 2 Product is not readily available but once acquired, is registered or not requiring registration.

Rank 1 Product requires registration through HSNO prior to use in New Zealand.

Neutralization: Downstream neutralization of the control compound would be a useful tool in some situations. Neutralization may not be necessary for biocides that degrade rapidly, or are rapidly removed from the water column. Neutralization is usually achieved by the addition of another chemical (e.g., potassium permanganate neutralizes rotenone). Chemical removal is also possible by sorption to a solid substrate or precipitation and collection. This option will be explored for a persistent chemical (e.g., copper), particularly if long-term use is likely.

Rank 1: Biocide can be neutralized OR, degrades rapidly OR can be removed from the aquatic ecosystem.

Rank 0: Biocide cannot be neutralized, OR is persistent and cannot be readily removed from the aquatic ecosystem.

Cost: The relative cost of the biocides is compared between the treatments for a one hour application to a 5 m^3/s river. The application rate is selected by an estimate of the chemical concentration that will cause the highest possible toxicity to *D. geminata* without causing fish mortality.

5. Summary and Recommendations

5.1. Stalk Disruption

Stage Two of the stalk degradation studies will allow for testing of the effective DSS in a larger scale system in New Zealand. Determination of a suitable suite of DSS for Stage Two will entail detailed analysis of cost/benefit, availability in New Zealand, toxicity, and the potential for bio-control using enzyme producing organisms co-inoculated with *D. geminata*. Identification of organisms that produce enzymes active against diatom stalks as a prelude to implementation of effective biological control in the natural environment of *D. geminata* will be a priority.

Based on preliminary work described herein, the parameters for optimisation of agents identified as efficient stalk degraders will need to be refined. As more information concerning the chemistry and organization of the *D. geminata* stalk becomes available, it will be possible to refine/expand the search for effective DSS. Trials will be expanded to include sequential treatments, (e.g., pre-swelling with TFA followed by other agents that can degrade expanded stalks or a series of enzymatic treatments). Bleaching or hot aqueous extractions may be effective pre-treatments.

Determination of which of the components in the enzyme mixtures tested are active against stalks will allow for expansion of the list of effective enzyme producers as well as potential bio-control organisms. Other enzyme preparations having similar components as those present in the crude extract from *P. funiculosum, A. niger and T. reesii* will be tested. The enzyme mixture from *T. villosus*, which contains lignin peroxidase, laccase, aryl—glucosidase and xylanase – effective for enzymatic delignification (Aleksanorova et al. 2000), will be tested.

5.2. Biocide control

5.2.1. Effectiveness

Out of ten of the top potential biocides identified and trialled thus far, four were effective to the degree that further work was warranted. Single exposure applications for 1 h in channels were effective for: Organic Interceptor^{TM} (~90% *D. geminata* cell mortality); chelated copper (>80% cell mortality); Hydrothol®-191 application (>60% mortality); whereas EDTA only caused approximately 40% mortality. All four biocide treatments also significantly decreased total biomass of *D. geminata* relative to the control treatment, and chelated copper had the greatest effect, suggesting that some disruption of stalk formation occurred. Statistical extrapolation of the concentration-

mortality relationship can be used to predict a high efficacy *D. geminata* kill in the case of Organic Interceptor™ and chelated copper. Presently chelated copper, a non-selective biocide, appears to provide the best potential for control measures, however Organic Interceptor™, a phytotoxic biocide, may be effective.

Proposed key development areas are:

1. Multiple dosing to improve kill efficiency.

2. Development of "cocktails" of formulated mixtures to test for improved effectiveness of these agents and sequential application of different biocides.

3. Test kill efficacy of different formulations of chelated copper agents.

5.2.2. Impacts

All biocides impacted on the viability of two types of non-target organisms to varying degrees (e.g., 4 mg Cu/L for 1 hour dose resulted in 35% mortality of the green alga, 100% mortality of the sensitive invertebrate (zooplankton) test species *D. magna*). However fish are the non-target species of most concern, as they are a key value in most of the *D. geminata* affected waterways and they will take the longest (perhaps years) to re-establish if killed by the *D. geminata* treatment. Chelated copper shows potential as a *D. geminata* control compound because fish can tolerate 1 h exposures to concentrations expected to control *D. geminata* (e.g., 100% survival of a 1 h 4 mg Cu/L exposure). It is expected that an in-river treatment of *D. geminata* will result in significant negative impacts on non-target algal species and, possibly some macroinvertebrates. However, these species are expected to rapidly re-establish (within several months) by immigration from upstream populations (i.e., unaffected tributaries). These impacts are expected to be insignificant in comparison to the long-term impact of continued *D. geminata* growth in these rivers. When applied correctly, the chelated copper compound is not thought to pose a health risk to humans. However, a thorough health impact assessment will be conducted prior to any widespread use in waterways.

5.2.3. Feasibility

Chelated copper is the most promising *D. geminata* control formulation and the next stage of investigations planned for August 2006 will establish the tolerance limits of fish species to the product. From here, the dose rate and techniques at which very high *D. geminata* mortality (>99%) can be achieved will be researched. In addition multiple doses and chemical combinations (that echo the development of booster biocides in

the antifouling arena) will be trialled. We also need to determine how *D. geminata* control rates achieved in the simplistic environments of the channels can be scaled up to a "real world" stream application where a multitude of factors will start to influence effectiveness (e.g., substrate type, variable water quality).

We have identified 3 key areas where chemical applications for *D. geminata* may be useful:

1. Controlled "routine" applications: with the objective of prolonged *D. geminata* growth limitation with minimal adverse effects on non-target species

2. Incursion applications: urgent applications of high chemical concentrations of biocide aimed at achieving complete control over limited areas at a time of first incursion in a waterway when the extent of infestation is very confined;

3. Control applications at sites of special significance: these would consist of a specially designed/targeted applications in areas of high value (e.g., trout spawning areas, water intakes). Special objectives will apply to these needs.

The majority of our biocide evaluation programme has, to date, been applied in the context of area (1) above, with the objective of obtaining very good control with minimal non-target effects. However, we recognise a key objective is also to have a rapid response ability in the near future for new incursions (e.g., when the first incursion is reported in the North Island). We consider that our present knowledge is such that chelated copper would be the compound of choice for this use, with a high likelihood of success in controlling the *D. geminata* if it has a very limited distribution. The application concentration would be increased with the 'threat' level posed by the incursion to provide increased certainty of prolonged control or eradication from the target waterway – recognising an increase in non-target effects. Other considerations relating to feasibility of using chelated copper for control applications are addressed below:

- History of use – copper has a long and current history of use as an aquatic algaecide in ponds and lakes and to a lesser extent in flowing waters in the USA. This practical expertise will be accessed by NIWA staff prior to any river trials.

- The chelated copper formulations tested in this project must be used, rather than the more toxic copper sulphate, in order to reduce toxicity to non-target species (i.e., especially fish, and invertebrates) whilst ensuring a high level of toxicity to *D. geminata*. There is a large selection of chelated copper

formulations available, all with different toxicological profiles; therefore our recommendations are limited to the chelated copper formulation tested in this project.

- Availability – copper compounds are readily available in bulk for use as an agricultural additive. A chemical supplier has already indicated the ability to supply 1000L at short notice. Approximately 5300 L would be required to treat a small river (e.g., 5 m^3/s) for 1 hour at 6 mg Cu/L.

- Ease of application – NIWA staff have extensive experience of the application of liquid herbicides to large water bodies. Herbicides are not normally applied to flowing water systems (such as those requiring treatment for *D. geminata* infestations), however NIWA routinely conducts large river studies of pollutants and their distribution/mixing in waterways together with tracing flow/mixing paths in backwaters, pools and eddies (using dyes). This experience makes us confident that we can develop protocols to dose rivers with *D. geminata* control compounds under a variety of flow conditions and achieve sufficient contact time with *D. geminata* mats to achieve effective control.

- Non-target safety (see 5.2.2 above)

- Low cost – early costings of chelated copper indicate that it will be very competitively priced compared with other aquatic herbicide formulations currently in use in New Zealand.

- Understanding of fate – copper is a persistent element and, as such, it is not suitable for ongoing use (years) as a control compound, however its fate in the environment is well understood. Copper will readily bind to organic matter (i.e., *D. geminata* mats, sediments, plants etc.) in river systems. This attribute is one of the factors why it has been chosen as a candidate for a rapid response *D. geminata* control compound, but will also cause it to bind readily to sediments and become non-bioavailable, thus greatly reducing its toxicity for other biota. NIWA intends to tailor *D. geminata* control techniques to the level of infestation in order to minimize the quantities of copper applied to river systems.

5.2.4. Duration of control

The duration required for effective *D. geminata* control as measured by regrowth at time intervals following treatment remains unknown, as a sufficiently high kill rate

has not yet been achieved at the relatively low biocide concentrations used so far in the channel/cobble trials. However, *D. geminata* cell viability and biomass decreased soon after treatment by approximately 60% and remained low for the monitoring period (28 days), suggesting that long-term control is feasible. NIWA is proposing to investigate this aspect of *D. geminata* control in August 2006 by conducting cobble trials using multiple doses of chelated copper over time. When a >95% initial kill rate is achieved, the affected substrates will be monitored for *D. geminata* regrowth.

5.2.5. Costs

Implementation costs will be developed as the control compound(s), concentrations and application techniques are finalised. Chelated copper is a relatively inexpensive formulation ($1-2/L) in comparison to other trialled products ($4/L-$18/L).

6. Acknowledgements

We thank Biosecurity New Zealand, particularly Christina Vieglais, for the opportunity to undertake this work. If it had not been for the generosity of Pioneer Generation Ltd. (CEO: Peter Dowling) this research would not have been possible. Pioneer Generation Ltd. has donated the land, water and many essential resources (including staff assistance, power etc.) that have allowed this project to be carried out. Water Dynamics Ltd. (in particular Guy Spence and Gerard Mulvey) and Jeremy Walsh are thanked for the design and installation of the MEF. Max Bothwell, Rex Lowe and Jan Stevenson provided advice and expertise during the trials, and design of the MEF. Excel Design & Build Ltd. (Glen & Craig Smith) are thanked for the construction of many aspects of the MEF. Bill Jarvie, Stuart Sutherland (Fish & Game) and Aaron Horrell (Clutha Fisheries Trust Inc.) are thanked for their invaluable assistance in the field. We are grateful to Russell Anderson for flying the artificial substrates from Cromwell to Monowai in difficult conditions. Peter Murdoch is acknowledged for feeding the fishes and cleaning screens during parts of the trial. Karen Robinson, Catherine Chagué-Goff, Amy Lagerstedt and Lindsay Hawke are thanked for the processing of field trial samples, Brendon Waugh assisted with sample counts during toxicity testing. Helen Hurren produced the map (Figure 1). The residents of Monowai (especially Mike & Christine O'Brien) are also thanked for their assistance in many aspects of this project. The field trials were conducted under resource consent N066-002 and NIWA Animal Ethics Committee permit no. 2006/84. We acknowledge the invaluable participation of students from Michigan Technological University: Michelle Riccio, Sarah Kiemle, Melba Apoya-Horton, Holly Burger, Marcus Carli, and Tendeukai Hungwe. Finally we would like to acknowledge the support of The Waiau Trust, Te Ao Marama Inc. and Environment Southland throughout this project.

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Appendix 1

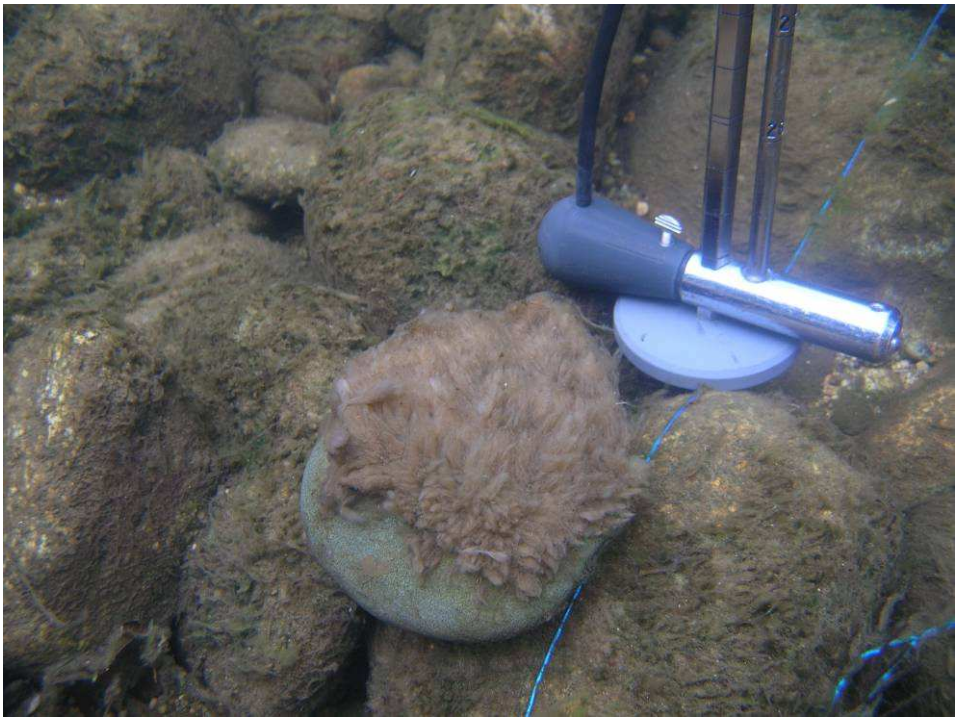
Water chemistry information for the water used in Stage 1 trials and at the MEF.

Parameter	Units	Data
Temperature	℃	12.1
Dissolved Oxygen	% Sat	100.3
Dissolved Oxygen	ppm	10.6
Instantaneous Discharge	m^3/sec	13.95
Visual Clarity	m	6.119
Turbidity	NTU	0.46
Hydrogen ion concentration	pH	7.35
Electrical conductivity	µS/cm @ 25℃	39.0
Ammonia (NH_4)	ppb	5
Nitrate ($N0_3$)	ppb	11
Total Nitrogen (Organic N+NO_3+NH_4)	ppb	83
Dissolved Reactive Phosphate (DRP)	ppb	0.6
Total Phosphate (TP)	ppb	3

Appendix 2

The application of a biocide during short-term screening trials (Stage 1).

Placing cobbles for longer-term trials along transect lines in the Monowai River (Stage 1).

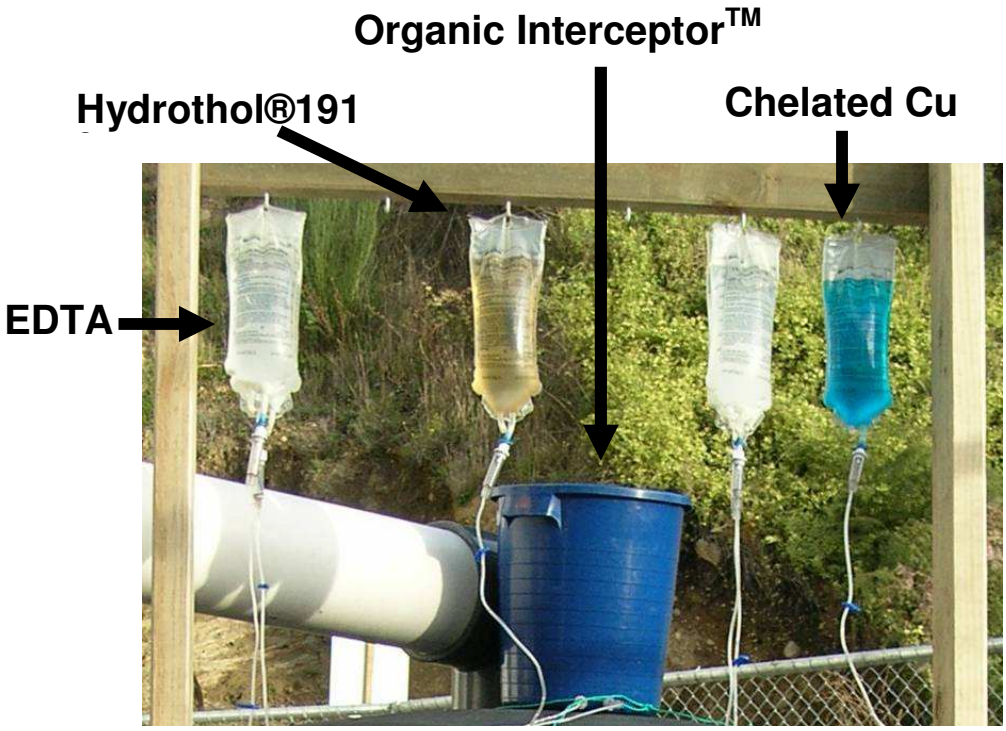


Measuring water depth and velocity of cobble substrates (longer-term trial) in the Monowai River (Stage 1).

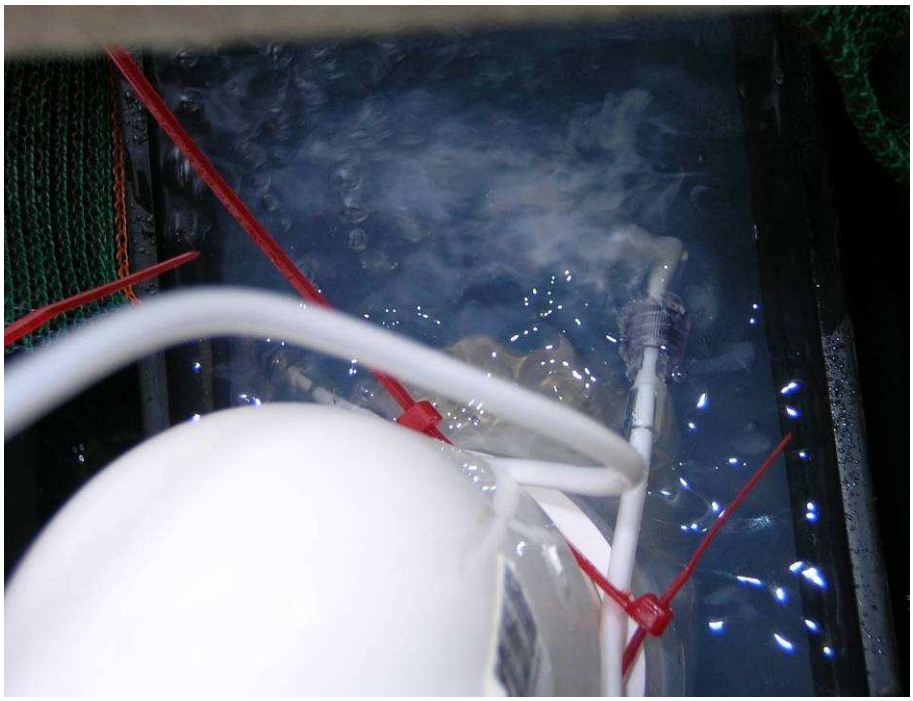
All substrates from longer-term trials were photographically documented (Stage 1).

Artificial substrates placed in a side channel of the Clutha River. Substrates provide a settlement surface for the growth of *D. geminata* (Stage 2 Phase 1).

Fully colonised artificial substrates prior to translocation into the channels at the MEF (Stage 2 Phase 1).



The four biocides being tested during field trials in Stage 2 Phase 1.



Application of Organic Interceptor™ to an experimental channel (Stage 2 Phase 1).

Fully colonised artificial substrates secured into experimental channels (Stage 2 Phase 1).

Application method: Biocides were applied using intravenous bags and were dripped into the channels over a one hour period (Stage 2 Phase 1).

Fish were enclosed in sections above the colonised substrates. Fish species were separated by partitioning sections of each channel with 3 mesh screens (Stage 2 Phase 1).

A multi-pipettor being used for sampling during toxicity testing in Hamilton (Stage 2 Phase 1).

The waterflea (*Daphnia magna*) used for laboratory toxicity testing (Stage 2 Phase 1).

Appendix 3

Derivation of chemical concentrations used in Stage 2 Phase 1 trials.

Organic Interceptor™ (OI)						
Stock soln	680	g Pine Oil/L Organic Interceptor				
therefore						
equivalent to	680	mg Pine Oil/mL Organic Interceptor				
Organic Interceptor™	**Rate**	**Amount of OI added over 1 h (mL)**	**Vol H2O through channel (L)**	**mL OI/L H2O**	**mg pine oil/L H2O**	**Comments**
	1	442	4430	0.0998	67.8	killed fish
	2	884	4430	0.1995	135.7	difficult to dissolve (used 10L H2O)
	3	1768	4430	0.3991	271.4	difficult to dissolve (used 10L H2O)
Hydrothol-191						
If stock (Hydrothol 191) is 240 g ae/L then equivalent to						
240 mg ae/mL where ae = acid equivalents						
Hydrothol-191	**Rate**	**Hydrothol added over 1 h (mL)**	**Vol H2O through channel (L)**	**ml Hydrothol/L H2O**	**mg ae/L**	**Comments**
	1	8.86	4430	0.002	0.48	96 h LC50s for fish are 0.079-0.82 mg ae/L
	2	17.72	4430	0.004	0.96	96 h LC50s for algae etc are 0.04-0.2 mg ae/L
	3	35.44	4430	0.008	1.92	
Chelated Cu						
Stock conc						
If initial stock is 20.36 g Cu/L						
then 20.36 mg/mL						
Chelated Cu	**Rate**	**Cu stock added over 1 h (mL)**	**Vol H2O through channel (L)**	**mL Stock/L H2O**	**mg Cu/L H2O**	**Comments**
	1	220	4430	0.0497	1.01	
	2	440	4430	0.0993	2.02	
	3	880	4430	0.1986	4.04	
EDTA						
EDTA	**Rate**	**g EDTA added over 1 h**	**L H2O thru channel in 60 min**	**g EDTA/L H2O**	**mg EDTA/L H2O**	**Comments**
	1	50	4430	0.0113	11.28668172	
	2	100	4430	0.0226	22.57336343	

Appendix 4

Summary of test conditions used in *Daphnia magna* (cladoceran) bioassay.

Project Name: Didymo Trials Test Initiations: 19/6/06-23/6/06	Project No: MAF06504
Reference Method:	ASTM (2004)
Test Protocol:	NIWA SOP 10.1 (NIWA, 1995) modified for short-term exposures of 1 h, 4 h or 24 h as detailed in methods.
Test Materials:	Organic Interceptor™, Hydrothol®191, Chelated copper, or EDTA
Test Organisms:	*Daphnia magna* (juveniles < 24h old)
Source:	Laboratory culture
Organisms/Container:	6-10 for controls, 7-10 for test solutions
Test Concentrations:	Control, and test materials as listed in Tables 2, 4, 6 and 8
Replicates:	3-8 for controls, 2 for each concentration of test material
Reference Toxicant:	Zinc sulphate
Test Duration:	48 hours
Sample pretreatment:	Nil.
Dilution Water:	*Daphnia magna* Culture Water (hardness 90 mg/L as $CaCO_3$)
Test Chambers:	55 ml polystyrene beakers
Lighting:	16: 8h light: dark
Temperature:	20 ± 1°C
Aeration:	No aeration
Chemical Data:	Temperature, pH, dissolved oxygen, salinity, cond..
Effect Measured:	Mortality
Test Acceptability:	Mean control mortality no greater than 10%, reference toxicant 48 h LC_{50} <±2 s.d. long term average.
Test Compliance:	Achieved

Appendix 5

Summary of test conditions used in *Pseudokirchneriella subcapitata* (green algae) microplate bioassay.

Project Name: Didymo Trials	Project No:MAF06504
Test Initiations: 19/6/06, 20/6/06	
Reference Method:	Environment Canada (1992) and USEPA (1987)
Test Protocol:	NIWA SOP 15.2 (Golding, 2004) modified for short-term exposures of 1 h, or 24 h as detailed in methods section.
Test Materials:	Organic Interceptor™, Hydrothol®191, Chelated copper, or EDTA
Test Organisms:	*Pseudokirchneriella subcapitata*
Source:	NIWA laboratory culture, (original source University of Texas, USA)
Organisms/Container:	10,000 cells
Test Concentrations:	Control, and test materials as listed in Tables 2, 4, 6 and 8
Replicates:	10 for controls, 5 for each concentration of test material once redistributed to microplates
Reference Toxicant:	Zinc sulphate
Test Duration:	72 hours
Sample pretreatment:	0.45 µm filtration
Dilution and Control Water:	Deionized water (0.2 µm filtration)
Test Chambers:	96 well polystyrene microplates
Lighting:	Continuous overhead lighting
Temperature:	24 ± 1°C
Aeration:	No aeration
Effect Measured:	Growth inhibition relative to controls
Test Acceptability:	Control coefficient no greater than 20%, at least 16 x cell growth increase in controls.
Test Compliance:	Achieved

Appendix 6

Observed structural changes in stalks due to treatments with various chemical degrading agents. [1]Active Ingredient - Cetylpyridinium chloride (.07%), [2] Active Ingredient - Sodium hypochlorite (5.25%), [3] Active Ingredient - Sodium hydroxide (.9%), [4,] Active Ingredient - Subtilisin (proteolytic enzyme) (40-43%), [5] Substrate as defined in Figure 4. Stalk structure as shown in Figures 2 and 3.

							Observed Structural Changes						
Type	Treatment	Molarity/ Units	pH	Temperature (°C)	Treatment Time	Substrate Tested	Scalloping [a]	Swelling [b]	Sloughing [c]	Unraveling [d]	Combination [e]	Dissolution [f]	No Apparent Effect
Antiseptic	Crest Pro-Health Mouthwash[1]	2.1E-5M	5	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Antiseptic	Crest Pro-Health Mouthwash[1]	2.1E-5M	5	22	1 hr	rehydrated[5]	-	-	-	-	-	-	x
Antiseptic	Crest Pro-Health Mouthwash[1]	2.1E-5M	5	22	5 hrs	rehydrated[5]	-	-	-	-	-	-	x
Biodegrader	Rid-X[4]	N/A	5	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Biodegrader	Rid-X[4]	N/A	5	22	1 hr	rehydrated[5]	-	-	-	-	-	-	x
Biodegrader	Rid-X[4]	N/A	5	22	5 hrs	rehydrated[5]	-	-	-	-	-	-	x
Biodegrader	Rid-X[4]	N/A	5	22	24 hrs	rehydrated[5]	-	-	-	-	-	-	x
Chemical	Acetic Acid	17M	4	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	HCl	12M	1	22	10 min	rehydrated[5]	-	-	-	-	-	+	
Chemical	HNO_3	16M	1	22	10 min	rehydrated[5]	-	-	-	-	-	+	
Chemical	TFA	13M	2	22	10 min	rehydrated[5]	-	+	-	-	-	-	
Chemical	TFA	13M	2	22	1 hr	rehydrated[5]	-	+	-	-	-	-	
Chemical	TFA	13M	2	22	5 hrs	rehydrated[5]	-	+	-	-	-	-	
Chemical	Ethanol (100%)	.22M	5	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	Ethanol (70%)	.15M	6	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NaOH	10M	14	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NaOH	1M	14	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NH_4OH	14M	11	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NH_4OH	14M	11	22	1 hr	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NH_4OH	14M	11	22	5 hrs	rehydrated[5]	-	-	-	-	-	-	x
Chemical	NH_4OH	14M	11	22	24 hrs	rehydrated[5]	+	-	-	-	-	-	
Chemical	$Bleach_2$	.0071M	N/A	22	10 min	fresh[6]	-	-	-	-	-	-	x
Chemical	$Bleach_2$	.0071M	N/A	22	1 hr	fresh[6]	-	-	-	-	-	-	x
Chemical	$Bleach_2$	.0071M	N/A	22	5 hrs	fresh[6]	+	-	-	-	-	-	
Chemical	$Bleach_2$	.0071M	N/A	22	24 hrs	fresh[6]	+	-	+	-	-	-	
Chemical	$Bleach_2$	.0071M	N/A	22	96 hrs	fresh[6]	-	-	-	-	+	+	
Chemical	$Bleach_2$	.0071M	N/A	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Chemical	$Bleach_2$	.0071M	N/A	22	1 hr	rehydrated[5]	-	-	-	-	-	-	x
Chemical	$Bleach_2$	.0071M	N/A	22	5 hrs	rehydrated[5]	+	-	-	-	-	-	
Chemical	$Bleach_2$	.0071M	N/A	22	24 hrs	rehydrated[5]	+	-	+	-	-	-	
Detergent	RBS_3	.045M	13	22	10 min	fresh[6]	-	-	-	-	-	-	x
Detergent	RBS_3	.045M	13	22	1 hr	fresh[6]	-	-	-	-	-	-	x
Detergent	RBS_3	.045M	13	22	5 hrs	fresh[6]	+	-	-	-	-	-	
Detergent	RBS_3	.045M	13	22	24 hrs	fresh[6]	+	-	+	-	-	-	
Detergent	RBS_3	.045M	13	22	96 hrs	fresh[6]	+	-	+	-	-	-	
Detergent	RBS_3	.045M	13	22	10 min	rehydrated[5]	-	-	-	-	-	-	x
Detergent	RBS_3	.045M	13	22	1 hr	rehydrated[5]	-	-	-	-	-	-	x
Detergent	RBS_3	.045M	13	22	5 hrs	rehydrated[5]	+	-	-	-	-	-	
Detergent	RBS_3	.045M	13	22	24 hrs	rehydrated[5]	+	-	-	-	-	-	
Steam	NaOH w/ steam	1M	13	97	10 mins	rehydrated	-	-	-	-	-	-	x
Steam	NaOH w/ steam	1M	13	97	1 hr	rehydrated	-	-	-	-	-	-	x
Steam	dH2O w/ steam	N/A	7	97	10 mins	rehydrated	-	-	-	-	-	-	x
Steam	dH2O w/ steam	N/A	7	97	1 hr	rehydrated	-	-	-	-	-	-	x
Chelator	EDTA	.2M	N/A	22	1.5 hrs	freeze-dried			+	+			
Hot Aqueous Extraction	hot water	N/A	7	90	1.5 hrs	freeze-dried	+			+			

Appendix 7

Structural changes evidenced by DIC microscopy due to interaction with selected degrading agents. Stalk structure defined as shown in Figures 2 and 3. Intact = native structure. Sloughing = separation of fibrous materials from stalk. Scalloping = series of semi-circular carvings on stalk periphery. Swelling stalk increase in diameter. Unravelling = similar to a multistrand rope unravelling. Dissolution = no stalk material remaining. Combination = two or more effects. Substrate as defined in Figure 4.

							% Observed Structural Stalk Changes						
Type	**Treatment**	**Molarity/Units**	**pH**	**Temperature (°C)**	**Treatment Time**	**Substrate**	*Dissolution*	*Scalloping*	*Swelling*	*Sloughing*	*Unravelling*	*Intact*	*Combination*
Chemical	Bleach	.0071M	N/A	22	24 hrs	rehydrated	0	8	0	2	0	90	0
Chemical	Bleach	.0071M	N/A	22	24 hrs	fresh	0	34	0	4	0	62	0
Biodegrader	Rid-X	N/A	5	22	5 hrs	rehydrated	0	10	0	0	0	90	0
Biodegrader	Rid-X	N/A	5	22	24 hrs	rehydrated	0	5	0	0	0	95	0
Biodegrader	Rid-X	N/A	5	22	10 min	rehydrated	0	0	0	0	0	100	0
Steam	DH2O w/ steam	N/A	7	97	10 min	rehydrated	0	0	0	0	0	100	0
Steam	DH2O w/ steam	N/A	7	97	1 hr	rehydrated	0	0	0	0	0	100	0
Steam	NaOH w/ steam	1M	14	97	10 min	rehydrated	0	0	0	0	0	100	0
Steam	NaOH w/ steam	1M	14	97	1 hr	rehydrated	0	0	0	0	0	100	0
Water	Heat + stirring (Enzyme Pre-Treatment 1)	N/A	7	22-130	2 hrs	freeze-dried	0	20	0	0	0	80	0
Water	Heat + stirring (Enzyme Pre-Treatment 1)	N/A	7	22-130	2 hrs	fresh	0	20	0	0	0	80	0
Buffer	Pre-Treatment 1+Acetate (Enzyme Pre-Treatment 2)	0.2 M	5	22	30 min	freeze-dried	0	32	0	5	0	47	16
Buffer	Pre-Treatment 1+Acetate (Enzyme Pre-Treatment 2)	0.2 M	5	22	30 min	fresh	0	0	0	31	0	69	0
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	10 min	freeze-dried	80	0	0	0	0	0	20
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	10 min	fresh	80	0	0	0	0	0	20
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	1hr	freeze-dried	90	0	0	0	0	0	10
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	1hr	fresh	90	0	0	0	0	0	10
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	5 hrs	freeze-dried	100	0	0	0	0	0	0
Enzymatic	Pre-Treatments 1 & 2+Cellulase from P. funiculosum+DNS+Heat	5 units	5	40-130-22-40-100	5 hrs	fresh	100	0	0	0	0	0	0
Enzymatic	Collagenase from C. histolyticum+ Buffer	5 units	7.5	37-100	10 min	freeze-dried	0	0	0	0	0	92.5	7.5
Enzymatic	Collagenase from C. histolyticum+ Buffer	5 units	7.5	37-100	1 hr	freeze-dried	0	0	0	0	0	75	25
Enzymatic	Collagenase from C. histolyticum+ Buffer	5 units	7.5	37-100	5 hrs	freeze-dried	0	0	0	0	0	70	30